The gut microbiota of the blattid cockroach *Shelfordella lateralis*

Bacterial and archaeal diversity, impact of diet and metabolic functions

Dissertation zur Erlangung des Doktorgrades der Naturwissenschaften

(Dr. rer. nat.)

am Fachbereich Biologie der Philipps-Universität Marburg

vorgelegt von

Christine Schauer

aus Landshut

Marburg/Lahn 2011

Die Untersuchungen zur folgenden Arbeit wurden von Februar 2008 bis Oktober 2011 am Max-Planck-Institut für terrestrische Mikrobiologie in Marburg unter Leitung von Prof. Dr. Andreas Brune durchgeführt.

Vom Fachbereich Biologie der Philipps-Universität Marburg als Dissertation angenommen am: 22.12.2011

Erstgutachter: Prof. Dr. Andreas Brune

Zweitgutachter: Prof. Dr. Roland Brandl

Tag der Dissputation: 22.12.2011

Erklärung

Ich versichere, dass ich meine Dissertation

„ **The gut microbiota of the blattid cockroach *Shelfordella lateralis*:**

Bacterial and archaeal diversity, impact of diet and metabolic functions"

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

Marburg, Oktober 2011

Danksagung

An erster Stelle möchte ich mich bei meinem Doktorvater **Prof. Dr. Andreas Brune** für die Überlassung des Themas sowie seine wertvollen Ideen, Gedanken und für die Freiheit bei der Bearbeitung des Themas herzlich bedanken.

Herrn **Prof. Dr. Roland Brandl** danke ich für die Übernahme des Zweitgutachtens und **Prof. Dr. Ralf Conrad** danke ich für die einmalige Möglichkeit in seiner Abteilung „Schädlinge“ als Forschungsobjekte zu etablieren.

Dr. Claire Thompson möchte ich sehr herzlich für die tolle Zusammenarbeit am Schabenprojekt danken. Ihre wertvollen Ratschläge waren stets eine große Hilfe.

Weiterer Dank gilt allen aktuellen und ehemaligen Mitgliedern der „**Termitentruppe**“ für die gute und fröhliche Arbeitsatmosphäre. Im Besonderen danke ich unserer technischen Assistentin **Katja Meuser** für die schöne Zeit und große Hilfe bei der Laborarbeit wie alltäglichen Dingen ohne die ein Labor nicht funktioniert.

Claudia Lüke, **Andreas Reim** und **Prof. Dr. Peter Frenzel** möchte für die Hilfestellung beim Arbeiten mit R danken. Sie haben mir das Einarbeiten in dieses vielseitige Programm wesentlich erleichtert.

Zusätzlich bedanke ich mich bei **Carsten Mettel** für seine Freundschaft, die interessanten Diskussionen und seine immer gern geleistete Hilfe in auch alltäglichen Dingen. Ich wünsche Dir von ganzem Herzen, dass es Dir bald wieder besser geht.

Mein letzter und größter Dank gilt meiner gesamten **Familie und Verwandten** für die immer gern geleistet Hilfe in großen wie in kleinen Dingen während des Studiums und der Doktorarbeit. Ohne Euch hätte ich diesen Weg nicht gehen können, und ich bin glücklich, dass Ihr mich all die Zeit begleitet habt.

Table of Contents

1 Introduction

The phylogeny of Dictyoptera: Termites, a group of social cockroaches

The order Dictyoptera consists of mantids (Mantodea), cockroaches (Blattodea) and termites (Isoptera), which are quite different from each other in diet and lifestyle.
The phylogenetic position of termites within the Dictyoptera remained for a long time unclear and the subject of much debate (Ware *et al.* 2008). The first attempt to resolve the Dictyoptera phylogeny was made in 1969, as Henning proposed a sister group relationship between Mantodea and Blattodea, the latter including cockroaches and termites. His attempt was mainly based on phylogenetic characteristics, already recognizing the important role of the wood-feeding cockroach *Cryptocercus* as evolutionary link between cockroaches and termites, as it shares several characteristics with termites like xylophagy and morphologically, behaviourally and physiologically similarities (Schal *et al.,* 1984). They also possess a cellulolytic gut microbiota, including cellulolytic flagellates that are only found in the guts of lower termites (Honigberg, 1969; Bobyleva, 1975; McKittrick, 1965, Cleveland *et al.,* 1934; Klass *et al.,* 2008). Similar to dampwood termites, one adult brood pair of *Cryptocercus* sp. lives together with several generations of offspring within a piece of rotten wood. Like termites, they are dependent on parental brood care like proctodeal trophalaxis, which is important to assure transfer of cellulolytic microbiota, especially flagellates, to their offspring (Nalepa, 1984). Many other similarities between cockroaches and termites indicated a close evolutionary relationship, like the presence of endosymbiotic *Blattabacterium* in the fat body of cockroaches as well as *Mastotermes darwiniensis*, the most basal lower termite (Lo *et al.,* 2003). Also *M. darwiniensis* is the only termite species, which lays its eggs in enclosed capsules, called oothecae, which are typical for cockroaches (Nalepa & Lenz, 2000).

In 2007, Inward & colleagues published a convincing comprehensive molecular study where they used five gene loci, two mitochondrial and three nuclear, for phylogenetic analysis. Here, termites were a monophyletic group within the Blattodea, linked to the blattid cockroaches by *Cryptocercus*, which appeared in this study to be closer to termites than to cockroaches (Figure 1). So *Cryptocercus* species were suggested as a model for termite evolution (Klass *et al.*, 2008).

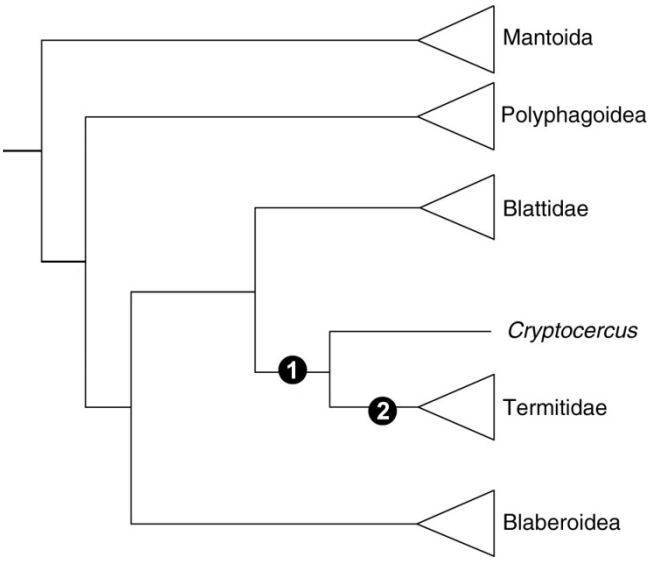

Figure 1. Simplified phylogenetic tree of Dictyoptera. Circles show evolutionary innovations.
1, wood-feeding, cellulolytic gut flagellates, proctodeal trophallaxis;
2, true soldier caste, overlapping generations, reproductive division of labour ('eusociality').
Based on Inward *et al.*, (2007).

Termite guts harbor an unique collection of microorganisms

Termites (Isoptera) are known for their obligate symbiosis with their gut microbiota, enabling them digestion of cellulosic diet. The most important task of this gut microbiota is the degradation of cellulosic material to acetate, which is the major carbon and energy source for the termite host (Breznak, 1984). Due to its unique functions, there is a great scientific interest to characterize this gut microbiota. So during the last years, the gut bacterial communities of all feeding-groups were targeted by molecular studies. While evolutionary lower termites feed mostly on

wood, higher termites have developed additional dietary specializations such as grass harvesting, soil feeding or fungus cultivation. During the last decades, extensive work was carried out to study the gut microbiota of the different feeding guilds (Hongoh *et al.*, 2003, Schmitt-Wagner *et al.*, 2003, Mackenzie *et al.*, 2007, Warnecke *et al.*, 2007). As most of these gut members resist cultivation attempts, characterization by molecular methods was carried out to find out about their role and function within the gut. These molecular studies have shown that a large proportion of the termite gut microbiota belongs to *Bacteriodetes* and *Firmicutes*, irrespective of the feeding group. The same was shown for *Cryptocercus* cockroaches (Berlanga *et al.*, 2009).

Evolutionary lower termites are especially known for a tripartite symbiosis with cellulolytic flagellates and symbiotic bacteria. Termite gut flagellates occur in the mostly anoxic and enlarged hindgut and carry out cellulose digestion. These flagellates are closely associated with various ecto- and endosymbiotic bacteria, which are assumed to carry out for example locomotion, hydrogen removal and recycling of nitrogen compounds to amino acids (Ohkuma, 2008). So, flagellate symbionts were found in many phyla like *Bacteroidetes*, *Proteobacteria*, *Synergistetes* or *Spirochaetes*. Also methanogenic archaea of the genus *Methanobrevibacter* were found to be associated with termite gut flagellates. Some flagellates species have a strong hydrogen-producing ability. This hydrogen can be utilized by *Methanobrevibacter* species, which use hydrogen and carbon dioxide for methane production.

Well examined flagellate symbionts are the *Endomicrobia*, formerly known as Termite Group I, which are abundant in the guts of lower termites and cryptocercid cockroaches (Ohkuma *et al.*, 2007). They occur endosymbiotic in *Trychonympha* flagellates and are supposed to supply the host with essential nitrogenous compounds like amino acids and cofactors (Ikeda-Ohtsubo *et al.*, 2007, Hongoh *et al.*, 2008). Higher termites were found to lack cellulolytic flagellates, but metagenomic studies found that *Spirochaetes* and *Fibrobacteres* were highly abundant in the gut, possessing glycohydrolases which could enable the degradation of cellulose and xylan to acetate, hydrogen and CO_2 (Warnecke *et al.*, 2007).

This essential gut microbiota was shown to be transferred between members of a termite colony via proctodeal trophallaxis, a social anus to mouth feeding between individuals of the same colony. This ensures not only a supply with nutrients, but also the transmission of essential gut microbiota; especially cellulolytic flagellates in lower termites and also semi-social *Cryptocercus* species (Nalepa *et al.*, 2001).

The gut community is shaped by host phylogeny and diet

Insects possess a characteristic gut microbiota that establishes early in development and can outnumber the hosts own somatic cells (Dillon & Dillon, 2004). A large comparative study on mammalian gut microbiota, examining the influence of host phylogeny and diet, found that gut microbial composition seemed to be shaped stronger by host phylogeny, as for example herbivorous pandas clustered together with other carnivore bears despite of their different diet (Ley *et al.* 2008). The same was observed for the analysed humans, where the gut microbiota of a vegetarian was not different to that of humans feeding a normal diet including meat. For insects, such large comparative studies are not available yet, but the gut microbiota composition of termites and bees has been found to be quite similar amongst individuals of the same species (Hongoh *et al.*, 2006, Mohr & Tebbe, 2006).

Nevertheless, host diet has been shown to influence gut microbial composition in earthworms and insects. Earthworms, feeding of different forms of plant litter and grass showed a change in gut microbial composition (Knapp *et al.*, 2009). It has also been observed in crickets that a change in diet is reflected in a change of fermentation products and bacterial composition (Santo Domingo *et al.*, 1998, Kaufmann & Klug, 1991). Experiments analysing the effect of high fiber diets on the gut microbiota of the omnivorous cockroach *Periplaneta americana,* showed a decrease in lactic acid bacteria together with a decrease of lactate and acetate within the foregut (Kane & Breznak, 1991).

Due to its high degree of dietary specialisation, a direct analysis of dietary impact on the gut microbial community of termites via a change of diet is not possible, but individuals belonging to the same colony or developmental caste in social insects

have been shown to harbour a similar bacterial composition, which was assumed to be due to feeding on the same diet as diet is locally collected or an increased exchange of gut microbiota between individuals of the same colony or special castes (Minkley *et al.,* 2006, Mohr & Tebbe, 2006).

Methanogenesis in insects

Termites have been shown to be a considerable source for the greenhouse gas methane. Due to their high abundance in tropical and subtropical regions, they were estimated to contribute up to 4% to the global annual methane emission (Sanderson, 1996). Methanogenesis in arthropods is known to be restricted to only few taxa: termites, cockroaches, millipedes and scarab beetles (Hackstein & Stumm, 1994). While in termites almost all analyzed species were found to emit methane, in cockroaches methanogenesis was shown to be restricted to half of the species (Brauman *et al.,* 1992, Hackstein & van Alen, 2011).

In termites, the amount of emitted methane was shown to differ strongly by diet. So a comparison of literature about methane emission of different termite species, wood-feeding termites were found to produce less methane than fungus growers and soil feeders (Sanderson, 1996). Sanderson estimated the coverage of measured termite species at that time point 1%. A recent review about methanogenesis in insects reported methane emission rates for different lower termite species between 0 and 1.3 µmol g^{-1} h^{-1}. The average methane emission rate for all measured lower termites was 0.2 µmol g^{-1} h^{-1} (Brune, 2010). Higher termites were shown to emit on average less methane than lower termites. Exceptions are the soil-feeding termites. There the average methane emission was twice as much as for the other feeding groups. Compared to that, the methane emission of cockroaches was much lower with an average rate of 0.05 µmol g^{-1} h^{-1}. Up to now, the factors which enable methanogenesis in insects are not known, but gut structure, diet, or even a genetic factor was discussed in recent literature (Hackstein & van Alen, 2011).

The cockroach gut – structure and function

Cockroaches belong to the oldest winged insects, as cockroach-like insects already appeared in the vegetation-rich Upper Carboniferous, 318 to 299 mya. They were assumed to have feed on rotten plant materials as fossilized cockroach fecal pellets were found to contain parenchyma (Nalepa *et al.*, 2001). Modern cockroaches are split into three subfamilies; the Blattoidea, the Blaberoidea and the Polyphagoidea; most of them are omnivores and not exclusively feeding on decaying materials anymore. Only few cockroach species are feeding on wood, as the wood-roach *Cryptocercus* (Blattidae, Cryptocercidae) and the genera *Salganea* and *Panesthia* (Blaberidae; Panesthiinae).

The digestive processes starts with grinding of food with the mouthparts, which do not show functional modifications or specialization, reflecting the broad diet of cockroaches (Beier, 1974). The ducts of the salivary glands end in the oral cavity, releasing a broad range of enzymes such as amylase, maltase, protease and invertase, as observed for the blaberid cockroach *Nauphoeta cinera* (Bland & House, 1971). Host cellulases are secreted from the salivary glands into the fore- and midgut lumen (Watanabe & Tokuda, 2000). Amongst cockroaches the Blattidae were found to produce the highest concentration of cellulases (Wharton & Wharton, 1965).

The first compartment of the cockroach digestive system is the foregut, which consists of the oesophagus and an enlarged crop. Here the grinded food is mixed with enzymes, predigested and stored, until it is passed on through the gizzard. The gizzard of Blattidae, in contrast to other cockroaches has strong teeth for food grinding (Beier, 1974). The crop ends with the proventriculus posterior, which functions as a valve, steering the food flow into the midgut. In blattid cockroaches, the midgut begins with eight gastric caeca. These blind-ending tubules enter the midgut directly after the gizzard and secret digestive enzymes into the lumen of the midgut. The enzymes secreted include beta-glucosidases, carbohydrases and amylases, but no lactases or pepsin. The gastric caeca are also the main place for glucose absorption (Treherne, 1957).

The epithelium of the tubular midgut is optimized for reabsorption of nutrients and also secretes digestive enzymes into the midgut lumen. The food bolus is separated from the epithelium by a protective chitinous layer, called the peritrophic membrane. This layer has pores with a diameter of about 0.15 µm, through which enzymes and other macromolecules, but not bacteria, can pass. The pH was shown to range close to neutral for several cockroach species, likely due to the buffering capacity of the secretions from the Malpighian tubules, which enter at the end of the midgut into the lumen. They perform excretory functions and contain ammonia, amino acids, potassium and other ions (Beier, 1974).

The major part of the hindgut is the colon with the highest bacterial fermentation rates and is accordingly a mostly anoxic compartment. Although the cockroach hindgut is enlarged, it does not reach the relative size and degree of compartmentalisation as observed in termites. The hindgut epithelium was shown to be permeable for ions, fatty acids, amino acids and products of bacterial fermentation, like acetate, propionate and butyrate (Hogan *et al.,* 1985). The last compartment is the rectum, where the water is reabsorbed and the feces formed to fecal pellets (Beier, 1974).

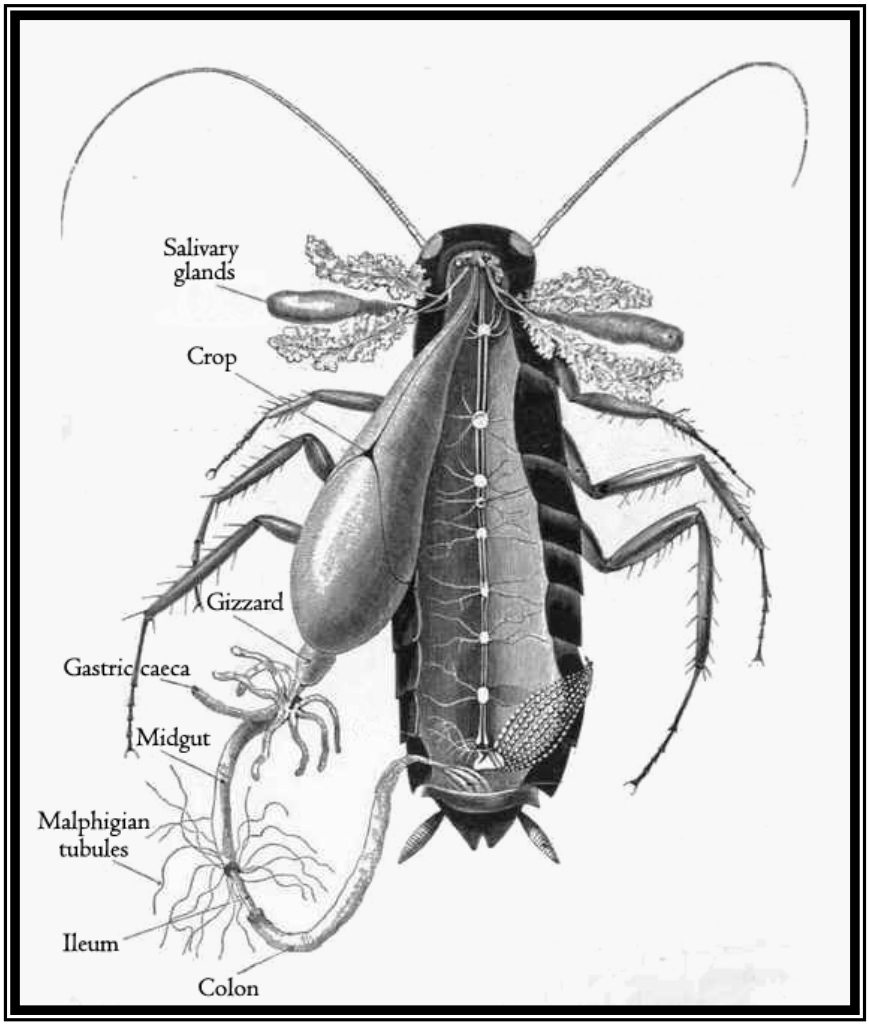

Figure 2. Schematic drawing of the intestine of the cockroach *Blatta orientalis* with the different parts of the gut named. Modified from Rolleston *et al.,* (1888).

Aims and goals of this study

The aim of this thesis is the first characterizations of the bacterial gut microbiota of an omnivorous cockroach. Therefore the blattid cockroach *S. lateralis* was used as a model organism, due to its close relationship to termites. Although neighbored by pest species like *Periplaneta americana* and *Blatta orientalis*, it has a low pest potential, as it is not able to run on smooth glass, making it more unlikely to escape. It is also easily available within Germany via many breeders.

The first goal was the characterization of the gut, including analysis of the different compartments, physiochemical conditions and metabolic products as well as bacterial

density and diversity along the different gut compartments. As the colon was found to harbor the highest density and diversity of bacteria, a clone library targeting the 16S rRNA gene prepared of pooled colonal samples was carried out. Samples of different individuals were pooled to cover variability in gut microbial composition between individuals, which were detected previously via T-RFLP fingerprinting. Phylogenetic analysis was carried out to examine, if the gut microbiota of a blattid cockroach reflects the close relationship between termites and cockroaches.

The second goal was the analysis of the dietary impact on the gut microbiota. As the colon showed the highest bacterial density and diversity, most analyses were focused on this compartment. Therefore individuals were kept on four different diets: a balanced chicken feed diet, a protein-rich soy diet and bran and bran-cellulose as high-fiber diets. Cellulolytic bacterial groups like *Fibrobacteres* or *Spirochaetes*, assumed to carry out cellulose digestion in higher termites, were not detected in the clone library. This could be due to a complete absence of this bacteria or abundance below detection limit. So a high amount of microcrystalline cellulose was added to enrich possible present cellulolytic but rare members of the cockroach gut microbiota. In order to estimate the influence of diet, the gut microbiota was fingerprinted by terminal restriction fragment length polymorphism (T-RFLP) and also analyzed via a high throughput pyrosequencing approach. The bacterial densities within the different gut compartments were counted, and gut metabolites were measured via high pressure liquid chromatography (HPLC).

The third goal was the examination of methanogenesis in *S. lateralis*. Methane emission rates were determined for intact individuals. Individuals were divided into methane emitting (ME) and non methane emitting (NME) individuals. The archaeal community within the hindgut of both types of cockroaches was determined. Also hydrogen was measured, to evaluate its importance as limiting factor on methanogenesis, as well as the effect of hydrogen addition to the headspace to determine the methanogenic potential of cockroaches. As different diets were shown to have an impact on methanogenesis in *P. americana* (Kane & Breznak, 1991), methane and hydrogen emission rates as well as the methanogenic potential were determined for all of the four diets, to examine a dietary impact.

References

1. **Beier, M.** 1974. Blattariae (Schaben). *In* J-G. Helmcke, D. Starck, and M. Beier (ed.), Handbuch der Zoologie, 2nd ed., vol. 4, Walter de Gruyter, Berlin.
2. **Berlanga, M., B. J. Paster, and R. Guerrero.** 2009. The taxophysiological paradox: changes in the intestinal microbiota of the xylophagous cockroach *Cryptocercus punctulatus* depending on the physiological state of the host. Int. Microbiol. 12: 227–236.
3. **Bland, K. P., and C.R. House.** 1971. Function of the salivary glands of the cockroach, *Nauphoeta cinerea*, Journal of Insect Physiology, 17: 2069-2084.
4. **Bobyleva, N. N.** 1975. Morphology and evolution of intestinal parasitic falgellates of the far-eastern roach *Cryptocercus relictus*. Acta Protozoologica. 14: 109-160.
5. **Brauman, A., M. D. Kane, M. Labat, and J. A Breznak.** 1992. Genesis of acetate and methane by gut bacteria of nutritionally diverse termites. Science. 257: 1384-1387.
6. **Breznak, J. A., and A. Brune.** 1994. Role of microorganisms in the digestion of lignocellulose by termites. Annu Rev Entomol 39: 453–487.
7. **Brune, A.** 2010. Methanogens in the digestive tract of termites. *In* Hackstein, J.H.P. (ed.), (Endo)symbiotic Methanogenic Archaea, Springer, Heidelberg, pp. 81-100.
8. **Cleveland, L. R., Hall, S. R., Saunders, E. P., and J. Collier.** 1934. The wood-feeding roach Cryptocercus, its Protozoa, and the symbiosis between Protozoa and roach. Mem. Am. Acad. Sci. 17: 185-342.
9. **Dillon, R. J. and V. M. Dillon.** 2004. The gut bacteria of insects: Nonpathogenic interactions. Annu. Rev. Entomol. 49: 71-92.
10. **Hackstein, J. H. P., and C. K. Stumm.** 1994. Methane production in terrestrial arthropods. Proc. Natl. Acad. Sci. U. S. A. 91: 5441-5445.
11. **Hackstein, J., and T. van Alen.** 2011. (Endo)symbiotic methanogenic archaea, Microbiology Monographs. 19: 115-142.
12. **Hennig, W.,** 1969. Die Stammesgeschichte der Insekten. Kramer-Verlag, Frankfurt.

13. **Hogan, M. E., M. Slaytor, and R. W. O'Brien.** 1985. Transport of volatile fatty acids across the hindgut of the cockroach *Panesthia cribrata* Saussure and the termite *Mastotermes darwiniensis* Froggatt. J. Insect Physiol. 31: 587-591.

14. **Hongoh, Y., L. Ekpornprasit, T. Inoue, S. Moriya, S. Trakulnaleamsai, M. Ohkuma, N. Noparatnaraporn, and T. Kudo.** 2006. Intracolony variation of bacterial gut microbiota among castes and ages in the fungus-growing termite *Macrotermes gilvus*. Mol. Ecol. 15: 505-516.

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

16. **Honigberg, B. M.** 1969. Protozoa associated with termites and their role in digestion. In: Krishna, K., Weesner, F. M. (Eds.), The Biology of Termites. vol. 2. Academic Press, New York, pp. 1-36.

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

18. **Inward, D., G. Beccaloni, and P. Eggleton.** 2007. Death of an order: a comprehensive molecular phylogenetic study confirms that termites are eusocial cockroaches. Biol. Lett. 3: 331–335.

19. **Kane, M. D., and J. A. Breznak.** 1991. Effect of host diet on production of organic acids and methane by cockroach gut bacteria. Appl. Environ. Microbiol. 57: 2628–2634.

20. **Kaufman, M. G.; and M. K. Klug.** 1991. The contribution of hindgut bacteria to dietary carbohydrate utilization by crickets (Orthoptera: Gryllidae). Comp. Biochem. Physiol. 98: 117-123.

21. **Klass, K-D., C. Nalepa, and N. Lo.** 2008. Wood-feeding cockroaches as models for termite evolution (Insecta: Dictyoptera): *Cryptocercus* vs. *Parasphaeria boleiriana*. Mol. Phylogenet. Evol. 46: 809-817.

