The gut microbiota of the cockroach *Shelfordella lateralis*

Primary colonization, succession, and metabolic response to microenvironmental conditions

Dissertation

zur Erlangung des Doktorgrades der Naturwissenschaften (Dr. rer. nat.)
am Fachbereich Biologie der Philipps-Universität Marburg

vorgelegt von
Dorothee Tegtmeier
aus Minden

Universitätsstadt Marburg, 2016

Die Untersuchungen zur vorliegenden Arbeit wurden von Oktober 2012 bis Mai 2016 am Max-Planck-Institut für terrestrische Mikrobiologie in Marburg unter der Leitung von Prof. Dr. Andreas Brune durchgeführt.

Vom Fachbereich Biologie der Philipps-Universität Marburg als Dissertation angenommen am: 19.05.2016

Erstgutachter: Prof. Dr. Andreas Brune

Zweitgutachter: Prof. Dr. Wolfgang Buckel

Tag der Disputation: 19.07.2016

Die in dieser Dissertation beschriebenen Ergebnisse sind in folgenden Publikationen veröffentlicht bzw. zur Veröffentlichung vorgesehen:

Tegtmeier D, Thompson CL, Schauer C, Brune A. 2016. Oxygen affects gut bacterial colonization and metabolic activities in a gnotobiotic cockroach model. Appl. Environ. Microbiol. **82:**1080–1089.

Tegtmeier D, Riese C, Geissinger O, Radek R, Brune A. 2016. *Breznakia blatticola* gen. nov. sp. nov. and *Breznakia pachnodae* sp. nov., two fermenting bacteria isolated from insect guts, and emended description of the family *Erysipelotrichaceae*. Syst. Appl. Microbiol. **39:**319–329.

Tegtmeier D, Mikaelyan A, Thompson CL, Brune A. Microbial community succession is linked to changes in gut environment and metabolic processes in the cockroach *Shelfordella lateralis*. (in Vorbereitung).

Tegtmeier D*, Belitz A*, Radek R, Heimerl T, Brune A. *Ereboglobus luteus* gen. nov. sp. nov., a novel propionigenic verrucomicrobium isolated from the gut of the cockroach *Shelfordella lateralis*, and emended description of the genus *Diplosphaera*. (in Vorbereitung).

*Diese Autoren haben zu gleichen Teilen beigetragen.

Table of Content

Summary

Cockroaches and their closest relatives, the termites, have a highly complex gut microbiota consisting of different microbial guilds, e.g. fermenting bacteria, methanogenic archaea and protists, which form a metabolic network within the gut. While the microbiota of termites has been studied for decades, little is known about the cockroach gut microbiota, especially about the primary colonization, the succession and metabolic interactions of gut microorganisms.

In order to investigate successive changes of the cockroach gut microbiota during host development, I analyzed the bacterial community composition (using high throughput sequencing of the 16S rRNA gene) and major metabolites in different instars of *Shelfordella lateralis*. First instar nymphs were often colonized by aerobic or oxygen-tolerant, lactate-producing taxa (e.g. *Enterobacteriaceae*, *Enterococcaceae* and *Lactobacillales*), which was associated with a strong lactate accumulation in the gut. By contrast, cockroaches from second instar to adulthood where consistently colonized by anaerobic taxa (e.g. *Ruminococcaceae*, *Lachnospiraceae* and *Rikenellaceae*). Methane emission of living cockroaches was absent in the first instar, relatively low in all other instars but high in adults. For hydrogen emission I found an opposite trend: Emission rates decreased during cockroach development, suggesting an increase in hydrogen consumption by methanogens. Cockroaches that were reared in isolation showed no methane emission but a similar gut microbiota than conventional ones, indicating that the majority of the microorganisms are acquired from the environment, whereas methanogens are likely transferred via coprophagy. Microsensor measurements revealed anoxic conditions in the hindguts of all but the first instar, which suggests that oxygen is an important factor shaping the microbiota of the first instar. The strong differences in gut microbiota and metabolism between first and later instars indicate that the microbiota is assembled by stochastic events and altered depending on the oxygen status of the gut. The results further show that those microorganisms colonize the gut that are picked up from the environment and are favored by the conditions in the gut.

While the composition of the gut microbiota has been studied in detail for many insects, a mechanistic understanding of the metabolic activities and interactions among individual microbes is still lacking. A detailed characterization of gut microorganisms requires pure cultures. However, bacteria are exposed to unique microenvironmental conditions in the gut that differ fundamentally from those in pure culture. Therefore, studies that investigate how far the metabolic properties of pure cultures (*in vitro*) reflect their activities in their natural

environment (*in situ*) are of particular interest. We established the cockroach *Shelfordella lateralis* as a gnotobiotic model in order to examine metabolic activities and biotic interactions of individual bacterial populations under *in situ* conditions. Germ-free cockroaches were successfully colonized with the autochthonous strains EbSL (a facultatively anaerobic species of *Enterobacteriaceae*) and FuSL (an obligately anaerobic *Fusobacterium* sp.). When monoassociated, both strains grew to high density, but population sizes of strain EbSL were higher than that of strain FuSL. In diassociation however, the population size of strain FuSL was even smaller than in monoassociation. Although microsensor measurements showed that strain EbSL completely consumed the oxygen in the gut, precolonization with the facultative anaerobe did not favor colonization by the obligate anaerobe. The results showed that strain FuSL is outcompeted by strain EbSL, possibly due to a better colonization success of the facultative anaerobe in the oxic zones of the gut. Comparison of the fermentation products of the cultures formed *in vitro* with those accumulated *in situ* indicated that the gut environment strongly affected metabolic activities of both strains. Pure cultures of strains EbSL and FuSL formed the typical products of mixed-acid or butyrate fermentation, whereas guts of gnotobiotic cockroaches accumulated mostly lactate and acetate. Shifts towards acetate or lactate were also confirmed *in vitro* when pure cultures were exposed to oxygen or high glucose concentrations (respectively), conditions similar to those present in the hindguts of germ-free cockroaches.

In order to gather more information about hitherto uncultivated representatives of the obligately anaerobic bacterial community in the guts of *S. lateralis* and other insects, we isolated further members of their gut microbiota and described the ultrastructure, physiology and metabolism of the strains in detail. The novel isolates are strictly anaerobic or slightly aerotolerant representatives of new genera of *Erysipelotrichaceae* (*Firmicutes*) and *Opitutaceae* (*Verrucomicrobia*) and have a purely fermentative metabolism. While former (strains ErySL and Pei061) showed products of a typical mixed acid fermentation, the latter (strain Ho45) fermented glucose to propionate and acetate. Growth and fermentations of the novel isolates were strongly influenced by oxygen and glucose concentrations in the medium. Since the closest relatives of the novel isolates were incorrectly described as aerobic or microaerophilic in the literature, we provide an emended description of the family *Erysipelotrichaceae* and the genus *Diplosphaera* (*Opitutaceae*).

Zusammenfassung

Schaben und ihre nächsten Verwandten, die Termiten, beherbergen komplexe mikrobielle Gemeinschaften in ihrem Verdauungstrakt. Die Darmmikrobiota besteht aus unterschiedlichen mikrobiellen Gilden z.B. gärende Bakterien, methanogene Archaeen und Protisten, die ein metabolisches Netzwerk im Darm bilden. Während die Darmmikrobiota von Termiten bereits seit Jahrzehnten intensiv untersucht wird, ist verhältnismäßig wenig über die mikrobiellen Gemeinschaften in Schaben, insbesondere über die Erstbesiedelung des Darms und die Sukzession und metabolischen Interaktionen der Darmmikroorganismen bekannt.

Um die sukzessiven Veränderungen der Darmmikrobiota im Laufe der Entwicklung der Schabe zu untersuchen, habe ich die bakterielle Zusammensetzung mittels Hochdurchsatz-Sequenzierung sowie wichtige Stoffwechselprodukte in verschiedenen Larvenstadien der Schabe *Shelfordella lateralis* analysiert. Schaben des ersten Larvenstadiums wurden häufig von aeroben oder aerotoleranten, Lactat produzierenden Bakterien (z.B. *Enterobacteriaceae*, *Enterococcaceae* und *Lactobacillaceae*) besiedelt, was von einer Lactatakkumulation im Darm begleitet wurde. Im Gegensatz dazu, waren Schaben ab dem zweiten Larvenstadium bis zum Adultstadium beständig von obligat anaeroben Bakterien (z.B. *Ruminococcaceae*, *Lachnospiraceae* and *Rikenellaceae*) besiedelt. Während Schaben des ersten Larvenstadiums keine Methanemission aufwiesen, zeigten die anderen Larvenstadien geringe und adulte Schaben hohe Methanemissionsraten. Wasserstoffproduktion hingegen zeigte einen gegenteiligen Trend: Die Emissionsraten gingen im Laufe der Entwicklung der Schabe deutlich zurück, was z.T. durch eine Wasserstoffzehrung der methanogenen Archaeen zu erklären ist. Schaben, die in Isolation aufgewachsen sind, zeigten keine Methanemission, jedoch eine ähnliche Darmmikrobiota wie konventionelle Schaben. Dies ist vermutlich darauf zurückzuführen, dass die meisten Mikroorganismen aus der unmittelbaren Umgebung aufgenommen werden, methanogene Archaeen jedoch über Koprophagie übertragen werden. Mikrosensor-Messungen ergaben, dass im Enddarm aller Schaben, mit Ausnahme des ersten Larvenstadiums, anoxische Bedingungen herrschen, was darauf schließen lässt, dass Sauerstoff die mikrobielle Besiedlung des Darms im ersten Larvenstadium stark beeinflusst. Die ausgeprägten Unterschiede zwischen dem ersten Larvenstadium und späteren Stadien deuten darauf hin, dass der Aufbau der Darmmikrobiota nach stochastischen Ereignissen erfolgt und sich die Zusammensetzung der Mikrobiota abhängig vom Sauerstoff-Status des Darms verändert. Die Ergebnisse zeigen außerdem, dass Mikroorganismen den Darm

besiedeln, die aus der Umwelt aufgenommen werden und durch die Bedingungen im Darm begünstigt werden.

Während die Zusammensetzung der Darmmikrobiota für viele Insektenarten schon ausführlich untersucht wurde, fehlt uns immer noch ein mechanistisches Verständnis über die Stoffwechselaktivitäten und Interaktionen der einzelnen Bakterienpopulationen. Für eine detaillierte Charakterisierung der Mikroorganismen werden Reinkulturen benötigt. Allerdings sind die Bakterien im Darm völlig anderen Bedingungen ausgesetzt als in Reinkultur. Daher sind Studien nötig, welche prüfen, in wie weit die Stoffwechselleistungen der Reinkulturen (*in vitro*) auch ihre eigentlichen Aktivitäten in ihrem natürlichen Standort (*in situ*) widerspiegeln. Wir haben die Schabe *Shelfordella lateralis* als gnotobiotisches Modellsystem etabliert, um Stoffwechsel und biotische Interaktionen einzelner Bakterienpopulationen unter *in-situ*-Bedingungen zu untersuchen. Keimfreie Schaben wurden erfolgreich mit den autochthonen Stämmen EbSL (ein fakultativ anaerober Stamm aus der Familie der *Enterobacteriaceae*) und FuSL (ein obligat anaerober *Fusobacterium*-Stamm) besiedelt. In Monoassoziation haben beide Stämme den Darm in hoher Dichte besiedelt, allerdings waren die Populationsgrößen von Stamm FuSL niedriger als die von Stamm EbSL. Weiterhin waren Populationsgrößen von Stamm FuSL in Diassoziation mit Stamm EbSL wesentlich niedriger als in Monoassoziation. Obwohl Messungen mit Sauerstoffelektroden zeigten, dass Stamm EbSL den Sauerstoff im Darm fast komplett verbraucht hat, hat die Vorbesiedlung mit dem fakultativ anaeroben Stamm die Besiedlung mit dem obligat anaeroben Stamm nicht begünstigt. Die Ergebnisse zeigen, dass Stamm FuSL von Stamm EbSL aus dem Darm verdrängt wird, vermutlich weil der fakultativ anaerobe Stamm erfolgreicher die oxischen Bereiche des Darms besiedeln kann. Ein Vergleich der Gärprodukte der Stämme *in vitro* mit denen *in situ* (im Darm) zeigte, dass die Umgebung im Darm die metabolischen Aktivitäten beider Stämme stark beeinflusst. Während Reinkulturen von Stamm EbSL und FuSL die typischen Produkte einer gemischten Säuregärung oder Buttersäuregärung bildeten, akkumulierten in den Därmen der gnotobiotischen Schaben hauptsächlich Lactat und Acetat. Solche Verschiebungen hin zum Acetat oder Lactat wurden *in vitro* bestätigt, wenn die Reinkulturen Sauerstoff oder hohen Glucosekonzentrationen, Bedingungen ähnlich wie im Darm der keimfreien Schaben, ausgesetzt wurden.

Um weitere Kenntnisse über bislang unkultivierte Vertreter der obligat anaeroben bakteriellen Gemeinschaft im Darm von *S. lateralis* und anderen Insekten zu gewinnen, haben wir weitere Mitglieder aus deren Darmmikrobiota in Reinkultur gebracht und die Ultrastruktur,

Physiologie und Metabolismus der Stämme im Detail untersucht. Bei den Isolaten handelt es sich um strikt anaerobe oder moderat aerotolerante Vertreter von neuen Gattungen in den Familien *Erysipelotrichaceae* (*Firmicutes*) sowie *Opitutaceae* (*Verrucomicrobia*), welche einen rein fermentativen Metabolismus aufweisen. Während erstere (die Stämme ErySL und Pei061) die typischen Produkte einer gemischten Säuregärung bildeten, vergärte letzterer (Stamm Ho45) Glucose zu Propionat und Acetat. Sauerstoff- und Glucosekonzentration im Medium beeinflussten deutlich das Wachstum und die Gärung der neuen Isolate. Da die nächsten Verwandten der neuen Isolate in der Literatur fälschlicherweise als aerob oder mikroaerophil beschrieben wurden, haben wir die Beschreibung der Familie *Erysipelotrichaceae* und der Gattung *Diplosphaera* (*Opitutaceae*) überarbeitet.

Chapter 1

General Introduction

Cockroaches: Phylogeny and evolution

Cockroaches (Blattodea), termites (Isoptera), and mantids (Mantodea) belong to the superorder Dictyoptera (Kristensen, 1981; Maekawa et al., 1999; Deitz et al., 2003). The cockroaches are split into the major families Corydiidae, Nocticolidae, Blattidae, Ectobiidae, and Blaberidae (Figure 1.1). Termites are the closest relatives of the blattid cockroaches and evolved from an omnivorous cockroach ancestor (Inward et al., 2007). Traditionally termites and cockroaches were considered as two distinct orders, but molecular phylogenetic analyses have shown that termites represent a monophyletic group within the order Blattodea (Inward et al., 2007; Legendre et al., 2008; Figure 1.1). Due to these findings, termites, which have evolved a colony-forming lifestyle, were considered as a family of eusocial cockroaches (Inward et al., 2007). Although it is now generally accepted that termites originated within the radiation of cockroaches, the order Isoptera was retained (Lo et al., 2007; Djernæs et al., 2012; Djernæs et al., 2015).

The split between the termite line and the blattid cockroaches occurred during the Middle Jurassic (150–170 Mya). Around this time, a common ancestor of termites and their sister group, the Cryptocercidae ("wood roaches") acquired cellulolytic flagellates, which gave them the ability to digest wood (lignocellulose) (Brune and Dietrich, 2015).

Like termites, cockroaches harbor a dense microbial community in their intestinal tract. Cockroaches of the family Blattidae are mostly omnivorous, but members of the Blaberidae are also able to feed on wood or leaf-litter (Pellens et al., 2002; Maekawa et al., 2003). The close relationship to termites, along with a complex and diverse gut microbiota, make cockroaches an interesting research subject in microbial ecology.

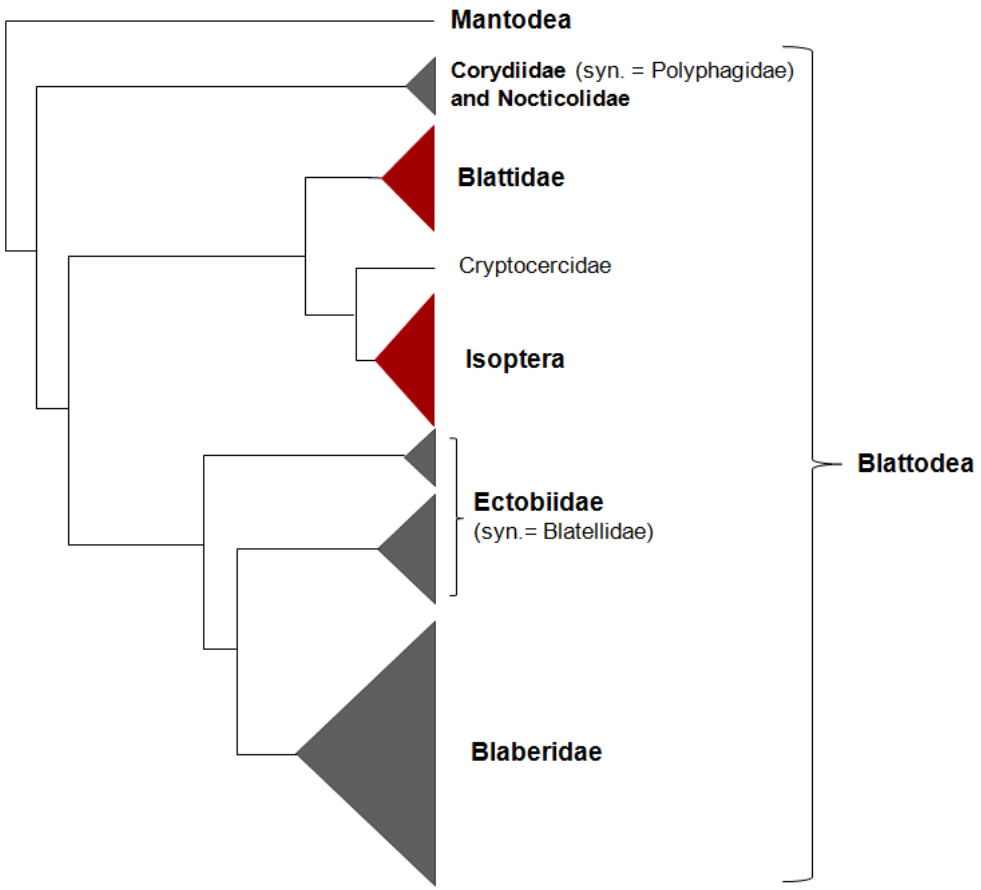

Figure 1.1. Simplified phylogenetic tree of the superorder Dictyoptera. The phylogeny is based on Inward et al., (2007), including the recent taxonomy (Beccaloni and Eggleton, 2011; Djernæs et al., 2012). The tree shows the position of the termites (Isoptera) within the order of cockroaches (Blattodea). Termites + Cryptocercidae are a sister group of the Blattidae family.

The cockroach gut: Structure, function and physiochemical conditions

The cockroach gut is divided into foregut, midgut and hindgut. The foregut consists of a large crop and a gizzard (proventriculus), which are lined with cuticle (Wigglesworth, 1972; Bignell, 1981). The gizzard of Blattidae has strong teeth for grinding food (Snodgrass, 1935; Bignell, 1981). The salivary glands release digestive enzymes such as amylases (Wigglesworth, 1972) and cellulases that are secreted into the foregut and midgut lumen (Watanabe and Tokuda, 2010).

The foregut is followed by a tubular midgut. The midgut epithelium has tall, columnar cells that are involved in the production and secretion of digestive enzymes and the absorption of nutrients (Bignell, 1981; Chapman, 2013). The absorptive surface of the midgut is protected by a chitinous layer, the peritrophic membrane, which separates the food bolus from the

epithelium. This layer has pores through which enzymes, peptides and small organic molecules (e.g. sugars and amino acids), but no lipids and large polysaccharides can pass (Chapman, 2013). The midgut carries eight gastric caeca on the anterior part. These blind-ending tubules secrete digestive enzymes like invertases, maltases, lipases and proteases, into the lumen of the midgut (Cornwell, 1968). Furthermore, the gastric caeca are the main place for glucose absorption (Treherne, 1957). Glucose and other monosaccharides are transported by passive diffusion that is facilitated by their conversion into trehalose in the haemolymph and fat body (Treherne, 1958).

The hindgut is an enlarged fermentation chamber. It consists of a small ileum, an anterior and posterior colon and a rectum, which are also lined with cuticle. The hindgut cuticle carries numerous spines that provide attachment sites for bacteria (Bignell, 1980). The cuticle in the hindgut is thinner than that in the foregut and permeable to water. Ions, fatty acids, amino acids and products of bacterial fermentation are resorbed and transported into the haemolymph. At the junction of the midgut and the hindgut lie the Malpighian tubules, which have an excretory and osmoregulatory function. In the rectum, water is absorbed by the rectal glands, and fecal pellets are excreted (Bignell, 1981).

The whole gut of the adult cockroach *Shelfordella lateralis* contributes to around 13% of the whole body weight of the cockroach (Schauer et al., 2012). Microsensor measurements with oxygen electrodes in guts of adult cockroaches have shown that the center of all gut compartments is anoxic. Furthermore, the redox potential is negative in all compartments except in the posterior hindgut at the thin passage from the colon to rectum. These conditions are accompanied by large and diverse populations of anaerobic microorganisms (Schauer et al., 2012). By contrast, in termites oxygen could be detected in all gut sections, with the exception of the enlarged paunch (Brune et al., 1995; Köhler et al., 2012). Some individuals of *S. lateralis* have shown a strong hydrogen accumulation in the midgut and the anterior hindgut. The pH is slightly acidic in the foregut, showing values around 5.8, and increases in the midgut and hindgut up to 6.8 (Schauer et al., 2012) (Figure 1.2).

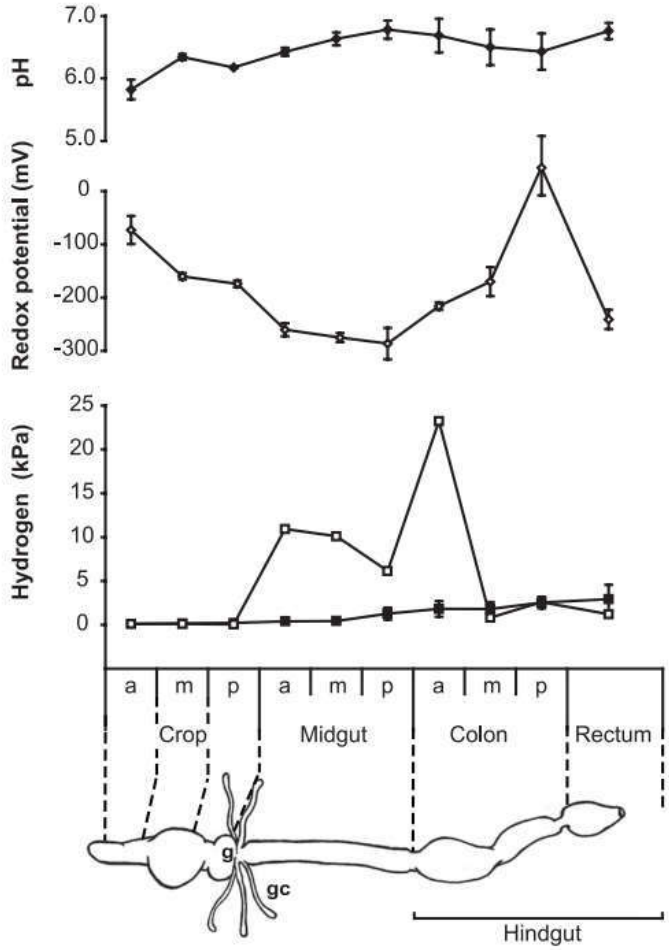

Figure 1.2. Physiochemical conditions in the gut of *S. lateralis* in each gut compartment. The gut is divided into crop with gizzard (g), midgut with gastric caeca (gc) and hindgut that consists of a colon and a rectum. pH values are indicated at the top, the redox potential in the middle and the hydrogen partial pressure at the bottom. Open squares show a typical profile of an individual with strong hydrogen accumulation and filled squares show values of individuals without hydrogen accumulation (from Schauer et al., 2012).

The cockroach gut microbiota

While the termite gut microbiota has been intensively studied (e.g. Schmitt-Wagner et al., 2003; Hongoh et al., 2006; Brune and Dietrich, 2015), little is known about the gut microbiota of cockroaches, especially about the acquisition and succession of its members and their individual functions in the gut ecosystem. Most studies on cockroach gut microbiota (e.g. Burgess et al., 1973; Cruden and Markovetz, 1984; Cloarec et al., 1992) are based on culture-dependent techniques, allowing only limited insights in the actual microbial diversity and abundance, since the majority of microorganisms is so far uncultivated. Many of these studies

focused on the cockroach as potential reservoir for pathogens, because often aerobic or facultatively anaerobic bacteria, including several pathogens, were isolated from the cockroach gut and were considered as abundant groups (e.g. Burgess et al., 1973; Cloarec et al., 1992).

The first culture-independent evaluations of the cockroach gut microbiota (Schauer et al., 2012; Schauer et al., 2014) revealed novel insights: Clone libraries and 454 pyrotag sequencing of 16S rRNA genes from the hindgut of *S. lateralis* indicated that the majority of the gut microbiota is comprised of hitherto uncultivated, presumably obligately anaerobic bacteria. However, most of the microbes previously isolated from cockroaches were of low abundance or even absent from the clone libraries. The most abundant phyla of the colonic gut microbiota are represented by *Bacteroidetes*, *Firmicutes* (mainly *Clostridia*) and *Proteobacteria*. *Fusobacteria*, *Planctomycetes* and *Actinobacteia* were also frequently present (Figure 1.3). *Spirochaetes* and *Fibrobacteres*, which are abundant phyla in the termite gut, were absent or of very low abundance in *S. lateralis*.

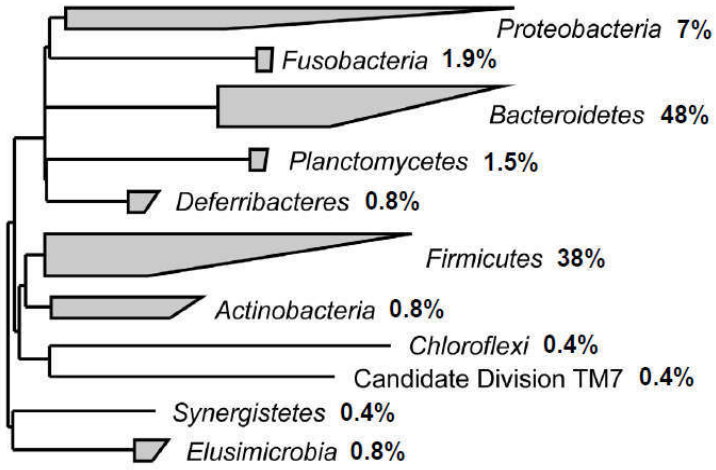

Figure 1.3. Maximum parsimony tree showing the phylum level affiliation of 251 full length 16S rRNA sequences obtained from the colon of *S. lateralis*. The relative abundance of each of the 11 detected phyla is displayed after the phylum name (modified from Schauer, 2011 and Schauer et al., 2012).

The composition of the gut microbiota of *S. lateralis* is quite similar to complex intestinal communities of mammals. The dominance of *Bacteroidetes* and *Firmicutes* is typical for omnivorous mammals like mice and humans but not for wood-feeding termites. Nevertheless, the gut microbiota of cockroaches exhibits also many common lineages with termites, which reflects their close relationship (Schauer et al., 2012; Dietrich et al., 2014). The majority of the

microbial diversity and abundance was found in the hindgut (Schauer et al., 2012), which is also characteristic for the termite gut microbiota (Schmitt-Wagner et al., 2003; Köhler et al., 2012).