22. **Knapp, B. A., S. M. Podmirseg, J. Seeber, E. Meyer, and H. Insam.** Diet-related composition of the gut microbiota of *Lumbricus rubellus* as revealed by a molecular fingerprinting technique and cloning, Soil Biology and Biochemistry, 41: 2299-2307.

23. **Ley, R. E., M. Hamady, C. Lozupone, P. J. Turnbaugh, R. R. Ramey, J. S. Bircher, M. L. Schlegel, T. A. Tucker, M. D. Schrenzel, R. Knight, and J. I. Gordon.** 2008. Evolution of mammals and their gut microbes. Science. 320: 1647–1651.
24. **Lo, N., C. Bandi, H. Watanabe, C. Nalepa, and T. Beninati.** 2003. Evidence for Cocladogenesis Between Diverse Dictyopteran Lineages and Their Intracellular Endosymbionts. Mol. Biol. Evol. 20: 907-913.
25. **Mackenzie, L. M., A. T. Muigai, E. O. Osir, W. Lwande, M. Keller, G. Toledo, and H. I. Boga.** 2007. Bacterial diversity in the intestinal tract of the fungus-cultivation termite *Macrotermes michaelseni* (Sjöstedt). Afric. J. Biotech. 6: 658-667.
26. **McKittrick, F. A.** 1965. A contribution to the understanding of cockroach-termite affinities. Ann. Entomol. Soc. Am. 58: 18-22.
27. **Minkley, N.; A. Fujita, A. Brune, W. H. Kirchner.** 2006. Nest specificity of the bacterial community in termite guts (Hodotermes mossambicus). Insect. Soc. 53: 339-344.
28. **Mohr, K. I., and C. C. Tebbe.** 2006. Diversity and phylotype consistency of bacteria in the guts of three bee species (Apoidea) at an oilseed rape field. Environ. Microbiol. 8: 258-272.
29. **Nalepa, C. A.,** 1984. Colony composition, protozoan transfer and some life history characteristics of the woodroach *Cryptocercus punctulatus* Scudder (Dictyoptera: Cryptocercidae). Behavioral Ecology and Sociobiology. 14: 273-279.
30. **Nalepa, C. A., and M. Lenz.** 2000. The ootheca of *Mastotermes darwiniensis* Froggatt (Isoptera: Mastotermitidae): homology with cockroach oothecae. Proc. R. Soc. Lond. B. 267: 1809-1813.
31. **Nalepa, C. A., D. E. Bignell, and C. Bandi.** 2001. Detritivory, coprophagy, and the evolution of digestive mutualisms in Dictyoptera. Insectes Sociaux, 48: 194-201.
32. **Ohkuma, M., S. Noda, Y. Hongoh, C. A. Nalepa, and T. Inoue.** 2008. Inheritance and diversification of symbiotic trichonymphid flagellates from a common ancestor of termites and the cockroach *Cryptocercus*. Proc. R. Soc. B 276:239–245.

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

34. **Sanderson, M. G.** 1996. Biomass of termites and their emissions of methane and carbon dioxide: A global database. *Global Biogeochem. Cycles*. 10: 543–557.

35. **Santo Domingo, J. W., M. G. Kaufman, M. J. Klug, W. E. Holben, D. Harris and J. M. Tiedje.** 1998. Influence of diet on the structure and function of the bacterial hindgut community of crickets. Blackwell Science Ltd. 7: 761-767.

36. **Schal, C., J-Y. Gautier, and W. J. Bell.** 1984. Behavioural ecology of cockroaches. Biol. Rev. 59: 209-254.

37. **Schmitt-Wagner, D., M. W. Friedrich, B. Wagner, and A. Brune.** 2003. Axial dynamics, stability, and interspecies similarity of bacterial community structure in the highly compartmentalized gut of soil-feeding termites (*Cubitermes* spp.). Appl. Environ. Microbiol. 69: 6018–6024.

38. **Treherne, J. E.** 1957. Glucose absorption in the cockroach. J. Exp. Biol. 34: 478-485.

39. **Ware, J. L., J. Litman, K-D. Klass, and L. A. Spearman.** 2008. Relationships among the major lineages of Dictyoptera: the effect of outgroup selection on dictyopteran tree topology. Syst. Entomology 33: 1365-3113.

40. **Warnecke, F., P. Luginbühl, N. Ivanova, M. Ghassemian, T. H. Richardson, J. T. Stege, M. Cayouette, A. C. McHardy, G. Djordjevic, N. Aboushadi, R. Sorek, S. G. Tringe, M. Podar, H. G. Martin, V. Kunin, D. Dalevi, J. Madejska, E. Kirton, D. Platt, E. Szeto, A. Salamov, K. Barry, N. Mikhailova, N. C. Kyrpides, E. G. Matson, E. A. Ottesen, X. Zhang, M. Hernández, C. Murillo, L. G. Acosta, I. Rigoutsos, G. Tamayo, B. D. Green, C. Chang, E. M. Rubin, E. J. Mathur, D. E. Robertson, P. Hugenholtz, and J. R. Leadbetter** 2007**.** Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. Nature 450: 560–565.

41. **Watanabe, H., and G. Tokuda. 2010.** Cellulolytic systems in insects. Annu. Rev. Entomol. 55: 609-632.

42. **Wharton, D. R. A., and M. L. Wharton. 1965.** The cellulose content of various species of cockroaches. J. Insect Physiology 11: 1401-1405.

2 The gut microbiota of *Shelfordella lateralis* reflects the close relationship between (blattid) cockroaches and termites

Christine Schauer, Claire L. Thompson, Andreas Brune*

Submitted to Applied Environmental Microbiology

Abstract

Termites and cockroaches share a close evolutionary relationship. The intestinal tract of wood-feeding termites harbors a remarkably diverse microbial community that is essential for the digestion of lignocellulose. However, surprisingly little is known about the gut microbiota of their closest relatives, the omnivorous cockroaches. Previous studies of the cockroach gut microbiota have employed only cultivation-based approaches and have focused mainly on cockroaches as pest species and their gut as a reservoir for human pathogens. Here, we present the first comprehensive molecular characterization of the bacterial microbiota in the gut of *Shelfordella lateralis*, a representative of the family Blattidae, the sister group of termites. Physiological parameters and metabolic activities of the gut were characterized and the bacterial communities within each gut compartment were compared using terminal-restriction-fragment length polymorphism (T-RFLP) analysis. We constructed a 16S rRNA gene clone library of the colon, the gut compartment with the highest density and diversity of bacteria. The colonic community was dominated by members of the *Bacteroidetes*, *Firmicutes* (mainly *Clostridia*) and some *δ-Proteobacteria*. *Spirochaetes* and *Fibrobacteres*, which are abundant members of termite gut communities, were conspicuously absent. Nevertheless, the many of the clones from the cockroach colon clustered with sequences previously obtained from the termite gut, whose species composition agrees with an evolutionary origin of termites among the cockroaches.

Authors' contribution: All analysis was performed and planned by C. Schauer. C. L. Thompson and A. Brune were involved in preparation of the manuscript.

Introduction

Comprehensive phylogenetic analysis of members of the lineage Dictyoptera shows that a close relationship exists between termites and cockroaches with termites being considered to be a family of social cockroaches (Inward *et al*. 2007). Termites are able to survive on diets such as lignocellulose due to the metabolic activities of their specialized gut microbiota. However, many cockroaches are omnivorous and subsist on a variable diet. While the diversity and community structure of microorganisms in the intestinal tracts of termites have been examined in detail for members of all major termite feeding guilds (Schmitt-Wagner *et al.,* 2003; Hongoh *et al.,* 2003; Shinzato *et al.,* 2007), an in-depth analysis of the gut microbiota of cockroaches using cultivation-independent molecular methods is so far lacking. Although the gut microbiota of the xylophagous cockroach, *Cryptocercus punctulatus*, has been characterized (Berlanga *et al.,* 2009), this species can be considered to be closer to termites given its similarity in respect to diet, sociality, phylogenetic descent and colonization by gut flagellates (Cruden & Markovetz, 1987; Inward *et al.,* 2007; Klass *et al.,* 2008; Ohkuma *et al.,* 2008).

Current knowledge regarding the gut microbiota of omnivorous cockroaches comes mainly from pest species such as *Periplaneta americana* and *Blatta orientalis*, which are considered to be reservoirs for human pathogens. However, all of these studies have been limited to microscopy and culture-based approaches, leading to the isolation of bacteria mainly from the genera *Enterobacter*, *Klebsiella*, and *Citrobacter* in the midgut and *Clostridium*, *Fusobacterium*, *Bacteroides*, *Serratia* and *Streptococcus* in the hindgut (Cruden & Markovetz, 1987). A similar inventory of isolates has been obtained in previous studies of termite guts (Thayer, 1976) and has served to illustrate the well-established bias of such cultivation-based approaches (Brune, 2006). The use of molecular techniques is therefore of vital importance in

surveys of microbial assemblages such as the cockroach gut where there is an obvious expectation of many unique and uncultivated microbial lineages.

Here we present a comprehensive analysis of the gut microbiota of *Shelfordella lateralis*, a member of the family Blattidae and a close relative of *P. americana* and *B. orientalis*. We characterized physicochemical parameters including pH, oxygen status and redox potential for each gut compartment and assessed activities of the microbial community in terms of fermentation products and hydrogen accumulation. Bacterial community structure was determined for each compartment by terminal-restriction-fragment length polymorphism (T-RFLP) analysis and a 16S rRNA clone library was constructed for the colon, the gut compartment with the highest density and diversity of microorganisms. We hypothesize that comparison of the microbial structure and physiochemical characteristics of the cockroach guts will provide further insight into their evolutionary relationship with termites.

Materials and Methods

Cockroaches and sample collection.

Shelfordella lateralis were obtained from a commercial breeder (J. Bernhard, Helbigsdorf, Germany). Cockroaches were fed chicken feed (Gold Plus, Versele-Laga, Deinze, Belgium) and maintained in a temperature controlled chamber at 25°C with 50% humidity. The guts of adult female cockroaches were dissected, the fat body was removed and whole gut weight was recorded. For analysis of single gut compartments, the gut was divided into five segments: crop, gastric caeca, midgut, colon and rectum.

Gut fermentation products and physiochemical parameters.

Fermentation products within the different gut compartments were measured by high pressure liquid chromatography (HPLC). Each gut compartment was homogenized in

200µl water, and centrifuged for 10 minutes at 14000 rpm. The supernatant was acidified with 1 volume 100mM H_2SO_4 and filtered (0.2 µm, ReZist, Whatman GmbH, Dassel, Germany). Gut metabolites were quantified by HLPC using a Grom Resin IEX column (8 µm, 250 x 4.6 mm i.d., Grom, Rottenburg, Germany), USA) with a refractive index detector (RID-10A, Shimatzu, Duisburg, Germany). Peak identity was verified using external standards. The presence of glucose was confirmed by a glucose oxidase assay (Sigma Aldrich, St Louis, USA). Prior to the assay, samples were deproteinized according to the protocol of Zeidler *et al.* (1976).

PH measurement.

PH measurements were taken as described previously (Brune *et al.*, 1995). Briefly, the guts of adult females (n = 3) were dissected and immediately embedded in Ringer's solution solidified with 0.5% agarose. Measurements were taken from the anterior, middle and posterior section of each gut compartment with the exception of the rectum, which was only measured in the middle. Electrode potentials were measured with a glass electrode (PH-50 electrode, Unisense, Aarhus, Denmark) against a reference Ag/AgCl electrode that was in contact with the agarose filled chamber via a KCl-filled agar bridge (3% agar in 1M KCl). The pH electrode was calibrated with standard pH solutions (4.0, 7.0, 9.0 and 11). Current was recorded with a voltmeter (VC444, Voltcraft, Hirschau, Germany).

Measurement of hydrogen, oxygen and redox potential.

Measurements were performed as described for pH with the exception that each gut was placed on top of a one cm layer of 2% agarose and suspended in Ringer's solution that was saturated with oxygen. The oxygen electrode (OX-10 electrode, Unisense, Aarhus, Denmark) was calibrated using Ringer's solution with oxygen partial pressures of zero and 20 kPa. The hydrogen electrode (H2-50 electrode, Unisense, Aarhus, Denmark) was calibrated using Ringer's solution with hydrogen partial pressures ranging from zero to 100 kPa. Redox potential was measured using a glass electrode (RD-10 (8-12 µm), Unisense, Aarhus, Denmark). The redox electrode

was calibrated with pH 4.0 and pH 7.0 buffer solutions, saturated with quinhydrone (1 g/ 100 ml pH solution).

DNA extraction of different gut compartments.

Prior to DNA extraction, each gut compartment was frozen in liquid nitrogen. DNA was extracted using the FastPrep system (Bio 101, Savant Instruments, Holbrook, USA) combined with phenol-chloroform extraction and ethanol precipitation as previously described (Ikeda-Ohtsubo *et al.,* 2007). Extracts were purified by repeating phenol/chloroform and ethanol precipitation three times in order to remove inhibitory substances present within the cockroach gut. DNA was dissolved in 10mM Tris buffer (pH 8) and stored at -20°C.

Bacterial abundance.

Bacterial cell density was measured as previously described (Schmitt-Wagner *et al*., 2003). Briefly, gut contents were diluted 1:100 in phosphate buffered saline (pH 7.2), stained with 4',6-diamidino-2-phenylindole (DAPI) and applied to 0.2 μm filters (Milipore, Billerica, USA) using a vacuum pump. For quantification, each filter was divided into quarters and five fields section per quarter were counted using a fluorescence microscope (Axiophot, Zeiss, Jena, Germany).

T-RFLP analysis of bacterial diversity.

T-RFLP profiles of 16S rRNA genes were generated as previously described (Egert *et al.,* 2003). Briefly, 16S rRNA genes were amplified using a 6-carboxyfluorescein-labeled forward primer 27f (Lane, 1991) and an unlabelled reverser primer, 907r (Muyzer *et al.,* 1995). Following digestion with *Msp*I, profiles were run on an automatic sequence analyzer (ABI 3130, Applied Biosystems, Carlsbad, USA). All samples were run in triplicate. Terminal restriction fragment (T-RF) sizes between 50-600bp with peak heights≥ 25 relative fluorescence units were used. The percentage

peak area was calculated for each T-RF. Bacterial phylotype richness was expressed as the total number of peaks within each profile. Diversity and community similarity were assessed by calculating the Shannon index (Shannon & Weaver, 1963) and the Morisita-Horn index (Horn, 1966). Evenness was assessed by calculating the Pielou index (Pielou, 1966). Nonmetric multidimensional scaling (NMDS) analysis was performed using R (version 2.10) and the VEGAN software package (Dixon, 2003).

Clone library of bacterial 16S rRNA genes.

A 16S rRNA gene clone library was constructed from colon DNA pooled from 6 adult female cockroaches. 16S rRNA genes were amplified using the primer pair 27f and 1492r (Lane, 1991) as described previously (Strassert *et. al.*, 2010). PCR products were purified using the MiniElute PCR purification kit (Qiagen, Hilden, Germany) and cloned using the pGem-T Cloning Kit (Promega, Mannheim, Germany) following the manufacturer's instructions. Clones were screened for the correct insert size and sequenced using the M13 primer set (GATC Biotech, Konstanz, Germany). Sequences were aligned with the SINA aligner tool and imported into the SILVA database (Pruesse *et al.*, 2007) using the ARB software package (Ludwig *et al.*, 2004). Sequences with greater than 97% similarity were assigned to the same phylotype. Aligned sequences were checked for chimeras using Bellerophon (Huber *et al.*, 2004). Three sequences from each phylotype were selected and used to calculate phylogenetic trees using the maximum likelihood and maximum parsimony methods.

Results

Gut structure and bacterial density in different gut compartments

The intestinal tract of *S. lateralis* consisted of five morphologically distinct gut compartments (Figure 1) that closely resembled the size and morphology of the gut of its close relative *Periplaneta americana* (Bracke *et al.*, 1979). The foregut comprised of the crop and a chitinized gizzard with gastric caeca. This was followed by the midgut and finally the hindgut which consisted of the colon and rectum. The gut of *S. lateralis* contributed up to 13% of the whole weight of the insect (597 ± 110 mg, n = 30). We observed that colon weight was slightly smaller than that of the crop and the midgut (Table 1).

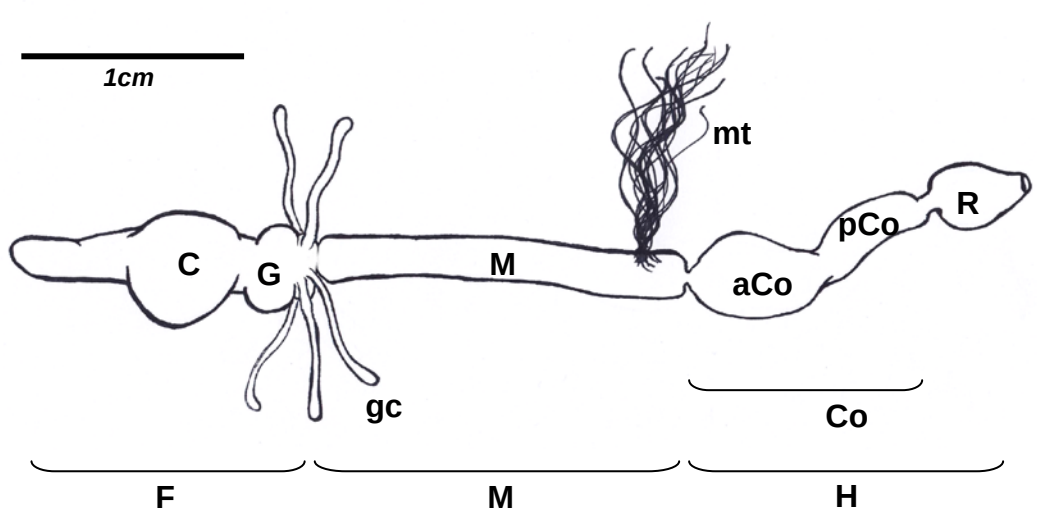

Figure 1. Gut morphology of *S. lateralis*. The gut consists of the foregut (F) with crop (C) and gizzard (G), the midgut (M) with gastric caeca (gc) and Malpighian tubules (mt), and the hindgut (H) with colon (Co) and rectum (R). The colon is divided into the enlarged anterior colon (aCo) and the tubular posterior colon (pCo).

Table 1. Gut weight, bacterial density and diversity within the gut compartments of *S. lateralis*.

	Crop	Gastr. Caeca	Midgut	Colon	Rectum
Gut weight[1]	22.8 ± 17.3	9.8 ± 5.2	20.5 ± 9.5	16.0 ± 6.6	7.5 ± 3.6
Bacterial cell density ($x10^7$)[2]	2.4 ± 4.7	2.7 ± 1.3	1.5 ± 1.3	5.6 ± 3.2	4.8 ± 4.1
Bacterial cell density /mg ($x10^6$)	2.2 ± 1.7	2.4 ± 1.2	2.0 ± 1.3	22.3 ± 15.9	15.3 ± 6.3
Average T-RFs[3]	8 ± 6	8 ± 5	8 ± 4	45 ± 20	24 ± 19
Shared TRFs [4]	1	3	2	15	0
Morisita-Horn SI	0.51 ± 0.42	0.96 ± 0.02	0.54 ± 0.25	0.35 ± 0.19	0.19 ± 0.27
Shannon index	0.51 ± 0.17	0.43 ± 0.22	0.48 ± 0.15	1.55 ± 0.11	1.14 ± 0.35
Pielou index	0.65 ± 0.21	0.39 ± 0.10	0.42 ± 0.10	0.86 ± 0.02	0.69 ± 0.14

All values are given as the mean with standard deviation

[1] fresh weight per compartment (n = 30)

[2] Bacterial cells per gut compartment (fresh weight) (n = 3)

[3] Total number of distinct T-RFs in profiles (n = 4)

[4] Number of T-RFs that occurring in all profiles from the same gut compartment

Physicochemical gut conditions

No significant difference was found in the pH values within the different gut compartments of *S. lateralis*. All compartments had a pH value ranging between 6 and 7 (Figure 2). Variability in pH values between individuals was highest in the colon where values between 6.2 and 7.0 were measured. Oxygen microsensor measurements indicated that anoxic zones were present at the center of each gut compartment. Measurement of redox potential indicated the presence of reducing conditions in all gut compartments. The only exception was the posterior colon of a single animal, where a potential of +104 mV was detected. Hydrogen accumulation inside each gut compartment was measured *in vivo*. Cockroaches were divided into two groups with respect to hydrogen content in the colon (Figure 2). The majority of individuals showed a low concentration of hydrogen within the colon, midgut and rectum, with values ranging between 0-6.5 kPa. However, in a few individuals, hydrogen concentration was high, particularly in the midgut and colon where it reached up to 23 kPa.

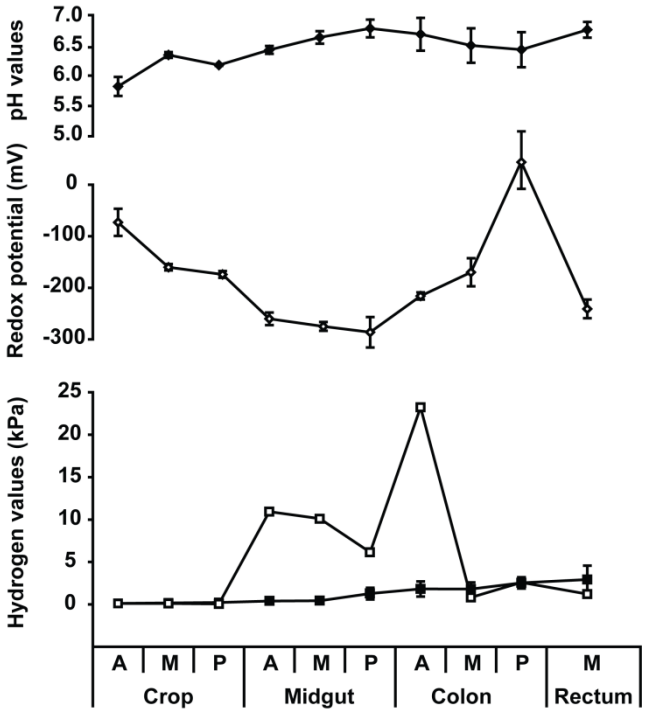

Figure 2. Physiochemical conditions in the gut of *S. lateralis*. Axial profiles of pH (n = 3), redox potential (n = 3) and hydrogen concentration (n =5) were measured using microsensors. Measurements were taken at the anterior (A), middle (M) and posterior (P) sections of the crop, midgut and colon and in the middle of the rectum. For hydrogen concentrations, non-shaded squares indicate the typical profile of a high hydrogen producing individual. All deviations are given as standard error of the mean.

Microbial fermentation products

Fermentation products within the different gut compartments were determined using HPLC (Figure 3). Acetate was high in all compartments, with an average of 600 nmol per compartment. The concentration of acetate was highest in the midgut with an average of 700 nmol per compartment. The concentration of lactate and glucose was highest in the crop and then decreased along the rest of the gut. Propionate was limited to the hindgut. Succinate was detected in crop and midgut (15.0 and 19.1 nmol/ compartment). However, the concentration of succinate was decreased in the colon (5 nmol/ compartment) and it was not present in the rectum. Ethanol concentration was high in the crop, but occurred only in half of the animals measured (Figure 3).

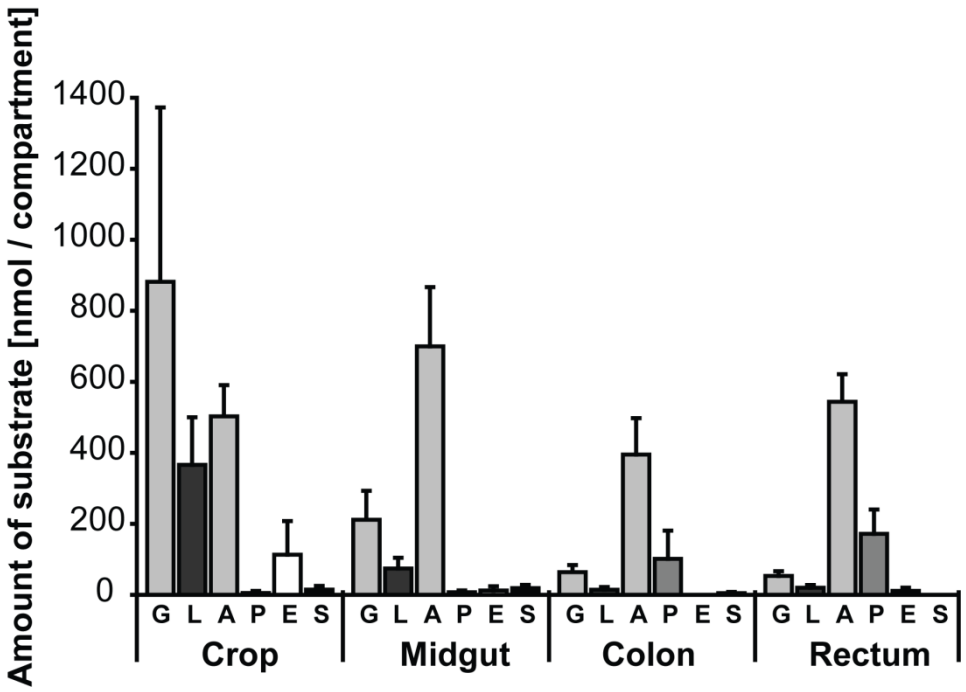

Figure 3. Concentrations of fermentation products within the different gut compartments of *S. lateralis* (*n* = 8). Glucose (G), lactate (L), acetate (A) propionate, (P), ethanol (E) and succinate (S) were detected. Deviations are given as standard error of the mean.