Besides numerous bacteria, the cockroach gut harbors also methanogenic archaea. However, in contrast to the bacterial community, the archaeal community is of low diversity. Two abundant methanogenic phylotypes, *Methanimicrococcus blatticola* and an uncultivated *Methanobrevibacter* species, were detected via 454 pyrosequencing in the guts of *S. lateralis* and several other cockroach species (Dietrich, 2015, doctoral thesis). These two phylotypes were also detected in the closely related cockroach *Periplaneta americana* (Gijzen et al., 1991; Sprenger et al., 2000). Furthermore, anaerobic ciliates (*Nyctotherus ovalis*), which harbor endosymbiotic *Methanobrevibacter* species, were found in large number in the guts of *P. americana* (Gijzen et al., 1991; Gijzen and Barugahare, 1992).

The metabolic network in the insect gut

Besides the endogenous enzymes of the hosts (see above), a variety of microorganisms are involved in the digestion of organic material in insect guts. In the termite gut, the anaerobic breakdown of plant fiber starts with the decomposition of polymers (e.g. cellulose and hemicellulose) to monomeric carbohydrates. In lower termites, flagellates efficiently break down phagocytized wood via cellulases (exoglucanases and endoglucanases) and hemicellulases (Hongoh, 2011; Brune and Dietrich, 2015). In wood-feeding higher termites, which lack cellulolytic flagellates, bacteria like *Fibrobacteres*, members of the TG3 phylum, and possibly also the *Spirochaetes*, are mainly involved in cellulose degradation (Mikaelyan et al., 2014). The depolymerization products (e.g. glucose) are further degraded by the polymer degraders or other primary fermenting bacteria to short chain fatty acids, lactate, ethanol, CO_2, and hydrogen (Brune, 2014; Brune and Dietrich, 2015). Acetate is a major fermentation product in termite and cockroach guts (Hungate 1943; Odelson and Breznak, 1983; Tholen and Brune, 2000; Schauer et al., 2012; Bauer et al., 2015) and also an important energy source for the host (Hungate 1943; Odelson and Breznak, 1983). Lactate, ethanol and succinate are important substrates for a further degradation by secondary fermenters, e.g. propionigenic bacteria (Tholen and Brune 2000; Boga et al., 2007). Hydrogen and CO_2 are the central substrates for methanogenic archaea and homoacetogenic bacteria, which perform the last step of the anaerobic breakdown.

Besides termites, millipedes, and scarab beetle larvae, cockroaches are the only known methane producing arthropods so far (Hackstein and Stumm, 1994). Due to their huge abundance in tropical and subtropical regions, termites contribute up to 4% to the global methane budget (Sugimoto et al., 2000). However, studies about the methanogenic degradation of organic matter in cockroaches, especially about the microbial guilds that are involved in this process, are rare. Schauer et al. (2014) found large differences in methane emission among individuals of *S. lateralis*, which is also in agreement with a significant individual variation of the microbial community composition in the gut. Hydrogenotrophic methanogenesis is the most common type of methane formation in termites. It is supposed to be the origin of methanogenesis, and other modes of methanogenesis subsequently evolved from this pathway (Bapteste et al., 2005). In this process, methane is formed from hydrogen and CO_2:

$$CO_2 + 4\ H_2 \rightarrow CH_4 + 2\ H_2O$$

The hydrogenotrophic methanogens *Methanobrevibacter curvatus, Methanobrevibacter cuticularis*, and *Methanobrevibacter filiformis* are so far the only described methanogens from termite guts (Leadbetter and Breznak, 1996; Leadbetter et al., 1998). In cockroaches, the hydrogen-dependent reduction of methanol by *Methanimicrococcus blatticola* (so far the only methanogenic isolate from cockroaches), probably contributes significantly to the methane budget (Sprenger et al., 2005):

$$CH_3OH + H_2 \rightarrow CH_4 + H_2O$$

Both types of methanogenesis require hydrogen as substrate. In lower termites the fermentation of carbohydrates by anaerobic flagellates constitutes a major hydrogen source in the gut (Odelson and Breznak, 1985; Pester and Brune, 2007). In higher termites, and probably other arthropods, which do not harbor flagellates, fermenting bacteria are responsible for the hydrogen emission (Brune, 2010).

Like flagellates in termites, anaerobic ciliates in cockroach guts are an important source for hydrogen. These ciliates possess hydrogenosomes, in which pyruvate is fermented to acetate and hydrogen (Müller, 1993). The ciliates acquired methanogenic endosymbionts that use hydrogen for the reduction of CO_2 to methane (Gijzen et al., 1991). Therefore, methane emission was found to be positively correlated with the amount of intestinal ciliates (Gijzen et al., 1991).

Methanol, another major substrate for methanogenesis, likely arises from the fermentative breakdown of the plant polymers pectin (Schink and Zeikus, 1980) and xylan (Paul et al.,

2012). However, it has not yet been determined which microbes are involved in pectin or xylan degradation in the cockroach gut.

Besides methanogenesis, hydrogen also fuels reductive acetogenesis (homoacetogenesis), whereby acetate is formed as the only end product from hydrogen and CO_2 (Diekert and Wolfarth, 1994):

$$2\ CO_2 + 4\ H_2 \rightarrow CH_3COO^- + H^+ + 2\ H_2O$$

Homoacetogens could also play an important role in hydrogen consumption, especially when methanogens are not present (Fonty et al., 2007). Homoacetogenesis is an important process in wood-feeding termites (Brauman et al., 1992) and the "wood roach" *Cryptocercus punctulatus* (Breznak and Switzer, 1986). However, in the omnivorous cockroach *Periplaneta americana*, homoacetogenesis is only a minor metabolic process (Kane and Breznak, 1991) and most of the CO_2 and hydrogen is incorporated in methane rather than acetate (Breznak and Switzer, 1986).

Microbial community assembly and succession

Most of the present studies have investigated the gut microbiota of adult cockroaches (e.g. Schauer et al., 2012; Schauer et al., 2014; 2014; Bauer et al., 2015), which have already established a climax community in their guts. By contrast, little is known about the acquisition of the microorganisms, their succession throughout the development of the host and the factors that shape community assembly.

Following hatching the cockroach nymphs typically acquire their gut microbiota from the environment, by ingestion of microorganisms together with the food source or by coprophagy (Klass et al., 2008; Nalepa, 2011; Nalepa, 2015) and the final composition and relative abundance of the gut microbiota of *S. lateralis* individuals was highly variable (Schauer et al., 2014). In the case of termites, however, it has been suggested that vertical inheritance is the primary force shaping the gut microbiota (Rahman et al., 2015). In lower termites, the symbiotic gut microbiota is transferred to the offspring by direct inoculation of nestmates (proctodeal trophallaxis) (e.g. Suarez et al., 2000; Nalepa, 2015), which results in highly similar microbial communities in individuals of the same species (Benjamino and Graf, 2016). In higher termites, also host phylogeny and diet were recently discussed as important

determinants of the bacterial community structure (Dietrich et al., 2014; Brune and Dietrich, 2015; Mikaelyan et al., 2015).

The gut microbiota is also influenced by the life cycle of the host. During larval development the insect gut is a very unstable habitat. In holometabolous insects, with distinct larval, pupal and adult stages, the gut and other organs are completely remodeled during metamorphosis, resulting in the elimination of the entire larval gut together with the gut microbiota (Moll et al., 2001; Engel and Moran 2013). Cockroaches and termites are hemimetabolous insects and undergo several nymphal stages, called instars, until adulthood (sexual maturity). During this nymphal phase the insect molts several times to reach the next instar. Since the cuticle of crop and hindgut is shed during the molt, this process causes a disruption or alteration of the gut microbiota as microbes, especially those that are attached to the cuticle, are (at least partially) lost (Engel and Moran 2013). However, mechanisms of recolonization in cockroaches and potential changes in the gut microbiota with the development of the host remain to be investigated.

Gnotobiotic model systems

While the analysis of the microbial community composition is a well-established procedure in microbial ecology, the functions and interactions of the individual members are difficult to elucidate. A detailed characterization of gut microorganisms requires pure cultures. However, the intestinal tract of insects comprises unique microenvironmental conditions (e.g. Engel and Moran, 2013; Brune and Dietrich, 2015). Therefore, metabolic properties of bacteria *in vitro* may not reflect their activities in their natural environment, the gut ecosystem. Nevertheless, studies with conventional animals with a complex gut microbiota do not allow investigations of specific metabolic processes or interactions of microorganisms separately from other intestinal processes. An experimental approach to investigate the metabolism and ecological interactions among community members requires the use of a suitable germ-free model.

Germ-free animals are free of all associated forms of life like bacteria, viruses, fungi, protozoa and other saprophytic or parasitic organisms (Gordon and Pesti, 1971). Such animals are specifically born and reared under aseptic conditions, which prevents the exposure to any kind of microorganisms and the acquisition of a gut microbiota. Germ-free (axenic) animals can be specifically inoculated with microorganisms, e.g. with indigenous (autochthonous) of foreign (allochthonous) microorganisms. Such gnotobiotic animals (with a known microbiota, Gordon

and Pesti, 1971) can be used to characterize the response of pure cultures of gut bacteria to their natural habitat (*in situ*) and their interactions with other strains, and may further allow to construct complex microbial networks.

Gnotobiotic studies focused mostly on mammals, particularly rats, mice, and piglets (Noack et al., 2000; Bäckhed et al., 2004; Samuel and Gordon, 2006; Kozakova et al., 2006). While germ-free mammals can be obtained only by Cesarean section, germ-free insects are easily generated by chemical surface sterilization of eggs. To date few well-established gnotobiotic insect model systems are available, e.g. the desert locus (Dillon and Charnley, 2002), mosquitoes (e.g. Micks and Ferguson, 1961; Hamilton and Bradley, 1977; Coon et al., 2014), the *Nasonia* wasp (Brucker and Bordenstein, 2013), or the fruit fly *Drosophila melanogaster* (e.g. Muyskens and Guillemin, 2008; Newell and Douglas, 2014). Most of these insects, e.g. fruit flies and mosquitoes are known to have a low diversity gut microbiota consisting mostly of aerobic and facultatively anaerobic bacteria (Wong et al., 2011; Coon et al., 2014). Moreover, insects like the desert locust have only a low bacterial density in their guts (Cazemier et al., 1997). However, processes like methanogenesis are only found in animals whose guts are densely colonized by complex microbial communities (Cazemier et al., 1997; Hackstein and Stumm, 1994).

Therefore, studies of insects with a highly abundant and diverse gut microbiota like termites and cockroaches are of particular interest. However, termites rely on their gut microbiota for food digestion and the early instars depend on brood care by colony members (Cleveland, 1924; Eutick et al., 1978; Nalepa, 1990; Nalepa 2015), which makes them unsuitable as gnotobiotic models. By contrast, their closest relatives, the non-social omnivorous cockroaches, do not obligately depend on parental care, though many species show aggregation behavior (Bell et al., 2007). All cockroaches possess an intracellular symbiont that is inherited via the germ line (Lo et al., 2003). These symbionts belong to the genus *Blattabacterium* and do not occur in the gut but colonize special cells of the fat body. They have an essential role in both nitrogen recycling and providing nutrients (Sabree et al., 2009), which makes the cockroach less dependent on gut bacterial symbionts than termites. Early studies by Doll et al. (1963) and Benschoter and Wrenn (1972) have already shown that the cockroach *Blatella germanica* can be reared and maintained under axenic conditions.

Studies with gnotobiotic insects require the use of suitable model strains that are able to robustly colonize the gut. Husseneder et al. (2004) and Husseneder and Grace (2005) have shown that an allochthonous GFP-labeled *E. coli* strain did not colonize the guts of

conventional termites, whereas an autochthonous GFP-labeled strain of *Enterobacter cloacae* persisted in the gut up to 11 weeks after inoculation. Similar studies with the collembolan *Folsomia candida* have shown that tagged *E. coli* disappeared from the gut after one day, but the indigenous species *Alcaligenes faecalis* persisted for more than 55 days in the collembolan gut (Thimm et al., 1998). Therefore, autochthonous strains likely have a better chance to colonize the gut than foreign ones and are preferred for gnotobiotic studies.

Aims of the study

The aim of this work was to study the primary colonization, the succession and the metabolism of microbial populations in cockroach guts. I wanted to identify factors that shape microbial community assembly, to investigate the metabolism of gut bacteria *in vitro* and to compare their activities to that in their natural environment *in situ*.

Acquisition and succession of the gut microbiota. My aim was to investigate the acquisition and succession of the microbiota in the cockroach gut and to identify factors which shape the community composition. I studied metabolic processes, like methane and hydrogen emission of different developmental stages of the cockroach *Shelfordella lateralis* via gaschromatography (GC) and analyzed gut metabolites using high performance liquid chromatography (HPLC). Furthermore, I determined the oxygen status of the gut in the early cockroach development using microsensors. To clarify if changes in metabolic processes and gut conditions are linked to changes in the gut microbiota, I analyzed the microbial diversity and density in cockroach guts using high Illumina-throughput sequencing and real-time PCR.

***In situ* activities of gut bacteria in a gnotobiotic cockroach model.** My aim was to study interactions of gut bacteria under *in situ* conditions and to investigate how far the metabolic properties of pure cultures reflect their activities in their natural environment.

I worked on the establishment of a gnotobiotic cockroach model and the isolation of autochthonous bacteria from cockroach guts in order to create synthetic microbial communities in their natural environment (*in situ*). One goal was to test the hypothesis if precolonization by facultatively anaerobic bacteria favors the colonization by obligate anaerobes. Therefore, I colonized germ-free cockroaches with facultatively and an obligately anaerobic fermenting bacteria and analyzed their colonization success via fluorescence microscopy and real-time PCR. The second aim of the study was to compare the metabolism of the strains *in vitro* with their activities in the gut environment (*in situ*) and to identify factors

which influence microbial metabolism. I analyzed the oxygen status of the gut using microsensors and determined metabolic product profiles via HPLC and GC.

Novel isolates from insect guts. In order to gain more insights into functions and metabolism of obligately anaerobic, hitherto uncultivated bacteria in the guts of cockroaches and other insects, my goal was to isolate and describe novel lineages of the insect gut microbiota. Using anaerobic cultivation techniques, I isolated new genera of *Erysipelotrichaceae* and *Opitutaceae*, and worked on their physiological and ultrastructural characterization. I tested the response of the isolates to different oxygen and glucose concentrations, which are factors that influence microbial activities like colonization success and metabolic product profiles in the gut environment (see chapter 2). Based on novel findings on the energy metabolism and the response to oxygen of close relatives of the novel isolates and an intensive literature research, we provided an emended description of the family *Erysipelotrichaceae* and the genus *Diplosphaera*.

References

1. **Bäckhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, Semenkovich CF, Gordon JI.** 2004. The gut microbiota as an environmental factor that regulates fat storage. Proc Natl Acad Sci USA **101:**15718–15723.
2. **Bapteste E, Brochier C, Boucher Y.** 2005. Higher-level classification of the Archaea: evolution of methanogenesis and methanogens. Archaea **1:**353–363.
3. **Bauer E, Lampert N, Mikaelyan A, Köhler T, Maekawa K, Brune A.** 2015. Physicochemical conditions, metabolites, and community structure of the bacterial microbiota in the gut of wood-feeding cockroaches (Blaberidae : Panesthiinae). FEMS Microbiol Ecol **91:**1–14.
4. **Beccaloni GW, Eggleton P.** 2011. Order Blattodea Brunner von Wattenwyl 1882, p 199–200. *In* Zhang, Z-Q (ed), Animal Biodiversity: An outline of higher-level classification and survey of taxonomic richness. Zootaxa 3148. Magnolia Press, Auckland, NZ.
5. **Bell WJ, Roth LM, Nalepa CA.** 2007. Cockroaches: Ecology, Behavior, and Natural History. Johns Hopkins University Press, Baltimore, Maryland.

6. **Benjamino J, Graf J.** 2016. Characterization of the core and caste-specific microbiota in the termite, *Reticulitermes flavipes*. Front Microbiol **7:**171. doi: 10.3389/fmicb.2016.00171
7. **Benschoter CA, Wrenn, RT.** 1972. Germfree techniques for establishment and maintenance of a colony of aseptic german cockroaches. Ann Entomol Soc Am **65**:641–644.
8. **Bignell DE.** 1980. An ultrastructural study and stereological analysis of the colon wall in the cockroach *Periplaneta americana*. Tissue Cell **12:**153–164.
9. **Bignell DE.** 1981. Nutrition and digestion, p 57–86. *In* Bell WJ, Adiyodi KG (ed), The American cockroach, Chapman & Hall, London,UK.
10. **Boga HI, Ji R, Ludwig W, Brune A.** 2007. *Sporotalea propionica* gen. nov. sp. nov., a hydrogen-oxidizing, oxygen-reducing, propionigenic firmicute from the intestinal tract of a soilfeeding termite. Arch Microbiol **187:**15–27.
11. **Brauman A, Kane MD, Labat M, Breznak JA.** 1992. Genesis of acetate and methane by gut bacteria of nutritionally diverse termites. Science **257:**1384–1387.
12. **Breznak, JA, Switzer JM.** 1986. Acetate synthesis from H_2 plus CO_2 by termite gut microbes. Appl Environ Microbiol **52:**623–630.
13. **Brucker RM, Bordenstein SR.** 2013. The hologenomic basis of speciation: Gut bacteria cause hybrid lethality in the genus *Nasonia*. Science **341:**667–669
14. **Brune A, Dietrich C.** 2015. The gut microbiota of termites: digesting the diversity in the light of ecology and evolution. Annu Rev Microbiol **69:**145–66.
15. **Brune A, Emerson D, Breznak JA.** 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.
16. **Brune A.** 2010. Methanogens in the digestive tract of termites, p 81–100. *In* Hackstein JHP (ed), (Endo)symbiotic Methanogenic Archaea. Springer, Heidelberg, Germany.
17. **Brune A.** 2014. Symbiotic digestion of lignocellulose in termite guts. Nat Rev Microbiol **12**:168–80.
18. **Burgess NRH, McDermott SN, Whiting J.** 1973. Aerobic bacteria occurring in the hind-gut of the cockroach, *Blatta orientalis*. J Hyg **71:**1–7.
19. **Cazemier AE, Op den Camp HJM, Hackstein JHP, Vogels GD.** 1997. Fibre digestion in arthropods. Comp Biochem Physiol **118:**101–109.

20. **Chapman RF.** 2013. The alimentary canal, digestion and absorption, p 46–79. *In* Simpson SJ, Douglas AE (ed), The insects: structure and function, 5th ed, Cambridge Univ. Press, Cambridge, UK.
21. **Cleveland LR.** 1924. The physiological and symbiotic relationships between the intestinal protozoa of termites and their host, with special reference to *Reticulitermes flavipes* Kollar. Biol Bull Mar Biol Lab **46:**178–227.
22. **Cloarec A, Rivault C, Fontaine F, Le Guyader A.** 1992. Cockroaches as carriers of bacteria in multi-family dwellings. Epidemiol Infec **109:**483–490.
23. **Coon KL, Vogel KJ, Brown MR, Strand MR.** 2014. Mosquitoes rely on their gut microbiota for development. Mol Ecol **23:**2727–2739.
24. **Cornwell PB.** 1968. Alimentary canal and digestion. p 117–33. *In* PB Cronwell (ed), The Cockroach, Hutchinson, London, UK.
25. **Cruden DL, Markovetz AJ.** 1984. Microbial aspects of the cockroach hindgut. Arch Microbiol **138:**131–139.
26. **Deitz LL, Nalepa C, Klass KD.** 2003. Phylogeny of the Dictyoptera Re-examined (Insecta). Entomologische Abhandlungen **61:**69–91.
27. **Diekert G, Wohlfarth G.** 1994. Metabolism of homoacetogens. Antonie Van Leeuwenhoek **66:**209–221.
28. **Dietrich CD.** 2015. Ecological and evolutionary drivers of microbial community structure in termite guts. Dissertation. Phillips-Universität Marburg.
29. **Dietrich C, Köhler T, Brune A.** 2014. The cockroach origin of the termite gut microbiota: patterns in bacterial community structure reflect major evolutionary events. Appl Environ Microbiol **80:**2261–9.
30. **Dillon RJ, Charnley AK.** 2002. Mutualism between the desert locust *Schistocerca gregaria* and its gut microbiota. Res Microbiol **153:**503–509.
31. **Djernæs M, Klass K-D, Picker MD, Damgaard J.** 2012. Phylogeny of cockroaches (Insecta, Dictyoptera, Blattodea), with placement of aberrant taxa and exploration of out-group sampling. Syst Entomol **37:**65–83.
32. **Djernæs M, Klass K-D, Eggleton P.** 2015. Identifying possible sister groups of Cryptocercidae+Isoptera: A combined molecular and morphological phylogeny of Dictyoptera. Mol Phylogenet Evol **84:**284-303
33. **Doll JP, Trexler PC, Reynolds LI, Bernard GR**. 1963. The use of peracetic acid to obtain germfree invertebrate eggs for gnotobiotic studies. Am Midl Nat **69:**231–239.

34. **Engel P, Moran NA.** 2013. The gut microbiota of insects – diversity in structure and function. FEMS Microbiol Rev **37:**699–735.
35. **Eutick ML, Veivers P, O'Brien RW, Slaytor M.** 1978. Dependence of the higher termite, *Nasutitermes exitiosus* and the lower termite, *Coptotermes lacteus* on their gut flora. J Insect Physiol **24:**363–368.
36. **Fonty G, Joblin K, Chavarot M, Roux R, Naylor G, Michallon F.** 2007. Establishment and development of ruminal hydrogenotrophs in methanogen-free lambs. Appl Environ Microbiol **73:**6391–6403.
37. **Gijzen HJ, Broers CA, Barugahare M, Stumm CK.** 1991. Methanogenic bacteria as endosymbionts of the ciliate *Nyctotherus ovalis* in the cockroach hindgut. Appl Environ Microbiol **57:**1630–1634.
38. **Gijzen HJ, Barugahare M.** 1992. Contribution of anaerobic protozoa and methanogens to hindgut metabolic activities of the American cockroach, *Periplaneta americana*. Appl Environ Microbiol **58:**2565-2570.
39. **Gordon HA, Pesti L.** 1971. The gnotobiotic animal as a tool in the study of host microbial relationships. Bacteriological Reviews **35:**390–429.
40. **Hackstein JHP, Stumm CK.** 1994. Methane production in terrestrial arthropods. Proc Natl Acad Sci USA **91:**5441–5445.
41. **Hamilton DR, Bradley RE.** 1977. An integrated system for the production of gnotobiotic *Anopheles quadrimaculatus*. J Invertebr Pathol **30:**318–324.
42. **Hongoh Y, Ekpornprasit L, Inoue T, Moriya S, Trakulnaleamsai S, Ohkuma M, Noparatnaraporn N, Kudo T.** 2006. Intracolony variation of bacterial gut microbiota among castes and ages in the fungusgrowing termite *Macrotermes gilvus*. Mol Ecol **15:**505–516.
43. **Hongoh Y.** 2011. Toward the functional analysis of uncultivable, symbiotic microorganisms in the termite gut. Cell Mol Life Sci **68:**1311–25.
44. **Hungate RE.** 1943. Quantitative analyses of the cellulose fermentation by termite protozoa. Ann Entomol Soc Am **36:**730–739.
45. **Husseneder C, Grace JK.** 2005. Genetically engineered termite gut bacteria (*Enterobacter cloacae*) deliver and spread foreign genes in termite colonies. Appl Microbiol Biotechnol **68:**360–367.
46. **Husseneder C, Grace JK, Oishi DE.** 2005. Use of genetically engineered *Escherichia coli* to monitor ingestion, loss, and transfer of bacteria in termites. Curr Microbiol **50:**119–123.

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

48. **Kane MD, Breznak JA.** 1991. Effect of host diet on production of organic acids and methane by cockroach gut bacteria. Appl Environ Microbiol **57:**2628–2634.

49. **Klass KD, Nalepa C, Lo N.** 2008. Wood-feeding cockroaches as models for termite evolution (Insecta: Dictyoptera): *Cryptocercus* versus *Parasphaeria boleiriana*. Mol Phylogenet Evol **46:**809–17.

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

51. **Kozakova H, Kolinska J, Lojda Z, Rehakova Z, Sinkora J, Zakostelecka M, Splichal I, Tlaskalova-Hogenova H.** 2006. Effect of bacterial monoassociation on brush-border enzyme activities in ex-germ-free piglets: comparison of commensal and pathogenic *Escherichia coli* strains. Microbes Infect **8:**2629–2639.

52. **Kristensen NP** 1981. Phylogeny of insect orders. A Rev Ent **26:**135–157.

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

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

55. **Legendre F, Whiting MF, Bordereau C, Cancello EM, Evans TA, Grandcolas P.** 2008. The phylogeny of termites (Dictyoptera: Isoptera) based on mitochondrial and nuclear markers: implications for the evolution of the worker and pseudergate castes, and foraging behaviors. Mol Phylogenet Evol **48:**615-627.

56. **Lo N, Bandi C, Watanabe H, Nalepa C, Beninati T.** 2003. Evidence for co-cladogenesis between diverse dictyopteran lineages and their intracellular endosymbionts. Mol Biol Evol **20:**907–913.

57. **Lo N, Engel MS, Cameron S, Nalepa CA, Tokuda G, Grimaldi D, Kitade O, Krishna K, Klass KD, Maekawa K, et al.** 2007. Save Isoptera: a comment on Inward et al. Biol Lett **3:**562–565.

58. **Maekawa K, Lo N, Rose HA, Matsumoto T.** 2003. The evolution of soil-burrowing cockroaches (Blattaria: Blaberidae) from wood-burrowing ancestors following an invasion of the latter from Asia into Australia. Proc Biol Sci **70:**1301–1307.
59. **Maekawa K, Kitade O, Matsumoto T.** 1999. Molecular phylogeny of orthopteroid insects based on the mitochondrial cytochrome oxidase II gene, Zoolog Sci **16:**175–184.
60. **Micks DW, Ferguson MJ.** 1961. Microorganisms associated with mosquitoes. III. Effect of reduction in the microbial ora of *Culex fatigans* Wiedemann on the susceptibility to *Plasmodium relictum* Grassi and Feletti. J Insect Pathol **3:**244–48.
61. **Mikaelyan A, Dietrich C, Köhler T, Poulsen M, Sillam-Dussès D, Brune A.** 2015. Diet is the primary determinant of bacterial community structure in the guts of higher termites. Mol Ecol **24:**5284–5295.
62. **Mikaelyan A, Strassert JFH, Tokuda G, Brune A.** 2014. The fibre-associated cellulolytic bacterial community in the hindgut of wood-feeding higher termites (*Nasutitermes* spp.). Environ Microbiol **16:**2711–2722.
63. **Moll RM, Romoser WS, Modrakowski MC, Moncayo AC, Lerdthusnee K.** 2001. Meconial peritrophic membranes and the fate of midgut bacteria during mosquito (Diptera: Culicidae) metamorphosis. J Med Entomol **38:**29–32.
64. **Müller M.** 1993. The hydrogenosome. J Gen Microbiol **139:**2879–2889.
65. **Muyskens JB, Guillemin K.** 2008. Bugs inside bugs: what the fruit fly can teach us about immune and microbial balance in the gut. Cell Host Micr **3:**118.
66. **Nalepa CA.** 1990. Early development of nymphs and establishment of hindgut symbiosis in *Cryptocercus punctulatus* (Dictyoptera: Cryptocercidae). Ann Entomol Soc Am **83:**786–789.
67. **Nalepa CA.** 2011. Altricial development in wood-feeding cockroaches: the key antecedent of termite eusociality, p 69–95. *In* Bignell DE, Roisin Y, Lo N (ed.), Biology of Termites: A Modern Synthesis. Springer, Dordrecht, Neth.
68. **Nalepa CA.** 2015. Origin of termite eusociality: Trophallaxis integrates the social, nutritional, and microbial environments. Ecol Entomol **40:**323–35.
69. **Newell PD, Douglas AE.** 2014. Among-species interactions determine the impact of gut microbiota on nutrient allocation in *Drosophila melanogaster*. Appl Environ Microbiol **80:**788–796.
70. **Noack J, Dongowski G, Hartmann L, Blaut M.** 2000. The human gut bacteria *Bacteroides thetaiotaomicron* and *Fusobacterium varium* produce putrescine and spermidine in cecum of pectin-fed gnotobiotic rats. J Nutr **130:**1225–1231.

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

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

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

74. **Pellens R, Grandcolas P, da Silva-Neto ID.** 2002. A new and independently evolved case of xylophagy and the presence of intestinal flagellates in the cockroach *Parasphaeria boleiriana* (Dictyoptera, Blaberidae, Zetoborinae) from the remnants of the Brazilian Atlantic forest. Can J Zool **80:**350–359.

75. **Pester M, Brune A**. 2007. Hydrogen is the central free intermediate during lignocelluloses degradation by termite gut symbionts. ISME J **1:**551–65.

76. **Rahman NA, Parks DH, Willner DL, Engelbrektson AL, Goffredi SK, Warnecke F, Scheffrahn RH, Hugenholtz P et al.** 2015. A molecular survey of Australian and North American termite genera indicates that vertical inheritance is the primary force shaping termite gut microbiomes. Microbiome **3:**1–16.

77. **Sabree ZL, Kambhampati S, Moran NA.** 2009. Nitrogen recycling and nutritional provisioning by *Blattabacterium*, the cockroach endosymbiont. Proc Natl Acad Sci USA **106:**19521–19526.

78. **Samuel BS, Gordon BI.** 2006. A humanized gnotobiotic mouse model of host archaeal-bacterial mutualism. Proc Natl Acad Sci USA 103, 10011–10016.

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

80. **Schauer C.** 2011. The gut microbiota of the blattid cockroach *Shelfordella lateralis*. Dissertation, Phillips-Universität Marburg.

81. **Schauer C, Thompson CL, Brune A.** 2012. The bacterial community in the gut of the cockroach *Shelfordella lateralis* reflects the close evolutionary relatedness of cockroaches and termites. Appl Environ Microbiol **78:**2758–2767.

82. **Schauer C, Thompson C, Brune A.** 2014. Pyrotag sequencing of the gut microbiota of the cockroach *Shelfordella lateralis* reveals a highly dynamic core but only limited effects of diet on community structure. PLoS One **9:**e85861. doi:10.1371/journal.pone.0085861.

83. **Schink B, Zeikus JG.** 1980. Microbial Methanol Formation: A major end product of pectin metabolism. Curr Microbiol **4:**387–389.
84. **Schmitt-Wagner D, Friedrich MW, Wagner B, Brune A.** 2003. Phylogenetic diversity, abundance, and axial distribution of bacteria in the intestinal tract of two soil-feeding termites (*Cubitermes* spp.). Appl Environ Microbiol **69:**6007–6017.
85. **Snodgrass, RE.** 1935. Priciples of Insect Morphology. McGraw-Hill, New York, NY.
86. **Sprenger WW, Hackstein JHP, Keltjens JT.** 2005. The energy metabolism of *Methanomicrococcus blatticola*: physiological and biochemical aspects. Antonie v. Leeuwenhoek **87:**289–299.
87. **Sprenger WW, van Belzen MC, Rosenberg J, Hackstein JHP, Keltjens JT.** 2000. *Methanomicrococcus blatticola* gen. nov., sp., nov., a methanol- and methylamine-reducing methanogen from the hindgut of the cockroach *Periplaneta americana*. Int J Syst Evol Microbiol **50:**1989–1999.
88. **Suarez ME, Thorne BL.** 2000. Rate, amount, and distribution pattern of alimentary fluid transfer via trophallaxis in three species of termites (Isoptera: Rhinotermitidae, Termopsidae). Ann Entomol Soc Am **93:**145–155.
89. **Thimm T, Hoffmann A, Borkott H, Munch JC, Tebbe CC.** 1998. The gut of the soil microarthropod *Folsomia candida* (Collembola) is a frequently changeable but selective habitat and a vector for microorganisms. Appl Environ Microbiol **64:**2660–69.
90. **Tholen A, Brune A.** 2000. Impact of oxygen on metabolic fluxes and *in situ* rates of reductive acetogenesis in the hindgut of the wood-feeding termite *Reticulitermes flavipes*. Environ Microbiol **2:**436–449.
91. **Treherne JE.** 1957. Glucose absorption in the cockroach. J Exp Biol **34:**478–485.
92. **Treherne JE.** 1958. The absorption of glucose from the alimentary canal of the locust *Schistocerca gregaria* (Forsk.) J Exp Biol **35:**297–306.
93. **Watanabe H, Tokuda G.** 2010. Cellulolytic systems in insects. Annu Rev Entomol **55:**609–632.
94. **Wong ACN, Ng P, Douglas AE.** 2011. Low diversity bacterial community in the gut of the fruitfly *Drosophila melanogaster*. Environ Microbiol **13:**1889–1900.
95. **Wigglesworth VB.** 1972. The Principles of Insect Physiology. 7th ed, Chapman and Hall, London, UK.

Chapter 2

Manuscript in preparation

Microbial community succession is linked to changes in gut environment and metabolic processes in the cockroach *Shelfordella lateralis*

Dorothee Tegtmeier, Aram Mikaelyan, Claire L. Thompson, and Andreas Brune

Department of Biogeochemistry, Max Planck Institute for Terrestrial Microbiology, Karl-von-Frisch-Str. 10, 35043 Marburg, Germany

Contributions

D.T. planned and designed the study, conducted the experiments, analyzed the data, discussed results, evaluated and visualized data and wrote the manuscript.

A.M. analyzed Illumina sequences, discussed results, evaluated and visualized data.

C.L.T. planned and designed the study, conducted preliminary analyses.

A.B. planned and designed the study, supervised the work, and discussed results.

Abstract

Cockroaches have a highly complex gut microbiota of bacteria, archaea, and protists. However, little is known about the primary colonization of the gut, the succession of the microbial communities during host development, and the dynamics of the different microbial guilds involved in the methanogenic degradation of organic matter. Here, we investigated successive changes of the bacterial gut microbiota and the major metabolites in different instars of *Shelfordella lateralis*. Acetate was the major fermentation product in the guts of all instars, but the first instar additionally accumulated large amounts of lactate. While methane emission of living cockroaches, which was absent in the first instar, was low in all other instars but high in adults, hydrogen emission decreased in every instar, suggesting an increasing hydrogen consumption by methanogens. Microsensor measurements revealed anoxic conditions in the hindguts of all but the first instar. Amplicon sequencing of bacterial 16S rRNA genes showed that the gut microbiota of the first instar was dominated by aerobic or oxygen-tolerant, lactate-producing taxa (e.g. *Enterobacteriaceae*, *Enterococcaceae* and *Lactobacillaceae*), whereas cockroaches from second instar to adulthood where consistently colonized by anaerobic taxa (e.g. *Ruminococcaceae*, *Lachnospiraceae* and *Rikenellaceae*). The strong differences in gut microbiota and metabolism between first and later instars suggest that oxygen is an important environmental driver of microbial community structure in cockroach guts. Despite a strong stochastic element during primary colonization and probably also after each molt of the insect, the microbiota of *S. lateralis* seems to be shaped by the selection of microorganisms that are favored by the conditions of the gut environment.

Introduction

Insects, particularly those feeding on a fiber-rich diet, possess a dense and complex microbiota in their intestinal tracts. The most famous example are termites, which harbor microbial symbionts in their gut that aid in the digestion of lignocellulose (Brune and Ohkuma, 2011; Brune, 2014). While the gut microbiota of termites has been intensively studied for all major feeding guilds (e.g. Hongoh et al., 2003; Warnecke et al., 2007; Dietrich et al., 2014; Brune and Dietrich, 2015; Mikaelyan et al., 2015*a*; Rahman et al., 2015), less is known about the microbiota in the intestinal tracts of cockroaches. Termites and omnivorous cockroaches are closely related (Inward et al., 2007), and they show also similarities in their gut microbiota. As in termites, the alimentary canal of cockroaches is colonized by complex microbial communities that are dominated by diverse lineages of presumably obligately and facultatively anaerobic bacteria (Schauer et al., 2012; Dietrich et al., 2014). Furthermore, cockroach guts harbor methanogenic archaea (Kane and Breznak, 1991; Brune, 2010) and ciliate protists with endosymbiotic methanogens (Gijzen et al., 1991).

In termites, the anaerobic breakdown of organic material starts with the depolymerization of plant fiber (e.g. cellulose and hemicellulose) to monomeric carbohydrates that are further degraded by primary fermenting bacteria to short chain fatty acids, lactate, CO_2 and hydrogen (Hongoh, 2011; Brune, 2014; Brune and Dietrich, 2015). While the short chain fatty acids are a major carbon and energy source for the host (Odelson and Breznak, 1983; Brune, 1998; Brune and Ohkuma, 2011), lactate is an important substrate for a further degradation by secondary fermenters, e.g. propionigenic bacteria (Tholen and Brune, 2000; Boga et al., 2007). Hydrogen is an important intermediate in the fermentative processes and the central substrate for methanogenic archaea or homoacetogenic bacteria, which perform the last step of the anaerobic breakdown (Kane and Breznak, 1991; Tholen and Brune, 2000; Brune, 2010). However, studies on the methanogenic degradation of organic matter in cockroaches, especially about the assembly of these metabolic networks and the dynamics of the involved microbial guilds are rare. Most of the existing studies have investigated the gut microbiota of adult cockroaches, which have already established a climax community in their guts, but little is known about the primary colonization of the gut and the microbiota of the nymphs.

In lower termites, the symbiotic gut microbiota is transferred to the offspring through the exchange of droplets of hindgut fluid by nestmates (proctodeal trophallaxis) (Suarez et al., 2000; Nalepa, 2015). This process introduces a strong deterministic component into

community assembly (Brune and Dietrich, 2015), resulting in highly similar microbial communities among individuals of the same colony (Benjamino and Graf, 2016). In higher termites, where proctodeal trophallaxis seems to be absent, the symbiotic microbiota may be transferred via the fecal route or through stomodeal feeding (Diouf et al., 2015). In line with this, a recent, large molecular survey of both lower and higher termites found that vertical inheritance was the primary force shaping termite gut microbiomes (Rahman et al., 2015).

The gut microbiota of non-social cockroaches, which do not practice brood care, is likely assembled by more stochastic events than that of termites. In agreement, variations in microbial community composition and the relative abundance of gut microbes between individuals of the same species were very high. The strong individual variation even masked the impact of dietary shifts on the gut microbiota (Schauer et al., 2014). Recent studies by Mikaelyan et al. (2016), who inoculated germ-free cockroaches with termite or mouse gut microbiota, have shown that the gut habitat of the host plays an important role in the selection of bacterial lineages from the environment. However, the mechanisms of community assembly and succession of the cockroach gut microbiota are far from clear and are less predictable as in termites.

In this study, we investigated the acquisition and succession of microbial communities in the gut during the development of the cockroach. We analyzed changes in metabolic processes, like the production of short chain fatty acids, hydrogen and methane in different developmental stages (instars) of the omnivorous cockroach *Shelfordella lateralis*. Furthermore, we determined the oxygen status of the gut in the early cockroach development using microsensors. To clarify if changes in metabolic processes and gut conditions are reflected by alterations in the gut microbiota, we analyzed the microbial diversity and density in cockroach guts using high throughput sequencing and real time PCR of the bacterial 16S rRNA gene.

Materials and Methods

Cockroach rearing

Shelfordella lateralis cockroaches were obtained from a commercial breeder (J. Bernhard, Helbigsdorf, Germany) and maintained as previously described (Schauer et al., 2012). In addition, *S. lateralis* were reared in isolation by separating oothecae from the conventional breed and placing them in separate containers. After hatching, these cockroaches were reared under normal conditions until adulthood but without any exposure to other conventional cockroaches.

Defining developmental stages (instars) of *S. lateralis*

After hatching, a cockroach nymph molts several times until it attains adulthood. The period from hatching until the first molt (first instar) was documented for 24 individuals. Head width was chosen to identify the number of instars, because the head capsule is strongly sclerotized (Chapman, 2013) and its size changes clearly only after the molt. 80 cockroaches of different body size were immobilized on ice in order to determine the head width for each individual. Additionally, body weight and body length, were documented from the same individuals. Routinely prior each experiment the instar was defined by measuring the head width unless indicated otherwise. All adults used for the analysis were females.

Detection of gut metabolites

For the detection of metabolites in the cockroach hindgut, cockroaches were dissected under a stereomicroscope using sterile forceps. The fat body surrounding each hindgut was carefully removed, and hindguts were homogenized in 150 µl water by sonication (ultrasonic processor UP50H, Hielscher Ultrasonics, Teltow, Germany). Samples were prepared and analyzed by HPLC with an ion-exclusion column (Resin H+, IEX, 8 µm, Grom, Rottenburg, Germany) and a refractive index detector as previously described in Schauer et al. (2012).

Hydrogen and methane emission of living cockroaches

Methane emission was measured by gas chromatography using a packed column (Porapack Q column, 80/100 mesh; 274 cm × 3.18 mm) and a methanizer coupled to a flame ionization detector.

Hydrogen emission by cockroaches was measured by gas chromatography with a packed Mol Sieve 5A column (80/100 mesh; 70 cm × 6.35 mm) and a reduction gas detector (RGD2, Trace Analytical, Menlo Park, CA, USA).

Prior the measurement the instar was defined by body weight (the weight which corresponds to the head width of the respective instar) to avoid a potential influence of the immobilization process on metabolic rates of the living animals. Cockroaches were measured in pools to mask the effect of the strong individual variation of the bacterial and methanogenic community (Schauer et al., 2014). For the measurement the cockroaches were placed in glass vials that were closed with rubber stoppers. The weight of the cockroaches (amount of individuals) was adjusted to the volume of the glass vial (10, 15, 30, 60 or 150 mL). The younger instars were measured in larger pools than the older instars. Stimulation of methane emission was tested via addition of 25% hydrogen to the headspace. All experiments were done in at least 3 replicates.

Gas production rates were determined from the linear increase of gas concentration; at least three time points were taken over a period of 5 to 6 h. In the rare cases when the slope slightly decreased at the end of the incubation, initial rates were used.

Microsensor measurements

Guts from nymphs (different instars) were dissected, placed in a chamber with a bottom layer of 2% agarose, and immediately embedded at a depth of approximately 2 mm in Ringer's solution solidified with 0.5% agarose. Axial profiles of intestinal oxygen and hydrogen concentrations at the gut center were measured with microsensors (10 or 25 µm tip diameter; Unisense, Aarhus, Denmark) as described previously (Brune et al., 1995).

DNA extraction

Cockroaches were dissected with sterile forceps. The fat body surrounding each hindgut was carefully removed, and five hindguts from the same instar were pooled.

For a quantification of bacteria, guts were homogenized in lysis buffer (SL1; Macherey-Nagel, Düren, Germany) by sonication (ultrasonic processor UP50H, Hielscher Ultrasonics, Teltow, Germany) and diluted when necessary according to gut weight that each sample contained approximately the same amount of gut material. Microbial cells were disrupted by beat beating (FastPrep-24; MP Biomedicals, Solon, OH, USA) for 45 s at 6.5 m s^{-1}. DNA was extracted with the NucleoSpin Soil kit (Macherey-Nagel, Düren, Germany) according to the

manufacturer's manual. Samples were used for subsequent quantitative real-time PCR (qPCR) analysis.

For Illumina sequencing, five hindguts from the same instar were pooled, homogenized, and microbial cells were disrupted by beat beating (see above). DNA was extracted with the FastDNA Spin Kit for Soil (MP Biomedicals) according to the manufacturer's manual.

Quantitative PCR

Standard curves were generated using purified 16S rRNA PCR products from strain EbSL (Tegtmeier et al., 2016). PCR products were checked photometrically for purity (Nano-drop; PeqLab, Erlangen, Germany) and quantified fluorimetrically (Qubit; Invitrogen, Eugene, OR). The number of bacterial 16S rRNA genes was determined by quantitative real-time PCR (qPCR) as described by Stubner (2002) using the primer pair 519fc (5′-CAGCMGCCGCGGTAANWC-3′) and 907r (5′-CCGTCAATTCMTTTRAGTT-3′) (Lane 1991; primer 519fc modified by Stubner 2002). Each sample was analyzed in duplicate with at least three independent determinations, which typically showed 10–30% deviation. All calibration curves were linear over a range of at least five orders of magnitude.

Detection of ciliates

The eukaryotic 18S rRNA gene was amplified using the Ciliophora-specific primer pair 384f (5′-YTBGATGGTAGTGTATTGGA-3′) and 1147b (5′-GACGGTATCTRATCG TCTTT-3′) as described in Dopheide et al. (2008). Three replicate samples of each instar and adults, each containing the DNA of five hindguts were analysed.

Additionally, hindgut homogenates were inspected for the presence of ciliates and methanogenic archaea under a fluorescence microscope (Zeiss, Axiophot) using an excitation wavelength of 420 nm (395–440 nm filter).

Illumina Sequencing

The construction of Illumina libraries was done as described previously in Mikaelyan et al. (2015*b*). Briefly, the V3–V4 region of the 16S rRNA gene was amplified from each sample using the primers M13-343Fmod and M13-784Rmod, which were based on the universal bacterial primers 343Fmod and 784Rmod (Köhler et al., 2012) respectively but additionally included universal M13-specific priming sites on their 5′ ends (Daigle et al., 2011). The cycling conditions for this PCR step were as described previously (Köhler et al., 2012). The

resulting amplicons, tagged with the M13 tails, were used as template for a subsequent PCR step using the Herculase II Fusion DNA Polymerase Kit (Agilent Technologies, USA). The cycling conditions for this step were as described previously (Mikaelyan et al., 2015*b*). Purified PCR products were mixed in equimolar amounts and sequenced commercially (paired-end; 2 × 350 nt; Illumina MiSeq; GATC Biotech, Konstanz, Germany). The quality of the final products was checked by gel electrophoresis.

Processing of sequence data

Only reads with a minimum length of 250 bp and no ambiguities were selected and sorted into separate fastq files using the sample-specific barcodes included in the sequences. For the iTag libraries, paired reads were merged and pairs with mismatches in the overlapping region were discarded using the mothur software suite (Schloss et al., 2009). After removal of barcodes and primers, the reads were screened for potentially chimeric sequences using the UCHIME implementation in mothur. Non-chimeric reads from each sample were clustered into operational taxonomic units (OTUs) using DNACLUST at a sequence identity threshold of 99%. Representative sequences from each OTU were aligned with the mothur aligner, using the Silva reference alignment (SSURef release 119) as a template.

Analysis of community structure

For the analysis of taxonomic composition of each sample, the OTUs in the de-replicated datasets were classified with the RDP classifier (Wang et al., 2007) implemented in the mothur software suite (Schloss et al., 2009) at a confidence cutoff of 80%, using DictDb v. 3.0 as reference database (Mikaelyan et al., 2015*a*). Sequences assigned to the genus *Blattabacterium*, an endosymbiont of cockroaches residing in the surrounding fat body (Lo et al., 2003), were not considered part of the cockroach gut microbiota and were removed before further analysis. Bray-Curtis distances between the communities were calculated at the genus level using the vegan package (Oksanen et al., 2015) in the R statistical software suite (R Core Team 2015). Datasets were subsampled to a thousand sequences, and distances were visualized using principal coordinates analysis (PCoA) implemented in vegan.

Results

Developmental stages (instars)

Following hatching, it took 10–14 days until the cockroaches molted and reached the second instar. In the course of the development, the head width of the cockroach increased in discrete steps (Figure 2.1), which allowed for the categorical sorting of the individuals into developmental stages (instars). According to head width, eight to ten different instars until adulthood were identified. Females usually showed one or two instars more until they attained adulthood and showed also a higher body weight (725 mg, n = 4) when compared to males (332 mg, n = 6). After sorting the individuals by increasing head width, the respective bodyweight increased in a continuous manner with the development of the cockroach, which also allowed for the definition of the instar. However, defining the instar based on body weight was not as accurate as by head width, as no discrete steps were recognizable. By contrast, body length did not increase continuously (Figure 2.1) and was therefore not applicable to define the instar.

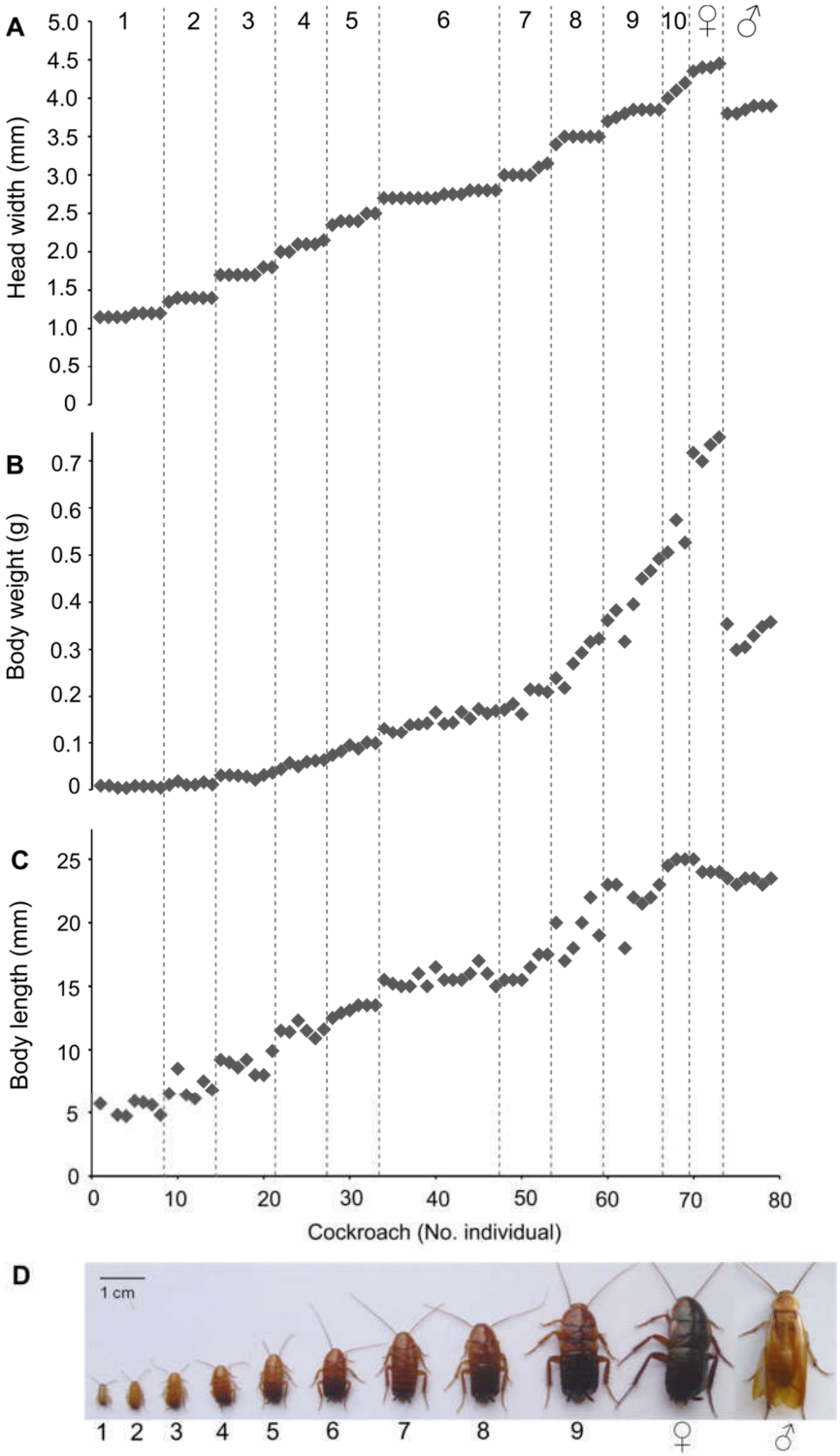

Figure 2.1. Head width (A), body weight (B) and body length (C) of *S. lateralis*. Individuals were sorted by increasing head width (except the adult males). Each step in (A) is considered as instar (separated by vertical lines). The number of the instar is given above the figure. Adult males were recognized by phenotype and not by head width, since they have a similar head width as ninth instar nymphs. Female and male adult cockroaches are indicated by symbols ♀ and ♂, respectively. The phenotype of the instars and adults (D) is given below the chart.

Gut metabolites

Acetate was the major fermentation product in all instars and adults. High amounts of lactate accumulated in the first instar, whereas in all other instars and adults lactate concentrations were comparatively low. In third instar nymphs and adults also small amounts of propionate were detected. Ethanol was found in minor amounts only in adults. Adults reared in isolation showed the same fermentation products than conventional adults, except an absence of propionate. Gut homogenates of all instars and adults contained glucose with highest concentrations and high variations among replicates in first instar nymphs (Figure 2.2). We found a strong correlation of glucose and lactate concentrations, as each replicate with a high glucose concentration showed also a high lactate concentration.

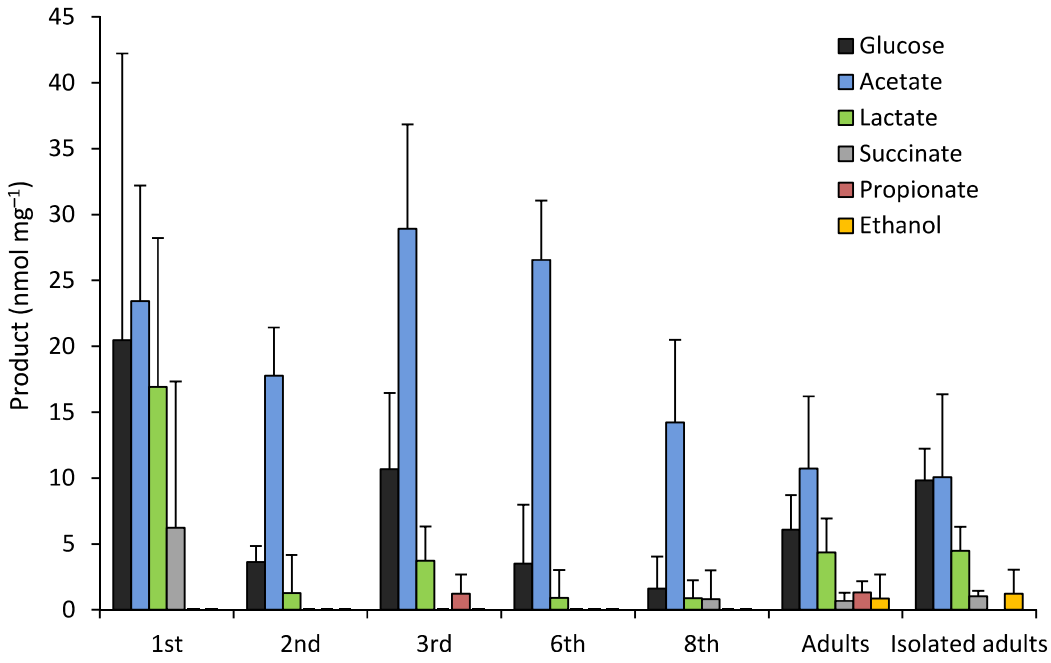

Figure 2.2. Hindgut concentrations of glucose and fermentation products in different instars and adults of *Shelfordella lateralis*. Bars represent means (+ standard deviation) of four to six replicate samples. Each sample consisted of the homogenized hindgut of individual cockroaches; in the case of the smaller nymphs, hindguts of ten (first instar) or five (second and third instar) individuals were pooled.