T-RFLP-based analysis of bacterial diversity

Microbial cell counts of the gut contents indicated that the colon contained the highest density of microorganisms in comparison to the other compartments (Table 1). We examined bacterial diversity in each gut compartment by T-RFLP analysis of bacterial 16S rRNA gene fragments from the gut homogenates of four individuals. Reproducibility was determined to exclude that measured differences in the T-RFLP profiles were due to technical artifacts. Samples that were run in triplicate had a Morisita-Horn similarity of 0.92 ± 0.08, indicating that profiles were reproducible and reflected the bacterial gut composition.

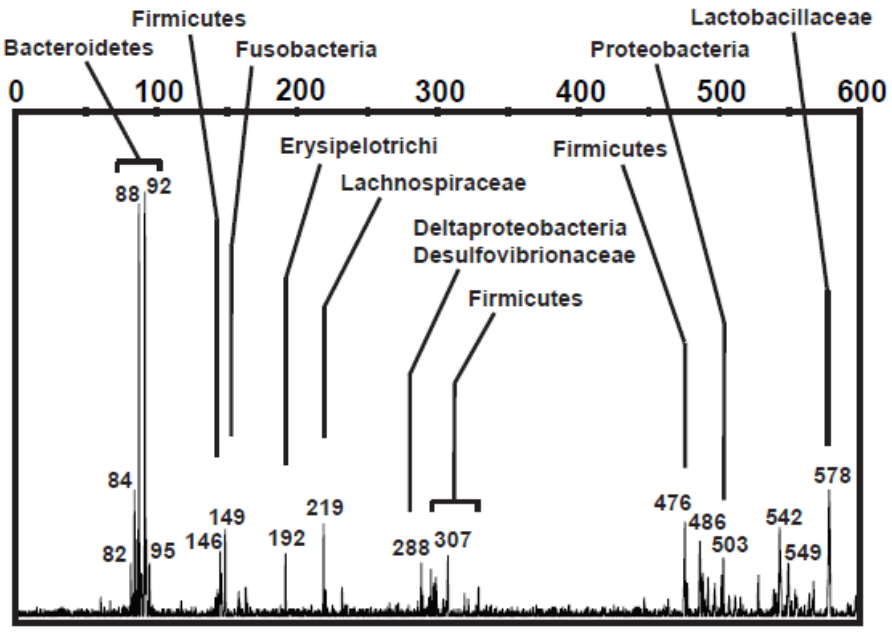

Figure 4. T-RFLP profile of 16S rRNA genes amplified from pooled colon DNA of *S. lateralis* (*n* = 6). Horizontal axis indicates the size (nucleotide base pairs) of the T-RFs. This sample was used to construct the 16S rRNA gene clone library of the colon. The identity of the peaks was determined by *in silico* digestion of clone sequences.

A total of 184 distinct TRFs were detected over all profiles, with phylotype richness varying considerably between different animals and gut compartments (Table 1). The highest number of T-RFs was found in the profiles from the colon and rectum (Table 1; Figure 5). In these compartments, evenness was also high but similarity between different individuals was low. Conversely, the crop, gastric caeca and midgut showed fewer T-RFs and a low evenness but had a high similarity between individuals. The crop, midgut and colon contained a number of common T-RFs that were present in profiles from all individuals (Table 1, Figure 4 & 5). However, profiles of the rectum from different individuals did not have any T-RFs in common.

Comparisons between gut compartments using nonmetric multidimensional scaling (NMDS) also showed that profiles of the gastric caeca and midgut clustered together and were closer to the profiles of the crop. Profiles obtained from the colon and rectum formed a separate cluster (Figure 6).

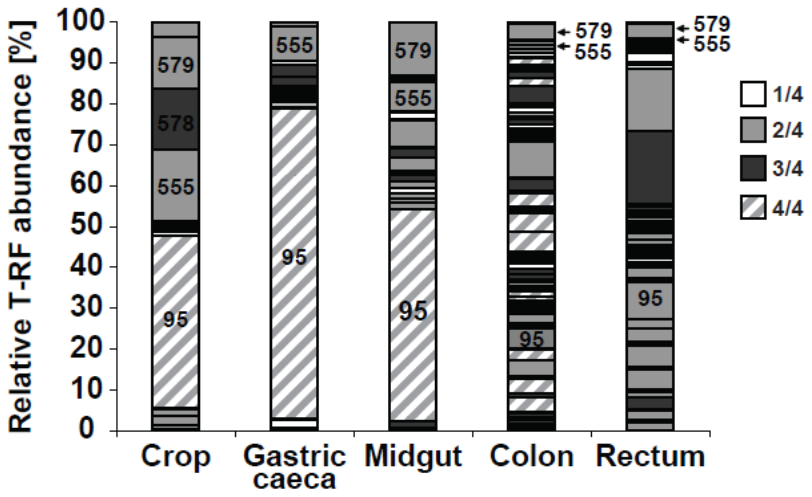

Figure 5. T-RFLP analysis of bacterial diversity within the gut compartments of *S. lateralis.* Each column represents an average of the profiles from four individuals and shading indicates the proportion of individuals containing a particular T-RF. T-RFs of interest are labelled with their size in base pairs.

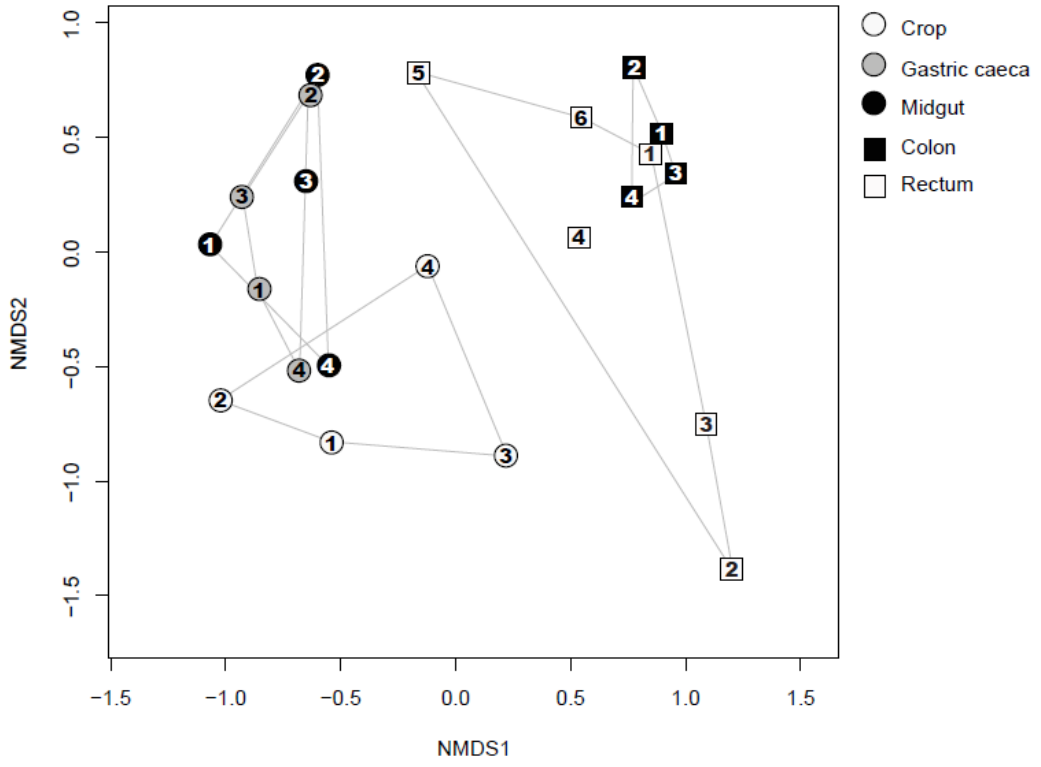

Figure 6. Nonmetric multidimensional scaling plot using Bray-Curtis similarities showing clustering of gut microbiota by compartments and the degree of individual variation. Symbols represent different gut compartments and gut compartments from same individuals are indicated by numbers. The stress value of the plot was 0.15 indicating the plot provides a good representation.

16S rRNA gene clone library of the colon bacterial community

A clone library of 16S rRNA genes was constructed from the colon samples of six adult cockroaches (Figure 5). A total of 265 randomly selected clones were sequenced; 14 were putative chimerae and excluded from further analysis. The remaining clones were assigned to 132 different phylotypes (> 97% sequence similarity).

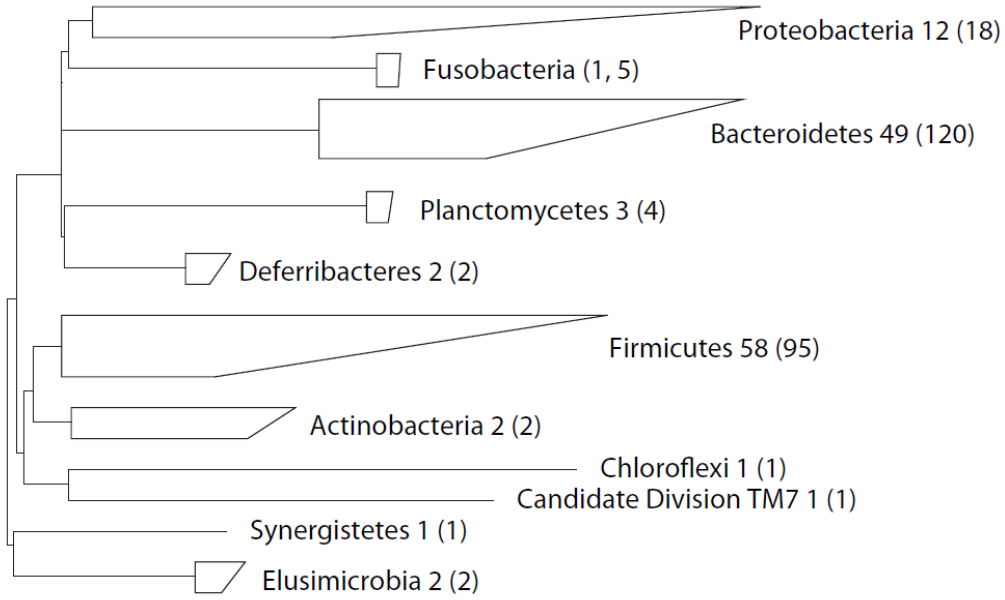

Figure 7. Maximum parsimony tree indicating the phylum level affiliation of the 251 full length 16S rRNA sequences obtained from the colon of *S. lateralis*. These sequences were assigned to 132 phylotypes (based on 97% sequence similarity criterion), belonging to 11 different bacterial phyla. Numbers give number of phylotypes and in brackets the number of clones assigned to each phylum.

Phylogenetic analysis revealed that the clones represented 11 bacterial phyla (Figure 7). Almost half of clones belonged to the *Bacteroidetes*, followed by the *Firmicutes* (mostly *Clostridia*), and diverse *Proteobacteria*. A few phylotypes clustered among the *Planctomycetes*, *Deferribacteres*, *Elusimicrobia*, *Actinobacteria*, *Fusobacteria Chloroflexi*, *Synergistetes*, and the candidate division TM7. Members of the *Spirochaetes* and *Fibrobacteres*, which are consistently found in the gut of termites, were absent from the clone library.

***Bacteroidetes*.** The 120 clones assigned to *Bacteroidetes* fell mostly within the order *Bacteroidales* (Figure 8). The most abundant phylotype was SL41 (5.2% of the library), which clustered together with SL35 (3.2%) and other phylotypes among a group consisting exclusively of termite and *Cryptocercus* clones in the Termite Group IV of *Bacteroidales* (Figure7). They represented a total of 28 clones in the library and were distantly related to bacteria in the genus *Parabacteroides* (92–94% sequence similarity to *Parabacteroides distasonis* from the mammalian gut). Another abundant group, represented by SL14 (4.0%) was loosely affiliated with clones from other intestinal sources, including *Bacteroides cellulosilyticus* (90–94% sequence similarity). Several phylotypes, with SL20 being the most abundant, formed a large cluster with clones in the Cluster V of *Bacteroidales* (4.4% of clones), which consists exclusively of clones from termites and *Cryptocercus punctulatus*, many of them representing symbionts of gut flagellates from Candidatus *Azobacteroides pseudotrichonymphae* (Hongoh *et al.,* 2008). Phylotypes SL48-51 clustered with sequences originating from the guts of higher termites within *Bacteroidales* Cluster I (Ohkuma *et al.,* 2002), distantly related (91–93% sequence identity) to bacteria in the genus *Alistipes*.

Numerous phylotypes represented minor clusters affiliated with the genus *Dysgonomonas*, *Tannerella*, and other clones or isolates from human or animal intestines, often with clones from termite guts as closest relatives. The clone library also contained 3 clones (phylotype SL65) affiliated with *Blattabacterium* sequences, obligate endosymbionts present in all cockroaches (Dasch *et al.,* 1984) with the sequence from *Blatta orientalis* as closest relative.

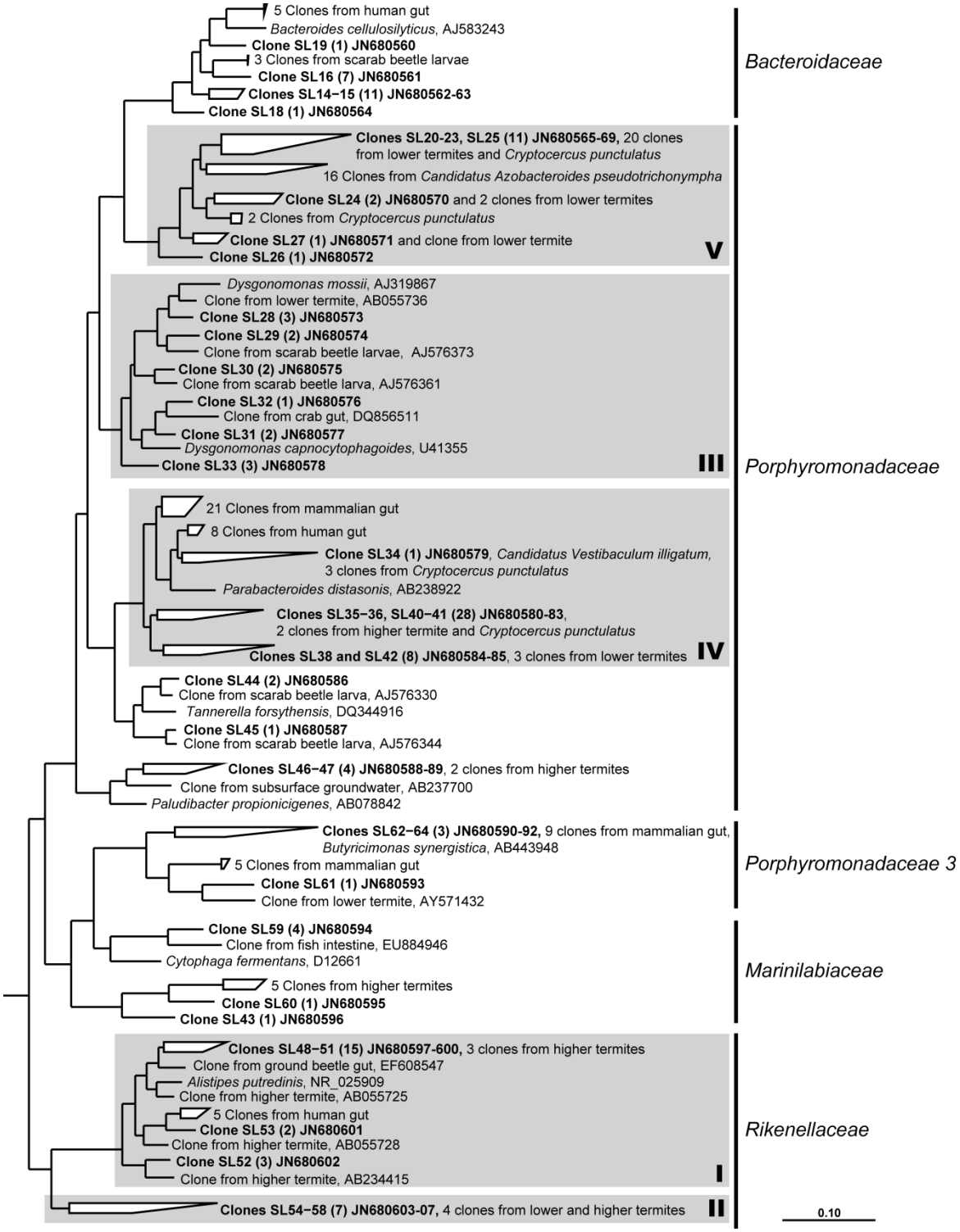

Figure 8. Phylogenetic position of 50 phylotypes obtained in this study belonging to the phylum *Bacteroidetes*. Termite Clusters identified by Ohkuma *et al*. (2002) are shaded. The tree was constructed using the maximum-likelihood and based on the analysis of 1815 valid alignment positions. The tree was rooted using sequences selected from other phyla. Phylotypes from this study are shown in bold. Numbers in brackets indicate the number of clones assigned to each phylotype or cluster. The scale bar represents a 10% estimated sequence divergence.

Clones assigned to *Firmicutes*. The majority of the 95 clones that belonged to *Firmicutes* fell into the class *Clostridia* (Figure 9). The most abundant group was represented by phylotypes SL84-90 (5.6% of the library) which clustered with uncultivated members of *Clostridiaceae* from the termite, ruminant and mammalian gut. Within the family *Lachnospiraceae*, two phylotypes, (SL82-83, 2.8%) were affiliated with *Clostridium piliforme* and clustered with sequences from the termite, millipede and scarab beetle gut. Five phylotypes (SL76-80, 2%) formed a cluster with *Clostridium hylemonae* as their closest relative and several clones from the gut of scarab beetle larvae (*Pachnoda epphipiata*). Within the family *Ruminococcaeceae*, the most abundant cluster consisted of phylotypes SL99–104 (4.4%) that were affiliated with *Clostridium leptum* (88–90% similarity) and sequences from the mammalian gut. Within the family *Veillonellaceae*, several phylotypes SL128-129 (2%) were neighbored by sequences from higher termites and a ground beetle with *Succinispira mobilis* as their closest relative. Five phylotypes (SL117-121, 2.8%) belonged to the *Enterococcaceae* with *Enterococcus asini*, *Enterococcus caselliflavus* and *Enterococcus malodoratus* as their closest cultivated relatives. Phylotypes SL122, SL124, and SL125 (2%) formed two clusters within the *Erysipelotrichi* with *Erysipelothrix inopenata* as their next cultivated relative.

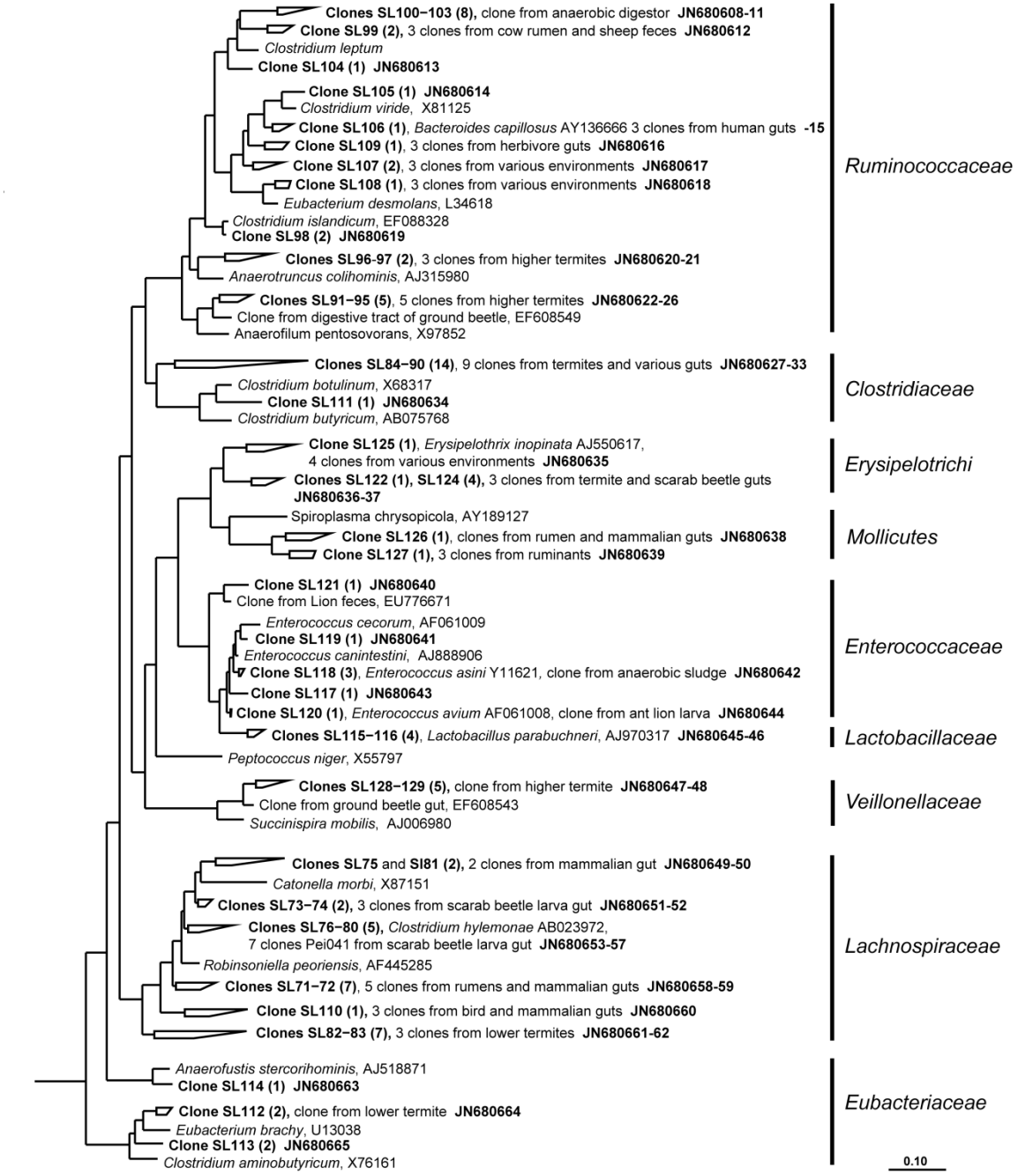

Figure 9. Phylogenetic position of 58 phylotypes obtained in this study belonging to the phylum *Firmicutes*. The tree was constructed using the maximum-likelihood and based on the analysis of 941 valid alignment positions. The tree was rooted using sequences selected from other phyla. Phylotypes from this study are shown in bold. Numbers in brackets indicate the number of clones assigned to each phylotype or cluster. The scale bar represents a 10% estimated sequence divergence.

Clones assigned to other phyla. The majority of the remaining clones (5.6% of the library) belonged to the phylum *Proteobacteria*. Most of them were *Deltaproteobacteria* of the family *Desulfovibrionaceae* and clustered with sequences previously obtained from the termite gut (Figure 10). A single clone of *Gammaproteobacteria* had 99% sequence similarity to *Escherichia blattae*, isolated from the cockroach gut.

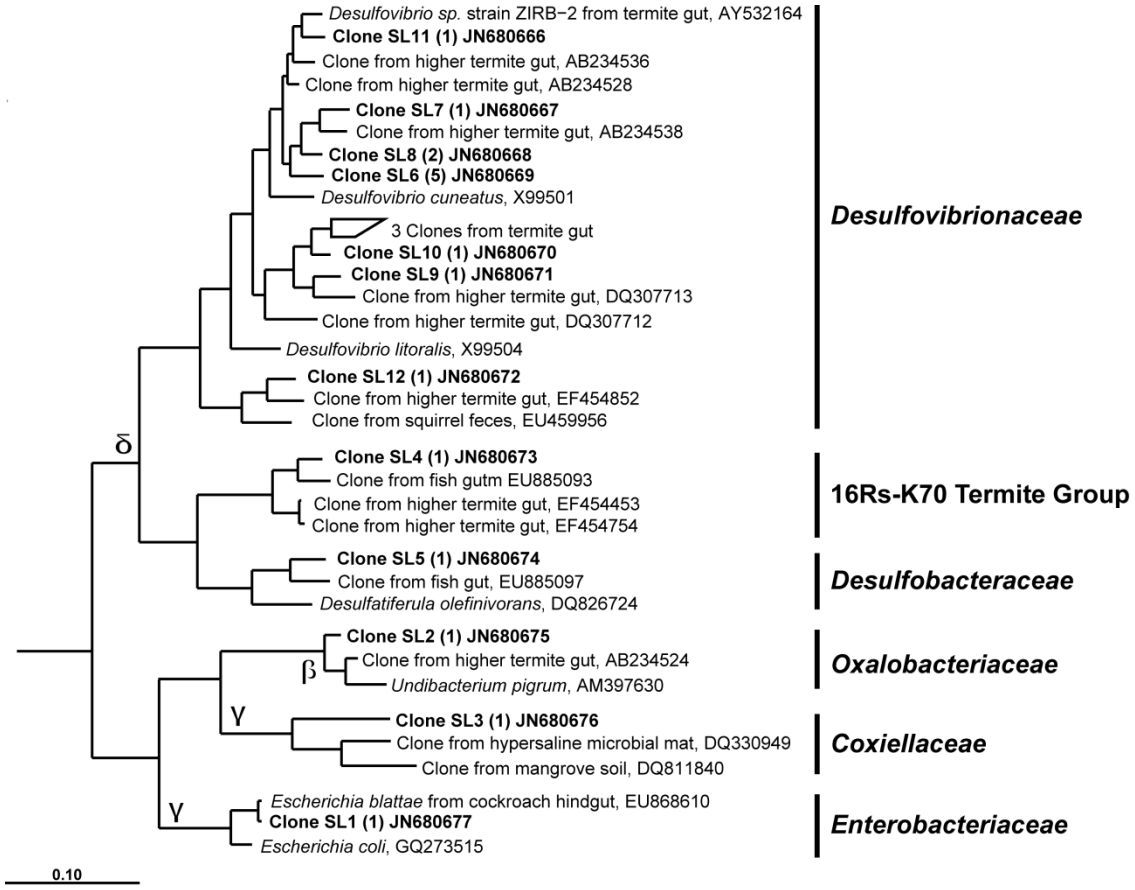

Figure 10. Phylogenetic position of 12 phylotypes obtained in this study belonging to the phylum *Proteobacteria*. The tree was constructed using the maximum-likelihood and based on the analysis of 1420 valid alignment positions. The tree was rooted using sequences selected from other phyla. Phylotypes from this study are shown in bold. Numbers in brackets indicate the number of clones assigned to each phylotype or cluster. The scale bar represents a 10% estimated sequence divergence.