Hydrogen and methane emission

Hydrogen emission of *S. lateralis* changed during the development. Hydrogen emission rates were highest in the first instar and decreased continuously in the different developmental stages; lowest rates were found in eighth instar nymphs and adults. Hydrogen emission rates

varied considerably between replicate experiments with different pools, suggesting the presence of large differences among individual cockroaches (Figure 2.3).

Methane emission was below the detection limit in the first instar. Cockroaches started to emit methane from the second instar, but methanogenesis was low and irregular until adulthood. No significant increase was observed between second and ninth instar (Figure S2.1). Adults showed highest methane emission rates. Addition of hydrogen to the headspace did not stimulate methanogenesis in the first, second and third instar, whereas pools of the sixth and eighth instar and adults showed a slight increase in methane emission.

Cockroaches reared in isolation never showed methane emission (Figure 2.3). Their hydrogen emission rates did not differ significantly from those of conventional cockroaches.

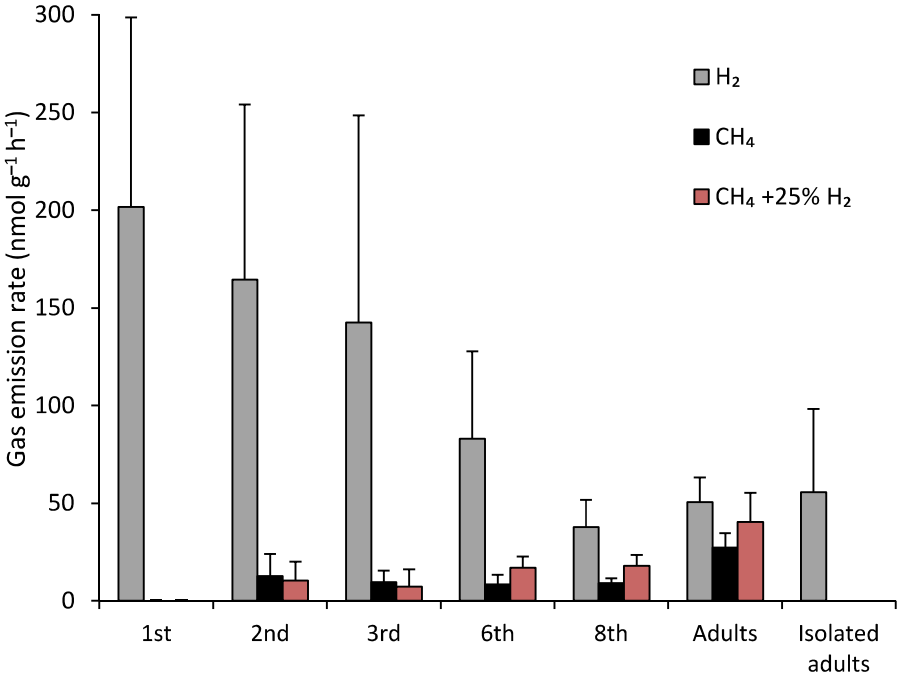

Figure 2.3. Hydrogen and methane emission rates of *S. lateralis* instars and adults. Methane was measured both before and after addition of hydrogen to the headspace (25% v/v). Bars represent means (+ standard deviation) of at least three replicate experiments with pools of 6–20 cockroaches.

Detection of ciliates

Ciliates were frequently observed in gut homogenates of adult cockroaches, often in large number. The ciliates showed strong F_{420}-autofluorescence typical for methanogenic archaea (Figure S2.2). No ciliates were observed in first instar nymphs. The presence of ciliates in adults and their absence from first instar nymphs was confirmed by PCR of the eukaryotic 18S rRNA gene of ciliates. Furthermore, PCR products for ciliates were absent in samples of the

second instar and adult cockroaches reared in isolation but present in some samples of the third (two out of three), sixth (one out of three) and eighth instar (two out of three).

Oxygen and hydrogen status of the gut

Axial profiles of oxygen showed the presence of oxygen in the guts of first instar cockroaches and virtually anoxic conditions in each gut compartment of the second and third instar. In the first instar, the oxygen partial pressure was highest in the posterior hindgut and generally low in the foregut, midgut and the anterior hindgut (lowest values in the anterior and posterior midgut) (Figure 2.4A).

Hydrogen was detected in the guts of all instars with highest partial pressures in the first instar, followed by the second and third instar. The hydrogen partial pressure was strongly variable among individuals of all instars and accumulated in most cases in larger amounts in the anterior hindgut but was very low in foregut and midgut (Figure 2.4B).

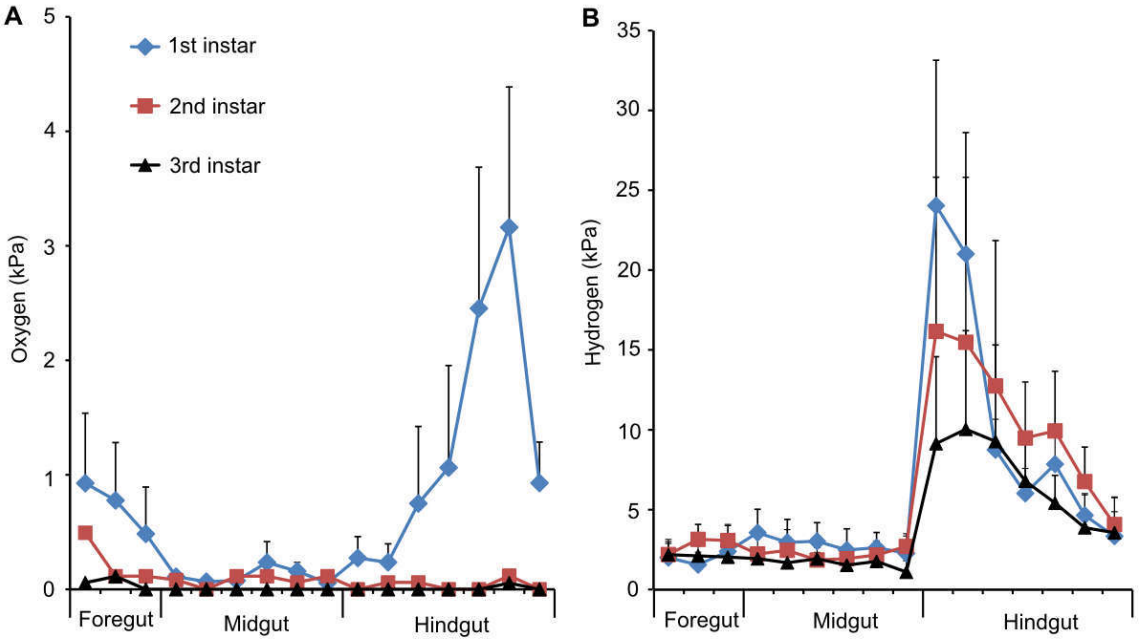

Figure 2.4. Partial pressures of oxygen (A) and hydrogen (B) in the guts of the first three instars of *Shelfordella lateralis*. Axial profiles were measured with microsensors and recorded at the gut center. Symbols are means (+ SEM) of replicate measurements with three guts. Error bars for the second and third instar in (A) were smaller than the symbols. Data for oxygen partial pressure in the first instar were published already by Tegtmeier et al. (2016).

Bacterial density and community structure

The bacterial density per hindgut increased from the first instar until adulthood. However, when based on gut weight, which significantly increased from the first instar to adulthood (from 0.2 ± 0.1 mg to 19.3 ± 4.9 mg), the bacterial density was highest in the first, second and third instar and decreased until adulthood (Figure 2.5).

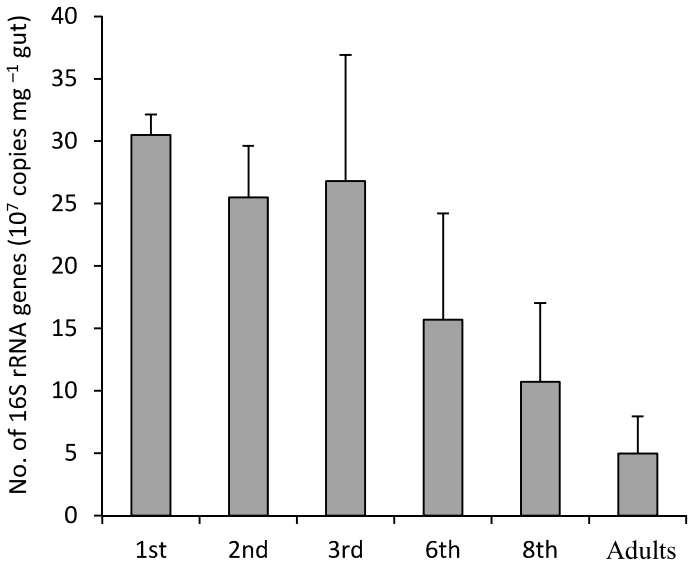

Figure 2.5. Quantification of bacteria in the hindguts of different instars via qPCR of the 16S rRNA gene with general *Bacteria*-primers. Values are means (+ standard deviation) of two to three replicate experiments with five hindguts each.

Quality processing of the amplicon libraries yielded 49,888–320,181 sequence reads per sample. Classification with the taxonomic framework of DictDb successfully assigned 75–96% of the reads to the genus level. The detailed results of the classification up to the genus level are shown in Table S2.1 (https://www.dropbox.com/s/6fydgndqv0li1mg/Table_S1.community_succession.xlsx?dl=0).

In all instars and adults the most important phyla were *Proteobacteria*, *Planctomycetes, Fusobacteria*, *Firmicutes* and *Bacteroidetes*. However, the abundance of these phyla varied among instars and adults, with strongest differences in the first instar. The abundance of *Proteobacteria* was much higher in first instar nymphs than in other instars and adults. The majority of the *Proteobacteria* in the first instar belong to *Enterobacteriaceae* (*Serratia* 1 and *Escherichia blattae* cluster) and *Pseudomonadaceae* (Insect cluster III). *Desulfovibrionaceae*

were present in all instars and adults with highest abundance in the sixth instar. The abundance of *Planctomycetes* was low in the first instar but higher in the third, sixth and eighth instar.

Firmicutes were abundant in all instars. However, *Lactobacillales* (*Lactobacillaceae*, *Enterococcaceae* and Cockroach cluster) were of higher abundance in first instar nymphs, whereas *Clostridiales* (especially *Ruminococcaceae* and *Lachnospiraceae*) were of higher abundance in the remaining instars and adults. *Bacteroidetes*, especially *Rikenellaceae*, *Porphyromonadaceae* V and *Bacteroidaceae*, were of lower abundance in the first instar and of higher abundance in the other instars and adults.

Variations among replicate pools were highest in the first instar. Pools of first instar guts were often dominated by one or two bacterial groups. In sample A *Pseudomonadaceae* was the most abundant family, whereas in sample C *Enterobacteriaceae* was the dominant group (35 and 44%, respectively; for details see Table S2.1). By contrast, the guts of cockroaches from the second instar to adulthood showed a more even distribution of the taxa. Nevertheless, variations in the gut microbiota were also present among pools of these cockroaches but less pronounced than in first instar nymphs (Figure 2.6).

No significant difference between the gut microbiota of conventional adults and isolated adults was found, except a higher abundance of the genus *Dysgonomonas* and the absence of a few groups like the "Termite cockroach cluster" (*Bacteroidete*s, *Porphyromonadaceae* V) and some groups within the *Clostridiaceae* (e.g. *Clostridium* 5 and Arthropod cluster) in isolated cockroaches.

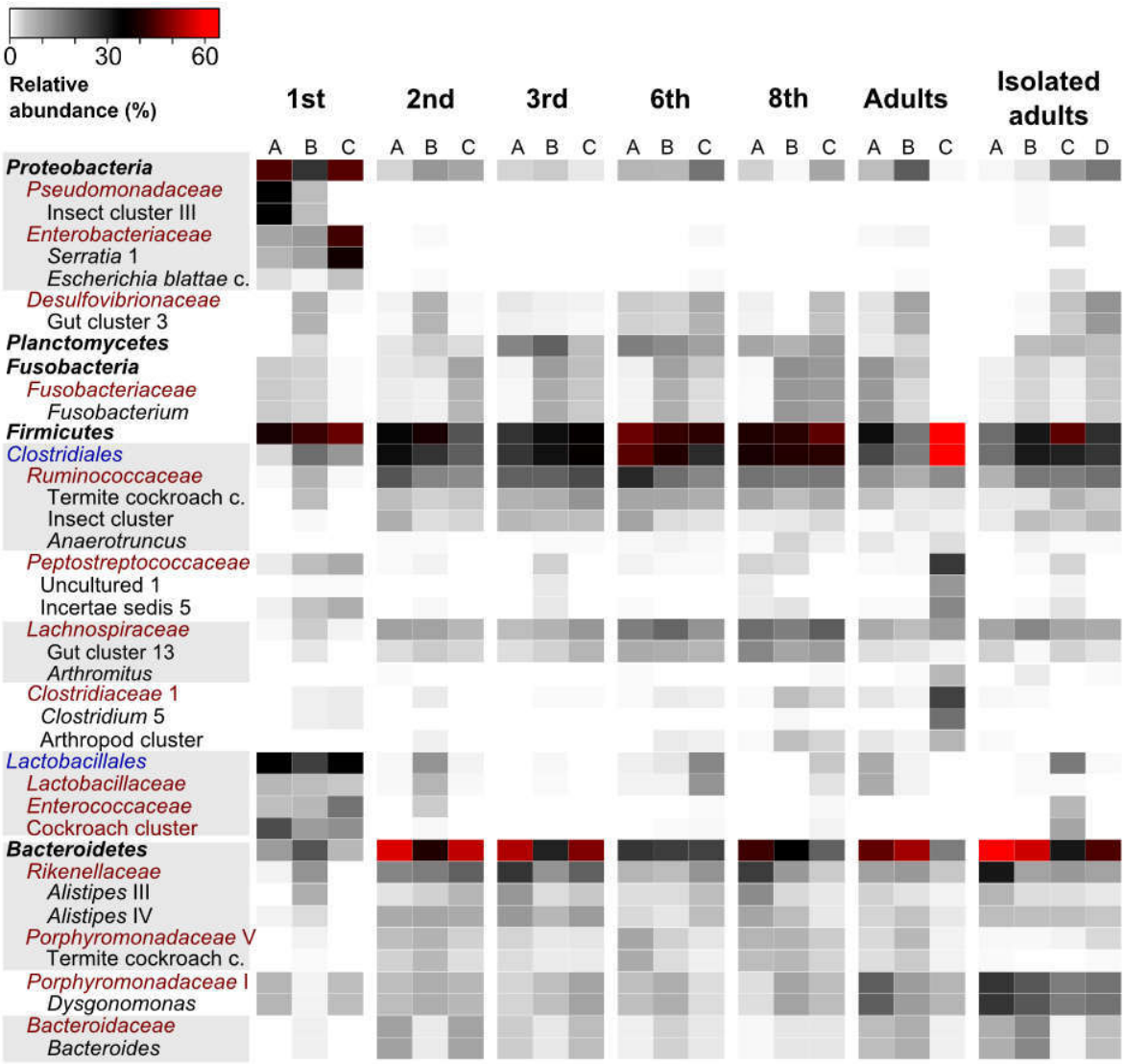

Figure 2.6. Relative abundance of selected taxa in hindguts of *S. lateralis* instars and adults. Each replicate sample (indicated by A, B, C and D) contained the DNA of five hindguts. Major phyla are marked in bold black and major orders and families are marked in blue and red, respectively. Important genera are placed below the families (c. = cluster). Groups which show pronounced successive changes are shaded in gray.

Successive changes during cockroach development were most pronounced from the first to second instar. *Enterobacteriaceae*, Cockroach cluster (*Lactobacillales*), *Pseudomonadaceae*, *Enterococcaceae* and *Lactobacillaceae*, which were the most abundant families in first instar nymphs, drastically decreased in the second instar. These families have disappeared completely from the gut in the third instar and remained almost absent until adulthood (except *Lactobacillaceae*) (Figure 2.7A).

Ruminococcaceae and *Rikenellaceae*, which were of very low abundance in the first instar, showed the strongest increase of all families until the third instar, followed by a decrease in

the late developmental stages. *Lachnospiraceae* increased strongly from first instar until eighth instar and decreased again in adults. Insect cluster (*Planctomycetes*) showed a strong increase during early development and a strong decrease in the late developmental phase. Other groups like *Porphyromonadaceae* 1 and *Clostridiaceae* 1 remained almost constant during nymphal development and increased in adults (Figure 2.7B).

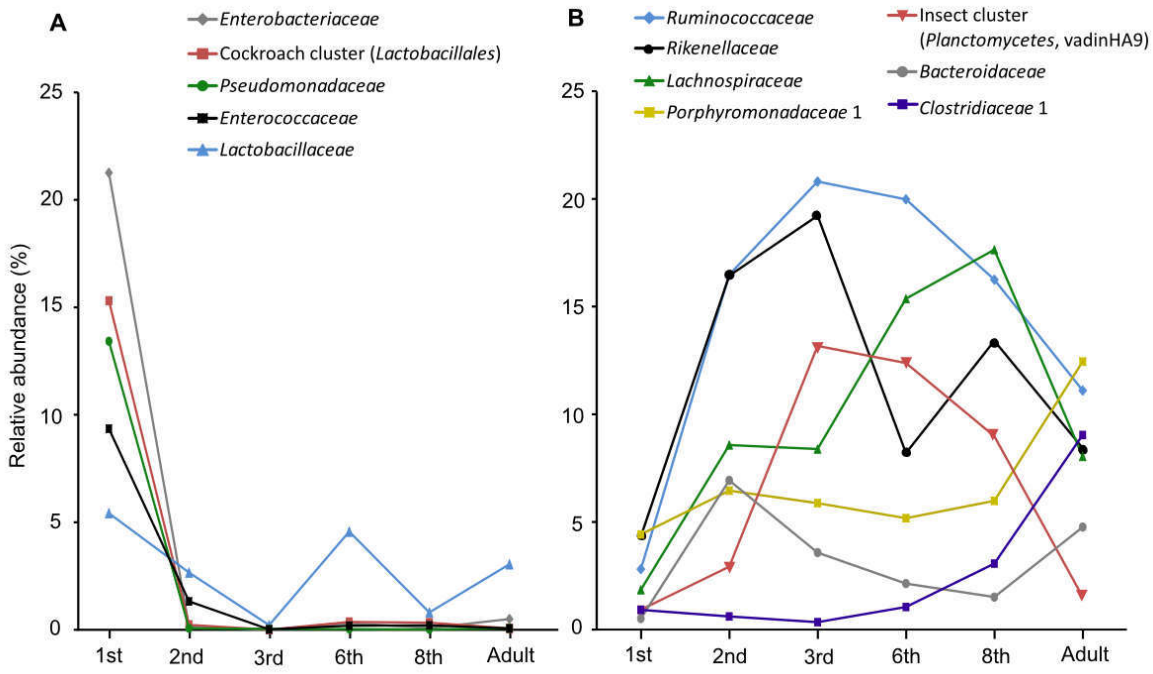

Figure 2.7. Successive changes of selected families in hindguts of *S. lateralis* during cockroach development. (A) Families that show a decrease and (B) families that show an increase in relative abundance over time. Symbols are means of the three replicate samples for each instar and adults (see Figure 2.6).

The community structure of the first instar was distinct from that of all other instars and adults. By contrast, the gut microbiota of the second, third, sixth and eighth instar as well as most of the adults shared more similarities. No clear clustering according to instar of the different replicates was distinguishable. Except two first instar samples, all other samples clustered more or less together (with no clear pattern). Replicates of the second, third, sixth, and eighth instar as well as isolated adults clustered closer together than replicates of the first instar and adults. Isolated adults clustered closely to second and sixth instar cockroaches (Figure 2.8).

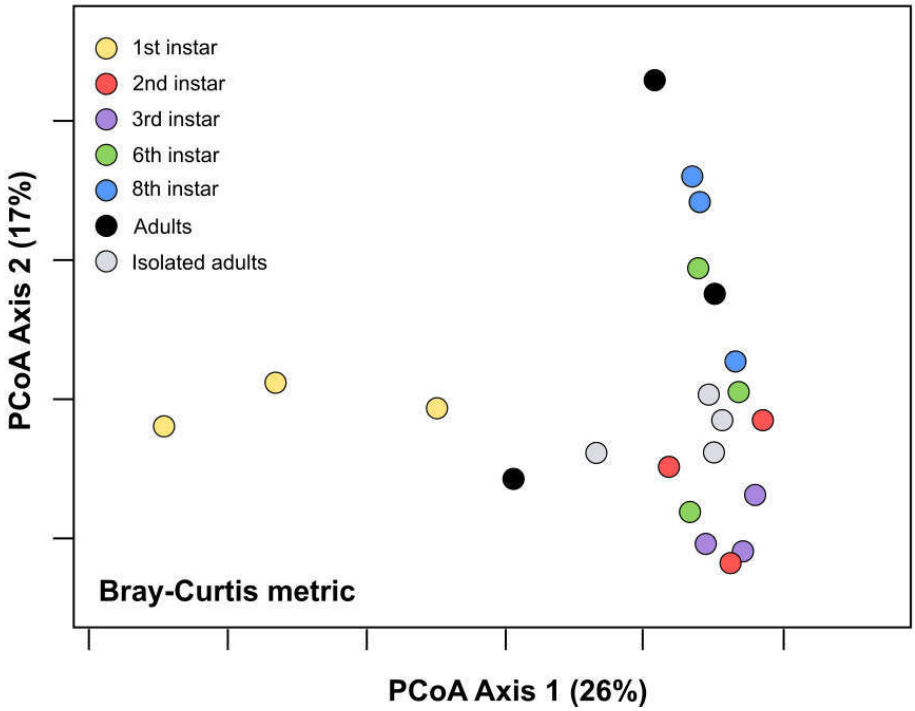

Figure 2.8. Principal coordinates analysis of pairwise comparisons of community structure (at the genus level) among *S. lateralis* instars and adult samples based on the Bray-Curtis metric.

Discussion

This is the first comprehensive study exploring successive changes in microbial diversity and metabolic processes in cockroach guts during the life cycle of the host. In the course of the early cockroach development, we found fundamental changes in the gut microbiota that were reflected by changes in metabolic processes and the oxygen status of the gut. Especially first instar nymphs were distinct from all other instars and adults with regard of different properties: A distinct gut microbiota, a strong accumulation of lactate in the hindgut, highest hydrogen emission rates, no methane emission, and oxic conditions in the hindgut.

Initial colonization and succession of bacterial communities

Following hatching, the initially germ-free guts of the nymphs are colonized by a diversity of microorganisms (bacteria, archaea and in the later development also by protists) as soon as the cockroach gets in contact with the environment. The gut microbiota of first instar cockroaches was often dominated by particular groups like *Enterobacteriaceae*, *Pseudomonadaceae*, *Enterococcaceae* and *Lactobacillales* (cockroach cluster and *Lactobacillaceae*) that were almost not present in all other instars and adults. Similarly, Mikaelyan et al. (2016) found that

Lactobacillaceae were very abundant (14–30%) in first instar conventional *S. lateralis*, but of much lower abundance in one month old nymphs and adults. These groups are strictly aerobic, facultatively aerobic or oxygen-tolerant taxa, which are likely fast growing pioneer species (r-strategists; Pianka, 1970; Andrew and Harris, 1986) that can colonize the oxygen containing guts of the first instar already in high density and more successfully than obligate anaerobes. Similarly, studies about the assembly of gut communities in mammals showed that first gut colonizers are typically facultative anaerobes (Favier et al., 2002; Gillilland et al., 2012). Furthermore, studies with germ-free cockroaches have shown that the gut of first instar *S. lateralis* was colonized by the facultatively anaerobic strain EbSL (*Enterobacteriaceae*) already within five days in high density ($29.9 \pm 15.7 \times 10^7$ 16S rRNA genes per mg gut; Tegtmeier et al., 2016), which was very close to the total bacterial density in conventional first instar nymphs (Figure 2.5). Also young *Pycnoscelus surinamensis* cockroaches with a small hindgut volume below 1µl showed already a high bacterial density and activity in their guts (Cazemier et al., 1997). Such a dense gut bacterial colonization of early instars of *S. lateralis* is likely possible due to the absence of ciliates, which occupy to thousands the hindguts of adult cockroaches (Gijzen et al., 1991; van Hoek et al., 1998; this study). Furthermore, other larger eukaryotes, e.g. nematodes, which we frequently observed in gut contents of adult cockroaches but not in first instar nymphs, are likely not part of the gut microbiota in the early developmental stages. Nematodes were also present in the hindgut of adult *Periplaneta americana* cockroaches, sometimes in high number (Hominick and Davey, 1973; Hominick and Davey, 1975). In termites it takes several molting cycles before the gizzard of the instars is of a diameter to allow passage of larger flagellates (Nalepa et al., 2001). Since blattid cockroaches have a dentated and strongly muscled gizzard (Bignell, 1981), larger eukaryotes may be damaged and not be able to colonize the hindguts of the early instars, which leaves more free space for bacterial colonization.

The early colonizers likely create a reduced environment in the gut, which favors colonization by obligate anaerobes, which already colonize the anoxic guts of the second instar and probably displace the early colonizers. This would explain why the gut microbiota of second instar nymphs showed more similarities with the microbiota of cockroaches from the third instar until adulthood, than with first instar nymphs. From second instar until adulthood the cockroaches were consistently colonized by anaerobic bacteria (e.g. *Ruminococcaceae*, *Lachnospiraceae* and *Rikenellaceae*), which have been previously found in large abundance in the anoxic guts of adult cockroaches (Schauer et al., 2012; Schauer et al., 2014; Bauer et al.,

2015; Mikaelyan et al., 2016). Also studies of the gut microbiota in larvae and adults of the higher termite *Nasutitermes arborum* showed a higher relative abundance of anaerobes in the final developmental stages, which may provide more favorable environmental conditions for growth of these groups than first instar larvae (Diouf et al., 2015).

Mechanisms of community assembly

There are two important mechanisms of microbial community acquisition in cockroaches: Vertical inheritance, by which the gut microbiota is transferred to the offspring, e.g. by coprophagy or stomodeal exchange (Bell et al., 2007) (although the latter has never been reported for blattid cockroaches) and random inoculation from the immediate environment, which implies that gut microbiota assembly follows more stochastic events (Brune and Dietrich, 2015). In both cases, most likely those microorganisms will be selected that are favored by the conditions in the gut habitat, which introduces also a deterministic component in community assembly (Mikaelyan et al., 2016).

The strong variations among replicates of the first instar indicate that following hatching, the initially germ-free gut is colonized by random inoculation with microbes from the immediate environment. This stochastic scenario likely gives rise to substantial variations between individuals. Schauer et al. (2014) already found strong variations in microbial community composition and metabolites among individual adult *S. lateralis*. In our analysis even pools of several individuals showed strong variations. Moreover, the prevalent dominance of one or two bacterial groups (e.g. *Enterobacteriaceae* or *Pseudomonadaceae*) in some samples suggests that those groups that are favored by the conditions in the gut, colonize the gut initially in high density. This is possible since no microorganisms that compete for space and nutrients are established yet. A similar scenario of community assembly in various environments was introduced by Curtis and Sloan (2004). They postulated that colonization from a high diversity reservoir (source of inoculums) depends on the ability of the microbe to colonize the niche and the random chance that it is picked up from the reservoir, which will often lead to a high variability in microbial community composition.

We also found variations in the gut microbiota among cockroaches from the second instar to adulthood, which are likely caused by frequent molts of the insect in the course of its development. Since the exoskeletal lining of crop and hindgut is shed during the molt, this process results in a disruption of the gut microbiota as microbes, especially those that are attached to the cuticle, are (at least partially) lost (Engel and Moran, 2013). The reinoculation

with microorganisms from a possibly large reservoir after each molt, introduces repeatedly a stochastic element in community assembly.

Since cockroaches reared in isolation had no contact to conventional colony members, no inheritance of gut microbiota was possible, except a possible inoculation from the surface of the ootheca. Coprophagy is a common behavior in wood-feeding cockroaches (Klass et al., 2008; Nalepa, 2011), and cockroach feces provide an important source of inoculum for all developmental stages, especially for the early ones (Nalepa et al., 2001; Bell et al., 2007). Since cockroaches reared in isolation had no access to feces of conventional colony members, we expected to find a more distinct gut microbiota in these cockroaches than in conventional ones. Surprisingly, this was not the case. Therefore, we suggest that the majority of the bacteria in the cockroach gut is acquired from the environment and that vertical inheritance and general coprophagy are not the dominant factors that shape community structure in *S. lateralis*. The few bacterial groups that were missing in isolated cockroaches (e.g. *Clostridium* 5 and Arthropod cluster) likely require a transfer via coprophagy. Similarly, a study by Sabree et al. (2012) has shown that the gut microbiota of the nonsocial cockroach *Periplaneta americana* was dominated by bacterial species common in the environment, whereas social species like termites were dominated by specialized communities of symbionts.

Microbial fermentation products

Like previously shown for adult *S. lateralis* (Schauer et al., 2012; Schauer et al., 2014), we found acetate as major fermentation product also in all nymphs. The strong accumulation of lactate in the guts of first instar cockroaches is justified, as lactate is a major fermentation product of the abundant groups like *Enterococcaceae* (Lory, 2014), *Enterobacteriaceae* (Tegtmeier et al., 2016) or *Lactobacillaceae* (Schleifer, 2009) in the first instar. Moreover, lactate might also accumulate in first instar guts, as lactate consuming secondary fermenting bacteria, e.g. propionic acid bacteria, are not established yet. A correlation between glucose levels and lactate formation is quite common, as shifts towards increased lactate formation in the presence of high glucose concentrations have also been reported for cultures of *Klebsiella aerogenes* (Teixeira de Mattos and Tempest, 1983) and several *Streptococcus* spp. (Thomas et al., 1979; Condon, 1987). A similar shift towards lactate has been observed also for strain EbSL (a mixed acid fermenting *Enterobacteriaceae* species) *in vitro*. Furthermore, when first-instar germ-free cockroaches, which showed high glucose concentrations in their guts, were colonized by strain EbSL, also high amounts of lactate accumulated (Tegtmeier et al., 2016).

Hydrogen emission of living cockroaches indicated high fermentation rates already in the early instars, which is in agreement with high bacterial colonization densities. Despite the obvious effects of oxygen on metabolic processes, the high hydrogen emissions rates of first instar cockroaches, further indicates the prevalence of fermentative processes even at microoxic conditions in the hindgut. Also pure cultures of strain EbSL showed an increase in hydrogen production in the presence of low oxygen concentrations (Tegtmeier et al., 2016).

Acquisition and succession of methanogens

The strong decrease in hydrogen emission rates with animal age indicated an increase of hydrogen consuming microorganisms, especially in adult cockroaches. Since adults emitted highest amounts methane, apparently methanogenic archaea fairly contribute to hydrogen consumption. A similar trend was also found for the ruminal microbiota, where hydrogen consumption capacities increased with animal age (Fonty et al., 2007). On the contrary, hydrogen accumulates in younger instars and methanogens play only a minor role in hydrogen consumption and are even absent in the first instar. Therefore, hydrogen is not a limiting factor for methanogenesis in early instars, which was further confirmed by our finding that methanogenesis could not be stimulated by the addition of external hydrogen (Figure 2.3).

However, the lower hydrogen emission rates in sixth and eighth instars cannot exclusively be explained with consumption by methanogenic archaea. The decrease of microbial density with the age of the cockroach indicates that the decrease in hydrogen emission results at least partially from a lower rate of formation due to a lower abundance of fermenting bacteria. Moreover, also a consumption of hydrogen by other microorganisms should be considered. Sulfate reducing bacteria (*Desulfovibrionaceae*) were of higher abundance in the sixth instar and may contribute to hydrogen consumption. Also homoacetogenic bacteria may contribute to hydrogen consumption. Nevertheless, homoacetogenesis is only a minor metabolic process in adults of the closely related cockroach *Periplaneta americana*, since most of the CO_2 and hydrogen was incorporated in methane rather than acetate (Breznak and Switzer, 1986; Kane and Breznak, 1991).

The acquisition of methanogenic archaea likely happens via consumption of feces from other cockroaches, which would explain why colonization of the gut by methanogens required contact to conventional cockroaches. Since methanogenic archaea are highly oxygen sensitive (Kiener and Leisinger, 1983; Sprenger et al., 2007; Wolfe and Metcalf, 2010), coprophagy possibly facilitates their transmission, as they could persist in anoxic gut contents. Absence of

methane emission in cockroaches reared in isolation has also been observed for *Eublaberus posticus* by Cruden and Markovetz (1984), who also suggested a transmission of methanogens via the fecal route.

The presence of oxygen in the guts of first instar cockroaches likely does not allow a proper colonization by methanogens. Furthermore, since methanogens grow very slow (Leadbetter and Breznak, 1996; Leadbetter et al., 1998), they are possibly not established in the first instar yet, which would explain why methane emission was absent in this early stage of development. The low and irregular methane emission rates during the nymphal development are probably caused by a disruption of the gut microbiota due to the frequent molts of the cockroach. Only in adults the methanogenic community can establish properly, as the microbial community is not affected by the molt of the insect anymore (Engel and Moran, 2013) and a stable metabolic network can be assembled.

In adult *Periplaneta americana* two abundant methanogenic phylotypes, *Methanimicrococcus blatticola* and a *Methanobrevibacter* species were found. The *Methanobrevibacter* species occurs endosymbiotically in the ciliate *Nyctotherus ovalis* (Gijzen et al., 1991; Sprenger et al., 2000). As methane emission was found to be positively correlated with the amount of intestinal ciliates (Gijzen et al., 1991), which can host more than 4000 methanogens per cell (Hackstein and Stumm, 1994), the absence of ciliates additionally explains the low methane emission rates in early instars.

References

1. **Andrews JH, Harris RF.** 1986. r- and K-selection and microbial ecology, p. 99–147. *In* Marshall KC (ed), Advances in microbial ecology. Volume 9. Plenum Press, New York, USA.
2. **Bauer E, Lampert N, Mikaelyan A, Köhler T, Maekawa K, Brune A.** 2015. Physicochemical conditions, metabolites, and community structure of the bacterial microbiota in the gut of wood-feeding cockroaches (Blaberidae : Panesthiinae). FEMS Microbiol Ecol **91:**1–14.
3. **Bell WJ, Roth LM, Nalepa CA.** 2007. Cockroaches: Ecology, Behavior, and Natural History. Johns Hopkins University Press, Baltimore, Maryland.

4. **Benjamino J, Graf J.** 2016. Characterization of the core and caste-specific microbiota in the termite, *Reticulitermes flavipes*. Front Microbiol **7:**171. doi: 10.3389/fmicb.2016.00171
5. **Bignell DE.** 1981. Nutrition and digestion, p 57–86. *In* Bell WJ, Adiyodi KG (ed), The American cockroach, Chapman & Hall, London, UK.
6. **Boga HI, Ji R, Ludwig W, Brune A.** 2007. *Sporotalea propionica* gen. nov. sp. nov., a hydrogen-oxidizing, oxygen-reducing, propionigenic firmicute from the intestinal tract of a soilfeeding termite. Arch Microbiol **187:**15–27.
7. **Breznak, JA, Switzer JM.** 1986. Acetate synthesis from H_2 plus CO_2 by termite gut microbes. Appl Environ Microbiol **52:**623–630.
8. **Brune A.** 1998. Termite guts: the world's smallest bioreactors. Trends Biotechnol **16:**16–21.
9. **Brune A.** 2010. Methanogens in the digestive tract of termites, p 81–100. *In* Hackstein JHP (ed), (Endo)symbiotic Methanogenic Archaea. Springer, Heidelberg, Germany.
10. **Brune A.** 2014. Symbiotic digestion of lignocellulose in termite guts. Nat Rev Microbiol **12**:168–80.
11. **Brune A, Dietrich C.** 2015. The gut microbiota of termites: digesting the diversity in the light of ecology and evolution. Annu Rev Microbiol **69:**145–66.
12. **Brune A, Emerson D, Breznak JA.** 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.
13. **Brune A, Ohkuma M.** 2011. Role of the termite gut microbiota in symbiotic digestion, p 439–475. *In* Bignell DE, Roisin Y, Lo N (ed), Biology of Termites: A Modern Synthesis. Springer, Dordrecht, Netherlands.
14. **Cazemier AE, Hackstein JHP, Op den Camp HJM, Rosenberg J, van der Drift C.** 1997. Bacteria in the intestinal tract of different species of arthropods. Microb Ecol **33:**189–197.
15. **Chapman RF.** 2013. Head, p 547–587. *In* Simpson SJ, Douglas AE (ed), The insects: structure and function, 5th ed, Cambridge Univ. Press, Cambridge, UK.
16. **Condon S.** 1987. Responses of lactic acid bacteria to oxygen. FEMS Microbiol Rev **46:**269–280.
17. **Cruden DL, Markovetz AJ**. 1984. Microbial aspects of the cockroach hindgut. Arch Microbiol **138:**131–139.

18. **Curtis TP, Sloan WT.** 2004. Prokaryotic diversity and its limits: microbial community structure in nature and implications for microbial ecology. Curr Opin Microbiol **7:**221 226.
19. **Daigle D, Simen BB, Pochart P.** 2011. High-throughput sequencing of PCR products tagged with universal primers using 454 Life Sciences systems. Curr Protoc Mol Biol **96:**1–14.
20. **Dietrich C, Köhler T, Brune A.** 2014. The cockroach origin of the termite gut microbiota: patterns in bacterial community structure reflect major evolutionary events. Appl Environ Microbiol **80:**2261–9.
21. **Diouf M, Roy V, Mora P, Frechault S, Lefebvre T, Hervé V, et al.** 2015. Profiling the succession of bacterial communities throughout the life stages of a higher termite *Nasutitermes arborum* (Termitidae, Nasutitermitinae) using 16S rRNA gene pyrosequencing. PLoS ONE **10:**e0140014 doi: 10.1371/journal.pone.0140014.
22. **Dopheide A, Lear G, Stott R, Lewis G.** 2008. Molecular characterization of ciliate diversity in stream biofilms. Appl Environ Microbiol **74:**1740–1747.
23. **Engel P, Moran NA.** 2013. The gut microbiota of insects – diversity in structure and function. FEMS Microbiol Rev **37:**699–735.
24. **Favier CF, Vaughan EE, De Vos WM, Akkermans ADL.** 2002. Molecular monitoring of succession of bacterial communities in human neonates. Appl Environ Microbiol **68:**119–226.
25. **Fonty G, Joblin K, Chavarot M, Roux R, Naylor G, Michallon F.** 2007. Establishment and development of ruminal hydrogenotrophs in methanogen-free lambs. Appl. Environ. Microbiol. **73:**6391–6403.
26. **Gijzen HJ, Broers CA, Barugahare M, Stumm CK.** 1991. Methanogenic bacteria as endosymbionts of the ciliate *Nyctotherus ovalis* in the cockroach hindgut. Appl Environ Microbiol **57:**1630–1634.
27. **Gillilland MG, Erb-Downward JR, Bassis CM, Shen MC, Toews GB, Young VB, Huffnagle GB.** 2012. Ecological succession of bacterial communities during conventionalization of germ-Free Mice. Appl. Environ. Microbiol.**78:**2359–2366.
28. **Hackstein JHP, Stumm CK.** 1994. Methane production in terrestrial arthropods. Proc Natl Acad Sci USA **91:**5441–5445.
29. **Hominick WM, Davey KG.** 1973. Food and spatial distribution of adult female pinworms parasitic in the hindgut of *Periplaneta americana* L. Int J Parasitol **3:**759–771.

30. **Hominick WM, Davey KG.** 1975. The effect of nutritional level of the host on space and food available to pinworms in the colon of *Periplaneta americana* L. Camp Biochem Physiol **51:**83–88.
31. **Hongoh Y.** 2011. Toward the functional analysis of uncultivable, symbiotic microorganisms in the termite gut. Cell Mol Life Sci **68:**1311–25.
32. **Hongoh Y, Ohkuma M, Kudo T.** 2003. Molecular analysis of bacterial microbiota in the gut of the termite *Reticulitermes speratus* (Isoptera; Rhinotermitidae). FEMS Microbiol Ecol **44:**231–242.
33. **Inward D, Beccaloni G, Eggleton P.** 2007. Death of an order: a comprehensive molecular phylogenetic study confirms that termites are eusocial cockroaches. Biol Lett **3:**331–335.
34. **Kane MD, Breznak JA.** 1991. Effect of host diet on production of organic acids and methane by cockroach gut bacteria. Appl Environ Microbiol **57:**2628–2634.
35. **Kiener A, Leisinger T.** 1983. Oxygen sensitivity of methanogenic bacteria. Syst Appl Microbiol **4:**305–312.
36. **Klass KD, Nalepa C, Lo N**. 2008. Wood-feeding cockroaches as models for termite evolution (Insecta: Dictyoptera): *Cryptocercus* versus *Parasphaeria boleiriana*. Mol Phylogenet Evol **46:**809–17.
37. **Köhler T, Dietrich C, Scheffrahn RH, Brune A.** 2012. High-resolution analysis of gut environment and bacterial microbiota reveals functional compartmentation of the gut in wood-feeding higher termites *Nasutitermes* spp. Appl Environ Microbiol **78:**4691–4701.
38. **Lane DJ.** 1991. 16S/23S rRNA sequencing, p 115–175. *In* Stackebrandt E, Goodfellow M (ed), Nucleic acid techniques in bacterial systematics. John Wiley & Sons Ltd, New York, NY.
39. **Leadbetter JR, Breznak JA.** 1996. Physiological ecology of *Methanobrevibacter cuticularis* sp. nov. and *Methanobrevibacter curvatus* sp. nov., isolated from the hindgut of the termite *Reticulitermes flavipes*. Appl Environ Microbiol **62:**3620–3631.
40. **Leadbetter JR, Crosby LD, Breznak JA**. 1998. *Methanobrevibacter filiformis* sp. nov., a filamentous methanogen from termite hindguts. Arch Microbiol **169:**287–292.
41. **Lo N, Bandi C, Watanabe H, Nalepa C, Beninati T.** 2003. Evidence for co-cladogenesis between diverse dictyopteran lineages and their intracellular endosymbionts. Mol Biol Evol **20:**907–913.

42. **Lory S.** 2014. The Family *Enterococcaceae*, p 75–77. *In* Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F (ed), The Prokaryotes. 4th ed, Springer, Berlin.
43. **Mikaelyan A, Dietrich C, Köhler T, Poulsen M, Sillam-Dussès D, Brune A.** 2015*b*. Diet is the primary determinant of bacterial community structure in the guts of higher termites. Mol Ecol **24:**5284–5295.
44. **Mikaelyan A, Köhler T, Lampert N, Rohland J, Boga H, Meuser K, Brune A.** 2015*a*. Classifying the bacterial gut microbiota of termites and cockroaches: a curated phylogenetic reference database (DictDb). Syst Appl Microbiol **38:**472–482.
45. **Mikaelyan A, Thompson CL, Hofer MJ, Brune A.** 2016. Deterministic assembly of complex bacterial communities in guts of germ-free cockroaches. Appl Environ Microbiol **82:**1256–1263.
46. **Nalepa CA.** 2011. Altricial development in wood-feeding cockroaches: the key antecedent of termite eusociality, p 69–95. *In* Bignell DE, Roisin Y, Lo N (ed.), Biology of Termites: A Modern Synthesis. Springer, Dordrecht, Neth.
47. **Nalepa CA.** 2015. Origin of termite eusociality: Trophallaxis integrates the social, nutritional, and microbial environments. Ecol Entomol **40:**323–35.
48. **Nalepa CA, Bignell DE, Bandi C.** 2001. Detritivory, coprophagy, and the evolution of digestive mutualisms in Dictyoptera. Insectes Sociaux **48:**194–201.
49. **Odelson DA, Breznak JA.** 1983. Volatile fatty acid production by the hindgut microbiota of xylophagous termites. Appl Environ Microbiol **45:**1602–1613.
50. **Oksanen JF, Blanchet G, Kindt R et al.** 2015. vegan: Community ecology package. R package version 2.3-0. Available from http://CRAN.R-project.org/package=vegan.
51. **Pianka E.** 1970. On r- and K-selection. American Naturalist. **104:**592–597.
52. **Rahman NA, Parks DH, Willner DL, Engelbrektson AL, Goffredi SK, Warnecke F, Scheffrahn RH, Hugenholtz P et al.** 2015. A molecular survey of Australian and North American termite genera indicates that vertical inheritance is the primary force shaping termite gut microbiomes. Microbiome, **3:**1–16.
53. **Sabree ZL, Huang CY, Arakawa G, Tokuda G, Lo N, Watanabe H, Moran NA.** 2012. Genome shrinkage and loss of nutrient-providing potential in the obligate symbiont of the primitive termite Mastotermes darwiniensis. Appl Environ Microbiol **78:**204–210.
54. **Schauer C, Thompson CL, Brune A**. 2012. The bacterial community in the gut of the cockroach *Shelfordella lateralis* reflects the close evolutionary relatedness of cockroaches and termites. Appl Environ Microbiol **78:**2758–2767.

55. **Schauer C, Thompson C, Brune A.** 2014. Pyrotag sequencing of the gut microbiota of the cockroach *Shelfordella lateralis* reveals a highly dynamic core but only limited effects of diet on community structure. PLoS One **9:**e85861. doi:10.1371/journal.pone.0085861.
56. **Schleifer K-H**, 2009. Family I. *Lactobacillaceae*, p 465. *In* De Vos P, Garrity GM, Jones D, Krieg NR, Ludwig W, Rainey FA, Schleifer KH, Whitman WB (ed), Bergey's Manual of Systematic Bacteriology. 2nd ed, Springer, New York.
57. **Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, et al.** 2009. Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol **75:**7537–7541.
58. **Sprenger WW, van Belzen MC, Rosenberg J, Hackstein JHP, Keltjens JT.** 2000. *Methanomicrococcus blatticola* gen. nov., sp., nov., a methanol- and methylamine-reducing methanogen from the hindgut of the cockroach *Periplaneta americana*. Int J Syst Evol Microbiol **50:**1989–1999.
59. **Sprenger WW, Hackstein JHP, Keltjens JT.** 2007. The competitive success of *Methanomicrococcus blatticola*, a dominant methylotrophic methanogen in the cockroach hindgut, is supported by high substrate affinities and favorable thermodynamics. FEMS Microbiol Lett **60:**266–275.
60. **Stubner S.** 2002. Enumeration of 16S rDNA of *Desulfotomaculum* lineage in ricefield soil by real-time PCR with Sybr Green detection. J Microbiol Methods **50:**155–164.
61. **Suarez ME, Thorne BL**. 2000. Rate, amount, and distribution pattern of alimentary fluid transfer via trophallaxis in three species of termites (Isoptera: Rhinotermitidae, Termopsidae). Annals of the Entomological Society of America **93:**145-155.
62. **Tegtmeier D, Thompson CL, Schauer C, Brune A.** 2016. Oxygen affects gut bacterial colonization and metabolic activities in a gnotobiotic cockroach model. Appl Environ Microbiol **82:**1080–1089.
63. **Teixeira de Mattos MJ, Tempest DW**. 1983. Metabolic and energetic aspects of the growth of *Klebsiella aerogenes* NCTC 418 on glucose in anaerobic chemostat culture. Arch Microbiol **134:**80–85.
64. **Tholen A, Brune A** 2000. Impact of oxygen on metabolic fluxes and *in situ* rates of reductive acetogenesis in the hindgut of the wood-feeding termite *Reticulitermes flavipes*. Environ Microbiol **2:**436–449.

65. **Thomas TD, Ellwood DC, Longyear MV.** 1979. Change from homo- to heterolactic fermentation by *Streptococcus lactis* resulting from glucose limitation in anaerobic chemostat cultures. J Bacteriol **138:**109–117.
66. **van Hoek AH, van Alen TA, Sprakel VS, Hackstei VS, Vogels GD.** 1998. Evolution of anaerobic ciliates from the gastrointestinal tract: phylogenetic analysis of the ribosomal repeat from *Nyctotherus ovalis* and its relatives. Mol Biol Evol **15:**1195–1206.
67. **Wang Q, Garrity GM, Tiedje JM, Cole JR** 2007. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol **73:**5261–5267.
68. **Warnecke F, Luginbühl P, Ivanova N, Ghassemian M, Richardson TH, Stege JT, et al**. 2007. Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. Nature **450:**560–565.
69. **Wolfe RS, Metcalf WW**. 2010. A vacuum-vortex technique for preparation of anoxic solutions or liquid culture media in small volumes for cultivating methanogens or other strict anaerobes. Anaerobe **16:**216–9.

Supplementary Material

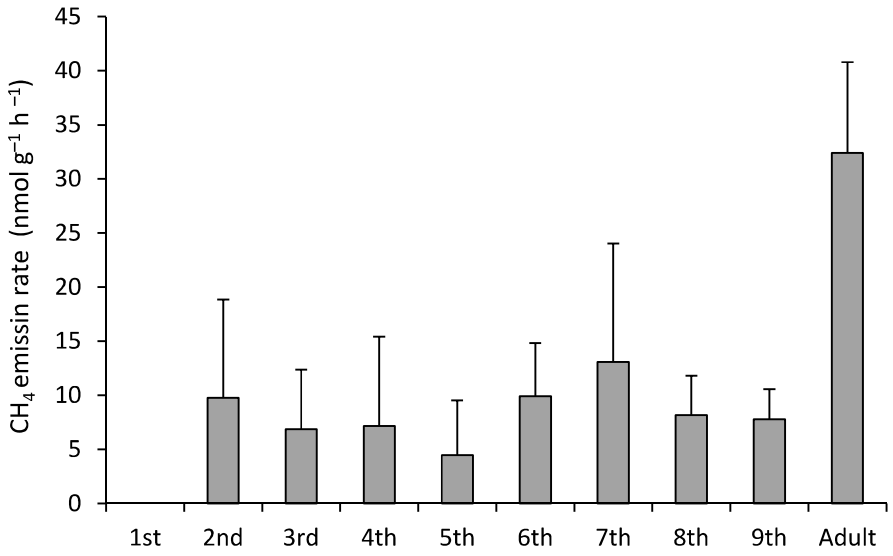

Figure S2.1. Methane emission rates of all instars and adult *S. lateralis*. Bars represent means (+ standard deviation) of three to eight replicate experiments with pools of 4–20 cockroaches (1st instar, n = 110; 2nd instar, n = 97; 3rd instar, n = 59; 4th instar, n = 25; 5th instar, n = 18; 6th instar, n = 43; 7th instar, n = 10; 8th instar, n = 45; 9th instar, n = 25; adults, n = 52).

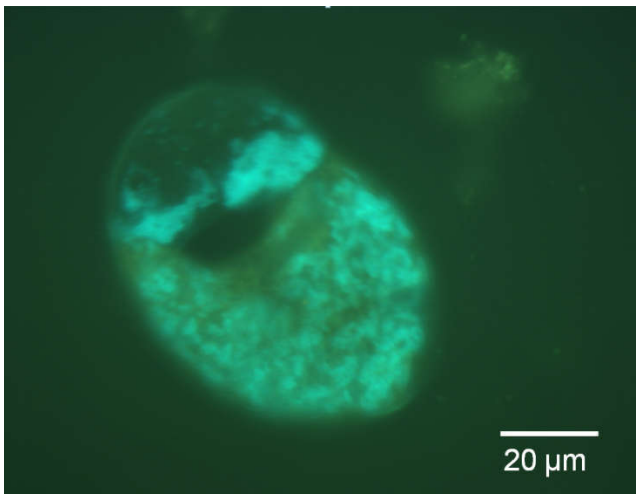

Figure S2.2. F_{420}-epifluorescence micrograph of a ciliate in the hindgut of *Shelfordella leteralis*, exhibiting the characteristic F_{420}-autofluorescence of methanogenic archaea.

Chapter 3

Tegtmeier et al. (2016) Appl. Environ. Microbiol. 82, 1080–1089

Oxygen affects gut bacterial colonization and metabolic activities in a gnotobiotic cockroach model

Dorothee Tegtmeier[1,2], Claire L. Thompson[1,2], Christine Schauer[1,2], and Andreas Brune[1,2]

[1] Department of Biogeochemistry, Max Planck Institute for Terrestrial Microbiology, Karl-von-Frisch-Str. 10, 35043 Marburg, Germany

[2] LOEWE Center for Synthetic Microbiology (SYNMIKRO), Philipps-Universität Marburg, Marburg, Germany

Contributions

D.T. planned and designed the study, isolated strain FuSL,* established the inoculation protocol of germ-free cockroaches, performed fluorescence microscopy (GFP and FISH) and qPCR analysis, analyzed gas emission rates* and fermentation products, performed microsensor measurements, analyzed data, discussed results, evaluated and visualized the data and wrote the manuscript.

C.L.T. planned and designed the study, established the sterilization protocol for generating germ-free cockroaches, isolated strain EbSL and transformed it with a GFP plasmid, supervised the diploma project of D.T., and analyzed and discussed results.

C.S. conducted preliminary tests for generating germ-free cockroaches.

A.B. planned and designed the study, supervised the work, discussed results, and wrote the manuscript.

*The isolation of strain FuSL and hydrogen emission rates measurements have been reported already in the diploma thesis of Dorothee Tegtmeier (Universität Kassel, 2012).

Abstract

The gut microbiota of termites and cockroaches represents complex metabolic networks of many diverse microbial populations. The distinct microenvironmental conditions within the gut and possible interactions among the microorganisms make it essential to investigate how far the metabolic properties of pure cultures reflect their activities in their natural environment. We established the cockroach *Shelfordella lateralis* as a gnotobiotic model and inoculated germ-free nymphs with two bacterial strains isolated from the guts of conventional cockroaches. Fluorescence microscopy revealed that both strains specifically colonized the germ-free hindgut. In diassociated cockroaches, the facultatively anaerobic strain EbSL (a new species of *Enterobacteriaceae*) always outnumbered the obligately anaerobic strain FuSL (a close relative of *Fusobacterium varium*), irrespective of the sequence of inoculation, which showed that precolonization by facultatively anaerobic bacteria does not necessarily favor colonization by obligate anaerobes. Comparison of the fermentation products of the cultures formed *in vitro* with those accumulated *in situ* indicated that the gut environment strongly affected the metabolic activities of both strains. Pure cultures formed the typical products of mixed-acid or butyrate fermentation, whereas guts of gnotobiotic cockroaches accumulated mostly lactate and acetate. Similar shifts towards more oxidized products were observed when pure cultures were exposed to oxygen, which corroborated the strong effects of oxygen on metabolic fluxes previously observed in termite guts. Oxygen microsensor profiles of the guts of germ-free, gnotobiotic, and conventional cockroaches indicated that both gut tissue and microbiota contribute to oxygen consumption and suggest that the oxygen status influences colonization success.