Other phyla were only scarcely represented. Phylotype SL13 (2%) belonged to the phylum *Fusobacteria* and showed >99% sequence similarity with *Fusobacterium varium*. Phylotypes SL66-68 (1.6%) belonged to an uncultured cluster of *Planctomycetes* from termite guts. Two clones (SL69-70) were assigned to *Deferribacteres* with *Mucispirillum schaedleri* as next cultivated neighbor. Two clones (phylotypes SL135-136) were affiliated with the phylum *Elusimicrobia* and fell into a lineage of putatively free-living *Endomicrobia* from termites and other cockroaches (Ikeda-Ohtsubo *et al.*, 2010). Two single clones (phylotypes SL130 and 131) were assigned to *Actinobacteria*, with *Propionibacterium granulosum* or sequences from the termite gut as closest relatives. Two others (phylotypes SL133 and 134) clustered with clones from mammalian guts among the candidate division TM7 or with clones from termite guts among the *Synergistetes*.

Discussion

Molecular characterization of the gut microbiota of the cockroach, *S. lateralis* revealed that it contains a diverse community of obligate anaerobes, with many sequences clustering with those previously obtained from the termite gut. This is in contrast to previous knowledge obtained from culture-dependent studies of the cockroach gut that isolated mostly facultative anaerobic genera (Cruden & Markovetz, 1987). Our study demonstrates that these groups do not represent abundant members of the cockroach gut microbiota. Instead, the composition of the cockroach gut community was reflective of both the phylogeny of the host and of its omnivorous lifestyle.

The gut environment of S. lateralis is typical of other omnivorous cockroaches

Analysis of gut morphology and physiochemical parameters of the gut of *S. lateralis* revealed that it is typical of other closely related omnivorous cockroaches such as *P. americana* and *B. orientalis*. It has been demonstrated that characteristics of the gut such as pH are conserved between different species of the same cockroach family (Vinokurov *et al.,* 2007). Our results fit with this, as both the general structure of the gut of *S. lateralis* as well as gut pH closely resembled that of the other blattid cockroaches, *P. americana* (Bignell, 1977) and *B. orientalis* (Greenberg *et al.*, 1970; Vinokurov *et al.,* 2007).

There is considerable uncertainty regarding the redox potential within the cockroach gut with both oxidizing conditions (Appel & Martin, 1990;Vinokurov *et al.,* 2007) and reducing conditions (Warhurst, 1964) previously being reported. We observed that the center of each gut compartment was anoxic and possessed a low redox potential. These findings are consistent with other features of the cockroach gut such as the accumulation of hydrogen and the presence of a large and diverse community of obligately anaerobic bacteria, specifically clostridia.

Our analysis of the microbial community in terms of fermentation products revealed similar fermentation products as previously detected in cockroaches (Kane & Breznak, 1991). We found lactate concentration to be highest in the crop of *S. lateralis*. This is confirms previous findings in *P. americana*, where the foregut was determine to be a site of high lactate production due to the activities of lactic acid bacteria (Kane & Breznak, 1991). Fragment sizes corresponding to *Lactobacillales* (555 and 579 bp) were abundant in T-RFLP profiles of the foregut of *S. lateralis*, suggesting that the activities of lactic bacteria may also play an important role in the microbial community of the crop.

The cockroach gut contains a highly diverse and individualistic community of microbes

Phylogenetic analysis of 16S rRNA genes revealed that the cockroach gut contains a highly diverse microbial community consisting of mainly uncultivated species. Each gut compartment was found to contain its own characteristic community with T-RFLP profiles of adjacent gut compartments such as the colon and rectum, clustering closest together (Figure 6). Our results demonstrate that isolates obtained from past attempts to cultivate the cockroach gut microbiota do not represent the most abundant bacterial species. These studies uncovered a large number of facultative anaerobes, some of which were considered to represent potential pathogens, giving rise to the idea of the cockroach gut as a reservoir for human disease (Burgess *et al.,* 1973; Cruden and Markovetz, 1987). In contrast to this, our results reveal that the cockroach gut is typical of colonic microbiota found previously in termites (Shinzato *et al.,* 2007) and omnivorous mammals (Leser *et al.,* 2002; Eckburg *et al.,* 2005) with the largest proportions of clones belonging to *Bacteroidetes* and *Firmicutes*.

Within the colon, the compartment with the highest density and diversity of bacteria, we observed a significant amount of variation between individuals, both in terms of microbial composition and in the gut parameters that arise from microbial activities such as hydrogen accumulation and the production of gut metabolites (Fig 2 & 3, Table 1). Hydrogen accumulation within the gut varied considerably between individuals, with as much as a 20-fold difference in colonic hydrogen concentration. Variation in microbial composition between individuals has previously been observed for the gut communities of many omnivores including humans (Zoetendal *et al.,* 1998) and pigs (Thompson *et al.,* 2008) and is considered to arise from the random acquisition of microbes from a large and diverse reservoir such as the environment (Curtis & Sloan, 2004).

The cockroach gut microbiota reflects the close relationship between cockroaches and termites

Host phylogeny along with gut morphology and diet are considered to be important factors that shape the structure and diversity of the gut communities of animals (Ley *et al.,* 2008). Termites and cockroaches are known to share a common evolutionary origin with termites having descended from an omnivorous cockroach ancestor (Inward *et al.,* 2007). Evidence of this shared evolutionary history has been previously observed in the gut microbiota of the wood feeding cockroach, *Cryptocercus punctulatus* (Berlanga *et al.,* 2009). This is unsurprising given that its lifestyle is more characteristic of a termite rather than being representative of the other members of the cockroach family.

Phylogenetic analysis of the gut microbiota of *S. lateralis*, a typical omnivorous cockroach, revealed that, despite different dietary habits, the gut microbiota of the omnivorous cockroach also reflects the close phylogenetic relationship between cockroaches and termites. In particular, the cockroach gut microbiota was similar to termites in terms of the types of bacterial species present rather than their overall abundance. About 30% of the clones from the library fell into clusters of sequences previously obtained from the termite gut, and most of the remaining clones clustered with sequences from other intestinal environments. These cockroach-termite clusters were distributed between multiple phyla. 27% and 24% of clones from *Firmicutes* and *Bacteroidetes*, respectively, were affiliated with termite clusters, despite that these phyla together represented more than two thirds of the clones in the library, Instead, the majority of clones from these phyla (about 65%) consistently clustered with sequences from other gut environments. Conversely, the majority of clones belonging to *Proteobacteria* belonged to termite clusters (67%), with all of these clusters occurring within *Desulfovibrio Cluster III* of *Deltaproteobacteria* (Figure 10). *Elusimicrobia, Synergistetes* and *Actinobacteria* all contained a single termite cluster while *Fusobacteria, Deferribacteres, Planctomycetes* and candidate division TM7 had no clusters.

Cockroach sequences clustered equally with both higher and lower termites. Some of these sequences represent bacterial lineages previously known to be termite-specific and our results show that their closest relatives are also present in the cockroach gut. Within the *Bacteroidetes*, all of the five previously identified Termite Clusters (Ohkuma *et al*., 2002) contained clones from *S. lateralis* (Figure 8). In particular, a number of clones from the cockroach gut were found to be closely related to sequences from bacterial symbionts of the cellulolytic termite gut flagellates. These symbionts are members of the phyla *Bacteroidetes and Elusimicrobia* and specifically colonize the surface and interior of the gut flagellates, respectively (Noda *et al.,* 2006; Ikeda-Ohtsubo *et al.,* 2007). The sequences detected in the cockroach are likely to be free-living relatives as, with the exception of *Cryptocercus punctulatus,* gut flagellates are not present in cockroaches including *S. lateralis*. Within *Elusimicrobia*, the occurrence of free-living forms within the guts of termites was previously proposed (Ikeda-Ohtsubo *et al.,* 2010). Our results agree with this as the sequences from the cockroach gut cluster with sequences considered to be free living such as those from defaunated, flagellate-free lower termites and flagellate-free higher termites.

The bacterial gut composition of the cockroach reflects its omnivorous lifestyle

In addition to being reflective of host phylogeny, the composition of the gut microbiota is also influenced by the omnivorous diet of the cockroach. Noticeably, members of phyla *Spirochaetes* and *Fibrobacteres*, considered to be involved in cellulose degradation in termites, were absent from the clone library. Spirochetes form an abundant group within the termite gut microbiota and in some cases constitutes up to half of all bacteria within the termite gut (Paster *et al.,* 1991; Hongoh *et al.,* 2005). Spirochetes have been previously observed in the guts of *C. punctulatus* (Breznak, 1984; Berlanga *et al.,* 2009) and the blaberid cockroach, *Eublaberus posticus* (Cruden & Markovetz, 1981). Members of the phylum *Fibrobacteres* are also assumed to be associated with cellulolytic activity and have been shown to be abundant in higher termites (Hongoh *et al.,* 2006; Warnecke *et al.,* 2007). While, the absence of both *Spirochaetes* and *Fibrobacteres* from the gut microbiota of *S.*

lateralis is likely reflective of its omnivorous lifestyle, a more expansive sequencing approach is required to determine whether they are rare or completely absent from the cockroach gut.

References

1. **Appel, H. M., and M. M. Martin.** 1990. Gut redox conditions in herbivorous lepidopteran larvae. J. Chem. Ecol. 16:3277–3290.
2. **Berlanga, M., B. J. Paster, and R. Guerrero.** 2009. The taxophysiological paradox: changes in the intestinal microbiota of the xylophagous cockroach *Cryptocercus punctulatus* depending on the physiological state of the host. Int. Microbiol. 12:227–236.
3. **Bignell, D. E.** 1977. Some observations on the distribution of gut flora in the American cockroach, *Periplaneta americana*. J. Invertebr. Pathol. 29:338–343.
4. **Bracke, J. W., D. L. Cruden, and A. J. Markovetz.** 1979. Intestinal microbial flora of the American cockroach. Appl. Environ. Microbiol. 38:945–955.
5. **Breznak, J. A.** 1984. Hindgut spirochetes of termites and *Cryptocercus puntulatus*, p. 67–70. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. Williams & Wilkins, Baltimore, MD.
6. **Brune, A.** 2006. Symbiotic associations between termites and prokaryotes, pp. 439–474. *In* M. Dworkin, ,S. Falkow, ,E. Rosenberg, K-H. Schleifer and E. Stackebrandt (eds.) (ed.), The Prokaryotes, 3rd ed., Volume 1: Symbiotic associations, Biotechnology, Applied Microbiology, vol. 1. Springer, New York.
7. **Brune, A., D. Emerson, and J. A. Breznak.** 1995. The termite gut microflora as an oxygen sink: microelectrode determination of oxygen and pH gradients in guts of lower and higher termites. Appl. Environ. Microbiol. 61:2681–2687.
8. **Burgess, N. R. H., S. N. McDermott, and J. Whiting.** 1973. Aerobic bacteria occurring in the hind-gut of the cockroach, *Blatta orientalis*. J. Hyg. 71:1–7.

9. **Cruden, D. L., and A. J. Markovetz.** 1981**.** Relative numbers of selected bacterial forms in different regions of the cockroach hindgut. Arch. Microbiol. 129:129–134.
10. **Cruden, D. L., and A. J. Markovetz.** 1987. Microbial ecology of the cockroach gut. Annu. Rev. Microbiol. 41:617–643.
11. **Curtis, T. P., and W. T. Sloan.** 2004. Prokaryotic diversity and its limits: microbial community structure in nature and implications for microbial ecology. Curr. Opin. Microbiol. 7: 221–226.
12. **Dasch, G. A., E. Weiss, and K. P. Chang.** 1984. Endosymbionts of insects, p. 811–833. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1, Williams and Wilkins, Baltimore, MD.
13. **Dixon, P.** 2003. VEGAN, a package of R functions for community ecology. J. Veg. Sci. 14:927–930.
14. **Eckburg, P. B., E. M. Bik, C. N. Bernstein, E. Purdom, L. Dethlefsen, M. Sargent, S. R. Gill, K. E. Nelson, and D. A. Relman.** 2005. Diversity of the human intestinal microbial flora. Science. 308**:**1635–1638.
15. **Egert, M., B. Wagner, T. Lemke, A. Brune, and M. W. Friedrich.** 2003. Microbial community structure in midgut and hindgut of the humus-feeding larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae). Appl. Environ. Microbiol. 69:6659–6668.
16. **Greenberg, B., J. Kowalski, and J. Karpus.** 1970. Micropotentiometric pH determinations of the gut of *Periplaneta americana* fed three different diets. J. Econ. Entomol. 63:1795–1797.
17. **Hongoh, Y., M. Ohkuma, and T. Kudo.** 2003. Molecular analysis of bacterial microbiota in the gut of the termite *Reticulitermes speratus* (Isoptera; Rhinotermitidae). FEMS Microbiol. Ecol. 44:231–242.
18. **Hongoh, Y., P. Deevong, S. Hattori, T. Inoue, S. Noda, N. Noparatnaraporn, T. Kudo, and M. Ohkuma.** 2006. Phylogenetic diversity, localization and cell morphologies of the candidate phylum TG3 and a subphylum in the phylum *Fibrobacteres*, recently found bacterial groups dominant in termite guts. Appl. Environ. Microbiol. 72:6780–6788.
19. **Hongoh, Y., P. Deevong, T. Inoue, S. Moriya, S. Trakulnaleamsai, M. Ohkuma, C. Vongkaluang, N. Noparatnaraporn, and T. Kudo.** 2005.

Intra- and interspecific comparisons of bacterial diversity and community structure support coevolution of gut microbiota and termite host. Appl. Environ. Microbiol. 71:6590–6599.

20. **Hongoh, Y., V. K. Sharma, T. Prakash, S. Noda, H. Toh, T. D. Taylor, T. Kudo, Y. Sakaki, A. Toyoda, M. Hattori, and M. Ohkuma.** 2008. Genome of an endosymbiont coupling N2 fixation to cellulolysis within protist cells in termite gut. Science. 322:1108–1109.
21. **Horn, H. S.** 1966. Measurement of "Overlap" in comparative ecological studies. Am. Nat. 100:419–424.
22. **Huber, T., G. Faulkner, and P. Hugenholtz.** 2004. Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. Bioinformatics. 20:2317–2319.
23. **Ikeda-Ohtsubo, W., M. Desai, U. Stingl, and A. Brune.** 2007. Phylogenetic diversity of "Endomicrobia" and their specific affiliation with termite gut flagellates. Microbiology. 153:3458–3465.
24. **Ikeda-Ohtsubo, W., N. Faivre, and A. Brune.** 2010. Putatively free-living '*Endomicrobia*'– ancestors of the intracellular symbionts of termite gut flagellates? Environ. Microbiol. Rep. 2:554–559.
25. **Inward, D., G. Beccaloni, and P. Eggleton.** 2007. Death of an order: a comprehensive molecular phylogenetic study confirms that termites are eusocial cockroaches. Biol. Lett. 3:331–335.
26. **Kane, M. D., and J. A. Breznak.** 1991. Effect of host diet on production of organic acids and methane by cockroach gut bacteria. Appl. Environ. Microbiol. 57:2628–2634.
27. **Klass, K-D., C. Nalepa, and N. Lo.** 2008. Wood-feeding cockroaches as models for termite evolution (Insecta: Dictyoptera): *Cryptocercus* vs. *Parasphaeria boleiriana.* Mol. Phylogenet. Evol. 46:809–817.
28. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115–175. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley and Sons Ltd., New York, N.Y.
29. **Leser, T. D., J. Z. Amenuvor, T. K. Jensen, R. H. Lindecrona, M. Boye, and K. Møller.** 2002. Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. Appl. Environ. Microbiol. 68:673–690.

30. **Ley, R. E., M. Hamady, C. Lozupone, P. J. Turnbaugh, R. R. Ramey, J. S. Bircher, M. L. Schlegel, T. A. Tucker, M. D. Schrenzel, R. Knight, and J. I. Gordon.** 2008. Evolution of mammals and their gut microbes. Science. 320:1647–1651.
31. **Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Förster, I. Brettske, S. Gerber, A. W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. König, T. Liss, R. Lüssmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, and K-H. Schleifer.** 2004. ARB: a software environment for sequence data. Nucleic Acids Res.32:1363–1371.
32. **Muyzer, G. A. Tesje, C. O. Wirsen, and H. W Jannasch.** 1995. Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. Arch. Microbiol. 164**:**165–172.
33. **Noda, S., T. Inoue, Y. Hongoh, M. Kawai, C. A. Nalepa, C. Vongkaluang, T. Kudo, and M. Ohkuma.** 2006. Identification and characterization of ectosymbionts of distinct lineages in *Bacteroidales* attached to flagellated protists in the gut of termites and a wood-feeding cockroach. Environ. Microbiol. 8:11–20.
34. **Ohkuma, M., S. Noda, Y. Hongoh, and T. Kudo.** 2002. Diverse bacteria related to the *Bacteroides* subgroup of the CFB phylum within the gut symbiotic communities of various termites. Biosci. Biotechnol. Biochem. 66:78–84.
35. **Ohkuma, M., S. Noda, Y. Hongoh, C. A. Nalepa, and T. Inoue.** 2008. Inheritance and diversification of symbiotic trichonymphid flagellates from a common ancestor of termites and the cockroach *Cryptocercus*. Proc. R. Soc. B 276:239–245.
36. **Paster, B. J, F. E. Dewhirst, W. G. Weisburg, L. A. Tordoff, G. J. Fraser, R. B. Hespell, T. B. Stanton, L. Zablen, L. Mandelco, and C. R. Woese.** 1991. Phylogenetic analysis of the spirochetes. J. Bacteriol. 173:6101-6109.
37. **Pielou, E. C.** 1966. The measurement of diversity in different types of biological collections. J. Theor. Biol. 13:131–144.

38. **Pruesse, E., C. Quast, K. Knittel, B. Fuchs, W. Ludwig, J. Peplies, and F. O. Glöckner.** 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res. 35:7188–7196.
39. **Schmitt-Wagner, D., M. W. Friedrich, B. Wagner, and A. Brune.** 2003. Axial dynamics, stability, and interspecies similarity of bacterial community structure in the highly compartmentalized gut of soil-feeding termites (*Cubitermes* spp.). Appl. Environ. Microbiol. 69:6018–6024.
40. **Shannon, C. E., and W. Weaver.** 1963. The mathematical theory of communication. University of Illinois Press, Urbana, Ill.
41. **Shinzato, N., M. Muramatsu, T. Matsui, and Y. Watanabe.** 2007. Phylogenetic analysis of the gut bacterial microflora of the fungus-growing termite *Odontotermes formosanus*. Biosci. Biotechnol. Biochem. 71:906–915.
42. **Strassert, J. F., M. S. Desai, R. Radek, and A. Brune.** 2010. Identification and localization of the multiple bacterial symbionts of the termite gut flagellate *Joenia annectens*. Microbiology. 156:2068–2079
43. **Thayer, D.W.** 1976. Facultative wood-digesting bacteria from the hind-gut of the termite *Reticulitermes hesperus*. J. Gen. Microbiol. 95:287–296.
44. **Thompson, C. L., B. Wang, and A. J. Holmes.** 2008. The immediate environment during postnatal development has long-term impact on gut community structure in pigs. ISME J. 2: 739–748.
45. **Vinokurov, K., Y. Taranushenko, N. Krishnan, and F. Sehnal.** 2007. Proteinase, amylase, and proteinase-inhibitor activities in the gut of six cockroach species. J. Insect Physiol. 53:794–802.
46. **Warhurst, D.C.** 1964. Growth and survival, *in vitro* and *in vivo* of *Endolimax blattae*, an entozoic amoeba of cockroaches. Ph.D Thesis. University of Leicester, U.K.
47. **Warnecke, F., P. Luginbühl, N. Ivanova, M. Ghassemian, T. H. Richardson, J. T. Stege, M. Cayouette, A. C. McHardy, G. Djordjevic, N. Aboushadi, R. Sorek, S. G. Tringe, M. Podar, H. G. Martin, V. Kunin, D. Dalevi, J. Madejska, E. Kirton, D. Platt, E. Szeto, A. Salamov, K. Barry, N. Mikhailova, N. C. Kyrpides, E. G. Matson, E. A. Ottesen, X. Zhang, M. Hernández, C. Murillo, L. G. Acosta, I. Rigoutsos, G. Tamayo, B. D.**

Green, C. Chang, E. M. Rubin, E. J. Mathur, D. E. Robertson, P. Hugenholtz, and J. R. Leadbetter 2007**.** Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. Nature. 450:560–565.

48. **Zeidler, R. B., P. Lee, and H .D. Kim.** 1976. Kinetics of 3-O-methyl glucose transport in red blood cells of newborn pigs. J. Gen. Physiol. 67:67–80.
49. **Zoetendal, E. G., A. D. L. Akkermans, and W. M. De Vos.** 1998. Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. Appl. Environ. Microbiol. 64: 3854–3859.

3 The response of gut microbiota of the cockroach *Shelfordella lateralis* to changes in diet

Christine Schauer, Claire Thompson, Andreas Brune*

In preparation for submission

Abstract

Despite their close evolutionary relationship, the dietary requirements of termites and cockroaches are distinctly different. While termites consume a highly specialized diet of lignocellulose, cockroaches are omnivorous and opportunistic feeders. Our previous analysis of the bacterial community in the colon of the cockroach *Shelfordella lateralis* revealed a diverse gut microbial community that reflected the close phylogenetic relationship between cockroaches and termites. However, a number of differences were apparent, including a lack of representatives from the phyla *Spirochaetes* and *Fibrobacteres,* which may be attributed to differences in diet between cockroaches and termites. Here, we examined the effects of different diets on the colonic gut microbiota of *S. lateralis.* Cockroaches were fed one of four different diets: chicken feed (balanced), soy (protein-rich), bran, and bran-cellulose (fiber-rich). Although colon weight was significantly greater in cockroaches that were fed a high fiber diet, there were no significant effects of diet on volatile fatty acid concentrations or bacterial cell density within the gut. Analysis of bacterial community structure by terminal-restriction-fragment length polymorphism and 454 pyrotagsequencing of 16S rRNA genes revealed a high individual variability but little impact of diet. This suggests that cockroaches are able to maintain a gut microbiota that is insensitive to dietary shifts.

Authors' contribution: All analysis was performed and planed by C. Schauer. C. L. Thompson and A. Brune were involved in preparation of the manuscript.

Introduction

Termites are able to survive on a highly specialized diet of lignocellulose due to the metabolic activities of their gut microbial communities. Although cockroaches are the closest phylogenetic neighbors to termites (Inward *et al.,* 2007), they differ significantly in their dietary requirements with many having an omnivorous lifestyle and surviving on a wide variety of substances that they scavenge from their environment. Our previous analysis of the bacterial community in the colon of the cockroach *Shelfordella lateralis* revealed that its composition reflected the close phylogenetic relationship between cockroaches and termites with many sequences clustering with those previously obtained from the termite gut (Schauer *et al.,* 2011). However, a number of differences were apparent, including an absence of members from the phyla *Spirochaetes* and *Fibrobacteres*. These phyla are associated with cellulolytic activity in termites and have been shown to be abundant in termite gut. The lack of bacterial representatives from these phyla may be attributed to differences in diet between cockroaches and termites. While much attention has been given to the role of the gut microbiota in the nutrition of termites (Breznak & Brune, 1994; Brune & Ohkuma, 2011), little is understood regarding the cockroach gut microbiota and the functional importance of its different microbial groups.

Previous studies that have examined the impact of dietary shifts on the cockroach gut microbiota have mostly examined microbial activities rather than direct changes in species composition. Studies of the gut microbiota of *Periplaneta americana*, a close relative of *S. lateralis*, have shown that a high-fiber diet increases methane production and volatile fatty acid concentrations (Zurek & Keddie 1998, Kane & Breznak, 1991) whereas reduction of the bacterial community using a broad spectrum antibiotic treatment has been shown to lower the concentration of volatile fatty acids as well as reduce the weight of the animal and impede its development (Zurek & Keddie 1998). These studies suggest the gut microbiota of the cockroach plays an important role in the nutrition of its host. However, the effects of diet in terms of changes to community composition and structure remain unknown.

Here we investigated the response of the gut microbiota of *S. lateralis* to high-protein and high-fiber diets. Changes to the bacterial community were assessed through terminal-restriction fragment length polymorphism (T-RFLP) analysis and pyrotagsequencing of 16S rRNA genes in addition to measurements of gut metabolites and gut morphology, We focused on changes in composition within the colonic community as this gut compartment contains the highest diversity and density of bacteria (Schauer *et al.*, 2011) and is considered to be an important site for the degradation of cellulose and hemicellulose (Bignell, 1977).

Material and Methods

Cockroaches and sample collection.

Cockroaches (*Shelfordella lateralis*) were obtained from a commercial breeder (J. Bernhard, Helbigsdorf, Germany). Cockroaches were maintained in a temperature controlled chamber at 25°C with 50% humidity. Cockroaches were fed one of four diets: a balanced diet of chicken feed (CF) (Gold Plus, Versele-Laga, Deinze, Belgium; 3% fiber, 16% protein), a high protein diet of soy meal (S) (Kornhaus, Cölbe, B. Ruppersberg KG, Germany; 7% fiber, 43% protein) or a fiber-rich diet of either wheat bran (B), (Spielberger-GmbH, Brackenheim Germany; 36% fiber, 15% protein) or wheat bran supplemented with 30% Cellulose powder (BC) (55% fiber, 11% protein, (Sigma-Aldrich, Steinheim). Food and water were provided *ad libitum*. For each diet, two separate feeding groups were established and maintained on each diet for 3 months. After three months, the gut was extracted from adult cockroaches, the gut compartments were weighed individually and colons were frozen at -20°C for further use. Significant differences between gut weights were determined by the Kruskal-Wallis nonparametric analysis of variance (ANOVA) in R (version 2.10, R development core team 2008).