Introduction

Many insects, particular those feeding on a fiber-rich diet, possess a dense and complex microbiota. The most prominent example are termites, whose ability to thrive on an entirely lignocellulosic diet depends on the digestive and nutritional contributions of microbial symbionts housed in their intestinal tract (Brune and Ohkuma, 2011; Brune, 2014). During recent years, the microbial community structure of many termites has been studied in detail, and evolutionary patterns in the gut microbiota of termites and their closest phylogenetic relatives, the cockroaches, are slowly emerging (Brune and Dietrich, 2015). Particularly the application of high-throughput sequencing techniques provides sufficient resolution and sampling depth to distinguish phylogenetic and environmental drivers of community structure (Dietrich et al., 2014; Mikaelyan et al., 2015*a*; Mikaelyan et al., 2015*b*).

The functional roles of individual community members and their interactions, however, are more difficult to elucidate, mostly due to their formidable resistance to cultivation. Metagenomic and metatranscriptomic approaches have provided first insights into functional potentials of the gut community (Warnecke et al., 2007; He et al., 2013; Liu et al., 2013), but owing to the lack of reference genomes for many deep-branching lineages of the gut microbiota, it remains difficult to assign functional genes to particular members of the respective communities. Improved binning strategies promise a solution for this problem in the near future (Laczny et al., 2015), but the elucidation of emergent community properties, such as specific interactions or metabolic networks, requires an entirely different approach. Even in cases where representative microorganisms have been brought into culture, our lack of knowledge about abiotic and biotic factors in the gut microenvironment makes it difficult to predict their metabolic activities *in situ*.

The intestinal tract of insects comprises unique microenvironmental conditions. It is therefore essential to investigate how far the *in vitro* metabolic properties of pure cultures reflect their *in situ* activities. Studies with termites have shown that especially the influx of oxygen, whose importance increases inversely proportional with the radius of a gut compartment (Brune, 1998), strongly affects fermentative processes in the entire hindgut community (Tholen and Brune, 2000), but this remains to be investigated with pure cultures. Early colonization of the (presumably oxic) gut and modalities of community succession are also unclear. Based on the observation that the first colonizers of mammalian guts are typically facultative anaerobes, it has been postulated that these bacteria create a reduced environment favorable for the

colonization of obligate anaerobes, which constitute the majority of the climax community (Favier et al., 2002; Gillilland et al., 2012)— a tempting hypothesis that awaits to be experimentally tested.

Experiments with germ-free animals inoculated with one or more strains of defined gut microorganisms would provide excellent opportunities to approach these questions. Such gnotobiotic animals (Gordon and Pesti, 1971) can be used to characterize the response of pure cultures of gut bacteria to their natural habitat and their interactions with other strains, and to construct complex microbial networks. However, such studies were so far restricted mostly to gnotobiotic mammals, particularly rats, mice, and piglets (e.g. Noack et al., 2000; Bäckhed et al., 2004; Samuel and Gordon, 2006; Kozakova et al., 2006).

While germ-free mammals can be obtained only by Cesarean section, germ-free insects are easily generated by chemical surface sterilization of eggs (Doll et al., 1963; Benschoter and Wrenn, 1972; Hamilton and Bradley, 1977; Dillon and Charnley, 2002). Unfortunately, termites are intractable as gnotobiotic models due to their eusociality and obligate dependence on their gut microbiota for food digestion. By contrast, their closest relatives, the non-social omnivorous cockroaches, do not depend on colony members and can be raised under axenic conditions. Moreover, their eggs are contained in egg cases (oothecae) that provide additional protection to the eggs, including potentially detrimental effects of the sterilization process (Doll et al., 1963; Benschoter and Wrenn, 1972). Surface sterilization of eggs does not remove the endosymbiotic *Blattabacterium* sp., an intracellular symbiont that occurs in all cockroaches and is inherited via the germ line (Lo et al., 2003). However, blattabacteria do not occur in the gut but colonize special cells of the fat body; they cannot be removed without severely affecting the well-being of the host because of their essential role in both nitrogen recycling and providing nutrients (Sabree et al., 2009).

We selected the cockroach *Shelfordella lateralis*, an omnivorous member of the cockroach family Blattidae, the sister group of termites (Inward et al., 2007), as a gnotobiotic model. The intestinal tract of *S. lateralis* and other cockroaches is colonized by complex microbial communities, which are dominated by diverse lineages of presumably obligately and facultatively anaerobic bacteria (Schauer et al., 2012; Bauer et al., 2015), but comprise also methanogenic archaea (Kane and Breznak, 1991; Brune, 2010) and ciliate protists (Gijzen et al., 1991). As in termites, the bacterial microbiota of cockroaches participates in the breakdown of food, supplies the host with short-chain fatty acids, and contributes to the host's

nutrition and normal development (Zurek and Keddie, 1996). The gut environment of cockroaches resembles that encountered in many termites (Schauer et al., 2012; Bauer et al., 2015), which explains why the gut microbiota of termites and cockroaches share several common lineages (Schauer et al., 2012; Dietrich et al., 2014) and may allow the use of cockroach guts as a surrogate environment for studying termite gut microbiota.

With this gnotobiotic cockroach model (Figure 3.1), we studied the colonization of the germ-free gut by a facultatively and an obligately anaerobic bacterium isolated from the same environment, using fluorescence microscopy and real-time PCR, and tested the effect of precolonization by one strain on the colonization success of the other. Moreover, we compared the effect of environmental factors on the metabolic product profiles of the strains *in vitro* with their activities in the gut environment *in situ*, including the effect of colonization on the oxygen status of the gut.

Materials and Methods

Generation of germ-free cockroaches

Shelfordella lateralis was obtained from a commercial breeder and maintained as previously described (Schauer et al., 2012). Oothecae were washed in water to remove dirt particles and to select those that floated at the surface, which is indicative of maturity. Only specimens without indentations or other damage were used for experiments.

Oothecae were surface-sterilized in a laminar flow workbench under aseptic conditions using the protocol described by Doll et al. (1963) with several modifications. After brief immersion in 0.1% sodium dodecylbenzenesulfonate, oothecae were placed in 2% peracetic acid solution for 5 min, rinsed in sterile water, and transferred to sterile 50-ml polypropylene tubes. The tubes were incubated at 25 °C. Cockroaches typically hatched from the oothecae within 1 month.

The efficiency of the sterilization protocol was initially evaluated by transferring freshly hatched cockroaches to 500-ml bottles containing Luria Bertani (LB) agar and sterile food (Corn flakes, Kelloggs, Hamburg, Germany). The agar surface was checked over several days for the absence of bacterial or fungal growth. To detect potential contaminants that escape cultivation, whole cockroaches and their feces were homogenized and disrupted by bead beating (FastPrep-24; MP Biomedicals, Irvine, CA, USA) for 45 s at 6.5 m s^{-1}. After DNA

extraction with the FastDNA SPIN kit for soil (MP Biomedicals) according to the manufacturer's manual, 16S rRNA genes were amplified using the *Bacteria*-specific primer pair 27f and 1492r (Lane, 1991); amplicons were purified and sequenced as described earlier (Strassert et al., 2010).

Once the protocol was firmly established, the axenic status of the cockroaches was routinely tested by sacrificing one hatchling of each ootheca; each sacrificed hatchling was crushed with sterile forceps and smeared onto the surface of an LB agar plate. The plates were incubated at 25 °C for several weeks and monitored for the absence of microbial growth. In the rare cases when a plate showed growth of bacteria or fungi, all results obtained with the batch of cockroaches from that ootheca were discarded. In addition, gut contents of individual germ-free cockroaches were randomly inspected for the absence of bacteria by phase-contrast microscopy.

Isolation of bacterial strains from cockroach hindguts

Pure cultures of numerically abundant gut bacteria were isolated from normal adult females of *S. lateralis* by plating serial dilutions of hindgut homogenates on solid media. Facultatively anaerobic bacteria were obtained on nutrient agar plates (Difco, Becton and Dickinson, Franklin Lakes, NJ, USA) and incubated under air at 30 °C. Obligately anaerobic bacteria were isolated on Fastidious Anaerobe Agar (Atlas, 2010) incubated in an anoxic jar under an atmosphere of N_2/CO_2 (80:20, v/v) at 25 °C. To identify the strains, DNA was extracted, and 16S rRNA genes were amplified and sequenced as described above. Two strains were selected for the colonization experiments in the gnotobiotic cockroaches: the facultatively anaerobic strain EbSL and the obligately anaerobic strain FuSL. Both strains have been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) culture collection (strain EbSL, DSM 100672; strain FuSL, DSM 100562).

Green fluorescent protein labeling of strain EbSL

Competent cells of strain EbSL were prepared following the protocol of Sharma and Schimke (1996). Briefly, overnight cultures were grown at 30 °C in YENB medium (0.75% yeast extract, 0.8% nutrient broth) and transferred to fresh medium (200 ml). In the exponential phase, the culture was chilled on ice and centrifuged at 4 °C for 10 min at 4,000 × *g*. Cells were washed twice in sterile distilled water and once in 10% glycerol, and finally resuspended

in 150 µL 10% glycerol. All solutions were kept on ice. Competent cells were stored at –80 °C prior to transformation.

Strain EbSL was transformed with plasmid pGFPuv (Clontech, Palo Alto, CA, USA), which carries genes for ampicillin resistance and green fluorescent protein (GFP) expression under the control of the *lacZ* promoter; the plasmid has a narrow host range that includes *Enterobacteriaceae* and contains no mobilizing or conjugation functions. After plasmid (500 ng) was added to a 50-µl suspension of competent cells of strain EbSL, the cells were transformed by electroporation in 0.2-cm gap electroporation cuvettes (Sigma-Aldrich) using an *E. coli* Gene Pulser (Bio-Rad) with settings of 25 µF, 2.5 kV, and 200 Ω. Cells were recovered in LB medium for 1 h at 30 °C and were then streaked on LB agar supplemented with ampicillin (100 µg/ml); plates were incubated overnight at 30 °C. Transformants were identified by their green fluorescence under UV light.

Plasmid stability was assessed by growing each transformed isolate in antibiotic-free medium overnight at 30 °C. Two independent cultures of each isolate were serially diluted and plated in triplicate onto LB agar and LB agar supplemented with ampicillin. The fraction of plasmid-containing cells was calculated as the number of colonies on LB-ampicillin plates divided by the number of colonies on LB plates. The retention of plasmid pGFPuv in strain EbSL was 95%.

Inoculation of germ-free cockroaches

Strain EbSL and strain FuSL were routinely grown in AM5 medium (Boga and Brune, 2003) containing 5 mM glucose, 0.2% yeast extract, 0.4% Casamino acids, 2 mM cysteine, and 1 mM dithiothreitol (but no ampicillin) and kept under a headspace of N_2/CO_2 (80:20, v/v). The entire inoculation procedure was carried out in a laminar flow workbench under aseptic conditions. Aliquots (200 µL) of cultures in the exponential growth phase (OD_{578} 0.4–0.45) were applied onto sterile filter paper strips, which were immediately placed into sterile 50-ml tubes with five newly hatched germ-free cockroaches. Inoculated cockroaches were incubated at 25 °C. One day after each inoculation, cockroaches were transferred to a fresh tube containing autoclaved wheat bran (Spielberger, Brackenheim, Germany) soaked with water. Incubations were terminated at different time points during the first instar, and batches were analyzed as described below.

Localization of bacteria within the gut

GFP-labelled strain EbSL was grown aerobically on LB medium with ampicillin overnight to allow maturation of the GFP fluorophore and inoculated into germ-free cockroaches as described above. Cells were localized in the gut by observing the intact foregut, midgut, and hindgut sections under a fluorescence microscope.

In the case of strain FuSL, pooled gut sections (foregut, midgut and hindgut; 5 each) were homogenized via sonication (ultrasonic processor UP50H, Hielscher Ultrasonics, Teltow, Germany), and the cells were detected using fluorescence *in situ* hybridization with the *Bacteria*-specific probe EUB338 (Amann et al., 1990) at 46 °C as previously described (Stingl and Brune, 2003).

Quantitative PCR

Cockroaches were dissected with sterile forceps, five hindguts from the same batch were pooled and homogenized, and microbial cells were disrupted by two cycles of beat beating (FastPrep-24; MP Biomedicals, Solon, OH, USA) for 45 s at 6.5 m s^{-1}. DNA was extracted with the NucleoSpin Soil kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's manual. Samples were used for subsequent quantitative real-time PCR (qPCR) analysis.

Standard curves were generated using purified 16S-rRNA PCR products of the target strains, which were checked photometrically for purity (NanoDrop; PeqLab, Erlangen, Germany) and quantified fluorimetrically (Qubit; Invitrogen, Eugene, OR, USA). The number of 16S rRNA genes of target strains was determined by qPCR as described by Stubner (2002) using primers specific for *Enterobacteriaceae* (5 -ATGGCTGTCGTCAGCTCGT-3 and 5 -CCTACTTCTTTTGCAACCCACTC-3) (Castillo et al., 2006) and *Fusobacteriaceae* (5 -GGATTTATTGGGCGTAAAGC-3 and 5 -GGCATTCCTACAAATATCTACGAA-3) (Boutaga et al., 2005), which matched the 16S rRNA gene sequences of the target strains. The total number of bacterial 16S rRNA genes in conventional cockroach guts was determined using the general *Bacteria* primer pair 519fc (5 -CAGCMGCCGCGGTAANWC-3) and 907r (5 -CCGTCAATTCMTTTRAGTT-3) (Lane 1991; primer 519fc modified by Stubner, 2002).

Each sample was analyzed in duplicate with at least three independent determinations, which typically showed 10–30% deviation. All calibration curves were linear over a range of at least six orders of magnitude. Cell densities of strains FuSL and EbSL in the hindgut were estimated

using the copy number of 16S rRNA genes in the genomes of *Enterobacter* spp. (seven) and *Fusobacterium nucleatum* (five) (Ribosomal RNA Database; Stoddard et al., 2015).

Fermentation products of pure cultures *in vitro*

Cultures of strains FuSL and EbSL were routinely grown in AM5 medium amended with 5 mM glucose, 0.1% yeast extract, 0.1% Casamino acids, 1 mM DTT as reducing agent, and 0.8 mg/L resazurin as redox indicator. Tubes were inoculated with 4% preculture and incubated at 30 °C. The influence of oxygen on growth and fermentation products was tested in non-reduced medium by adding different amounts of sterile oxygen to the headspace of cultures incubated on a rotary shaker.

Growth was determined photometrically by following the increase in optical density at 578 nm (OD_{578}) using a culture tube photometer (Spectronic 20^{+}, Milton Roy; path length ca. 1.3 cm); optical densities > 0.8 were calculated after appropriate dilution. After centrifugation of the fully grown cultures, the cell-free supernatants were acidified with H_2SO_4 (50 mM final concentration) and analyzed by HPLC with an ion-exclusion column (Resin H+, IEX, 8 µm, Grom, Rottenburg, Germany) and a refractive index detector (Schauer et al., 2012). The hydrogen concentration in the culture headspaces was analyzed by gas chromatography with a thermal conductivity detector (Schuler and Conrad, 1990).

For computation of electron recoveries, all metabolites were formally oxidized to CO_2, and the number of electrons theoretically released from the respective amounts of products was compared with that of the amount of substrate consumed (Tholen et al., 1997). The amount of glucose assimilated into biomass was estimated using the turbidity of the culture (OD_{578} = 0.1 corresponds to a dry weight of 30 mg l^{-1}) and an elemental composition of $C_4H_8O_2N$ for bacterial cells (Mayberry et al., 1968), which corresponds to 6.9 mmol glucose per g cell mass.

Detection of metabolites *in situ*

For the detection of metabolites in the cockroach hindgut, cockroaches (first-instar nymphs, nine days after inoculation) were dissected under a stereomicroscope using sterile forceps. The fat body surrounding each hindgut was carefully removed, and ten hindguts were pooled and homogenized in 150 µl water by sonication (ultrasonic processor UP50H, Hielscher Ultrasonics, Teltow, Germany). Samples were prepared and analyzed by HPLC as previously described in Schauer et al. (2012).

Hydrogen emission by living cockroaches (first-instar nymphs, seven days after inoculation) was measured by gas chromatography with a packed Mol Sieve 5A column (80/100 mesh; 70 cm × 6.35 mm) and a reduction gas detector (RGD2, Trace Analytical, Menlo Park, CA, USA). For the measurement, pools of ca. ten cockroaches were placed in 15-ml glass vials closed with a rubber stopper. Using the respiratory activity of *S. lateralis* (Schauer et al., 2014), it was estimated that the oxygen concentration in the vials decreased by 0.3–1.1% per hour of incubation. Hydrogen production rates were determined from the linear increase of hydrogen concentration; at least three time points were taken over a period of 5 to 6 h. In the rare cases when the slope slightly decreased at the end of the incubation, initial rates were used.

Oxygen microsensor measurements

Guts from first instar nymphs were dissected, placed in a chamber with a bottom layer of 2% agarose, and immediately embedded at a depth of approximately 2 mm in Ringer's solution solidified with 0.5% agarose. Axial profiles of intestinal oxygen concentrations at the gut center were measured with microsensors (10 or 25 µm tip diameter; Unisense, Aarhus, Denmark) as described previously (Brune et al., 1995).

Nucleotide sequence accession numbers

The 16S rRNA gene sequences of strain EbSL and strain FuSL have been submitted to GenBank under accession numbers KU043525 and KU043524, respectively.

Results

Establishment of the sterilization protocol

Based on previous protocols for the axenic rearing of insects (Doll et al., 1963; Benschoter and Wrenn, 1972; Hamilton and Bradley, 1977), we tested several biocides for their applicability in the surface sterilization of *S. lateralis* oothecae. Highest hatching rates were obtained when oothecae were exposed to 2% peracetic acid for 5 min; since these conditions yielded sterile hatchlings, they were subsequently used as the standard protocol. Sterilization was not reliable at lower concentrations of peracetic acid (0.5%), and longer exposure times (10 or 20 min) severely reduced the hatching rate. Preliminary experiments showed that oothecae treated with benzalkonium chloride (10%, 10 min) often showed microbial growth,

whereas no living cockroaches hatched from oothecae treated with sodium hypochlorite (0.25%, 5 min).

The oothecae subjected to the standard protocol yielded an average of 10 ± 3.6 hatchlings (n = 22), which was not significantly different from the number of hatchlings obtained from untreated controls (11 ± 3.1; n = 20). However, surface sterilization significantly influenced the number of oothecae that yielded healthy hatchlings (36% of treated oothecae vs. 92% of untreated controls; n = 50 each). No bacterial 16S rRNA genes were amplified from the hatchling feces. The only 16S rRNA gene amplified from DNA extracts of whole cockroaches was that of a *Blattabacterium* sp. (99% sequence similarity to the *Blattabacterium* sp. from *Periplaneta americana*, a close relative of *S. lateralis*), which was expected because of the omnipresence of this essential, maternally transmitted endosymbiont in the fat body of cockroaches. This was in agreement with the results of phase-contrast microscopy of gut homogenates, which confirmed the general absence of bacteria from the gut but always showed a small number of large, rod-shaped cells (4–6 µm long and 1–1.5 µm wide) with the same morphology as the blattabacteria located in the fat body tissue surrounding the gut.

The routinely conducted controls, in which one hatchling from each clutch was crushed with sterile forceps and smeared onto the surface of an LB agar plate, showed bacterial or fungal growth in only 2% of all oothecae; in such cases, the results obtained with that batch were discarded.

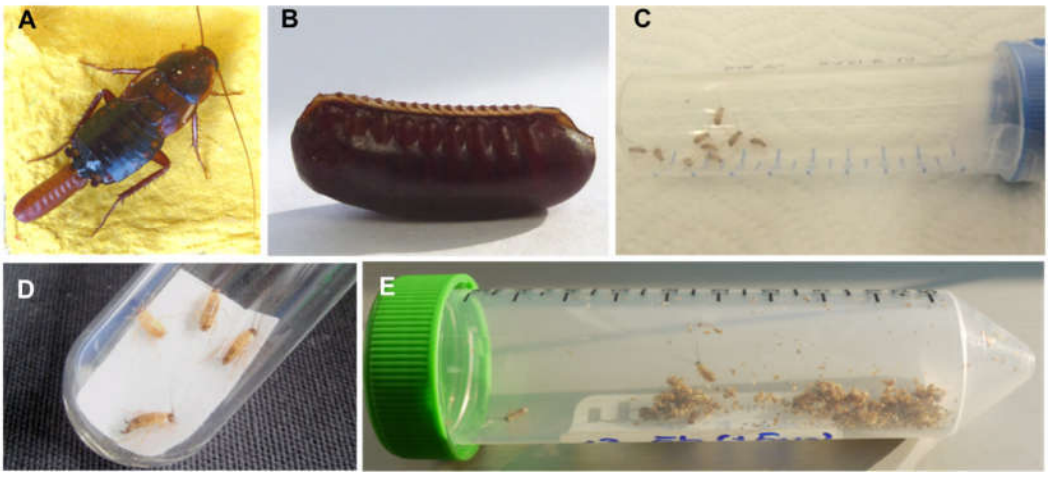

Figure 3.1. *Shelfordella lateralis* as a gnotobiotic model system. (A) Adult female with ootheca; (B) ootheca sterilized with 2% peracetic acid; (C) germ-free hatchlings; (D) germ-free cockroaches on filter paper strips soaked with bacterial culture; and (E) gnotobiotic cockroaches in containers with autoclaved wheat bran.

Isolation of bacterial strains from the cockroach gut

Representative isolates of numerically abundant hindgut bacteria of *S. lateralis* were obtained by serial dilution of hindgut homogenates on solid media. We selected one facultatively anaerobic and one strictly anaerobic strain for further investigations.

The facultatively anaerobic strain EbSL is a hitherto uncultured representative in the family *Enterobacteriaceae*. 16S rRNA gene sequencing showed 95–97% sequence similarity to that of described species in the genera *Pantoea* and *Cronobacter* (formerly *Enterobacter*) and *Shimwellia*; strain EbSL represents a new genus of *Enterobacteriaceae* and will be described in a separate study.

The obligately anaerobic strain FuSL shares more than 99% sequence similarity with *Fusobacterium varium* and showed the phenotypic properties of *Fusobacterium* species previously isolated from cockroach guts, including a pleomorphic cell shape during growth in rich media (Foglesong et al., 1984).

Colonization of germ-free cockroaches

Germ-free cockroaches were inoculated with pure cultures of strains EbSL and FuSL, either in monoassociation or diassociation. Phase-contrast microscopy already indicated a dense colonization of the hindgut compartment, but gut particles interfered with an accurate localization.

Fluorescence microscopy of mono-colonized guts showed that both strains preferentially colonized the hindgut (Figure 3.2A–C); only very few cells were observed in the foregut or midgut section. In hindgut homogenates of diassociated cockroaches, the majority of the cells hybridizing with the *Bacteria*-specific probe also showed GFP fluorescence (Figure 3.2D), which indicated that strain EbSL was more abundant than strain FuSL. Again, almost no cells were detected in the foregut and midgut.

These observations were in agreement with the quantitative assessment of 16S rRNA genes by qPCR, which indicated that the estimated cell density of strain EbSL in diassociated cockroaches was always an order of magnitude higher than that of strain FuSL, irrespective of the sequence of inoculation and the time of incubation (Table 3.1). While the cell density of strain FuSL was about five times higher in monoassociation than in diassociation with strain EbSL, the colonization success of strain EbSL was not significantly affected by the presence of strain FuSL. The number of 16S rRNA genes in monoassociated cockroaches was in the

same range as the total number of bacterial 16S rRNA genes in conventional cockroaches, where *Enterobacteriaceae* and *Fusobacteriaceae* formed only a small fraction of the entire community (Table 3.1).

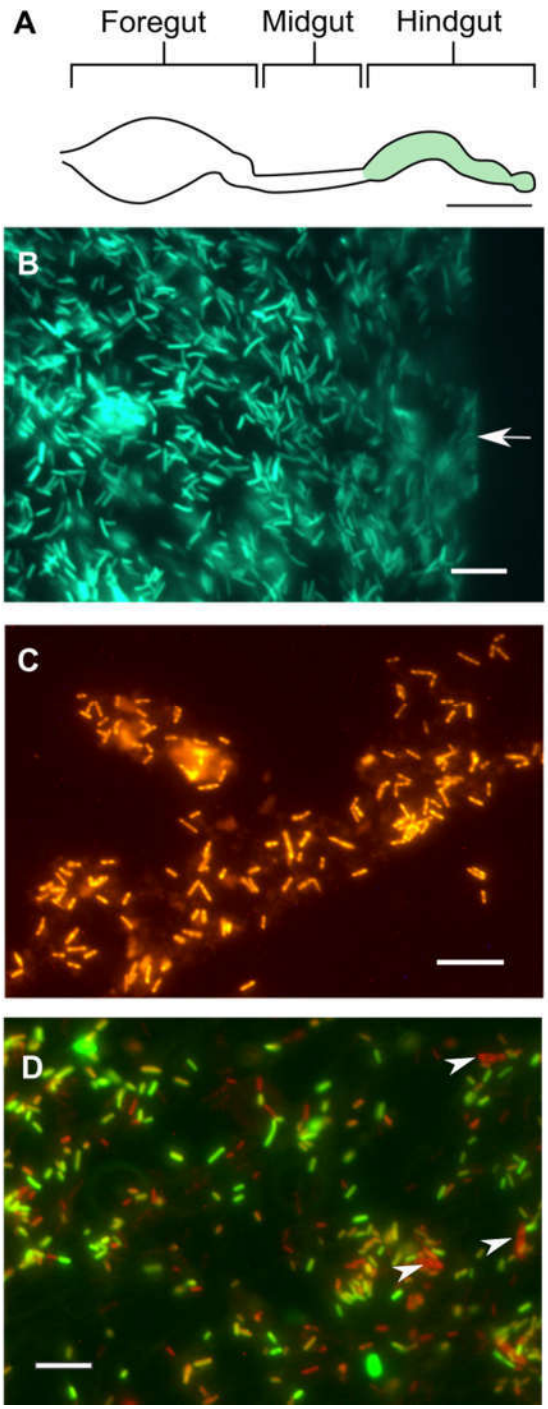

Figure 3.2. Epifluorescence micrographs of hindguts colonized with strains EbSL and FuSL. (A) Scheme illustrating the structure of the intestinal tract of *Shelfordella lateralis* and the exclusive location of fluorescent cells throughout the hindgut. (B) Periphery of an intact hindgut colonized by GFP-labeled strain EbSL (green); the gut wall is indicated by an arrow. (C) Hindgut homogenate of cockroaches colonized by strain FuSL, hybridized with a Cy3-labeled *Bacteria*-specific oligonucleotide probe (orange). (D) Hindgut homogenate of cockroaches diassociated with both strains; the image is an overlay of GFP fluorescence and FISH signal. Cells of *Blattabacterium* sp. are indicated by arrowheads. Scale bars are ca. 2 mm (A) and 10 µm (B–D).

Table 3.1. Quantification of strains EbSL and FuSL in the gut of gnotobiotic cockroaches via qPCR with family-specific primers. Values are means (± standard deviation) of three replicate experiments with five hindguts each. Results with conventional cockroaches are shown for comparison.

Inoculum	Incubation time (no. of days)	No. of 16S rRNA genes (10^7 copies mg^{-1} gut)		Cell density[a] (10^7cells mg^{-1} gut)	
		EbSL	FuSL	EbSL	FuSL
Strain EbSL[b]	5	29.9 ± 15.7		4.3 ± 2.2	
	7	39.8 ± 6.4		5.7 ± 0.9	
	10	40.4 ± 1.2		5.8 ± 0.2	
Strain FuSL[b]	7		8.4 ± 3.8		1.7 ± 0.8
	10		8.7 ± 2.5		1.7 ± 0.5
EbSL + FuSL[c]	9	32.9 ± 14.0	2.08 ± 0.4	4.7 ± 2.0	0.4 ± 0.1
FuSL + EbSL[c]	9	36.3 ± 12.2	1.43 ± 0.4	5.1 ± 1.7	0.3 ± 0.1
Conventional[d]	9	0.1 ± 0.1	0.3 ± 0.4	0.01 ± 0.01	0.06 ± 0.08

[a] Estimated using the rRNA gene copy number of seven for *Enterobacter* spp. and five for *Fusobacterium nucleatum*.

[b] Cockroaches monoassociated with either strain EbSL or strain FuSL.

[c] Cockroaches diassociated with strains EbSL and FuSL, inoculated in the given order (the second strain two days after the first).

[d] Conventional cockroaches (first instar), two replicate experiments; total bacterial 16S rRNA genes were between 8.2 and 24.8 × 10^7 per mg gut.

In situ activities in gnotobiotic cockroaches

The metabolic activities of strains EbSL and FuSL within the cockroach gut were assessed by comparing the metabolites in gut homogenates of gnotobiotic cockroaches with those of germ-free and conventional cockroaches. In all cases, the homogenates contained high concentrations of glucose, which indicated that dietary starch is depolymerized by host enzymes. Glucose levels were similar in gut homogenates of germ-free and gnotobiotic cockroaches but lower in that of conventional animals. In monoassociated cockroaches, the prevailing fermentation products in gut homogenates were acetate and lactate; in some batches of cockroaches associated with strain EbSL, the gut homogenates also contained low amounts of ethanol and succinate. Gut homogenates of conventional cockroaches contained acetate and

lactate, but lactate accumulated in lower amounts. In gut homogenates of germ-free cockroaches, only low amounts of lactate were detected (Table 3.2).

Hydrogen production by the microbiota was assessed by measuring hydrogen emission of cockroaches *in vivo*. Both conventional and gnotobiotic cockroaches emitted hydrogen, albeit at different rates (Table 3.2). Hydrogen emission rates of monoassociated cockroaches were in the same range as those in diassociation (70 ± 23 nmol g^{-1} h^{-1}), with strong variations among replicates. The hydrogen emission of conventional cockroaches, however, was significantly higher. As expected, germ-free cockroaches did not emit any hydrogen.

Table 3.2. Gut metabolites and hydrogen emission rates of gnotobiotic *S. lateralis* monoassociated with strain EbSL or strain FuSL, and germ-free and conventional cockroaches of the same age group.

	Gut metabolite (nmol mg^{-1})[a]					Hydrogen emission rate (pmol mg^{-1} h^{-1})[b]
	Glucose	Acetate	Lactate	Ethanol	Succinate	
Strain EbSL	28.8 ± 3.8	13.3 ± 6.1	15.8 ± 4.2	4.4 ± 7.7	1.5 ± 2.6	76 ± 78
Strain FuSL	31.2 ± 10.6	20.8 ± 5.5	14.0 ± 2.6	–[c]	–	58 ± 39
Conventional	7.2 ± 1.9	22.9 ± 2.1	6.6 ± 3.5	–	–	235 ± 145
Germ-free	36.2 ± 12.3	–	3.6 ± 2.9	–	–	–[d]

[a] Values are means (± standard deviation) of four replicate experiments, using homogenates of ten hindguts with an average fresh weight of 0.2 ± 0.06 mg per gut.

[b] Values are means (± standard deviation) of three to four replicate experiments with pools of ca. ten cockroaches, each with a fresh weight of 8.0 ± 2.3 mg per cockroach.

[c] Below the detection limit (< 1 nmol mg^{-1}).

[d] Below the detection limit (< 1 pmol mg^{-1} h^{-1}).

Influence of cultivation conditions on metabolic activities *in vitro*

When pure cultures of strains EbSL and FuSL were grown *in vitro* on glucose under anoxic conditions (Table 3.3), their fermentation products differed substantially from those produced *in situ* in the gut of monoassociated cockroaches.

The major fermentation products of the strictly anaerobic strain FuSL in pure culture were butyrate, acetate, and hydrogen. Already with 1% oxygen in the headspace, the growth rate, cell yield, and butyrate production decreased significantly, whereas acetate production slightly

increased (Table 3.3). Oxygen was completely consumed in fully grown cultures, as indicated by the reduced status of the redox indicator resazurin at the end of the incubation. With 2% oxygen in the headspace, however, growth, glucose consumption and product formation ceased almost completely; the medium was still oxidized at the end of the incubation. The high electron recovery under anoxic conditions (Table 3.3) can be explained by the substantial amounts of products (3 mM acetate and 1.5 mM butyrate) produced by the amino-acid fermenting strain FuSL already on basal medium (0.1% Casamino acids and 0.1% yeast extract). After subtraction of the products formed in the absence of glucose, the electron recovery decreased to 110%, yielding a reaction stoichiometry of 0.60 mol butyrate, 0.47 mol acetate, and 1.12 mol H_2 per mol glucose.

The major fermentation products of the facultatively anaerobic strain EbSL in pure culture were formate, ethanol, acetate, succinate, and hydrogen, which are typical of mixed-acid fermentations. In contrast to the strictly anaerobic strain FuSL, the cell yield of strain EbSL increased with the oxygen concentration in the headspace, and less formate and ethanol were produced (Table 3.3). Glucose was consumed completely at all oxygen concentrations. The electron recoveries under anoxic conditions were much lower than in the case of strain FuSL and decreased to 90% when the amounts of fermentation products formed on basal medium were subtracted (1 mM acetate and minor amounts of formate, ethanol, and succinate), resulting in corrected reaction stoichiometries of 0.95 mol formate, 0.65 mol ethanol, 0.53 mol acetate, 0.11 mol succinate, and 0.12 mol H_2 per mol glucose.

The fermentation products were also influenced by the glucose concentration in the medium (Table 3.4). In the case of strain EbSL, lactate was entirely absent in cultures grown on 5 mM glucose but increased at higher glucose concentrations. Strain FuSL also produced more lactate at higher glucose concentrations, but the effect was less pronounced.

Table 3.3. Growth and fermentation products of strains EbSL and FuSL cultivated on basal medium with 5 mM glucose at different oxygen concentrations in the headspace. Values are means of duplicate cultures (typically less than 10% deviation).

Oxygen concentration [a]	Turbidity (OD_{578})[b]	Glucose dissim. (mM)[c]	Product (mM)[d]							Electron recovery (%)[e]
			Formate	Ethanol	Succinate	Acetate	Hydrogen	Butyrate	Lactate	
Strain FuSL										
0%	0.65	3.7	–[f]	–	–	5.3	6.4	4.4	0.2	166
1%	0.32	4.3	–	–	–	6.1	6.4	2.1	0.2	102
2%	0.01	0.8	–	–	–	1.0	0.3	–	–	46
Strain EbSL										
0%	0.56	3.9	5.4	3.6	1.1	3.7	0.6	–	–	108
1%	0.61	3.7	2.1	4.0	1.2	4.5	2.4	–	–	122
2%	0.62	3.7	1.5	3.3	1.0	4.3	2.4	–	–	108
4%	0.66	3.6	1.1	2.8	0.9	4.7	2.1	–	–	101
8%	0.78	3.4	0.4	1.5	0.6	4.7	1.0	–	–	83
21%	2.38	2.0[g]	–	–	0.4	0.9	–	–	–	26

[a] Initial values.

[b] Values include cell mass formed on basal medium (0.1% Casamino acids and 0.1% yeast extract).

[c] Dissimilated glucose, accounting for the amount of glucose assimilated into biomass.

[d] Values include the products formed on basal medium (0.1% Casamino acids and 0.1% yeast extract).

[e] Electron recoveries in fermentation products, based on dissimilated glucose.

[f] Below the detection limit (< 0.02 mM)

[g] Corrected for the large amount of cell mass formed on basal medium under oxic conditions (OD_{578} = 0.93).

Table 3.4. Metabolic products of strains EbSL and FuSL cultivated with different glucose concentrations under anoxic conditions. Hydrogen was not determined. Values are means of duplicate cultures (less than 10% deviation).

Glucose (mM)	Products (mol per mol glucose)[a]					
	Formate	Ethanol	Succinate	Acetate	Butyrate	Lactate
Strain FuSL						
5	–[b]	–	–	0.47	0.60	0.03
10	–	–	–	0.22	0.56	0.10
15[c]	–	–	–	0.20	0.53	0.11
Strain EbSL						
5	0.95	0.65	0.11	0.53	–	–
10	0.68	0.76	0.19	0.59	–	0.36
15	0.39	0.62	0.20	0.39	–	0.70

[a] Calculated from consumed glucose, after subtraction of products formed on basal medium (0.1% Casamino acids and 0.1% yeast extract).

[b] Below the detection limit (< 0.02 mM).

[c] Only 12.3 mM glucose was consumed.

Oxygen status of the gut

Microsensor measurements revealed strong differences in oxygen status in the different regions of agarose-embedded guts of conventional, germ-free, and gnotobiotic cockroaches (Figure 3.3). In all cockroaches, oxygen partial pressures were generally low in the foregut and midgut compartment, often under the detection limit of the sensor (ca. 0.15 kPa). In germ-free and conventional guts, the axial oxygen profiles were more variable than in gnotobiotic guts. Highest partial pressures were encountered in the posterior hindgut, where the oxygen levels in germ-free cockroaches surpassed those in gnotobiotic and conventional cockroaches. Guts colonized with strain EbSL consistently exhibited only very low oxygen concentrations in all compartments.

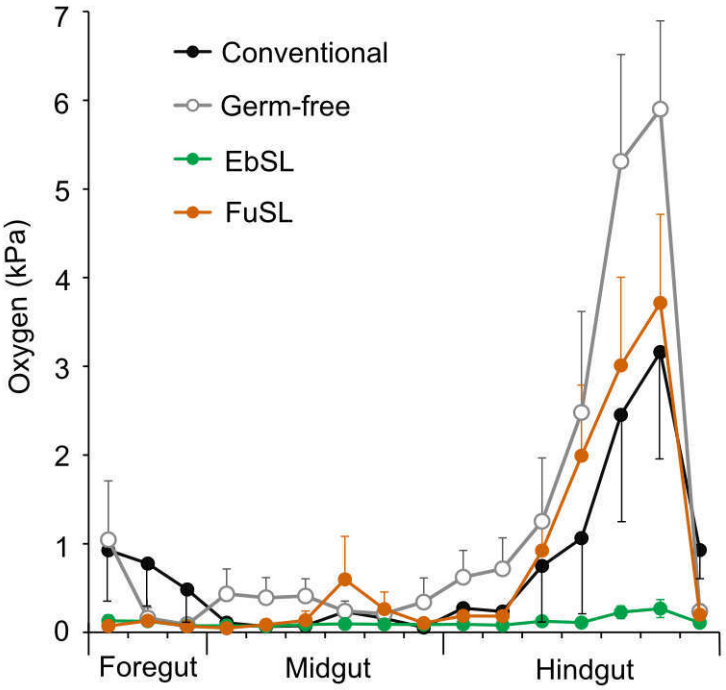

Figure 3.3. Oxygen partial pressure in the guts of gnotobiotic, germ-free, and conventional first instar cockroaches. Axial profiles were measured with microsensors at the gut center. Symbols are means (+ SEM) of replicate measurements with eight different guts. Error bars are shown in one direction only for clarity.

Discussion

Our gnotobiotic cockroach model offered the unique opportunity to study the effect of the gut environment on a defined microbiota of autochthonous gut bacteria. The fermentation product patterns of the model strains under *in situ* conditions, which differed from those observed in anoxic cultures, were elicited *in vitro* by the influence of oxygen and glucose. Microsensor measurements confirmed the assumption that the colonization of the gut with a facultative anaerobe creates an anoxic environment. However, precolonization with the facultatively anaerobic strain EbSL did not favor colonization by the obligately anaerobic strain FuSL, which suggested that the differences in their colonization success are most likely due to their different responses to oxygen.

Model organisms specifically colonize the germ-free hindgut

GFP labeling is a useful tool to localize bacterial strains in the gut. Husseneder and Grace (2005) isolated an indigenous strain of *Enterobacter cloacae* from termite guts and introduced a GFP label to monitor its fate after inoculation into the conventional gut microbiota of

termites, where it persisted up to 11 weeks after inoculation. In our study, the GFP label allowed us to accurately localize strain EbSL in the gnotobiotic cockroach gut without any interference of gut tissue or food particles (Figure 3.2B).

The exclusive colonization of the germ-free cockroach hindgut by strains EbSL and FuSL suggests that only this gut compartment provides a favorable environment for microbial colonization. This is in agreement with observations in conventional cockroaches, where the hindgut shows the highest density and diversity of bacteria among all gut compartments (Cruden and Markovetz, 1984; Schauer et al., 2012). It is likely that the colonization of foregut and midgut is suppressed by the high activities of digestive enzymes in these compartments (Bignell, 1981; Terra and Ferreira, 1994; Chapman, 2013).

The colonization densities of strains EbSL and FuSL in monoassociation and diassociation are much higher than those of all *Enterobacteriaceae* and *Fusobacteriaceae* in the hindgut of conventional cockroaches, and in the case of strain EbSL even surpassed the total cell counts in the hindguts of conventional adults ($2.2 \pm 1.6 \times 10^7$ cells/mg gut; Schauer et al., 2012). The high colonization densities in gnotobiotic cockroaches is most likely explained by the absence of other microorganisms, particularly the ciliate protists, which occupy a substantial portion of the hindgut volume in conventional cockroaches (Gijzen et al., 1991).

Colonization sequence does not explain colonization success

Although colonization by strain EbSL created a mostly anoxic environment in the hindgut, precolonization with this strain did not enhance the colonization success of strain FuSL. This suggests that an early colonization with a facultative anaerobe does not necessarily favor colonization by obligate anaerobes. On the contrary, colonization densities of strain FuSL in the presence of strain EbSL were even lower than in monoassociation, which suggested that the obligate anaerobe is outcompeted by the facultative anaerobe, irrespective of the sequence of colonization. The basis for this phenomenon is not entirely clear, and it is possible that the situation in this simple gnotobiotic model system differs from that in a complex community. Since the fermentation product concentrations in the two strains are similar, the higher growth yields of strain EbSL could be explained by its capacity for respiration. However, glucose does not appear to be a limiting factor in the gnotobiotic gut, so that also antagonistic effects resulting in the suppression of strain FuSL cannot be excluded. Similarly, in diassociated gnotobiotic rats, the relative abundance of *Fusobacterium varium* was almost an order of magnitude lower than that of *Bacteroides thetaiotaomicron* (Noack et al., 2000).

In view of the complete growth inhibition of the obligately anaerobic strain FuSL already at 2% oxygen in the headspace, its capacity to robustly colonize the hindguts of germ-free cockroaches in monoassociation is remarkable. The decreased oxygen partial pressure in hindguts colonized with strain FuSL in comparison to germ-free guts indicated that strain FuSL is able to remove at least some of the oxygen diffusing into its habitat. However, its oxygen tolerance is lower than that of *Fusobacterium nucleatum*, which survives prolonged exposure to air (Loesche, 1969) and can grow in dense cultures in a chemostat even under atmospheric oxygen partial pressure (Diaz et al., 2000).

Colonization of the germ-free hindgut with strain FuSL is probably facilitated by the low oxygen partial pressure in the anterior hindgut, which is below 1 kPa even in germ-free cockroaches, most likely due to the respiratory activity of the gut epithelium. Oxygen consumption by the gut tissue appears to be significant and is probably responsible for the low oxygen partial pressures in the midgut and the production of low amounts of lactate in the hindgut of germ-free nymphs, which is likely caused by a switch to anaerobic metabolism in the gut tissue due to a limiting oxygen supply.

The further reduction of oxygen partial pressure in the hindgut after successful colonization by strain FuSL indicated that the strain itself is able to reduce oxygen, which is confirmed by the results obtained *in vitro*. The removal of oxygen by non-respiratory activities is a common phenomenon in anaerobes (e.g. De Vries et al., 1978; Chen et al., 1993; Karnholz et al., 2002; Riebe et al., 2009) and has been documented for lactic acid bacteria, homoacetogens, and methanogens isolated from the intestinal tracts of termites (Tholen et al., 1997; Boga and Brune, 2003; Tholen et al., 2007; Leadbetter and Breznak, 1996; Graber and Breznak, 2004); many obligate anaerobes possess enzymes that detoxify oxygen or oxygen radicals (Jenney et al., 1999; Brioukhanov and Netrusov, 2007).

Metabolic activities *in situ* are controlled by oxygen

The strong differences between the fermentation products in anoxic cultures and in association with cockroaches cannot be explained by the selective resorption of metabolites by the gut wall. Fermentation products such as acetate that are formed at high rates will inevitably accumulate at the gut center, no matter how efficiently they are resorbed at the gut epithelium (Tholen and Brune 2000). Conversely, metabolites such as butyrate and formate, which do not accumulate in gnotobiotic cockroaches, lack the concentration gradients required for an efficient diffusive transport towards the epithelium.

Therefore, the differences between the product patterns observed under *in situ* conditions and in anoxic cultures must be caused by different rates of formation, which indicates that the metabolism of both strains is strongly affected by microenvironmental factors in the gut habitat. The most obvious factor is the presence of oxygen, whose influx rates into the small guts of first instar nymphs must be substantial (Ebert and Brune, 1997; Brune, 1998). A strong effect of inflowing oxygen on the fermentative processes has been documented for termites, where hindgut fermentations shift to more oxidized products when intact guts are incubated under oxic conditions (Tholen and Brune 2000).

This effect would explain the absence of butyrate and the strong accumulation of acetate in cockroaches colonized by strain FuSL, which is confirmed by the effect of oxygen on butyrate production *in vitro*. A shift from butyrate to acetate has been described also for the more oxygen-tolerant *Fusobacterium nucleatum* when incubated under oxic conditions (Diaz et al., 2000). The strong influence of oxygen on hindgut metabolism is corroborated by the absence of formate and the low concentrations of ethanol observed in cockroaches colonized with strain EbSL and the corresponding effects of oxygen *in vitro*. In this case, the decreased formate production may result from the inhibitory effects of oxygen on pyruvate-formate lyase, as has been reported for *Streptococcus mutans* and *Streptococcus sanguis* (Abbe et al., 1982; Yamada et al., 1985; Takahashi et al., 1987). However, the increase in hydrogen production by strain EbSL in the presence of low oxygen concentrations *in vitro* also suggests an enhanced turnover of formate owing to an increased formate-hydrogen lyase activity *in situ*.

The high proportions of lactate among the fermentation products formed *in situ* in the hindguts of monoassociated cockroaches may also be caused by the high concentrations of free glucose. When grown on 5 mM glucose, strain EbSL formed no lactate, and strain FuSL formed only low amounts. However, at higher glucose concentrations of 10 or 15 mM, which resemble the *in situ* conditions in the guts of conventional cockroaches, strain EbSL formed increasing amounts of lactate; in the case of strain FuSL, increasing glucose concentrations had the same but less pronounced effect. Such shifts towards increased lactate formation in the presence of non-limiting concentrations of glucose have been described also for chemostat cultures of *Klebsiella aerogenes* (Teixeira de Mattos and Tempest, 1983) and several *Streptococcus* spp. (Thomas et al., 1979; Condon, 1987) and are in agreement with the production of lactate by *Fusobacterium varium* grown at high glucose concentrations (Resmer and White, 2011). In conventional cockroaches, the accumulation of lactate was less pronounced, which suggested

that the normal gut microbiota, like that of termites, comprises active lactate-consuming populations (Tholen and Brune, 2000).

Despite the obvious effects of oxygen on the metabolic processes of both strains, the hydrogen emissions of the gnotobiotic cockroaches underscores the prevalence of anaerobic processes in the hindgut. The *in vivo* emission of hydrogen is in agreement with the increase in hydrogen production in the presence of low oxygen concentrations observed *in vitro*. This opens interesting perspectives also for future studies, because a production of hydrogen allows coupling fermentative processes with hydrogenotrophic processes and the creation of synthetic methanogenic microbial communities, which may provide new insights into methanogenesis in insects and the factors limiting the colonization by methanogenic archaea in the intestinal tracts of both invertebrates and mammals (Brune, 2010; St-Pierre et al., 2015).

Lastly, the gnotobiotic cockroach model could provide a valuable tool in "synthetic microbial ecology" by helping to identify factors governing community assembly in cockroaches. It will likely improve also our understanding of community ecology and metabolic interactions in the intestinal tract of the closely related termites, where the eusociality of the host and an obligate dependence on its gut microbiota prohibits gnotobiotic studies.

Acknowledgements

This work was supported within the LOEWE program of the State of Hessen (Center for Synthetic Microbiology) and by the Max Planck Society. CLT received a postdoctoral fellowship of the Alexander von Humboldt Foundation. We thank Katja Meuser for excellent technical assistance.

References

1. **Abbe K, Takahashi S, Yamada T.** 1982. Involvement of oxygen sensitive pyruvate formate-lyase in mixed-acid fermentation by *Streptococcus mutans* under strictly anaerobic conditions. J Bacteriol **152:**175–182.
2. **Amann R, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA.** 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl Environ Microbiol **56:**1919–1925.
3. **Atlas, RM.** 2010. Handbook of microbiological media, p 2036. 4th ed. Taylor and Francis. Boca Raton, Florida.
4. **Bäckhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, Semenkovich CF, Gordon JI.** 2004. The gut microbiota as an environmental factor that regulates fat storage. Proc Natl Acad Sci U S A **101:**15718–15723.
5. **Bauer E, Lampert N, Mikaelyan A, Köhler T, Maekawa K, Brune A.** 2015. Physicochemical conditions, metabolites, and community structure of the bacterial microbiota in the gut of wood-feeding cockroaches (Blaberidae : Panesthiinae). FEMS Microbiol Ecol **91:**1–14.
6. **Benschoter CA, Wrenn, RT.** 1972. Germfree techniques for establishment and maintenance of a colony of aseptic german cockroaches. Ann Entomol Soc Am **65:**641 644.
7. **Bignell DE.** 1981. Nutrition and digestion, p 57–86. *In* Bell WJ, Adiyodi KG (ed), The American cockroach, Chapman & Hall, London.
8. **Boga HI, Brune A.** 2003. Hydrogen-dependent oxygen reduction by homoacetogenic bacteria isolated from termite guts. Appl Environ Microbiol **69:**779–786.
9. **Boutaga K, van Winkelho AJ, Vandenbroucke-Grauls CMJE, Savelkoul PHM.** 2005. Periodontal pathogens: a quantitative comparison of anaerobic culture and real-time PCR. FEMS Immunol Med Microbiol **45:**191–199.
10. **Brioukhanov AL, Netrusov AI.** 2007. Aerotolerance of strictly anaerobic microorganisms and factors of defense against oxidative stress: a review. Appl Biochem Microbiol **43:**567–582.
11. **Brune A, Dietrich C.** 2015. The gut microbiota of termites: digesting the diversity in the light of ecology and evolution. Annu Rev Microbiol **69:**145–66.

12. **Brune A, Emerson D, Breznak JA.** 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.
13. **Brune A, Ohkuma M.** 2011. Role of the termite gut microbiota in symbiotic digestion, p 439–475. *In* Bignell DE, Roisin Y, Lo N (ed), Biology of termites: A modern synthesis. Springer, Dordrecht, Netherlands.
14. **Brune A.** 1998. Termite guts: the world's smallest bioreactors. Trends Biotechnol **16:**16–21.
15. **Brune A.** 2010. Methanogenesis in the digestive tracts of insects, p 707–728. *In* Timmis, K.N. (ed.), Handbook of hydrocarbon and lipid microbiology, Springer, Heidelberg.
16. **Brune A.** 2014. Symbiotic digestion of lignocellulose in termite guts. Nat Rev Microbiol **12:**168–180.
17. **Castillo M, Martín-Orú SM, Manzanilla EG, Badiola I, Martín M, Gasa J.** 2006. Quantification of total bacteria, enterobacteria and lactobacilli populations in pig digesta by real-time PCR. Vet Microbiol **114:**165–170.
18. **Chapman RF.** 2013. The alimentary canal, digestion and absorption, p 46–79. *In* Simpson SJ, Douglas AE (ed), The insects: structure and function, 5th ed, Cambridge Univ. Press, Cambridge, UK.
19. **Chen L, Liu MY, Legall J, Fareleira P, Santos H, Xavier AV.** 1993. Purification and characterization of an NADH-rubredoxin oxidoreductase involved in the utilization of oxygen by *Desulfovibrio gigas*. Eur J Biochem **216:**443–448.
20. **Condon S.** 1987. Responses of lactic acid bacteria to oxygen. FEMS Microbiol Rev **46:**269–280.
21. **Cruden DL, Markovetz AJ.** 1984. Microbial aspects of the cockroach hindgut. Arch Microbiol **138:**131–139.
22. **De Vries W, Donkers C, Boellaard M, Stouthamer AH.** 1978. Oxygen metabolism by the anaerobic bacterium *Veillonella alcalescens.* Arch Microbiol **119:**167–174.
23. **Diaz PI, Zilm PS, Rogers AH.** 2000. The response to oxidative stress of *Fusobacterium nucleatum* grown in continuous culture. FEMS Microbiol Letts **187:**31–34.
24. **Dietrich C, Köhler T, Brune A.** 2014. The cockroach origin of the termite gut microbiota: patterns in bacterial community structure reflect major evolutionary events. Appl Environ Microbiol **80:**2261–9.

25. **Dillon RJ, Charnley AK.** 2002. Mutualism between the desert locust *Schistocerca gregaria* and its gut microbiota. Res Microbiol **153:**503–509.
26. **Doll JP, Trexler PC, Reynolds LI, Bernard GR**. 1963. The use of peracetic acid to obtain germfree invertebrate eggs for gnotobiotic studies. Am Midl Nat **69:**231–239.
27. **Ebert A, Brune A.** 1997. Hydrogen concentration profiles at the oxic-anoxic interface: a microsensor study of the hindgut of the wood-feeding lower termite *Reticulitermes flavipes* (Kollar). Appl Environ Microbiol **63:**4039–4046.
28. **Favier CF, Vaughan EE, De Vos WM, Akkermans ADL.** 2002. Molecular monitoring of succession of bacterial communities in human neonates. Appl Environ Microbiol **68:**119–226.
29. **Foglesong MA, Cruden DL, Markovetz AJ.** 1984. Pleomorphism of Fusobacteria isolated from the cockroach hindgut. J Bacteriol **158:**474–480.
30. **Gijzen HJ, Broers CA, Barugahare M, Stumm CK.** 1991. Methanogenic bacteria as endosymbionts of the ciliate *Nyctotherus ovalis* in the cockroach hindgut. Appl Environ Microbiol **57:**1630–1634.
31. **Gillilland MG, Erb-Downward JR, Bassis CM, Shen MC, Toews GB, Young VB, Huffnagle GB.** 2012. Ecological succession of bacterial communities during conventionalization of germ-free mice. Appl Environ Microbiol **78:**2359–2366.
32. **Gordon HA, Pesti L.** 1971. The gnotobiotic animal as a tool in the study of host microbial relationships. Bacteriol Rev **35:**390–429.
33. **Graber JR, Breznak JA.** 2004. Physiology and nutrition of *Treponema primitia*, an H_2-CO_2-acetogenic spirochete from termite hindguts. Appl Environ Microbiol **70:**1307–1314.
34. **Hamilton DR, Bradley RE.** 1977. An integrated system for the production of gnotobiotic *Anopheles quadrimaculatus*. J Invertebr Pathol **30:**318–324.
35. **He S, Ivanova N, Kirton E, Allgaier M, Bergin C, Scheffrahn RH, Kyrpides NC, Warnecke F, Tringe SG, Hugenholtz P.** 2013. Comparative metagenomic and metatranscriptomic analysis of hindgut paunch microbiota in wood- and dung-feeding higher termites. PLoS One **8:**e61126. doi:10.1371/journal.pone.0061126.
36. **Husseneder C, Grace JK.** 2005. Genetically engineered termite gut bacteria (*Enterobacter cloacae*) deliver and spread foreign genes in termite colonies. Appl Microbiol Biotechnol **68:**360–367.