Gut metabolites within the colon of cockroaches of different diet groups.

Gut metabolites were quantified by high pressure liquid chromatography (HPLC) using a Grom Resin IEX column (8 µm, 250 x 4.6 mm i.d., Grom, Rottenburg, Germany), USA) with a refractive index detector (RID-10A, Shimatzu, Duisburg, Germany) as previously described (Schauer *et al.*, 2011). Colons were weighed, homogenized in 200µl water and centrifuged for 10 minutes at 14000 rpm. Prior to analysis, supernatants were acidified with 1 volume 100mM H_2SO_4 and filtered (0.2 µm, ReZist, Whatman GmbH, Dassel, Germany). Peak identity was verified using external standards. Significant differences in concentrations of gut metabolites were determined by the Kruskal-Wallis nonparametric analysis of variance (ANOVA) in R (version 2.10, R development core team 2008).

Bacterial abundance.

Bacterial cell density was measured as previously described (Schmitt-Wagner *et al.*, 2003; Schauer *et al.,* 2011). Briefly, gut contents from the colon were diluted 1:100 in phosphate buffered saline (pH 7.2), stained with 4',6-diamidino-2-phenylindole (DAPI) and applied to 0.2 µm filters (Milipore, Billerica, USA) using a vacuum pump. For quantification, each filter was divided into quarters and five fields section per quarter were counted using a fluorescence microscope (Axiophot, Zeiss, Jena, Germany).

T-RFLP analysis.

DNA was extracted from colons of cockroaches by phenol/ chloroform extraction and ethanol precipitation as previously described (Schauer *et al.*, 2011). T-RFLP profiles of 16S rRNA genes from the colon were generated following the protocol of Egert *et al*. (2003) with minor modification as described in Schauer *et al.* (2011). Briefly; 16S rRNA genes were amplified using the primers 27f and 907r (Lane, 1991; Muyzer *et al.,* 1995) and digested with *Msp*I. Profiles were generated on an ABI 3130 (Applied Biosystems, Carlsbad, CA, USA) with an internal standard (MapMarker 1000,

BioVentures Inc., Murfreesboro, TN, USA). Terminal restriction fragment sizes between 50-600 bp with peak heights of ≥ 25 fluorescence units were used and all samples were run in triplicate. The percentage peak area was calculated for each T-RF and T-RFs under 1% rejected. Bacterial phylotype richness was expressed as the total number of peaks within each profile. Similarities between T-RFLP profiles were calculated using the Morisita-Horn index (Horn, 1966). Nonmetric multidimensional scaling (NMDS) analysis was performed using R (version 2.10, R development core team 2008) and the VEGAN software package (Dixon, 2003).

Pyrotagsequencing of 16S rRNA genes.

Colon DNA was pooled from 5 individuals for each diet and from 10 individuals of the termites *Nasutitermes corniger*, *Reticulitermes santonensis* and *Zootermopsis nevadensis*. Pyrosequencing was done as previously described (Köhler T., C. Dietrich, R. H. Scheffrahn, and A. Brune 2011, in prep.). Briefly, 16S rRNA genes were amplified using the primers $343F_{mod}$ (TACGGGWGGCWGCA) and $748R_{mod}$ (GGGTMTCTAATCCBKTT). PCR products were pooled and 454 pyrosequencing was performed at GATC Biotech (Konstanz, Germany). Sequences were classified as described previously (Köhler *et al.*, 2011 in prep.). Heat maps were constructed using the *heatmap.2* function implemented in the R package "gplot" (version 2.7.4, Warners et al. 2009) in R (version 2.10.0, R development core team 2008).

Results

Gut weight

No significant differences were observed in whole body weight between diet groups. Whole body weight was 597 ± 117 mg for chicken feed-fed cockroaches (n = 30), 555 ± 176 mg for soy-fed cockroaches (n = 9), 556 ± 126 mg for bran-fed cockroaches (n = 20) and 591 ± 129 mg for bran-cellulose-fed cockroaches (n = 20). Significant differences in gut weight were observed between cockroaches fed different diets

(Figure 1). Cockroaches fed a fiber-rich diet of either bran or bran-cellulose, had a significantly increased colon weight in comparison to those fed either a high-protein or a balanced diet ($P < 0.001$). Crop weight was only increased in individuals fed bran-cellulose ($P < 0.001$).

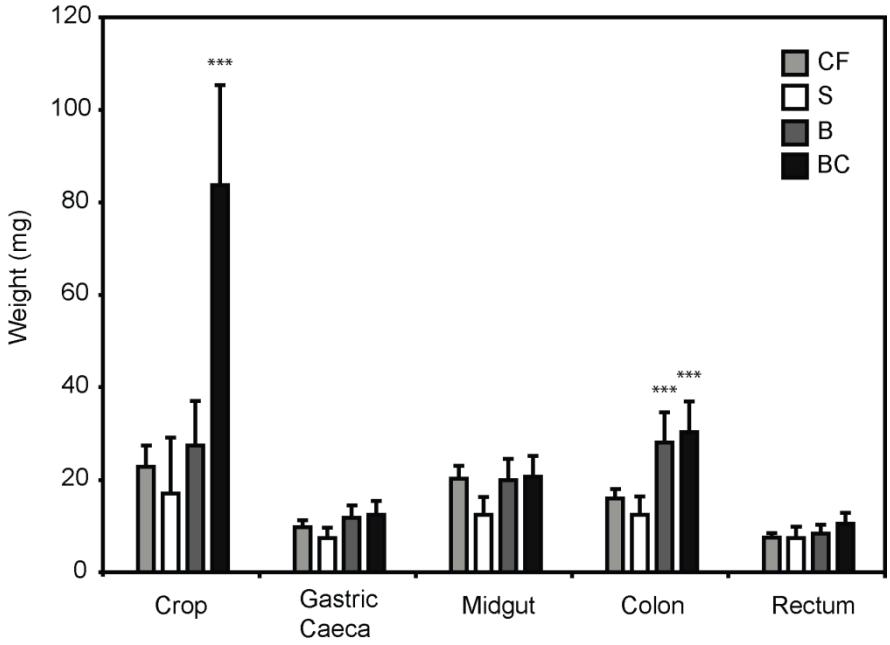

Figure 1. Weight of gut compartments in Shelfordella lateralis fed different diets. Fresh weight (mg) of the crop, gastric caeca, midgut, colon and rectum are given with standard error of the mean. n = 30 for chicken feed (CF), n = 9 for soy (S), n = 20 for bran (B) and n = 20 for bran-cellulose (BC). Significance was determined using a Kruskal-Wallis nonparametric ANOVA test. ***: $P < 0.001$

Gut metabolites

Fermentation products were assessed within the colons of animals from each diet group (Figure 2). No significant difference in gut metabolites was observed for the diet groups with similar fermentation products detected for all diets. HPLC analysis indicated that acetate was the major product for all diet groups; however it was highest in soy-fed individuals. Glucose, lactate and propionate were also detected in all diet groups. While the concentration of lactate was similar for each diet, propionate appeared to be highest for those animals fed a balanced diet of chicken feed. Low concentrations of succinate, malate and butyrate were detected in a few individuals fed chicken feed, soy and bran-cellulose.

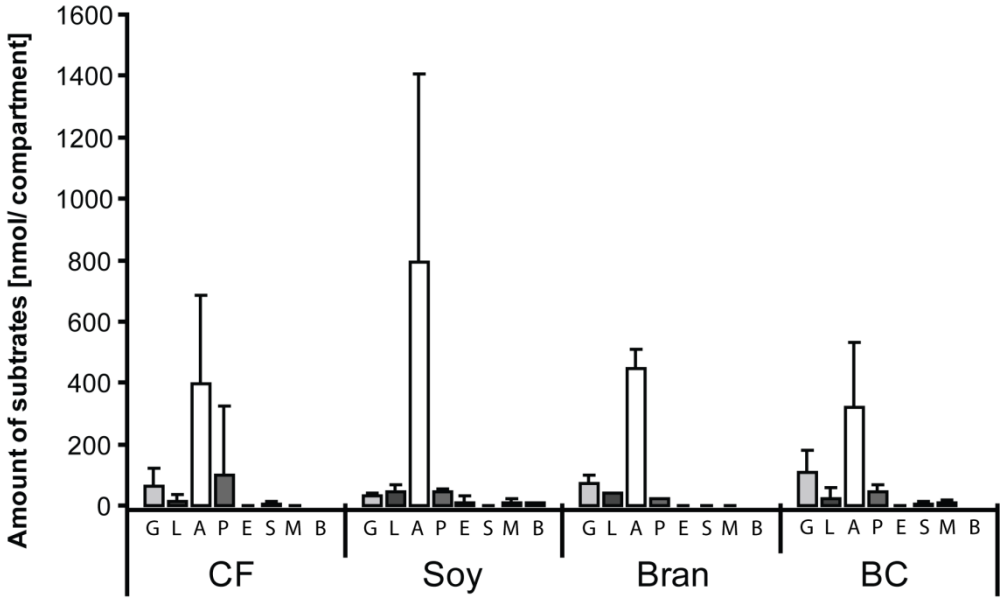

Figure 2. Differences in content of gut metabolites in gut fluid of adult *S. lateralis* fed different diets. Glucose (G), lactate (L), acetate (A) propionate, (P), ethanol (E) and succinate (S) were detected. Deviations are given as standard error of the mean. Significance was determined using a Kruskal-Wallis nonparametric ANOVA test.

Microbial community structure

DAPI counts of bacterial cell density within the colons of cockroaches fed different diets indicated that density was highest in individuals fed a high-protein diet of soy (Table 1), followed by those fed a high-fiber diet of bran-cellulose. Density was lowest for cockroaches fed a balanced diet of chicken feed.

Table 1: Bacterial density and diversity within the colon of Shelfordella lateralis fed different diets

	Chicken feed	Soy	Bran	Bran-cellulose
Average T-RFs[1]	24 ± 7	24 ± 5	26 ± 4	22 ± 5
Shared TRFs[2]	6	4	10	4
Morisita Horn index	0.58 ± 0.2	0.46 ± 0.29	0.49 ± 0.2	0.38 ± 0.19

All values are given as the mean with standard deviation

[1] Total number of distinct T-RFs in profiles (n = 4)

[2] Number of T-RFs that occurring in all profiles from the same gut compartment

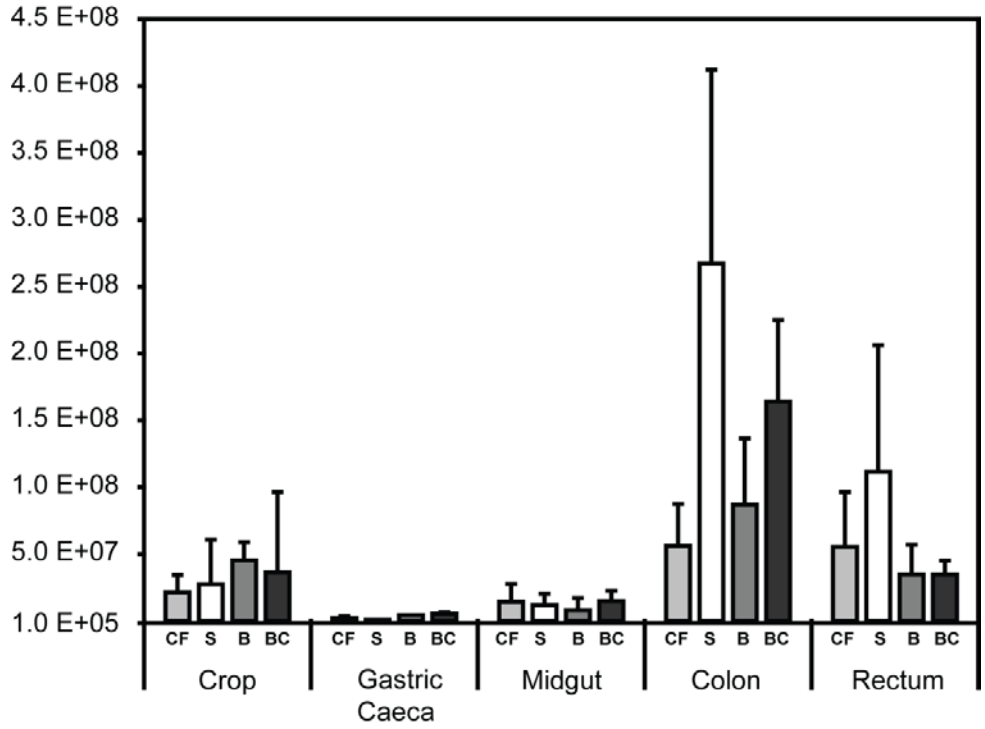

Figure 2. Number of DAPI stained cells within different gut compartments of adult *S. lateralis* fed different diets. Chicken feed (CF), soy (S), bran (B) and bran-cellulose (BC) were detected. n = 3 for all samples. Deviations are given as standard error of the mean.

The colonic communities of cockroaches from the four diet groups were assessed by T-RFLP analysis. A total of 126 distinct T-RFs were identified across all profiles. Community diversity was not significantly different between the diets as the average number of T-RFs was similar in the profiles from all diet groups (Table 1). Community similarity, as calculated by the Morisita Horn index, was low between individuals fed the same diet. This individual variation was also evidenced by the low proportion of T-RFs shared amongst individuals of the same diet. Despite the high variation between individuals, three T-RFs (83–84, 87–89, 92bp) were present in all profiles regardless of diet. *In silico* digest of 16S rRNA sequences previously obtained from the colon of *S. lateralis* indicated that these T-RFs likely correspond to members of the phylum *Bacteroidetes*.

NMDS analysis of T-RFLP profiles showed only marginal separation of profiles based on diet (Figure 3A). However, when feeding groups (two per diet) were accounted for then profiles from the same feeding group cluster more closely than those from the same diet but different feeding group (Figure 3B).

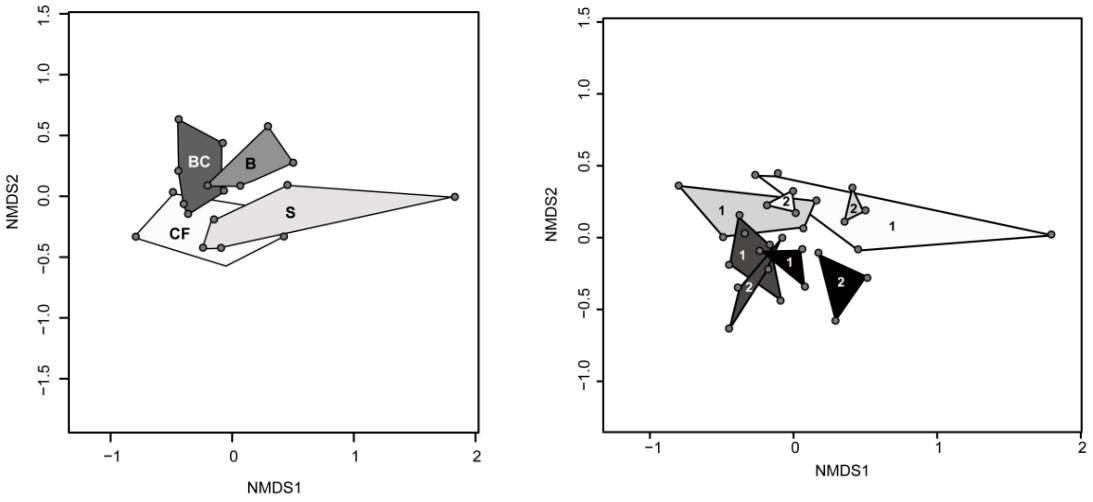

Figure 3. Nonmetric multidimensional scaling (NMDS) plot using Bray-Curtis similarities showing clustering of colon T-RFLP profiles according to diet (A) and diet and feeding group (B). For each diet, two separate feeding groups were established. The stress value of the plot was 0.2 indicating the plot provides a good representation.

Microbial composition using pyrotagsequencing

Over 42000 sequences were obtained for all different diets with pyrotagsequencing. About 2000 were from the sample of chicken feed fed individuals, 20000 of soy fed individuals, 12000 of bran fed individuals and 7500 of bran-cellulose fed individuals. Comparisons at the phylum level between cockroaches fed different diets showed that the diet did not to have a big influence on bacterial distribution and diversity. Similar numbers of phyla were present for each feeding group with 20 different phyla for bran-cellulose, 19 for soy 18 for chicken feed and bran. The most abundant phyla were similar between all diet groups (Figure 4). The majority of sequences fell within the *Firmicutes* (36–52% of sequences), followed by *Bacteroidetes* (12–25%), *Proteobacteria* (7–18%), *Fusobacteria* (2-15%) and *Planctomycetes* (2–5%). Candidate division TM7 which was increased in bran and bran-cellulose fed animals (2-3%) compared to those fed soy or chicken feed (0.3–0.4%). Members of *Synergistetes* were lower in abundance in bran-cellulose fed individuals (0.4%), compared to the other diets (1–6%).

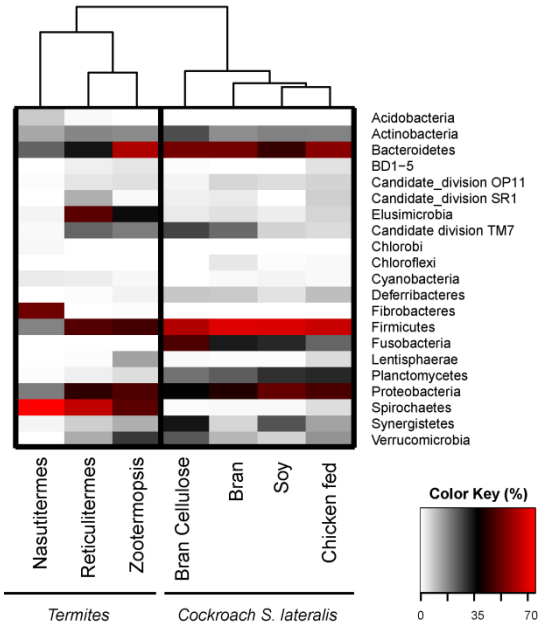

Figure 4. Comparison of 454 pyrosequencing data from guts of termites and the cockroach *S. lateralis* fed different diets. Heat map showing the relative abundance of bacteria at phylum-level within the gut of cockroaches fed different diets in comparison to the lower termite *Nasutitermes corniger* and higher termites *Reticulitermes santonensis* and *Zootermopsis nevadensis*.

In comparison to sequences obtained from termites, members of the phylum *Spirochaetes* were low in abundance for all cockroaches (0.03–0.3%) (Figure 4). No increase in abundance was observed for cockroaches fed a diet of bran-cellulose (0.05%) compared to cockroaches fed chicken feed (0.3%). Members of *Fibrobacteres* were absent in chicken feed-fed individuals and were extremely low in abundance in the other diet groups (0.008–0.01%). In comparison to a previous Sanger sequenced 16S rRNA clone library (Schauer *et al.*, 2011), the 454 sequencing approach detected nine new phyla belonging to the cockroach colon, namely *Acidobacteria*, BD1-5, *Candidate division* SR1, *Candidate division* OP11, *Cyanobacteria*, *Fibrobacteres*, *Lentisphaerae*, *Spirochaetes* and *Verrucomicrobia*. All phyla were rare (less than 1%) with exception of *Verrucomicrobia* (0.46–3%).

Discussion

Cockroaches and termites share a close evolutionary relationship with termites having evolved from an omnivorous cockroach ancestor. Despite their close evolutionary relationship, the dietary requirements of these two groups differ considerably.

Termites are able sustain themselves on a highly specialized diet of lignocellulose, due to the metabolic activities of their gut microbiota. Conversely, many cockroaches are omnivorous and subsist on a variable diet. Our previous study revealed that the cockroach possesses a highly diverse gut microbial community whose composition reflects the host's close relationship to termites. Here, we examined the response of the cockroach gut microbiota to changes in diet.

Individual variation masks the influence of diet

Analysis of the colonic gut microbiota of cockroaches fed different diets by T-RFLP analysis revealed significant variation between individuals in terms of community composition and structure. Our attempts to identify individual T-RFs that corresponded to a particular diet failed as this variation masked the effect of the different diets. We therefore assessed these samples using an alternative technique, pyrotagsequencing that has a taxonomic resolution far superior to T-RFLP. Pyrotagsequencing revealed that the cockroach gut is more diverse than previously shown by Sanger sequencing (Schauer *et al.*, 2011) with nine new phyla detected (Figure 4). These new phyla can be considered rare as they were all less than 1% in abundance. Although abundance of most phyla were similar between diet groups, Candidate division TM7 was increased ten-fold in cockroaches fed a high-fiber diet of either bran and bran-cellulose (Figure 4). These diets also had an impact on gut morphology with increased colon weight present in individuals fed a high-fiber diet (Figure 1). Our results confirm the finding of Zurek & Keddie (1998) who also observed no difference in the overall body of cockroaches fed a high fiber diet. However, unlike them, we did not observed any increase in the concentrations of gut metabolites.

Our previous study on the gut microbiota of *S. lateralis* revealed differences in the structure and composition of the microbial community between individual cockroaches fed the same diet as well as variation in parameters arising from microbial activities such as production of hydrogen and gut metabolites (Schauer *et al.*, 2011). Curtis and Sloan (2004) postulated that microbial communities of physically identical environments will differ in composition when they are formed

from a large and diverse reservoir of micro-organisms. In the absence of parental care, omnivorous cockroaches such as *S. lateralis* acquire their gut microbiota from their immediate environment. Environmental micro-organisms capable of colonising the cockroach gut will therefore be acquired at random, resulting in variation between the gut microbiota of different cockroaches. Such variation has also been observed in the gut communities of many other omnivores including humans (Zoetendal *et al.,* 1998) and pigs (Simpson *et al.,* 1999; Thompson *et al.,* 2008). Early exposure to micro-organisms from the environment is important in shaping community composition and creating variation in composition (Thompson *et al.,* 2008; Yin *et al.,* 2010). In the case of *S. lateralis*, although the gut community may be partly shaped by diet, any diet-drive changes become difficult to discern as the initial gut community will vary between individuals and these different communities will respond to dietary changes in different ways.

Termites are capable of digesting lignocellulose due to the metabolic activities of their gut microbiota. Our previous study of the colon of *S. lateralis* revealed similarities between the gut microbiota of cockroaches and termites with a third of 16S rRNA sequences from the gut microbiota of *S. lateralis* clustering closely with sequences previously obtained from the termite gut (Schauer *et al.,* 2011). Despite this similarity, feeding cockroaches with either a high fiber diet (bran) or a cellulose-enriched diet (bran-cellulose) did not result in a more “termite-like” gut microbiota and no increase was observed in the abundance of microbial groups that are involved in cellulose degradation in the termite gut (Figure 4). Specifically, the abundance of members from the phyla *Spirochaetes* and *Fibrobacteres* remained low in cockroaches fed these diets compared to a more balanced diet (chicken feed). Furthermore, the low abundance of these phyla in the pyrosequencing data explains their absence in the previous 16S rRNA gene clone library of the gut microbiota of *S. lateralis* (Schauer *et al.,* 2011).

Immediate environment influences the composition of the cockroach gut microbiota

Analysis of T-RFLP profiles by NMDS indicated only minimal separation based on diet (Figure 3A). However, when profiles were further separated into different feeding groups (two feeding groups per diet) then profiles from the same feeding group

clustered closer together (Figure 3B). This is despite the fact that in this study, all cockroaches originated from the same breeder where they were kept in a single group. These results; together with the high level of individual variation observed, suggest that the immediate environment is an important source of micro-organism for the cockroach gut microbiota and has a larger influence on the diversity and structure of the gut community than dietary change.

Even within these feeding groups, however, significant variation in the composition of the gut microbiota occurred. Analysis of T-RFLP profiles indicated that the gut microbiota of different cockroaches had very few peaks in common (Table 1). Out of the 126 distinct T-RFs detected in T-RFLP profiles, only three were found to occur in all profiles. As T-RFLP only detects the most abundant members of a bacterial community (Li *et al.,* 2007), this suggests that these members do not constitute a core microbiota. In order to examine the cockroach gut microbiota for the presence of a core community, a more in-depth sequencing approach such as pyrotagsequencing was required. Pyrosequencing of 16S rRNA genes demonstrated that at phylum level 85% of taxa within the cockroach colon were shared (17 out of 20 phyla present in all diet groups). However, only 39% (138 out of 357) of groups were in common at genus level. This agrees with previous studies where individual differences within the gut microbiota manifest themselves as only slight differences in abundance at phylum level with increasing variation in community composition at the finer taxonomic levels such as species and genus (Ley *et al.,* 2006).

The absence of a larger core group of micro-organisms in the cockroach colon suggests that a high level of functional redundancy is likely. Such redundancy is thought to be a feature of the animal gut microbiota (Dethlefsen *et al.,* 2008; Ley *et al.,* 2006) with the concept of a core microbiome that contains similar genes encoding for various metabolic functions. This core microbiome provides functional stability, maintaining gut homeostasis while actual taxonomic diversity may vary significantly (Turnbaugh *et al.,* 2009). It is due to this functional redundancy that there has been significant difficulty in predicting the response of such communities to dietary changes or disease (Gross *et al.,* 2010: Scanlan *et al.,* 2006).

References

1. **Bignell, D. E.** 1977. Some observations on the distribution of gut flora in the American cockroach, *Periplaneta americana*. J. Invertebr. Pathol. 29:338–343.
2. **Breznak, J. A., and A. Brune.** 1994. Role of microorganisms in the digestion of lignocellulose by termites. Annu Rev Entomol 39: 453–487.
3. **Brune, A., and M. Ohkuma. 2011.** Role of the termite gut microbiota in symbiotic digestion, p. 439–475. *In*: D.E. Bignell, Y. Roisin and N. Lo (ed.), Biology of termites: a modern synthesis. Springer, Heidelberg, Germany
4. **Curtis, T. P., and W. T. Sloan.** 2004. Prokaryotic diversity and its limits: microbial community structure in nature and implications for microbial ecology. Curr Opin Microbiol 7: 221–226.
5. **Dethlefsen, L., S. Huse, M. L. Sogin, and D. A. Relman.** 2008. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. PLoS Biol. 6**:**e280.
6. **Dixon, P.** 2003. VEGAN, a package of R functions for community ecology. J. Veg. Sci. 14:927–930.
7. **Egert, M., B. Wagner, T. Lemke, A. Brune, and M. W. Friedrich.** 2003. Microbial community structure in midgut and hindgut of the humus-feeding larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae). Appl. Environ. Microbiol. 69:6659–6668.
8. **Gross, G., D. M. Jacobs, S. Peters, S. Possemiers, J. van Duynhoven, E. E. Vaughan, and T. van de Wiele.** 2010. In vitro bioconversion of polyphenols from black tea and red wine/grape juice by human intestinal microbiota displays strong interindividual variability. J Agric Food Chem.58:10236-10246.
9. **Horn, H. S**. 1966. Measurement of "Overlap" in comparative ecological studies. Am. Nat. 100:419–424.
10. **Inward, D., G. Beccaloni, and P. Eggleton.** 2007. Death of an order: a comprehensive molecular phylogenetic study confirms that termites are eusocial cockroaches. Biol. Lett. 3:331–335.

11. **Kane, M. D., and J. A. Breznak.** 1991. Effect of host diet on production of organic acids and methane by cockroach gut bacteria. Appl. Environ. Microbiol. 57:2628–2634.
12. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115–175. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley and Sons Ltd., New York, N.Y.
13. **Ley, R. E., D. A. Peterson, and J. I. Gordon.** 2006. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell. 124:837–848.
14. **Li, F., M. A. Hullar, and J. W. Lampe.** 2007. Optimization of terminal restriction fragment polymorphism (TRFLP) analysis of human gut microbiota. J. Microbiol. Methods 68**:**303–311
15. **Muyzer, G. A. Tesje, C. O. Wirsen, and H. W Jannasch.** 1995. Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. Arch. Microbiol. 164**:**165–172.
16. **R Development Core Team.** 2008. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.
17. **Scanlan, P. D., F. Shanahan, C. O'Mahony, and J. R. Marchesi.** 2006. Culture-independent analyses of temporal variation of the dominant fecal microbiota and targeted bacterial subgroups in Crohn's disease**.** J. Clin. Microbiol. 44:3980–3988.
18. **Schauer, C., C. L. Thompson, and A. Brune.** 2011. The gut microbiota of *Shelfordella lateralis* reflects the close relationship between blattid cockroaches and termites. Unpublished.
19. **Schmitt-Wagner, D., M. W. Friedrich, B. Wagner, and A. Brune.** 2003. Axial dynamics, stability, and interspecies similarity of bacterial community structure in the highly compartmentalized gut of soil-feeding termites (*Cubitermes* spp.). Appl. Environ. Microbiol. 69:6018–6024.
20. **Simpson, J. M., V. J. McCracken, B. A. White, H. R. Gaskins, and R. I. Mackie.** 1999. Application of denaturant gradient gel electrophoresis for the analysis of the porcine gastrointestinal microbiota. J. Microbiol. Methods. 3:167–179.

21. **Thompson, C. L., B. Wang, and A. J. Holmes.** 2008. The immediate environment during postnatal development has long-term impact on gut community structure in pigs. ISME J 2: 739–748.
22. **Turnbaugh, P. J., M. Hamady, T. Yatsunenko, B. L. Cantarel, A. Duncan, R. E. Lay, M. L. Sogin, W. J. Jones, B. A. Roe, J. P. Affourtit, M. Egholm, B. Henrissat, A. C. Heath, R. Knight, and J. I. Gordon.** 2009. A core microbiome in obese and lean twins. Nature. 457: 480–484.
23. **Warnes. G. R., B. Bolker, L. Bonebakker, R. Gentleman, W. Huber, A. Liaw, T. Lumley, M. Maechler, A. Magnusson, S. Moeller, M. Schwartz, and B. Venables.** 2009. gplots: Various R programming tools for plotting data. R package version 2.7.4. http://CRAN.R-project.org/package=gplots
24. **Yin, Y., F. Lei, L. Zhu, S. Li, Z. Wu, R. Zhang, G. F. Gao, B. Zhu and X. Wang.** 2010. Exposure of different bacterial inocula to newborn chicken affects gut microbiota development and ileum gene expression. ISME J. 4:367–376.
25. **Zoetendal, E. G., A. D. L. Akkermans and W. M. De Vos.** 1998. Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. Appl Environ Microbiol 64: 3854–3859.
26. **Zurek, L., and B. A. Keddie.** 1998. Significance of methanogenic symbionts for development of the American cockroach, *Periplaneta americana*. J Insect Physiol. 44:645–651.

4 Individual variation in methanogenesis is not linked to archaeal composition within the colon of the blattid cockroach *Shelfordella lateralis*

Abstract

Methanogenesis in arthropods is limited in occurrence to few taxa, namely termites, scarab beetles, millipedes and cockroaches. Methanogenic archaea located in the insect hindgut are responsible for the production of methane. The factors which enable an insect to harbor large amounts of methanogenic archaea are still not clear. Here, we examine methanogenesis in *S. lateralis* under the influence of different diets. Therefore cockroaches were fed one of four different diets: chicken feed (balanced), soy (protein-rich), bran and bran-cellulose (fiber-rich). Diet had no impact on methane emission rates, but hydrogen was significantly higher in individuals fed a high fiber diet. Incubation of individuals under a headspace containing 25% hydrogen had the highest increase in methane emission in chicken feed individuals. Individuals fed a high fiber had lower increased or decreased methane emission rates. In all diets, individuals not capable of methane emission were found, although 16S rRNA of methanogenic archaea was detected in the hindguts of all cockroaches. *Methanomicrococcus blatticola* and an uncultivated *Methanobrevibacter* were found to be the only methanogenic archaea in *S. lateralis* which relative abundance was quite variable in methane and non methane emitting cockroaches. Ciliates from the cockroach hindgut were shown to be associated with methanogenic archaea via F_{420} auto fluorescence. Sequences of picked ciliates were all belonging to *Nyctotherus ovalis* and the associated methanogenic archaea all belonged to uncultivated *Methanobrevibacter*. So methanogenesis in *S. lateralis* was shown to differ due to individual variation and diet.

Introduction

While the guts of most vertebrates have been shown to contain methanogenic archaea and so emit methane, methanogenesis in arthropods is restricted to only few taxa: termites, cockroaches, millipedes and scarab beetles (Hackstein & Stumm, 1994). Nearly all species of termites and scarab beetles were found to emit methane, in opposite to millipedes and cockroaches, where around half of species were emitting methane (Brauman *et al.*, 1992, Hackstein & Stumm, 1994). In *Blatella germanica* even populations of methane emitting as well as non methane emitting individuals were found (Hackstein, 1994). Methane emission was shown to be carried out by methanogenic archaea, which were only found in the hindgut compartments for cockroaches, scarab beetles and termites (Gijzen *et al.*, 1991; Kane & Breznak, 1991; Egert *et al.*, 2005; Brune, 2010). However, the factors that enable these insect taxa to harbor methanogenic archaea have up to now stayed unclear. Several key factors were named which could determine the ability of methanogenesis in these diverse arthropod groups. It was suggested that the ability to perform methanogenesis is the result of either diet, gut differentiation or an unknown genetic factor (Hackstein & van Alen, 2011).

Diet has been considered to be an important factor for methanogenesis. However, a strict correlation of diet with methanogenesis is unlikely as in termites nearly all of the different feeding guilds were found to emit methane, while other insect species that consume similar plant and fiber-rich diets, like crickets, locusts and stick insects, do not (Hackstein & Stumm, 1994; Brune, 2010). Also in millipedes, which are litter-feeders, non-methane emitting and methane emitting species were detected, but litter-feeding cockroaches of the family Ectobiinae were found to produce no methane at all (Hackstein & van Alen, 2011).

Although diet was shown not to be a strict determining factor for methane emission, diet was shown to influence the amount of emitted methane as well as the number of methanogenic ciliates within individuals of the cockroach *P. americana*. Methane production and ciliate number was found to be increased in cockroaches fed a high-fiber diet (Gijzen et al. 1991). Also formate was demonstrated as a methanogenic substrate, as methane emission rate was enhanced when added to isolated cockroach

hindguts. Also in Kane & Breznak (1991) an increase in methane emission was observed after feeding a high-fiber diet to individuals of *P. americana*. They also observed that larvae emitted more methane than adults.

Hackstein suggested in his review in 2011 that the ability to perform methanogenesis could be based on a genetic property of the host. This was grounded on the observation that when in vertebrates once the ability to host large numbers of methanogens is lost in lower taxonomic levels (e.g. species level); it was never observed to be acquired again. In arthropods, the wide distribution of taxa which show methane emission does not support this idea. Althoug termites and cockroaches were shown to be close related (order Dictyoptera), scarab beetles (order Coleoptera) belonging to the class Insecta and millipedes of the class Diplopoda are not.

Gut morphology was assumed to be a possible factor, affecting methanogenesis. Especially as termite and scarab beetle hindguts are known for being highly enlarged and predominantly anoxic, containing a microbiota aiding digestion of an otherwise for the host indigestible diet (Figure 1, Brune & Friedrich 2000; Lemke *et al.,* 2003). By contrast, the hindguts of millipedes and cockroaches appear less enlarged in relation to the residual gut than observed in termites or scarab beetles (Figure 1). This leads to the conclusion that a highly enlarged hindgut appears not necessary for the ability to harbor methanogenic archaea. As methanogenic archaea were considered to be strict anaerobes, the presence of large anoxic areas within the hindgut could be important for a large abundance of methanogenic archaea (Brune *et al.,* 1995; Thauer *et al.,* 2008; Chapter 2).

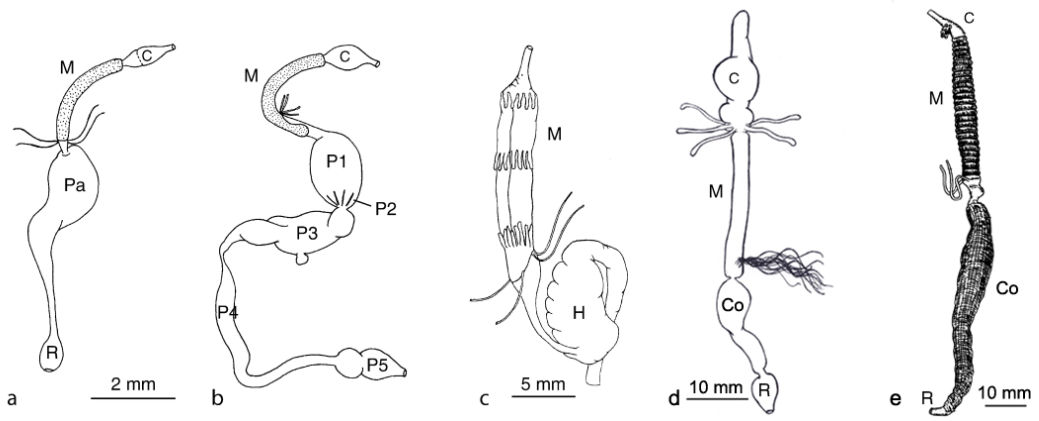

Figure 1. Schematic drawings of the intestinal tract of a) a lower termite b) a higher termite c) a scarab beetle (*Pachnoda ephippiata*) d) a cockroach (*Shelfordella lateralis*) and e) a millipede (*Orthoporus ornatus*). C = crop, M = midgut, Pa = hindgut paunch, P1-P5 different gut compartments of higher termites, Co = colon, H = Hindgut, R = rectum (Panel a) and b) reproduced from Brune 2011, Panel c) reproduced from Werner 1926a, Panel e) reproduced from Crawford et al. 1983 and scale bar added).

The composition of methanogenic archaea in insect guts was shown to differ for different hosts (Brune, 2011). In lower termites, most methanogenic archaea belonged exclusively to the order *Methanobacteriales* and were found to colonize the hindgut epithelium as well as the surface of flagellates. By contrast, higher termites typically possess archaea from the orders *Methanomicrobiales* and *Methanosarcinales*. Only the gut of the soil-feeding higher termite, *Cubitermes orthognathus* contains all taxa of methanogenic archaea, excluding the *Methanopyrales* but including the *Thermoplasmatales* (an order assumed to be methanogenic).

In opposite to termites and scarab beetles, the archaeal composition of cockroaches is up to now not well analyzed by molecular techniques. There are hardly any data of archaeal species available for blattid cockroaches and wood-feeding cockroaches of the genus *Cryptocercus*. Blaberid wood-feeding cockroaches as *Panesthia angustipennis* and *Salganea taiwanensis* have been previously shown to harbor a highly diverse archaeal community, containing *Thermoplasmatales* and *Methanosarcinales* (Hara et al., 2002).

In blattid cockroaches and also in millipedes, ciliates belonging to the genus *Nyctotherus* are frequently detected in the hindgut. These ciliates were further identified as *Nyctotherus ovalis* in blattid cockroaches and *Nyctotherus velox* in julid

millipedes (Van Hoek *et al.*, 1998). *Nyctotherus ovalis* was shown to harbor endosymbiotic methanogenic archaea belonging to *Methanobrevibacter*. These archaea are interspersed between the hydrogenosomes of the ciliate, implying a strong hydrogen dependency. Also the number of ciliates has been shown to strongly correlate with the amount of emitted methane (Gijzen *et al.*, 1991). The free-living methanogenic archaea *Methanobrevibacter blatticola* was isolated from the hindgut wall of *P. americana*, where it was shown to produce methane by the reduction of methanol and methylated amines with molecular hydrogen (Sprenger *et al.*, 2000; and 2007).

As only little is known about the archaeal community of omnivorous cockroaches, our study aim was to test *S. lateralis* for its ability to emit methane. Therefore methane and hydrogen rates were measured via gas chromatography and the methanogenic potential accessed via incubation under an atmosphere of 25% hydrogen. As we detected methane emitting and non-methane emitting individuals, the archaeal community structure of both types of cockroaches were analyzed on individual level via cloning and sequencing of archaeal 16S rRNA genes. As diet in regards to fiber content was shown to considerably influence the methane emission rate (Kane & Breznak, 1991), methane emission was also measured for animals fed either a balanced, a protein-rich or a fiber-rich diet.

Material and methods

Cockroaches and sample collection.

S. lateralis cockroaches were obtained from a commercial breeder (J. Bernhard, Helbigsdorf, Germany) and maintained in a temperature controlled chamber at 25°C with 50% humidity. Cockroaches were split into four diet groups: a balanced diet of chicken feed (CF) (Gold Plus, Versele-Laga, Deinze, Belgium; 3% fiber, 16% protein), a high protein diet of soy meal (S) (Kornhaus, Cölbe, B. Ruppersberg KG, Germany; 7% fiber, 43% protein) or a fiber-rich diet of either wheat bran (B),

(Spielberger-GmbH, Brackenheim Germany; 36% fiber, 15% protein) or wheat bran supplemented with 30% Cellulose powder (BC) (55% fiber, 11% protein, (Sigma-Aldrich, Steinheim). Food and water were provided *ad libitum*. For each diet, two separate feeding groups were established in separated boxes and maintained on each diet for at least 3 months.

Measurement of methane and hydrogen emission of whole individuals.

Gas emission of live cockroaches was analyzed by separate placing of each cockroach into a 15ml rubber-stoppered glass vial. Gas emissions were measured every 30 minutes with a sample volume of 250 µl. Methane emission was stimulated via the addition of 25% hydrogen to the headspace.

H_2 was measured by gas chromatography using a packed column (Mol Sieve 5A, 80/100 mesh; 70 cm $\times$ 6.35 mm) and a reduction gas detector (RGD2, Trace Analytical, Calif., USA) like described in Schuler and Conrad 1990.
CH_4 and CO_2 were quantified using a gas chromatography system (Shimadzu GC-8A, Kyoto, Japan) fitted with a flow ionization detector (FID) and coupled to a methanisator (FUSI electric, Germany). Signal processing and chromatogram integration were done with the Peak Simple software (version 2.66, SRI Instruments, Torrence, USA).
For all gas measurements, the system was routinely calibrated with certified standards (CO_2 and CH_4: 0.01, 0.02, 0.083 µmol; H_2: 0.0082, 0.0165, 0.0206, 0.041, 0.165 µmol), always resulting in a linear response. All calibration gases were obtained from Messer (Sulzbach, Germany).

Cloning and sequencing of archaea and ciliates from the cockroach colon.

Individual cockroaches were found as either methane emitting or non-methane emitting. In order to construct an archaeal 16S rRNA gene clone library, colonal DNA of four ME and three NME cockroaches was extracted following the procedure as outlined in Chapter 2. Ciliates were collected from the hindgut by micropipetting using an inverted microscope (see Ikeda-Ohtsubo et *al.*, 2007) followed by DNA extraction.

Archaeal 16S rRNA genes were amplified using the universal archaeal primers Arch109f (5'-AC (GT) GCT CAG TAA CAC GT-3') and Arch 912r (5'-CTC CCC CGC CAA TTC CTT TA-3') as described in Großkopf *et al.* (1998) and Lueders & Friedrich (2000). Eukaryotic 18S rRNA was amplified from ciliates with universal eukaryotic primers 384f (5'-YTB GAT GGT AGT GTA TTG GA-3') and 1147b (5'-GAC GGT ATC TRA TCG TCT TT-3') as described in Dopheide *et al.* (2008).

PCR products were purified using the MiniElute PCR purification kit (Qiagen, Hilden, Germany) and cloned using the pGem-T Cloning Kit (Promega, Mannheim, Germany) following the manufacturer's instructions. Ten clones were picked from each individual, screened for the correct insert size and sequenced using the M13 primer set (GATC Biotech, Konstanz, Germany). 62 archaeal clones and 8 eukaryotic clones were aligned using ARB (Ludwig *et al.*, 2004) and sequences with more than 97% sequence similarity were assigned to the same phylotype. Aligned sequences were checked for chimeras using Bellerophon (Huber *et al.*, 2004). Three archaeal sequences were detected as being chimeric and removed from further analysis. Phylogenetic trees were calculated using the maximum likelihood method. The eukaryotic sequences from picked ciliates were identified by an online blast search (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Significant differences between gut weights were determined by parametric ANOVA or the Kruskal-Wallis nonparametric analysis of variance (ANOVA) in R (version 2.10, R development core team 2008).

Results

Not all cockroaches are capable of methane emission

Methane emission of individuals maintained on a chicken feed diet differed between individuals (Figure 2). Seven of eleven measured cockroaches emitted methane at rates of 0.0255 to 0.1649 µmol g^{-1} h^{-1} and were classified as methane emitting (Figure 2). The remaining four individuals showed no detectable methane in the headspace and were classified as non methane emitting. The groups of non methane emitting and methane emitting individuals differed significantly from each other due to methane emission rates (ANOVA, $p = 0.035$).

After addition of 25% hydrogen to the headspace, methane was detectable within the headspace of all eleven individuals and the methane emission rates were considerably increased (Figure 2). For previously non methane emitting individuals, methane emission rates were increased to 0.0026 and 0.038 µmol g^{-1} h^{-1}. For previously methane emitting individuals, methane emission rates were almost doubled to 0.0546 and 0.2353 µmol g^{-1} h^{-1}. Although non methane emitting and methane emitting individuals showed a clear increase in methane emission rates after hydrogen stimulation, this increase was not significant.

Carbon dioxide emission was similar for non methane emitting and methane emitting individuals and not influenced by hydrogen addition (ANOVA, $p = 0.2445$). In both treatments, the rates ranged between 12.6 to 56.0 µmol g^{-1} h^{-1}, respectively (Figure 2).

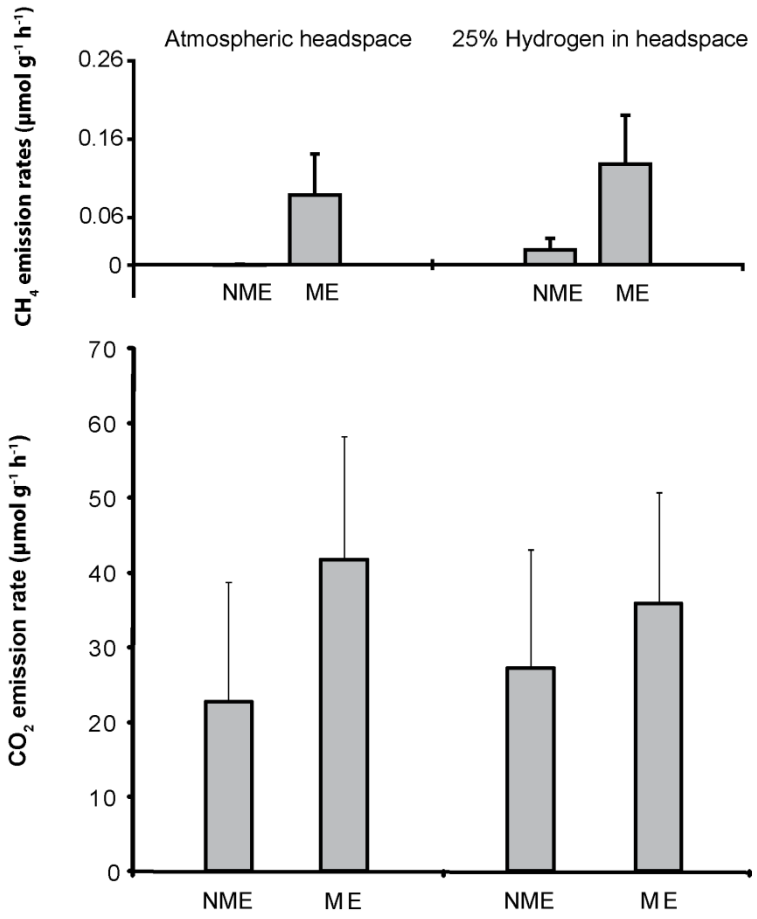

Figure 2. Methane and carbon dioxide emission rates of cockroaches, divided into non methane emitting (NME, n = 4) and methane emitting (ME, n = 7) cockroaches. All cockroaches were fed a chicken feed diet.

Archaeal community structure of methane and non methane emitting cockroaches

Analysis of 59 clones showed the presence of two different archaeal phylotypes within the colon of *S. lateralis*, both belonging to the phylum *Euryarchaeota*. Phylotype Sla1 (21 clones) belonged to the genus *Methanomicrococcus* and showed 99% sequence similarity to *Methanomicrococcus blatticola* (Figure 3A). It was also neighbored by clones previously obtained from the guts of soil feeding termites. The second phylotype Sla2 (38 clones) was assigned to the genus *Methanobrevibacter* and showed over 99% sequence similarity with an uncultivated clone from the soil-feeding termite *Cubitermes fungifaber* (Figure 3B).

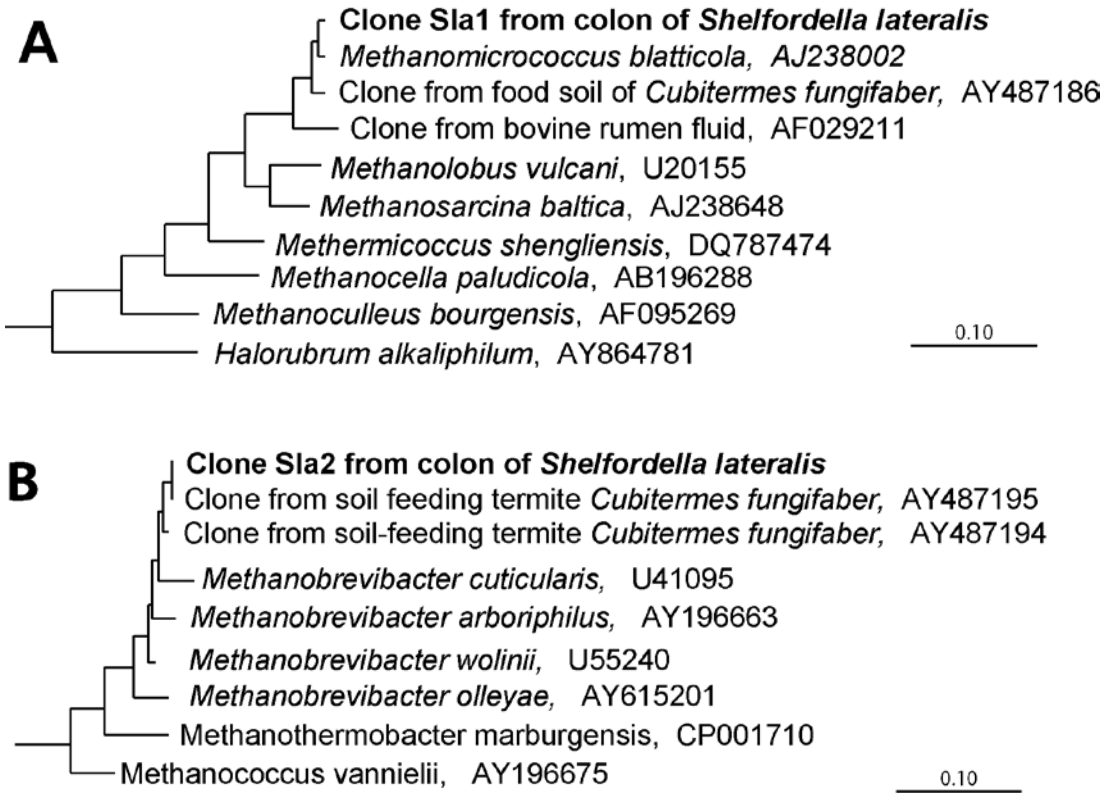

Figure 3. Maximum likelihood trees showing the phylogenetic position of archaeal 16S rRNA sequences obtained from the hindgut of *S. lateralis*. The nodes were reproducibly present in all phylogenetic analyses with maximum-likelihood algorithms. The scale bars represent 10% estimated sequence divergence. Tree was rooted with *Desulfurococcus mobilis* (X06188). **A)** Assignment of Phylotype Sla1 to *Methanomicrococcus blatticola*. **B)** Assignment of Phylotype Sla2 to *Methanobacteriales.*

No differences were observed between non methane emitting and methane emitting cockroaches in their colonal archaeal composition (Figure 4). For example, the abundance of clone Sla1 belonging to *Methanomicrococcus blatticola* ranged between 14-93% in ME cockroaches and between 15-100% in NME cockroaches and showed no significant difference when compared to each other (unpaired t test, $p = 0.258$).

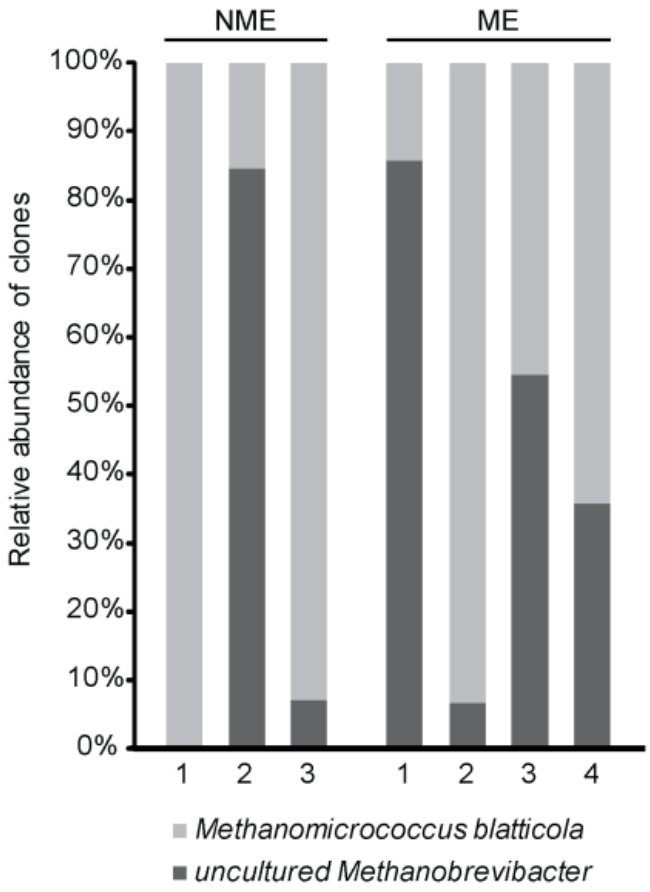

Figure 4. Distribution of archaeal clones in non methane emitting (NME) and methane emitting (ME) cockroaches. Number of sampled clones for each individual were n = 9 with exception of individual 3 of ME individuals where six clones were analyzed. All cockroaches were fed a chicken feed diet.

All clones from picked hindgut ciliates had a similarity of 95% to *Nyctotherus ovalis*. Sequences of archaeal 16S rRNA gene from the same sample of picked ciliates displayed 99% sequence similarity to the previously obtained sequences of uncultured *Methanobrevibacter*. In addition, we observed that these ciliates from the hindgut of *S. lateralis* (Figure 5A) contained endosymbionts that also showed the characteristic autoflorescence for methanogenic archaea due to the cofactor F_{420} (Figure 5B) which is necessary as electron carrier from hydrogen to carbon dioxide.

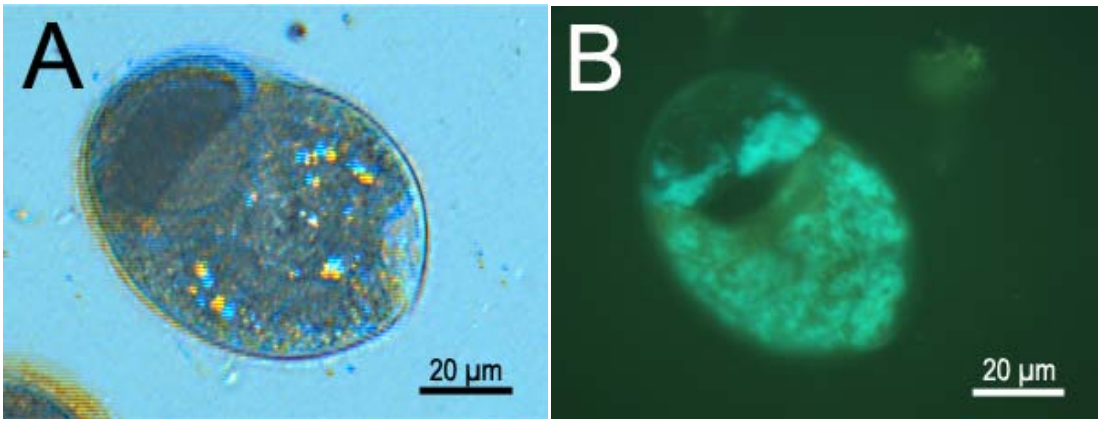

Figure 5. Phase contrast micrograph (A) and epifluorescence micrograph (B) of a *Nyctotherus ovalis* ciliate from the hindgut of the cockroach *S. lateralis*. Autofluorescence of the cofactor F_{420} is evident indicating the presence of methanogenic archaea as endosymbionts of the ciliate (B).

To estimate the diet impact on methane and hydrogen emission of the cockroaches, the gas emission rates of cockroaches fed a different diet were compared. No significant differences were found between methane emission rates of different dietary groups, as methane emission rates ranged between 0.054 ± 0.048 µmol g^{-1} h^{-1} for chicken feed individuals, 0.058 ± 0.050 µmol g^{-1} h^{-1} for soy fed individuals, 0.031 ± 0.022 µmol g^{-1} h^{-1} for bran fed individuals and 0.035 ± 0.026 µmol g^{-1} h^{-1} for bran-cellulose fed individuals (Figure 6). After incubation with 25% hydrogen in the headspace, all methane emission rates increased strongly. The highest methane emission rates were measured for individuals fed a chicken feed diet, with rates between 0.391 ± 0.204 µmol g^{-1} h^{-1}. This was also highly significant in the statistical test (Kruskal-Wallis rank sum test, p-value < 0.0001).

Hydrogen was significantly higher in individuals fed a high fiber diet of bran when compared to other diets. Gas emission rates of bran fed individuals were 0.208 ± 0.155 µmol g^{-1} h^{-1} (Kruskal-Wallis rank sum test, p-value < 0.001). The hydrogen emission rates of the other diets were much lower, with rates for bran-cellulose-fed individuals of 0.036 ± 0.060 µmol g^{-1} h^{-1}, soy-fed individuals of 0.021 ± 0.015 µmol g^{-1} h^{-1} and chicken feed fed individuals of 0.017 ± 0.013 µmol g^{-1} h^{-1}.

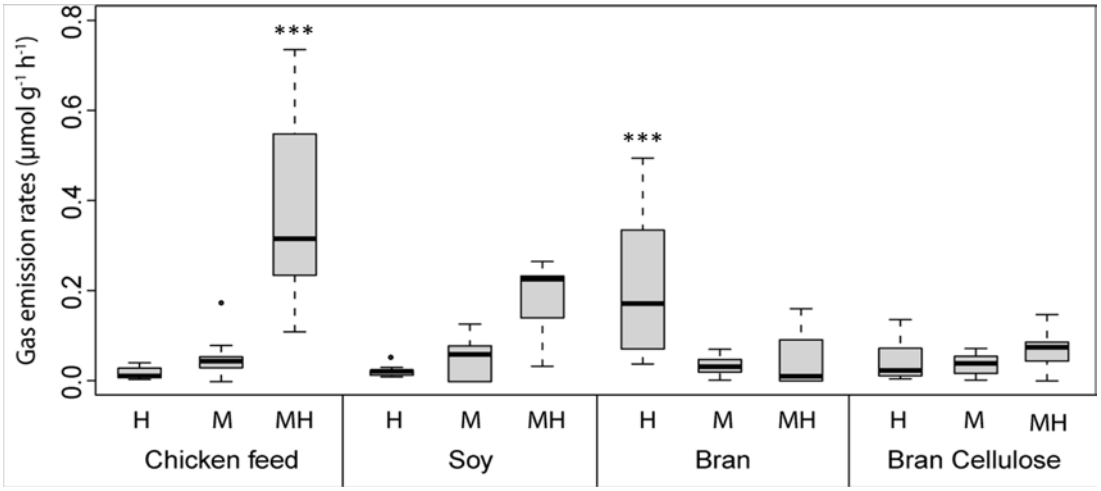

Figure 6. Average gas emission rates of individuals fed different diets. (H = hydrogen, M = methane and MH = methane after addition of 25% hydrogen to the headspace, $n_{chicken\ feed} = 10$, $n_{soy} = 9$, $n_{bran} = 10$ and $n_{bran\text{-}cellulose} = 8$). Significance was determined using a Kruskal-Wallis nonparametric ANOVA test. ***: $P < 0.001$

Discussion

Methanogenesis and hydrogen production in *S. lateralis* is highly variable between individuals

Methanogenesis in *S. lateralis* varied strongly between individuals. Methane emissions rates up to 0.178 µmol g^{-1} h^{-1} were measured. This is similar to what was previously detected for termites (0 - 1 µmol g^{-1} h^{-1}) (Bignell, 2010). By contrast, some of the measured individuals were not able to produce methane without hydrogen stimulation. No significant differences were observed for the emission of carbon dioxide (Figure 2). Non methane emitting cockroaches were not detected in previous studies of *P. americana*, a close relative of *S. lateralis* (Zurek & Keddie, 1998; Kane & Breznak, 1991). Kane and Breznak detected large variation in methane emission rates of individuals with 0.030 ± 0.024 µmol g^{-1} h^{-1}, however all of these individuals were found to emit methane. Conversely, Hackstein detected non methane emitting individuals within the methane emitting species *Periplaneta americana* and *Blatella germanica*, referring to them as amethanogenic strains (Hackstein, 2011). Our results confirmed these findings, as also non methane emitting individuals were detected within a population of mainly methane emitting cockroaches.

The cockroach hindgut harbors a variable community of two different archaeal species

The archaeal hindgut communities harbored only two different archaeal species. The proportion of these two phylotypes did not reflect an individual's status as either non methane emitting or methane emitting. Both phylotypes have been previously detected in cockroaches before and it could be assumed that they inhabit spatially separated niches within the hindgut. The free-living *M. blatticola* was isolated from the gut wall of *P. americana* and produces methane by reduction of methanol and methylated amines with molecular hydrogen (Sprenger *et al.*, 2000, and 2007). The colonisation of the gut wall requires a high hydrogen affinity as well as oxygen

tolerance, as these regions are assumed to be determined by decreasing hydrogen and an increasing oxygen gradient, already demonstrated for different termite species (Brune, 1995). As hydrogen and an anoxic center were detected in the hindgut of *S. lateralis*, these conditions are also likely to apply for cockroaches (Figure 2, Chapter 2). *M. blatticola* has been shown to have a higher hydrogen affinity than other methylotropic methanogens and uses methanol as an electron sink, which is thermodynamically advantageous at low hydrogen concentrations, giving it a competitive advantage over other archaea (Brune, 2011; Sprenger, 2007). So it would be well adapted to the low hydrogen concentration prevailing at the gut wall.

The second archaeal species was an uncultivated *Methanobrevibacter*, known as an endosymbiont of the ciliate *N. ovalis* (Gijzen *et al.*, 1991). We observed these ciliates to be present in the hindgut of *S. lateralis* in large numbers (data not shown). These ciliates harboured a dense population of endosymbiotic methanogenic archaea, as evidenced by their characteristic F_{420} autofluorescence (Figure 5B). In the ciliate, the *Methanobrevibacter* sp. symbionts were shown to be located between hydrogenosomes, implicating a hydrogen dependency for methanogenesis (Gijzen *et al.*, 1991).

Clone libraries targeting the archaeal 16S rRNA gene of single hindguts of methane and none methane emitting individuals revealed that methanogenic archaea were present also in non methane emitting individuals (Figure 4). It was shown previously, that methane emission rates correlates strongly with the number of ciliates in the hindgut, which were observed as high abundant in the hindguts of *P. americana* (Zurek & Keddie, 1998; Gijzen *et al.*, 1991; Gijzen & Barughare, 1992). As methanogenesis was shown to be strongly correlated with the number of ciliates in previous studies, it seems to be mainly determined by the activity of the endosymbiotic *Methanobrevibacter* species. The contribution to entire methane emission of the wall associated *M. blatticola* still remains to be analysed. In our analysis of *S. lateralis*, two non methane emitting individuals had none or less than 10% endosymbiotic uncultured *Methanobrevibacter* (Figure 4), which could display an also low amount of ciliates, resulting in hardly detectable methane emission rates. In order to assess whether the absence of methane emission in non methane emitting

individuals is due to a low number of ciliates, the amount of ciliates in the hindgut has to be assessed for non methane emitting individuals.

Correlation of methane emission, hydrogen content and methanogenic potential

The methane emission rates of cockroaches fed a chicken feed diet, were increased from zero in non methane emitting cockroaches to 18.5 ± 0.9 µmol g^{-1} h^{-1} and nearly doubled in methane emitting cockroaches (Figure 2), suggesting a hydrogen limitation within the hindgut. Hydrogen limitation in methanogenesis has been previously observed in termites as hydrogen appears to be a key substrate of methanogenesis in termite guts (Pester & Brune, 2007). To test for a direct correlation between methane emission, methanogenic potential and available hydrogen, each value was measured in single individuals raised on different diets. The methanogenic potential of the cockroach gut was analysed by the addition of 25% hydrogen to the headspace.

Methane emission rates were not influenced by diet, but hydrogen emission rates differed strongly between the diets, as bran fed individuals had five to ten times increased rates compared to rates measured for the other diets (Figure 6). In opposite to the findings of Cruden and Markovetz (1987), hydrogen was found in all analysed individuals, perhaps due to a lower detection limit of the used electrodes for measuring. Already in the second Chapter of this thesis, hydrogen content differed within the hindguts of chicken feed individuals when measured with microelectrodes (Chapter 2, Figure 2). In contrast to methane emission rates, where individual variation was large but no differences according to diet were found, hydrogen differed strongly due to diet. This could be explained with an accumulation of hydrogen in the gut due to fiber degradation.

When looking at the methanogenic potential, the methane emission of individuals fed chicken feed had the largest increase, followed by soy-fed individuals (Figure 6). The lowest or even negative effect of hydrogen stimulation on methane emission in the high fiber diets could be due to the observation of already high hydrogen values in

these diets. The assumption that sulphate-reducing bacteria could consume redundant hydrogen (Zurek & Keddie, 1998) seems not likely for *S. lateralis*, as hydrogen accumulated to high concentrations in the guts of individuals fed a fiber rich diet. Sulphate-reducing bacteria, such as *Desulfovibrionaceae* and *Desulfobacteriaceae* were detected in the clone library, but no increase in these groups was observed in fiber rich diets via pyrosequencing approach (Chapter 3, Figure 4).

Acknowledgements

I would like to thank Dorothee Tegthmeier for performing the phylogenetic analysis of the methanogenic archaea and the sequencing of the hindgut ciliate.

References

1. **Bignell, D. E.** 2010. Termites. In: Reay, D.; Smith, P.; van Amstel, A. (ed.), Methane and climate change, Earthscan, pp. 62-73.
2. **Breznak, J.A., and J. M. Switzer.** 1986. Acetate synthesis from H_2 plus CO_2 by termite gut microbes. Appl. Environ. Microbiol. 52: 623-630.
3. **Brune, A.** 2010. Methanogenesis in the digestive tracts of insects. In: Timmis, K.N. (ed.), Handbook of Hydrocarbon and Lipid Microbiology, vol. 8. Springer, Heidelberg, pp. 707-728.
4. **Brune, A., and M. Friedrich.** 2000. Microecology of the termite gut: structure and function on a microscale. Curr. Opin. Microbiol. 3: 263-269.
5. **Brune, A., D. Emerson, and J. A. Breznak.** 1995. The termite gut microflora as an oxygen sink: microelectrode determination of oxygen and pH gradients in guts of lower and higher termites. Appl. Environ. Microbiol. 61: 2681–2687.
6. **Cruden, D. L., and A. J. Markovetz.** 1987. Microbial ecology of the cockroach gut. Annu. Rev. Microbiol. 41: 617–643.
7. **Dopheide, A., G. Lear, R. Stott, and G. Lewis.** 2008. Molecular Characterization of Ciliate Diversity in Stream Biofilms. Appl. Environ. Microbiol. 74: 1740-1747.

8. **Egert, M., B. Wagner, T. Lemke, A. Brune, and M. W. Friedrich.** 2003. Microbial community structure in midgut and hindgut of the humus-feeding larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae). Appl. Environ. Microbiol. 69: 6659–6668.
9. **Egert, M., U. Stingl, L. Dyhrberg Bruun, B. Wagner, A. Brune, and M. W. Friedrich.** 2005. Structure and topology of microbial communities in the major gut compartments of *Melolontha melolontha* larvae (Coleoptera: Scarabaeidae). Appl. Environ. Microbiol. 71: 4556-4566.
10. **Gijzen, H.J., and M. Barugahare.** 1992. Contribution of anaerobic protozoa and methanogens to hindgut metabolic activities of the American cockroach, *Periplaneta americana*. Appl. Environ. Microbiol. 58: 2565-2570.
11. **Gijzen, H.J., C.A. Broers, M. Barugahare and Stumm, C.K.** 1991. Methanogenic bacteria as endosymbionts of the ciliate *Nyctotherus ovalis* in the cockroach hindgut. Appl. Environ. Microbiol. 57: 1630-1634.
12. **Grosskopf, R., P. H. Janssen, and W. Liesack.** 1998. Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval. Appl. Environ. Microbiol. 64: 960-969.
13. **Hackstein, J.H.P., C. K. Stumm.** 1994. Methane production in terrestrial arthropods. Proc. Natl. Acad. Sci. U. S. A. 91: 5441-5445.
14. **Hackstein, J.H.P.; T. A. van Alen.** 2010. Methanogens in the gastro-intestinal tract of animals. In: Hackstein, J.H.P. (ed.), (Endo)symbiotic Methanogenic Archaea, vol. . Springer, Heidelberg, pp. 115-142.
15. **Hara, K.; N. Shinzato, M. Seo, T. Oshima, and A. Yamagishi.** 2002. Phylogenetic analysis of symbiotic archaea living in the gut of xylophagous cockroaches. Microb. Environ. 17: 185-190.
16. **Huber, T., G. Faulkner, and P. Hugenholtz.** 2004. Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. Bioinformatics. 20: 2317-2319.
17. **Ikeda-Ohtsubo, W., Desai, M., Stingl, U. and A. Brune.** 2007. Phylogenetic diversity of ‘Endomicrobia’ and their specific affiliation with termite gut flagellates. Microbiology. 153: 3458-3465.

18. **Kane, M. D., and J. A. Breznak.** 1991. Effect of host diet on production of organic acids and methane by cockroach gut bacteria. Appl. Environ. Microbiol. 57: 2628–2634.
19. **Lemke, T., U. Stingl, M. Egert, M. W. Friedrich, and A. Brune.** 2003. Physicochemical conditions and microbial activities in the highly alkaline gut of the humus-feeding larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae). Appl. Environ. Microbiol. 69: 6650-6658.
20. **Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Förster, I. Brettske, S. Gerber, A. W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. König, T. Liss, R. Lüssmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, and K-H. Schleifer.** 2004. ARB: A software environment for sequence data. Nucleic Acids Res. 32: 1363–1371.
21. **Lueders, T., and M. Friedrich.** 2000. Archaeal population dynamics during sequential reduction processes in rice field soil. Appl. Environ. Microbiol. 66: 2732-2742.
22. **Pester, M., and A. Brune.** 2007. Hydrogen is the central free intermediate during lignocellulose degradation by termite gut symbionts. ISME Journal 1: 551-565.
23. **Schuler, S., and R. Conrad.** 1990. Soils contain two different activities for oxidation of hydrogen. FEMS Microbiology Letters, 73: 77-83.
24. **Sprenger, W. W. ; M. C. van Belzen, J. Rosenberg, J. H. Hackstein, and J. T. Keltjens.** 2000. *Methanomicrococcus blatticola* gen. nov., sp. nov., a methanol- and methylamine-reducing methanogen from the hindgut of the cockroach *Periplaneta americana*. Int. J. Syst. Evol. Microbiol. 50: 1989-99.
25. **Sprenger, W. W., J. H. Hackstein, and J. T. Keltjens.** 2007. The competitive success of *Methanomicrococcus blatticola*, a dominant methylotrophic methanogen in the cockroach hindgut, is supported by high substrate affinities and favorable thermodynamics. FEMS Microbiol. Ecol. 60: 266-275.
26. **Thauer, R. K.; A-K. Kaster, H. Seedorf, W. Buckel, R. Hedderich.** 2008. Methanogenic archaea: ecologically relevant differences in energy conservation. Nat. Rev. Microbiol. 6: 579-591.

27. **van Hoek, A. H., T. A. van Alen, V. S. Sprakel, J. H. Hackstein, and G. D. Vogels.** 1998. Evolution of anaerobic ciliates from the gastrointestinal tract: phylogenetic analysis of the ribosomal repeat from *Nyctotherus ovalis* and its relatives. Mol. Biol. Evol. 15: 1195-1206.
28. **Zurek, L., and B. A. Keddie.** 1998. Significance of methanogenic symbionts for development of the American cockroach, *Periplaneta americana*. J. Insect. Physiol. 44: 645–651.

5 Discussion

This thesis contains detailed results about the gut of an omnivorous cockroach and fills the gap of knowledge regarding its gut microbiota. Several studies have targeted the cockroach gut as a potential reservoir for human pathogens (Baumholtz *et al.*, 1997). As some cockroaches are pests in human buildings, many studies focused on the cockroach's ability to harbor and spread pathogens in its environment and regarded them as a potential danger for human health. So former studies neglected the typical gut microbiota of cockroaches and isolation of their non pathogenic members occurred accidentally (Burgess *et al.*, 1973). Here we identified the bacterial and archaeal gut communities of a blattid cockroach. We were able to show that the close relationship of cockroaches and termites is reflected in some members of the hindgut microbiota.

Gut physiology of *S. lateralis*

Gut physiological parameters of *S. lateralis* were consistent with what was shown for other omnivorous cockroaches (Cruden & Markovetz, 1987). All gut compartments had an anoxic center with low redox potentials. This favors fermentative digestion of substrates and was also reflected in the detected gut metabolites, which was mainly acetate. Hydrogen concentration was low along the gut with 0-6.5 kPa, with a slight increase in the hindgut. Individual variation was evident by the presence of individuals which contained very high hydrogen concentrations in the hindgut, up to 23 kPa.

Bacterial gut community had highest density and diversity in the colon

Each gut compartment contained bacteria within the magnitude of 10^7 cells per compartment. Large differences in terms of the bacterial community structure were observed between the anterior gut, including crop and midgut, and the posterior gut with colon and rectum. Bacterial diversity was low in crop, gastric caeca and midgut and profiles were quite similar to each other and between different individuals. The microbial communities of these compartments contained mainly *Lactobacillales* and *Bacteroidetes.* Accordingly in these compartments a high lactate concentration was found. This agrees with the findings, that the foregut is an important site of lactate production and lactic acid fermentation is an important process for the metabolism in the foregut (Kane & Breznak, 1991). The colon and rectum showed the highest bacterial density and diversity, but also displayed a large degree of individual variation, as amounts of shared TRFs between individuals were very low when compared by T-RFLP fingerprinting, as TRFs could be assigned in this study to family level. The variability of profiles between individuals suggests that the colonal gut microbiota establishes itself from a large pool of bacteria, some of them probably with redundant functions. Microbial communities of physically identical environments have been hypothesized to differ in composition when they are formed from a large and diverse reservoir of micro-organisms (Curtis & Sloan, 2004). In the absence of parental care, e.g. proctodeal trophallaxis, omnivorous cockroaches such as *S. lateralis* have to acquire their gut microbiota from their direct environment. Environmental micro-organisms capable of colonising the cockroach gut will therefore be acquired at random, resulting in variation between the gut microbiota of different individuals. Such variation has also been observed in the gut communities of many other omnivores including humans (Zoetendal *et al.,* 1998) and pigs (Simpson *et al.*, 1999; Thompson *et al.*, 2008).

Colonal microbiota reflects gut environment and host phylogeny, but no dietary shifts

The bacterial microbiota of the colon consisted mainly of *Bacteroidetes* and *Firmicutes*, especially *Clostridia*, latter reflecting the mainly anaerobic environment in this compartment. The predominance of these two phyla is commonly found in various other intestinal environments, such as wood-feeding insects like *Cryptocercus punctulatus* or lower termites like *Reticulitermes speratus* (Berlanga *et al.,* 2009; Hongoh *et al.,* 2003). *Bacteroidetes* and *Firmicutes* were also predominant in guts of mammals, belonging to completely different feeding groups, like herbivores, carnivores or insectivores (Ley *et al.,* 2008).

The presence or absence of less abundant phyla in this study appeared more reflective of the omnivorous diet of *S. lateralis*. *Spirochaetes* and *Fibrobacteres*, assumed to be cellulose digesters in higher termites, were not detected in our analysis. Phylogenetic analysis revealed that two clones were affiliated with a cluster of *Endomicrobia*, assumed to occur free-living and not associated with flagellates (Ikeda-Ohtsubo *et al.*, 2010). Sequences of this cluster were shown to be wide-spread in other environments and not restricted to animal guts (Herlemann *et al.,* 2007). Also the metabolism of the first and only cultivated free-living representative of this phylum, isolated from the gut of a scarab beetle, shows not a strict specialization for cellulosic diets (Herlemann *et al.,* 2007 and 2009, Geissinger *et al.,* 2009). Clones of putatively free-living *Endomicrobia* were obtained also from *P. americana*, showing that this bacteria can also be found within the gut of an omnivore insect (Ikeda-Ohtsubo *et al.,* 2010). The affiliation of two clones with putatively free-living *Endomicrobia* from termites and other cockroaches also supports the conclusion that *Endomicrobia* were present already in Dictyopteran ancestors of termites and not acquired together with a cellulosic diet and cellulolytic flagellates in *Cryptocercus* wood-roaches and lower termites (Ikeda-Ohtsubo *et al.,* 2010).

Another putatively free-living relative of a flagellate endosymbiont was found within the *Bacteroidetes* (Noda *et al.,* 2006). *Azobacteroides pseudotrichonymphae* was found to be an endosymbiont of the flagellate *Pseudotrichonympha grassii* and accounts for two third of the prokaryotic cells in the gut of the lower termite

Coptotermes formosanus. Here it supplies the flagellate host with nitrogen (Hongoh, 2010). As no large cellulolytic ciliates were observed in the hindgut of *S. lateralis* via light microscopy or cloning of 18S rRNA, the clones assigned with the *Azobacteroides pseudotrichonymphae* cluster are assumed to be putatively free-living. Like assumed for *Endomicrobia*, the free-living ancestors of this endosymbiont could be acquired already in cockroach ancestors.

Phylogenetic analysis revealed that 30% of the clones were reflective of the close relatedness between cockroaches and termites. These clones fell into clusters consisting exclusively of sequences obtained from termite guts, but showed no preference for clustering with sequences from higher or lower termites or clones obtained from the gut of *Cryptocercus punctulatus*. For example, a large proportion of clones could be assigned to termite clusters I-V in the *Bacteriodetes* (Ohkuma *et al.*, 2002). This shows that some of these clusters might not be termite specific, but are likely to represent lineages of Dictyopteran specific gut bacteria. Therefore, a sampling of more cockroach and mantis species could reveal some of these clusters as specific for the whole order Dictyoptera and not only for termites.

About two thirds of clones clustered with sequences obtained from animal intestines, showing a specialization to this habitat. After hatching germ-free, with exception of endosymbiotic bacteria within the cockroach tissue, the gut microbiota is acquired completely new from the environment. The domination of so-called gut lineages, bacterial lineages which are found regularly in the guts of animals, displays a preference of these bacterial lineages to colonize animal intestines.

The occurrence of clones affiliated to other environments than guts could be due to the uptake of environmental bacteria by the cockroach host. The low abundance of these clones in the clone library indicates that most of the incidentally up taken bacteria cannot persist in the cockroach gut. As gut microbial communities of many insect orders are not well examined by molecular methods, an increased sampling of more insect species could reveal these clones of *S. lateralis* belonging to not yet detected gut lineages.

Effects of different diets on the colonal gut microbiota

The third chapter examines the variability of this gut microbiota under the influence of different diets. In our study the gut bacterial composition was not observed to be changed by different diets, neither when analyzed with ordination on the basis of T-RFLP profiles nor through a pyrosequencing analysis, where bacteria could be assigned down to species level. A possible change of gut microbial composition caused by different diets could be masked by the large degree of individual variation, which was shown in the chapter before.

The pyrotagsequencing approach detected nine additional phyla, which were not observed by Sanger sequencing. These new phyla can be considered rare as they were all less than 1% in abundance. Most phyla were similar distributed between diet groups, except Candidate division TM7 which was ten times increased in cockroaches fed a high-fiber diet of either bran or bran-cellulose. Members of *Synergistetes* were lower in abundance in bran-cellulose fed individuals compared to other diets. In the case of *S. lateralis*, a possible dietary impact on the gut microbiota was probably masked by pooling of samples for the pyrosequencing approach.

T-RFLP profiles of single individuals also display a large degree of individual variation. As profiles were highly reproducible, a methodical error for the differences between individuals is unlikely. In ordination analysis, the effect of a collective housing overlaid the influence of diet, which was only slightly visible. According to that, differences in T-RF abundance could not be linked to a particular diet as the variation between individuals was larger than the effect of different diets. Comparison of T-RFLP profiles showed only few shared peaks within all individuals fed the same diet, like already observed for the individuals raised on chicken feed in the second chapter. There were only three out of 126 distinct T-RFs shared between all profiles. Therefore, the formation or existence of a core microbiota for a special diet seems unlikely for the omnivorous *S. lateralis*.

The pyrosequencing approach detected 85% shared taxa on phylum level. Going down to genus level divided the shared taxa to half. This agrees with previous studies where individual differences in community composition occurred mainly at species

and genus level, due to a large degree of functional redundancy of gut microbiota (Ley *et al.,* 2006). The absence of a larger core microbiota on the cockroach colon suggests a high level of functional redundancy. The pyrosequencing approach was successful in detection of rare phyla, but for detection of the most abundant phyla, the Sanger sequencing was also sufficient. Pooling of samples increases coverage of the diversity, but may mask individual differences.

Although the phylogenetic analysis revealed that some members of the cockroach and termite gut microbiota are reflective of the common evolutionary history of their hosts, feeding a high fiber diet (bran) or a cellulose-enriched diet (bran-cellulose) did not enrich cellulose digesting bacteria, like *Spirochaetes* or *Fibrobacteres*. Furthermore, the low abundance of these phyla in the pyrosequencing data with less than 40 sequences out of 40000 explains their absence in the clone library. Also the abundance of these phyla was low in all different diets.

Individual variation of methanogenesis in *S. lateralis*

As shown for the gut bacterial composition, methanogenesis in *S. lateralis* also differed strongly between individuals. In previous studies, individuals of *P. americana* fed a balanced diet were found to produce methane with rates about 0.03 µmol g^{-1} h^{-1} (Kane & Breznak, 1991, Zurek & Keddie, 1998). This was confirmed as *S. lateralis* emitted methane within the same range, but did not achieve the high rates observed in termites, which can reach rates of up to 1 µmol g^{-1} h^{-1} (Bignell, 2010).
Individuals with no detectable methane production were observed in all diets. This was not due to a lack of methanogenic archaea in the hindgut, as two types of methanogenic archaea were detected within the colons of all analyzed cockroaches for the chicken feed diet. This methanogenic archaea were identified as *Methanomicrococcus blatticola* and an uncultivated *Methanobrevibacter* species. These two species presumably inhabit two completely different niches within the cockroach gut, as *M. blatticola* was isolated from the gut-wall of *P. americana* and produces methane via reduction of methanol and methylated amines with molecular hydrogen (Sprenger et al. 2000 and 2007). The *Methanobrevibacter* species was

shown to occur endosymbiotic within the ciliate *N. ovalis*, which was also observed in the hindgut and assumed to produce methane from hydrogen and carbon dioxide.

Individuals maintained on different diets differed not in methane emission. Hydrogen emission was shown to be significantly increased in bran fed individuals and slightly increased in individuals fed a bran-cellulose diet. Methanogenic potential was largest in individuals fed chicken feed, followed by individuals maintained on a soy diet. This implies that low fiber diets were hydrogen limited and high fiber diets led to an accumulation of hydrogen. The slight decrease of methane emission in high fiber diets after hydrogen stimulation could be due to an accumulation of hydrogen leading to a disturbance of anaerobic fermentative processes within the gut due to disadvantageous conditions caused by a high hydrogen partial pressure.

The idea that sulfate reducing bacteria could consume spare hydrogen was postulated by Zurek and Keddie in 1998 and not observed for bran fed individuals. Sulfate reducing bacteria were at least present in the colon, shown by the clone library, but seemed not able to consume the accumulating hydrogen in high fiber diets. This could be due to a possible sulfate limitation in high fiber diets, inhibiting the hydrogen consumption of sulfate reducing bacteria in the cockroach colon.

The results of the experiments showed, that methanogenesis in the cockroach *S. lateralis* is quite variable, displayed in the ability to emit methane and the amount of measured methane and hydrogen. Diet seemed to have an impact on methanogenic potential and hydrogen content, but not on the methane emission. Also the contribution of the two detected archaeal phylotypes to whole methane emission is still unclear. For further insight into the mechanism of methanogenesis in cockroaches, more work is needed.

References

1. **Akhmanova, A., F. Voncken, T. van Alen, A. van Hoek, B. Boxma, G. Vogels, M. Veenhuis, and J. H. P Hackstein.** 1998. A hydrogenosome with a genome. Nature. 396: 527- 528.
2. **Baumholtz, M. A., L. C. Parish, J. A. Witkowski, and W. B. Nutting.** 1997. The medical importance of cockroaches. International Journal of Dermatology. 36: 1365-4632.
3. **Berlanga, M., B. J. Paster, and R. Guerrero.** 2009. The taxophysiological paradox: changes in the intestinal microbiota of the xylophagous cockroach *Cryptocercus punctulatus* depending on the physiological state of the host. Int. Microbiol. 12:227–236.
4. **Bignell, D. E..** 2010. Termites. In: Reay, D.; Smith, P.; van Amstel, A. (ed.), Methane and climate change, Earthscan, pp. 62-73.
5. **Brune, A.** 2010. Methanogenesis in the digestive tracts of insects. In: Timmis, K.N. (ed.), Handbook of Hydrocarbon and Lipid Microbiology, vol. 8. Springer, Heidelberg, pp. 707-728.
6. **Burgess, N. R. H., S. N. McDermott, and J. Whiting.** 1973. Aerobic bacteria occurring in the hind-gut of the cockroach, *Blatta orientalis*. J. Hyg. 71: 1–7.
7. **Cruden, D. L., and A. J. Markovetz.** 1987. Microbial ecology of the cockroach gut. Annu. Rev. Microbiol. 41: 617–643.
8. **Curtis, T. P., and W. T. Sloan.** 2004. Prokaryotic diversity and its limits: microbial community structure in nature and implications for microbial ecology. Curr Opin Microbiol 7: 221–226.
9. **Geissinger, O., Herlemann, D. P. R., Mörschel, E., Maier, U. G., and A. Brune.** 2009. The Ultramicrobacterium "Elusimicrobium minutum" gen. nov., sp. nov., the first cultivated representative of the Termite Group 1 phylum. Appl. Environ. Microbiol. 75: 2831-2840.
10. **Gibson, G. R., S. Macfarlane and G.T. Macfarlane.** 1993. Metabolic interactions involving sulphate-reducing and methanogenic bacteria in the human large intestine. FEMS Microb. Ecol. 12: 117-125.

11. **Gijzen, H.J.; and M. Barugahare.** 1992. Contribution of anaerobic protozoa and methanogens to hindgut metabolic activities of the American cockroach, *Periplaneta americana*. Appl. Environ. Microbiol. 58: 2565-2570.
12. **Gijzen, H.J.; C.A. Broers, M. Barugahare and Stumm, C.K.** 1991. Methanogenic bacteria as endosymbionts of the ciliate *Nyctotherus ovalis* in the cockroach hindgut. Appl. Environ. Microbiol. 57: 1630-1634.
13. **Herlemann, D. P. R., Geissinger, O., Ikeda-Ohtsubo, W., Kunin, V., Sun, H., Lapidus, A., Hugenholtz, P. and Brune, A.** 2009. Genomic analysis of "Elusimicrobium minutum," the first cultivated representative of the phylum "Elusimicrobia" (formerly Termite Group 1)**.** Appl. Environ. Microbiol. 75: 2841-2849.
14. **Herlemann, Daniel P. R., Geissinger, Oliver and A. Brune. 2007.** The Termite Group I phylum is highly diverse and widespread in the environment. Appl. Environ. Microbiol. 73: 6682-6685.
15. **Hongoh, Y.** 2010. Diversity and genomes of uncultured microbial symbionts in the termite gut, Biosci. Biotechnol. Biochem. 74: 1145-1151.
16. **Hongoh, Y., M. Ohkuma, and T. Kudo.** 2003. Molecular analysis of bacterial microbiota in the gut of the termite *Reticulitermes speratus* (Isoptera; Rhinotermitidae). FEMS Microbiol. Ecol. 44: 231-242.
17. **Ikeda-Ohtsubo, W., Desai, M., Stingl, U. and A. Brune.** 2007. Phylogenetic diversity of 'Endomicrobia' and their specific affiliation with termite gut flagellates. Microbiology. 153: 3458-3465.
18. **Ikeda-Ohtsubo, W.; N. Faivre, and A. Brune.** 2010. Putatively free-living *Endomicrobia* - ancestors of the intracellular symbionts of termite gut flagellates? Environ. Microbiol. 2: 554-559.
19. **Kane, M. D., and J. A. Breznak.** 1991. Effect of host diet on production of organic acids and methane by cockroach gut bacteria. Appl. Environ. Microbiol. 57: 2628–2634.
20. **Ley, R. E., D. A. Peterson, and J. I. Gordon.** 2006. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell. 124: 837–848.
21. **Ley, R. E., M. Hamady, C. Lozupone, P. J. Turnbaugh, R. R. Ramey, J. S. Bircher, M. L. Schlegel, T. A. Tucker, M. D. Schrenzel, R. Knight, and J.**

I. Gordon. 2008. Evolution of mammals and their gut microbes. Science. 320: 1647–1651.

22. **Noda, S., T. Inoue, Y. Hongoh, M. Kawai, C. A. Nalepa, C. Vongkaluang, T. Kudo, and M. Ohkuma.** 2006. Identification and characterization of ectosymbionts of distinct lineages in *Bacteroidales* attached to flagellated protists in the gut of termites and a wood-feeding cockroach. Environ. Microbiol. 8: 11–20.
23. **Ohkuma, M., S. Noda, Y. Hongoh, and T. Kudo.** 2002. Diverse bacteria related to the *Bacteroides* subgroup of the CFB phylum within the gut symbiotic communities of various termites. Biosci. Biotechnol. Biochem. 66: 78–84.
24. **Sanderson, M. G.** 1996. Biomass of termites and their emissions of methane and carbon dioxide: A global database. Global Biogeochem. Cycles. 10: 543–557.
25. **Simpson, J. M., V. J. McCracken, B. A. White, H. R. Gaskins, and R. I. Mackie.** 1999. Application of denaturant gradient gel electrophoresis for the analysis of the porcine gastrointestinal microbiota. J. Microbiol. Methods. 3: 167–179.
26. **Sprenger, W. W. ; M. C. van Belzen, J. Rosenberg, J. H. Hackstein, and J. T. Keltjens.** 2000. *Methanomicrococcus blatticola* gen. nov., sp. nov., a methanol- and methylamine-reducing methanogen from the hindgut of the cockroach *Periplaneta americana*. Int. J. Syst. Evol. Microbiol. 50: 1989-99.
27. **Sprenger, W. W.; J. H. Hackstein, and J. T. Keltjens.** 2007. The competitive success of *Methanomicrococcus blatticola*, a dominant methylotrophic methanogen in the cockroach hindgut, is supported by high substrate affinities and favorable thermodynamics. FEMS Microbiol. Ecol. 60: 266-275.
28. **Thompson, C. L., B. Wang, and A. J. Holmes.** 2008. The immediate environment during postnatal development has long-term impact on gut community structure in pigs. ISME J. 2: 739–748.
29. **Zoetendal, E. G., A. D. L. Akkermans and W. M. De Vos.** 1998. Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. Appl. Environ. Microbiol. 64: 3854–3859.

30. **Zurek, L., and B. A. Keddie.** 1998. Significance of methanogenic symbionts for development of the American cockroach, *Periplaneta americana*. J. Insect Physiol. 44: 645–651.

Summary

This thesis focuses on the conditions prevailing in the gut compartments of the cockroach *Shelfordella lateralis* and gives new insights into the composition of associated bacterial, archaeal and eukaryotic microorganisms. While the symbiotic gut microbiota of termites has been focused by many studies due to its cellulolytic function, the gut microbiota of the others members of the order Dictyoptera, cockroaches and mantis, remained mostly unexamined. Therefore, the gut microbiota of the blattid cockroach *S. lateralis* was characterized via molecular techniques, including a deep pyrosequencing approach.

Crop and midgut were in all individuals mainly colonised by lactic acid bacteria and *Bacteroidetes*. This was also in agreement with the high lactate concentrations found in these compartments, which was consistent over all analysed individuals. By contrast, the bacterial composition of the hindgut, including colon and rectum, differed strongly between individuals. The colon, as hotspot of bacterial diversity, harboured mainly members of *Bacteroidetes*, *Firmicutes* as well as *δ-Proteobacteria*. A large proportion of clones belonged to groups isolated from anaerobic environments, for example Clostridia, reflecting the anoxic center detected within each gut compartment. *Spirochaetes* and *Fibrobacteres*, assumed to carry out cellulose digestion in higher termites, were not detected via Sanger sequencing, but showed a low abundance in pyrosequencing approach, reflecting the omnivorous diet of *S. lateralis*. A third of clones clustered with sequences previously obtained from the termite gut, reflecting the common evolutionary history of cockroaches and termites.

Different diets (chicken feed as balanced, soy as protein-rich and bran and bran-cellulose as fiber-rich diets) were used to examine the influence of diet. A possible dietary impact was masked by the large degree of individual variation. In ordination analysis, the colonal fingerprinting profiles of individuals showed a tendency to cluster after diets, but the effect of housing environment was overlaid, indicating that the available pool of bacteria for gut microbiota acquisition plays a superior role. Pyrosequencing analysis detected no differences in gut microbial composition, but

found nine additional phyla, which were less abundant in all diets and so counted to the rare phyla. Analysis of bacterial community structure by terminal-restriction-fragment length polymorphism and 454 pyrotagsequencing of 16S rRNA genes revealed a high individual variability but little impact of diet, suggesting that cockroaches are able to maintain a gut microbiota that is insensitive to dietary shifts. This was also supported by the results that volatile fatty acid concentrations were not significantly changed by different diets.

Methanogenesis in arthropods is limited in occurrence to few taxa, namely termites, scarab beetles, millipedes and cockroaches. In this study, most individuals emitted methane. Some individuals emitted only detectable amounts of methane after stimulation with hydrogen. Two species of methanogenic archaea were found in the hindgut, namely *Methanomicrococcus blatticola* and an uncultivated *Methanobrevibacter*. The relative abundance of both types was quite variable in cockroaches with and without methane emission. Ciliates observed in the cockroach hindgut belonged to *Nyctotherus ovalis* and were associated endosymbiotic with uncultivated *Methanobrevibacter*. Diet had no impact on methane emission rates, but hydrogen was significantly higher in individuals fed a high fiber diet. Chicken feed fed individuals had the highest increase in methane emission after hydrogen stimulation, whereas individuals fed a high fiber diet showed a low or negative response of the methane emission rate. In all different diets, individuals not capable of methane emission were found.

Zusammenfassung

Die vorliegende Arbeit befasst sich mit dem Darm, seiner Physiology und der assoziierten Mikrobiota anhand der Schabe *Shelfordella lateralis*. Während die symbiotische Darmmikrobiota von Termiten in vielen Studien wegen ihrer Fähigkeit des Zelluloseabbaus untersucht wurde, existieren kaum molekulare Daten über die Darmmikrobiota anderer Mitglieder der Ordnung Dictyoptera, nämlich Schaben (Blattodea) und Gottesanbeterinnen (Mantodea). Das grundlegende Ziel dieser Arbeit war daher die Charakterisierung der Darmmikrobiota der Schabenart *S. lateralis* (Blattidae) anhand molekularer Techniken, einschließlich der hochauflösender Pyrosequenzierung.

Kropf und Mitteldarm waren in allen Individuen hauptsächlich von Milchsäurebakterien und *Bacteroidetes* besiedelt. Diese Beobachtung stimmte mit der hohen Laktatkonzentration in diesen Darmabschnitten überein, welche in allen Tieren gemessen wurde. Im Gegensatz dazu unterschied sich die Bakterienzusammensetzung von Dick- und Enddarm stark zwischen Individuen. Der Dickdarms, als sogenannter „Hotspot“ der bakteriellen Diversität, war überwiegend von *Bacteroidetes*, *Firmicutes* und Deltaproteobakterien besiedelt. Viele dieser Bakterien gehören zu Gruppen welche typisch für anaerobe Habitate sind, zum Beispiel Vertreter der Klasse der *Clostridia*, was die anaeroben Zentren widerspiegelt welche für jeden Darmabschnitt gezeigt wurden. *Spirochaetes* und *Fibrobacteres*, welche als die Zellulose abbauenden Gruppen innerhalb der höheren Termiten vermuten werden, konnten durch die Sequenzierung nach Sanger nicht nachgewiesen werden, wurden aber in niedriger Anzahl durch die Pyrosequenzierung nachgeweisen, was die omnivore Ernährung dieser Schabenart wiederspiegelt. Trotz der unterschiedlichen Ernährungsweise fielen 30 % der Sequenzen in Kluster, die ausschließlich aus Termitendarmsequenzen bestanden, welches die gemeinsame evolutionäre Entwicklung von Schaben und Termiten aus einem gemeinsamen Vorfahren wiederspiegelt.

Unterschiedliche Nahrung (Hühnerfutter als ausgewogene Ernährung, Soyaschrot als proteinreiche Nahrung, und Kleie und Kleie mit Zellulosezusatz als faserreiche

Nahrung) wurde benutzt, um den Einfluß von Nahrung auf die Zusammensetzung der Darmmikrobiota zu untersuchen. Ein möglicher Einfluß der Nahrung wurde durch individuelle Variabilität überlagert. Ordinationsanalysen der Darmprofile klustern nach Nahrung, welchem aber ein stärkeres Klustern nach Herkunft aus einem bestimmten Haltungsumgebung zugrundelag. Das deutet auf die Wichtigkeit der aus der Umwelt verfügbaren Bakterien, aus denen die Darmmikrobiota rekrutiert wird und anscheinend einen wesentlicheren Einfluß auf ihre Endzusammensetzung hat als die Art der Nahrung. Die Pyrosequenzierung zeigte ebenfalls keine großen Unterschiede in der Darmmikrobiota, brachte aber neun zusätzlich Phyla zu Tage, welche in sämtlichen Nahrungsgruppen zahlenmäßig sehr gering waren und somit zu den spärlich vertretenen Phyla gezählt wurden. Übereinstimmend wurde durch beide molekulare Methoden gezeigt, dass die Schabendarmnikrobiota starken individuellen Schwankungen unterliegt, auf die unterschiedliche Nahrung keinen großen Einfluß zu haben scheint. Damit übereinstimmend wurden auch keine Unterschiede in der Konzentration kurzkettiger Fettsäuren gefunden.

Methanogenese in Arthropoden ist auf wenige Arten beschränkt, namentlich Termiten, Blatthornkäfer (die sogenannten Pillendreher), Tausendfüßler und Schaben. In dieser Studie produzierten die meisten Individuen Methan, aber einige produzierten nur nach einer Zugabe von Wasserstoff detektierbare Mengen von Methan. Es wurden zwei Arten methanogener Archeen im Dickdarm gefunden, *Methanomicrococcus blatticola* und ein noch unkultivierter Vertreter der *Methanobrevibacter*. Dabei wurden keine Unterschiede in der Zusammensetzung dieser zwischen Methan produzierenden und nicht Methan produzierenden Schaben gefunden. Die im Dickdarm beobachteten Ciliaten gehörten zu der Spezies *Nyctotherus ovalis*. Die mit ihnen assoziierten Archeen wurden als uncultivierte Vertreter der *Methanobrevibacter* identifiziert. Dabei wurde für *S. lateralis* gezeigt, dass Nahrung keinen Einfluß auf die Menge des ausgestossenen Methans hatte, jedoch waren die gemessenen Wasserstoffkonzentrationen in den Kleie gefütterten Tieren am höchsten. Tiere die mit Hühnerfutter gefütter wurden zeigten den stärksten Anstieg in Methan Emission nach einer Inkubation mit Wasserstoff, wogegen Tiere die mit einer faserreichen Nahrung gefüttert wurden, keinen Anstieg der Methanemission zeigten oder sogar weniger Methan ausstießen. In allen Nahrungsgruppen wurden Tiere gefunden, die nicht zur Methanproduktion befähigt waren.

Contribution by other people to this work

Unless otherwise stated, all experiments were planned, conducted and evaluated by myself under the supervision of Prof. Andreas Brune.

Cloning and sequencing of archaeal 16S rRNA genes for the phylogenetic analysis of the archaeal composition in ME and NME individuals and sequencing of the 18 S rRNA gene of the hindgut ciliate were conducted by an undergraduate student Dorothee Tegthmeier, who did the analysis under my supervision during her practicum between Mai and September 2010. In this practicum also the light microscopy pictures of the ciliates were made.

Curiculum Vitae

Name	Christine Schauer
Address	Lindenstraße 22, D-84166 Adlkofen
Born	09.05.1980
Place of birth	Landshut, Germany

Academic studies

2008–2011	Max Planck Institute for Terrestrial Microbiology Marburg German Doctor of natural science
2007	Diploma thesis at the Universität Regensburg at the Faculty of Evolution, Behavior and Genetics "Verwandtenerkennung bei der Kompasstermite *Amitermes meridionalis*", Supervisor Prof. Dr. Judith Korb.
2005–2006	Field work and sample collection for the Diploma thesis in Darwin, Norther Territory, Australia
2000–2005	Universität Regensburg Diploma studies in Biology Main subjects: zoology, genetics, medical microbiology

School education

1986–1990 Volkshochschule Adlkofen
1990–1997 Gymnasium Seligenthal, Landshut
1997–1998 Christophorusschule, Braunschweig
1998–2000 Abitur at the IGS West, Braunschweig