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

38. **Jenney FE Jr, Verhagen MFJM, Cui X, Adams MWW.** 1999. Anaerobic microbes: oxygen detoxification without superoxide dismutase. Science **286:**306–309.

39. **Kane MD, Breznak JA.** 1991. Effect of host diet on production of organic acids and methane by cockroach gut bacteria. Appl Environ Microbiol **57:**2628–2634.

40. **Karnholz A, Kusel K, Gossner A, Schramm A, Drake HL.** 2002. Tolerance and metabolic response of acetogenic bacteria toward oxygen, Appl Environ Microbiol **68:**1005–1009.

41. **Kozakova H, Kolinska J, Lojda Z, Rehakova Z, Sinkora J, Zakostelecka M, Splichal I, Tlaskalova-Hogenova H.** 2006. Effect of bacterial monoassociation on brush-border enzyme activities in ex-germ-free piglets: vcomparison of commensal and pathogenic *Escherichia coli* strains. Microbes Infect **8:**2629–2639.

42. **Laczny CC, Sternal T, Plugaru V, Gawron P, Atashpendar A, Margossian HH, Coronado S, van der Maaten L, Vlassis N, Wilmes P.** 2015. VizBin - an application for reference-independent visualization and human-augmented binning of metagenomic data. Microbiome **3:**1. doi:10.1186/s40168-014-0066-1.

43. **Lane DJ.** 1991. 16S/23S rRNA sequencing, p 115–175. *In* Stackebrandt E, Goodfellow M (ed), Nucleic acid techniques in bacterial systematics. John Wiley & Sons Ltd, New York, NY.

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

45. **Liu N, Zhang L, Zhou H, Zhang M, Yan X, Wang Q, Long Y, Xie L, Wang S, Huang Y, Zhou Z.** 2013. Metagenomic insights into metabolic capacities of the gut microbiota in a fungus-cultivating termite (*Odontotermes yunnanensis*). PLoS One **8:**e69184. doi:10.1371/journal.pone.0069184.

46. **Lo N, Bandi C, Watanabe H, Nalepa C, Beninati T.** 2003. Evidence for co cladogenesis between diverse dictyopteran lineages and their intracellular endosymbionts. Mol Biol Evol **20:**907–913.

47. **Loesche WJ.** 1969. Oxygen sensitivity of various anaerobic bacteria. Appl Microbiol **18:**723–727.

48. **Mayberry WR, Prochazka GJ, Payne WJ.** 1968. Factors derived from studies of aerobic growth in minimal media. J Bacteriol **96:**1424–1426.

49. **Mikaelyan A, Dietrich C, Köhler T, Poulsen M, Sillam-Dussès D, Brune A.** 2015*b*. Diet is the primary determinant of bacterial community structure in the guts of higher termites. Mol Ecol **24:**5284–5295.

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

51. **Noack J, Dongowski G, Hartmann L, Blaut M.** 2000. The human gut bacteria *Bacteroides thetaiotaomicron* and *Fusobacterium varium* produce putrescine and spermidine in cecum of pectin-fed gnotobiotic rats. J Nutr **130:**1225–1231.

52. **Resmer KL, White RL.** 2011. Metabolic footprinting of the anaerobic bacterium *Fusobacterium varium* using 1H NMR spectroscopy. Mol Biosyst **7:**2220–2227.

53. **Riebe O, Fischer RJ, Wampler DA, Kurtz DM Jr, Bahl H**. 2009. Pathway for H_2O_2 and O_2 detoxification in *Clostridium acetobutylicum*. Microbiology **155:**16–24.

54. **Sabree ZL, Kambhampati S, Moran NA**. 2009. Nitrogen recycling and nutritional provisioning by *Blattabacterium*, the cockroach endosymbiont. Proc Natl Acad Sci U S A **106:**19521–19526.

55. **Samuel BS, Gordon BI**. 2006. A humanized gnotobiotic mouse model of host archaeal-bacterial mutualism. Proc Natl Acad Sci U S A **103:**10011–10016.

56. **Schauer C, Thompson C, Brune A.** 2014. Pyrotag sequencing of the gut microbiota of the cockroach *Shelfordella lateralis* reveals a highly dynamic core but only limited effects of diet on community structure. PLoS One **9:**e85861. doi:10.1371/journal.pone.0085861.

57. **Schauer C, Thompson CL, Brune A**. 2012. The bacterial community in the gut of the cockroach *Shelfordella lateralis* reflects the close evolutionary relatedness of cockroaches and termites. Appl Environ Microbiol **78:**2758–2767.

58. **Schuler S, Conrad R.** 1990. Soils contain two different activities for oxidation of hydrogen. FEMS Microbiol Ecol **73:**77-84.

59. **Sharma RC, Schimke RT.** 1996. Preparation of electrocompetent *E. coli* using salt-free growth medium. Biotechniques **20:**42–44.

60. **Stingl U, Brune A.** 2003. Phylogenetic diversity and whole-cell hybridization of oxymonad flagellates from the hindgut of the wood-feeding lower termite *Reticulitermes flavipes*. Protist **154:**147–155.

61. **Stoddard SF, Smith BJ, Hein R, Roller BRK, Schmidt TM.** 2015. *rrn*DB: improved tools for interpreting rRNA gene abundance in bacteria and archaea and a new foundation for future development. Nucleic Acids Res **43:**D593–598.

62. **St-Pierre B, Cersosimo LM, Ishaq SL, Wright AD.** 2015. Toward the identification of methanogenic archaeal groups as targets of methane mitigation in livestock animals. Front Microbiol **6:**776. doi:10.3389/fmicb.2015.00776.

63. **Strassert JF, Desai MS, Radek R, Brune A.** 2010. Identification and localization of the multiple bacterial symbionts of the termite gut flagellate *Joenia annectens.* Microbiology **156:**2068–2079.

64. **Stubner S.** 2002. Enumeration of 16S rDNA of *Desulfotomaculum* lineage in ricefield soil by real-time PCR with Sybr Green detection. J Microbiol Methods **50:**155–164.

65. **Takahashi N, Abbe K, Takahashi-Abbe S, Yamada T.** 1987. Oxygen sensitivity of sugar metabolism and interconversion of pyruvate formate lyase in intact cells of *Streptococcus mutans* and *Streptococcus sanguis*. Infect Immun **55:**652–656.

66. **Teixeira de Mattos MJ, Tempest DW**. 1983. Metabolic and energetic aspects of the growth of *Klebsiella aerogenes* NCTC 418 on glucose in anaerobic chemostat culture. Arch Microbiol **134:**80–85.

67. **Terra WR, Ferreira C.** 1994. Insect digestive enzymes: properties, compartmentalization and function. Comp Biochem Physiol B **109:**1–62.

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

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

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

71. **Thomas TD, Ellwood DC, Longyear MV.** 1979. Change from homo- to heterolactic fermentation by *Streptococcus lactis* resulting from glucose limitation in anaerobic chemostat cultures. J Bacteriol **138:**109–117.

72. **Warnecke F, Luginbühl P, Ivanova N, Ghassemian M, Richardson TH, Stege JT, Cayouette M, McHardy AC, Djordjevic G, Aboushadi N, et al**. 2007. Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. Nature **450:**560–565.

73. **Yamada T, Takahashi-Abbe S, Abbe K.** 1985. Effects of oxygen on pyruvate formate-lyase in situ and sugar metabolism of *Streptococcus mutans* and *Streptococcus sanguis*. Infect Immun **47:**129–134.

74. **Zurek L, Keddie BA**. 1996. Contribution of the colon and colonic bacterial flora to metabolism and development of the American cockroach *Periplaneta americana* L. J Insect Physiol **42:**743–748.

Chapter 4

Tegtmeier et al. (2016) Syst. Appl. Microbiol. 39, 319–329

Breznakia blatticola **gen. nov. sp. nov. and** ***Breznakia pachnodae*** **sp. nov., two fermenting bacteria isolated from insect guts, and emended description of the family** ***Erysipelotrichaceae***

Dorothee Tegtmeier[1], Cornelius Riese[1], Oliver Geissinger[1], Renate Radek[2] and Andreas Brune[1*]

[1] Department of Biogeochemistry, Max Planck Institute for Terrestrial Microbiology, Karl-von-Frisch-Straße 10, 35043 Marburg, Germany

[2] Institute of Biology/Zoology, Free University of Berlin, Königin-Luise-Strasse 1–3, 14195 Berlin, Germany

Contributions

D.T. planned and designed the study, isolated strain ErySL*, supervised the bachelor project of Cornelius Riese, performed growth tests, analyzed fermentation products, performed electron microscopy and phylogenetic analysis, discussed results, evaluated and visualized the data and wrote the manuscript.

C.R. performed growth tests, analyzed fermentation products and physiological properties of the strains, performed electron microscopy.*

O.G. isolated strain Pei061, conducted preliminary analyses.*

R.R. performed ultra thin sections and electron microscopy.

A.B. planned and designed the study, supervised the work, discussed results, wrote the manuscript.

*The isolation of strains ErySL and Pei061 and preliminary analysis have been reported already in the diploma thesis of Dorothee Tegtmeier (Universität Kassel, 2012) and Oliver Geissinger (Universität Konstanz, 2004) and the BSc thesis of Cornelius Riese (Universität Marburg, 2014).

Abstract

Two novel, obligately anaerobic *Firmicutes* from the family *Erysipelotrichaceae* were isolated from the intestinal tracts of a cockroach (strain ErySL, *Shelfordella lateralis*) and a scarab beetle larva (strain Pei061, *Pachnoda ephippiata*). Phylogenetic analysis indicated that the strains belong to a monophyletic group of hitherto uncultured bacteria from insect guts that are only distantly related to any described species (< 90% 16S rRNA gene sequence similarity). Ultrastructural analysis revealed a Gram-positive cell envelope and, in the case of strain ErySL, a wide electron-lucent space between the cytoplasmic membrane and cell wall. In older cultures, cells formed pleomorphic rods with a thicker peptidoglycan layer. Both strains were obligately anaerobic and fermented glucose to formate, ethanol, and acetate as major products, but strain Pei061 tolerated up to 1% oxygen in the headspace. The same type of metabolism was observed with *Erysipelothrix inopinata*, except that the latter grew, albeit poorly, even under air. However, previous claims of a microaerophilic or facultatively anaerobic metabolism in the genus *Erysipelothrix* could not be substantiated. Based on phenotypic and phylogenetic evidence, we propose to classify the isolates as members of a new genus, *Breznakia blatticola* gen. nov. sp. nov. and *Breznakia pachnodae* sp. nov., with strain ErySL (= DSM 28867^T = JCM 30190^T) and strain Pei061 (= DSM 16784^T = JCM 30191^T) as type strains, and provide an emended description of the family *Erysipelotrichaceae*.

Introduction

The bacterial family *Erysipelotrichaceae* belongs to a deep-branching clade among low-G+C Gram-positive bacteria. Members of this family comprise a number of misnamed species in the genera *Clostridium, Eubacterium,* and *Streptococcus* that had been recognized as the 'walled relatives' of mycoplasmata (Weisburg et al., 1989) and were later classified as members of the clostridial Cluster XVI (Collins et al., 1994). Based on 16S rRNA gene sequence analysis, members of the *Erysipelotrichaceae* are now considered to form a separate line of descent (*Erysipelotrichia*) within the phylum *Firmicutes* (Ludwig et al., 2009). Interestingly, a recent multi-locus sequence analysis of sequenced genomes substantiated the common evolutionary root with the *Mollicutes* (phylum *Tenericutes*) (Davis et al., 2013).

Recently, the taxonomy of the family *Erysipelotrichaceae* has undergone major changes. New genera have been described to accommodate several generically misnamed species (De Maesschalck et al., 2014) and two new isolates (De Maesschalck et al., 2014; Kanno et al., 2015). Other family members have been reclassified into the new families *Coprobacillaceae* (representing the former clostridial clusters XVII and XVIII; Collins et al., 1994) and *Turicibacteraceae* (Verbarg et al., 2014).

Most of the strains in the *Erysipelotrichaceae* have been isolated from intestinal environments, and others are pathogens that cause infectious diseases (Verbarg et al., 2014). In addition, cultivation-independent studies have revealed an abundant presence of hitherto uncultured lineages of *Erysipelotrichaceae* in the intestinal tracts of mammals (e.g. Greiner and Bäckhed, 2001; Han et al., 2010; Alcaide et al., 2012) and insects (e.g. Egert et al., 2003; Köhler et al., 2012; Schauer et al., 2012), which suggests that they are common members of their gut microbiota. Reports on the increased abundance of uncultured *Erysipelotrichaceae* in human patients with colorectal cancer (Chen et al., 2012) or inflammation-related disorders (Dinh et al., 2015) and in animals with diet-induced obesity (Turnbaugh et al., 2008; Fleissner et al., 2010) nurtured a growing interest in this family, but due to the lack of isolates, little is known about their biological function and metabolic activities in the gut ecosystem (Kaakoush, 2015).

Also knowledge about the ultrastructure and metabolism of existing isolates of the family *Erysipelotrichaceae* is quite limited, and further investigations on this subject are highly desirable. Of particular interest are the principle mode of energy metabolism and the relationship of the strains to oxygen. The fermentation products formed by the different isolates indicate a diversity of fermentative pathways, ranging from homolactic fermentation

to butyrate fermentation to mixed acid fermentation (e.g. Robertson and McCullough, 1968; Moore et al., 1976; Barnes et al., 1977; Kanno et al., 2015). While the majority of the species are described as obligately anaerobic (e.g. Smith and King, 1961; Cato et al., 1974; Moore et al., 1976; Barnes et al., 1977; Kageyama and Benno, 2000; Greetham et al., 2004; Kanno et al., 2015), members of the genus *Erysipelothrix* grow under air and have been described as microaerophilic or facultative anaerobic (Jones et al., 1986; Verbarg et al., 2004). Based on the latter reports, also major reference books describe members of the family *Erysipelotrichaceae* as aerobic or microaerophilic to facultatively anaerobic with a respiratory and weak fermentative metabolism (Stackebrandt, 2009; Verbarg et al., 2014). However, the absence of evidence for aerobic respiration in the original descriptions and the reported lack of quinones and cytochromes also in members of the genus *Erysipelothrix* clearly underscore the need for further analysis of their relationship to oxygen.

Here, we describe the isolation, physiology, and ultrastructure of novel members of the family *Erysipelotrichaceae* isolated from the intestinal tract of insects. We evaluated previous reports on other family members and provide new experimental data on the metabolism of *Erysipelothrix inopinata* (described as facultatively anaerobic; Stackebrandt, 2009) and *Faecalicoccus pleomorphus* (described as obligately anaerobic; Barnes et al., 1977) and their relationship to oxygen. Based on both phenotypic and phylogenetic evidence, we propose that the isolates be classified as two species of a novel genus and provide a corrected and emended description of the family *Erysipelotrichaceae*.

Materials and Methods

Microbiological media

Microorganisms were routinely cultivated in anoxic bicarbonate-buffered mineral medium AM-5 supplemented with various vitamins and other growth factors (Boga and Brune, 2003) under an atmosphere of N_2/CO_2 (80:20, v/v). The medium contained (per liter) 1 g NaCl, 0.5 g KCl, 0.4 g $MgCl_2 \cdot 6\ H_2O$, 0.1 g $CaCl_2 \cdot 2\ H_2O$, 0.3 g NH_4Cl and 0.2 g KH_2PO_4. After autoclaving, the medium was cooled under N_2/CO_2 (80:20, v/v) atmosphere, and the following supplements (final concentrations) were added from sterile stock solutions, following the procedure described by Widdel and Pfennig (1981): $NaHCO_3$ (30 mM); 1 ml 7-vitamin solution (Pfennig, 1978) (10 mg biotin, 25 mg calcium pantothenate, 50 mg thiamine, 50 mg *p*-aminobenzoic acid, 100 mg nicotinic acid, and 250 mg pyridoxamine, 50 mg vitamin B_{12}

per liter); 1 ml trace element solution SL 11 (Eichler and Pfennig, 1986) (2 g $FeC1_2 \cdot 4\ H_2O$, 70 mg $ZnCl_2$, 100 mg $MnCl_2 \cdot 4\ H_2O$, 190 mg $CoCl_2 \cdot 6\ H_2O$, 2 mg $CuCl_2 \cdot 2\ H_2O$, 24 mg $NiCl_2 \cdot 6\ H_2O$, 36 mg $Na_2MoO_4 \cdot 2\ H_2O$, 6 mg H_3BO_3, 5.2 g Na_2-ethylene-diamine-tetraacetate per liter), 1 ml selenite-tungstate solution (Lechner, 2009) (0.5 g NaOH, 3 mg $Na_2SeO_3 \cdot 5\ H_2O$, 4 mg $Na_2WO_4 \cdot 2\ H_2O$ per liter); menadione (vitamin K_3, 2.5 µM); lipoic acid (1 µM); folic acid and riboflavin (50 µg/L each); isobutyric, 2-methylbutyric, *n*-valeric, and isovaleric acids (25 µM each); phenylacetic, phenylpropionic, 4-hydroxyphenylacetic, and 3-indolylacetic acids (5 µM each), and resazurin (0.8 mg/L) as redox indicator. The pH was adjusted to 7.2 with HCl. The medium was stored in 100-ml screw-cap bottles, which were completely filled (no gas headspace).

For most experiments, the AM-5 medium was further amended with yeast extract and Casamino acids (0.1% each), and cysteine and DTT (1 mM each) were added as reducing agents. This "basal medium" routinely received glucose (5 mM) as substrate and was dispensed (4.5 ml) into 16-ml rubber-stoppered culture tubes, which were gassed with N_2/CO_2 (80:20, v/v). Tubes were inoculated with overnight cultures (0.5 ml) and routinely incubated at 25 °C.

For some experiments, strains were grown in phosphate-buffered mineral medium MM-5 (Lemke et al., 2003) under an atmosphere of N_2. The medium contained (per liter) 1 g NaCl, 0.5 g KCl, 0.1 g $MgCl_2 \cdot 6\ H_2O$, 0.015 g $CaCl_2 \cdot 2\ H_2O$, 0.3 g NH_4Cl, 0.15 g KH_2PO_4 and 0.15 g Na_2SO_4. The medium was prepared and amended with the same supplements as described for the medium AM-5, except that it was cooled and dispensed under N_2-atmosphere and the $NaHCO_3$ solution was replaced with sodium phosphate buffer (pH 7.0; final concentration 20 mM).

Alternatively, strains were cultivated on Fastidious Anaerobe Agar, a complex nutrient-rich medium for anaerobes (Atlas, 2010), with slight modifications. The medium contained (per liter) 23 g peptone, 5 g NaCl, 1 g soluble starch, 0.4 g $NaHCO_3$, 1 g glucose, 0.5 g cysteine, 0.25 g sodium pyrophosphate, 1 g L-arginine, 0.01 g hemin, and 12 g agar. After autoclaving, menadione (final concentration, 2.5 µM) was added from a sterile stock solution.

Isolation and cultivation

An adult female of *Shelfordella lateralis* (Blattodea: Blattidae) and a third-instar larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae) were taken from insect populations that were maintained in our laboratory in the context of other studies (Andert et al., 2009; Schauer et al.,

2012). The specimens were dissected, and midgut or hindgut sections were placed in 16-ml culture tubes with 1 ml substrate-free AM5 medium (Boga and Brune, 2003) reduced with 1 mM dithiothreitol (DTT). After adding a small amount of 2-mm glass beads, the tubes were closed with a rubber stopper, gassed with N_2/CO_2 (80:20, v/v), and vortexed for 10 min. Pure cultures were obtained from gut homogenates by serial dilution in AM5 medium and subsequent isolation on solid media. Strain ErySL was isolated on Fastidious Anaerobe Agar (Atlas, 2010) incubated in an anoxic jar under N_2/CO_2 (80:20, v/v). Strain Pei061 was isolated by two consecutive agar dilution series (Pfennig and Trüper, 1981) in AM5 medium amended as detailed above.

Erysipelothrix inopinata (DSM 15511^T) and *Faecalicoccus pleomorphus* (DSM 20574^T) were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany.

Growth and nutrition

Growth was tested on D-fructose, D-galactose, *N*-acetyl-glucosamine, D-maltose, D-mannitol, D-ribose, D-melibiose, salicin, D-raffinose, D-trehalose, D-cellobiose (each 5 mM), L-lactate, L-malate, D-gluconate (each 10 mM) and Casamino acids and yeast extract (each 0.5%). The temperature range for growth was determined at 5, 10, 15, 20, 25, 30, 37, 40, and 45 °C. To determine the pH range of growth, MM5 medium was adjusted to the desired pH using alternative buffer systems (final concentration 20 mM): sodium acetate, pH 4 and 5; sodium phosphate, pH 6.0, 7.0, and 11.0; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 8.0, and sodium carbonate, pH 9 and 10. Oxygen tolerance was tested in culture tubes with non-reduced medium that received different amounts of sterile-filtered (0.2 µm pore size) oxygen in the headspace and were incubated on a roller mixer (60 rpm). In all cases, growth was determined photometrically by following the increase in optical density at 578 nm (OD_{578}) using a culture tube photometer (Spectronic 20$^+$, Milton Roy; path length ca. 1.3 cm).

For dry weight determination, strains were grown in 1 liter AM-5 basal medium with 5 mM glucose. The cells were harvested by centrifugation (14,000 × *g*; 10 min), washed with ammonium acetate solution (20 mM), and dried overnight at 80 °C. Elemental composition of the cell pellets was determined by HEKAtech GmbH (Wegberg, Germany) using an Euro EA – CHNS elemental analyzer and (for P content) atomic emission spectrometry (DIN ISO 22036). Oxygen was calculated as the elemental mass balance of C, H, N, S, and P; ash content was neglected.

Other tests

Gram staining was performed according to Murray et al. (1994) with *Bacillus subtilis* strain JH642 and *Escherichia coli* strain DH5α as controls. Motility was tested in stab tubes containing Luria Bertani medium reduced with 1 mM DTT with 0.5% agar, incubated under a headspace of N_2/CO_2 (80:20, v/v). Oxidase activity was tested with liquid cultures using oxidase test strips (Bactident, Merck, Darmstadt), using *E. coli* and *B. subtilis* as controls. Catalase activity was tested with bacterial colonies grown on agar plates in an anoxic jar (see above); colonies that formed gas bubbles after addition of a drop of H_2O_2 (3%) were considered catalase positive; *E. coli* and *Clostridium mangenotii* (DSM 1289) were used as controls.

Metabolic products

Cell-free supernatants of fully grown cultures were acidified with H_2SO_4 (50 mM final concentration), and microbial fermentation products were analyzed by HPLC with an ion-exclusion column and a refractive index detector (Schauer et al., 2012). Hydrogen concentrations in the culture headspace were analyzed by gas chromatography using a thermal conductivity detector (Schuler and Conrad, 1990). Since the bicarbonate buffer did not allow analysis of CO_2, the calculation of carbon balances involved the assumption that acetate production and ethanol production were each accompanied by the formation of one CO_2 and formate production by the consumption of one CO_2. For the calculation of electron balances, all metabolites were formally oxidized to CO_2, and the total number of electrons released from the measured amounts of products was compared with that released from the amount of substrate dissimilated (Tholen et al., 1997). These calculations yielded the electrons of dissimilated glucose recovered in fermentation products expressed on a per cent basis (electron recovery).

Light and electron microscopy

For bright-field and phase-contrast light microscopy of Gram stains or unfixed cells, an Axiophot photomicroscope (Zeiss, Oberkochen, Germany) was used. For transmission electron microscopy (TEM), cells were fixed with glutardialdehyde and postfixed with osmium tetroxide before embedding. Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined with a Philips EM 208 transmission electron microscope (for details, see Zheng et al., 2016). For negative staining, fixed cells were pipetted onto a film-

coated copper grid, washed twice with water, contrasted with uranyl acetate, and examined with a JEOL JEM-2100 transmission electron microscope.

Phylogenetic analysis

16S rRNA genes were amplified using *Bacteria*-specific primers and sequenced by Sanger sequencing (Strassert et al., 2010). Sequences were imported into the DictDb reference database (version 3.0; Mikaelyan et al., 2015), which contained all type strains included in the Living Tree Project (Yarza et al., 2008), and aligned against the existing alignment using the *fast aligner* included in the ARB software package (version 5.5; Ludwig et al., 2004). The alignment was manually corrected, and highly variable base positions were excluded using a frequency-based filter (minimum similarity 50%) calculated for all high-quality full-length sequences of *Erysipelotrichia* (1463 sequence positions). A maximum-likelihood tree was constructed using the fastDNAml algorithm included in ARB (100 bootstraps); tree topology was tested using maximum-parsimony analysis.

The 16S rRNA gene sequences of strain ErySL (KU043523) and strain Pei061 (AJ629069) have been submitted to GenBank (http://www.ncbi.nlm.nih.gov).

Fatty acid analyses

Strain ErySL was grown in AM5 basal medium amended with 10 mM glucose. Whole-cell fatty acids were analyzed by the Identification Service, Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany, using the method of Miller (1982) and Kuykendall et al. (1988). Fatty acid methyl ester mixtures were separated by gas chromatography with the Sherlock Microbial Identification System (MIDI, Newark, DE, USA) and analyzed using the Microbial Identification software package (BHIBLA library 3.80).

Results

Isolation and morphological characterization

Strains ErySL and Pei061 were obtained from serially diluted gut homogenates of *Shelfordella lateralis* (hindgut) and *Pachnoda ephippiata* (midgut), respectively. Both cultures were derived from single colonies isolated from the penultimate (strain ErySL) or ultimate (strain Pei061) dilution step that yielded bacterial colonies within one week of incubation and were

subjected to further dilution series to ensure clonality of the cultures. Both strains formed white colonies with smooth, well-defined edges on Fastidious Anaerobe Agar; in the case of strain ErySL, the colonies were slightly sunk into the agar surface.

The cells of both strains were rod-shaped with a diameter of 0.3–0.4 µm and a variable length (0.8–2.5 µm) (Figure 4.1). Most of the cells remained connected in chains (Figure 4.1A, B, G, and H). Chains formed by strain Pei061 often had a twisted, rope-like appearance (Figure 4.1G). At higher cell density, cultures of both strains appeared cloudy, and the cells aggregated and sank to the bottom of the tubes. The sedimented cells had a viscous consistency and could be resuspended by vortexing. Both strains were non-motile and stained Gram positive.

Transmission electron microscopy of strain ErySL showed different cell forms: single rods, chains, and coiled cells (Figure 4.1A, B, and D). Strain Pei061 consists of fewer single cells but more and longer chains (Figure 4.1G, H, and J). The cell envelope of strain ErySL consists of a thick electron-dense layer (10–20 nm) with an underlying electron-lucent layer (7–11 nm) (Figure 4.1C and E). The electron-dense layer in the cell envelope of strain Pei061 was even thicker (15–25 nm), but the electron-lucent layer was much thinner (3 nm) or absent (Figure 4.1I and K). Both strains also formed cells with a thickset, irregular appearance characterized by a much thicker cell envelope, and in the case of strain ErySL, a wide electron-lucent layer (Figure 4.1F and L). Such pleomorphic cells were observed also by phase-contrast microscopy and dominated in older cultures of both strains.

While cross walls of strain ErySL joined the longitudinal walls at sharp angles (Figure 4.1E), the joints of strain Pei061 were more rounded (Figure 4.1K). Dividing cells of strain ErySL typically formed a straight septum (Figure 4.1D), whereas the septum of strain Pei061 was curved (Figure 4.1J). Both strains had a relatively homogeneous cytoplasm with condensed areas, most likely representing the nucleoid. Structures resembling flagella or endospores were never observed.

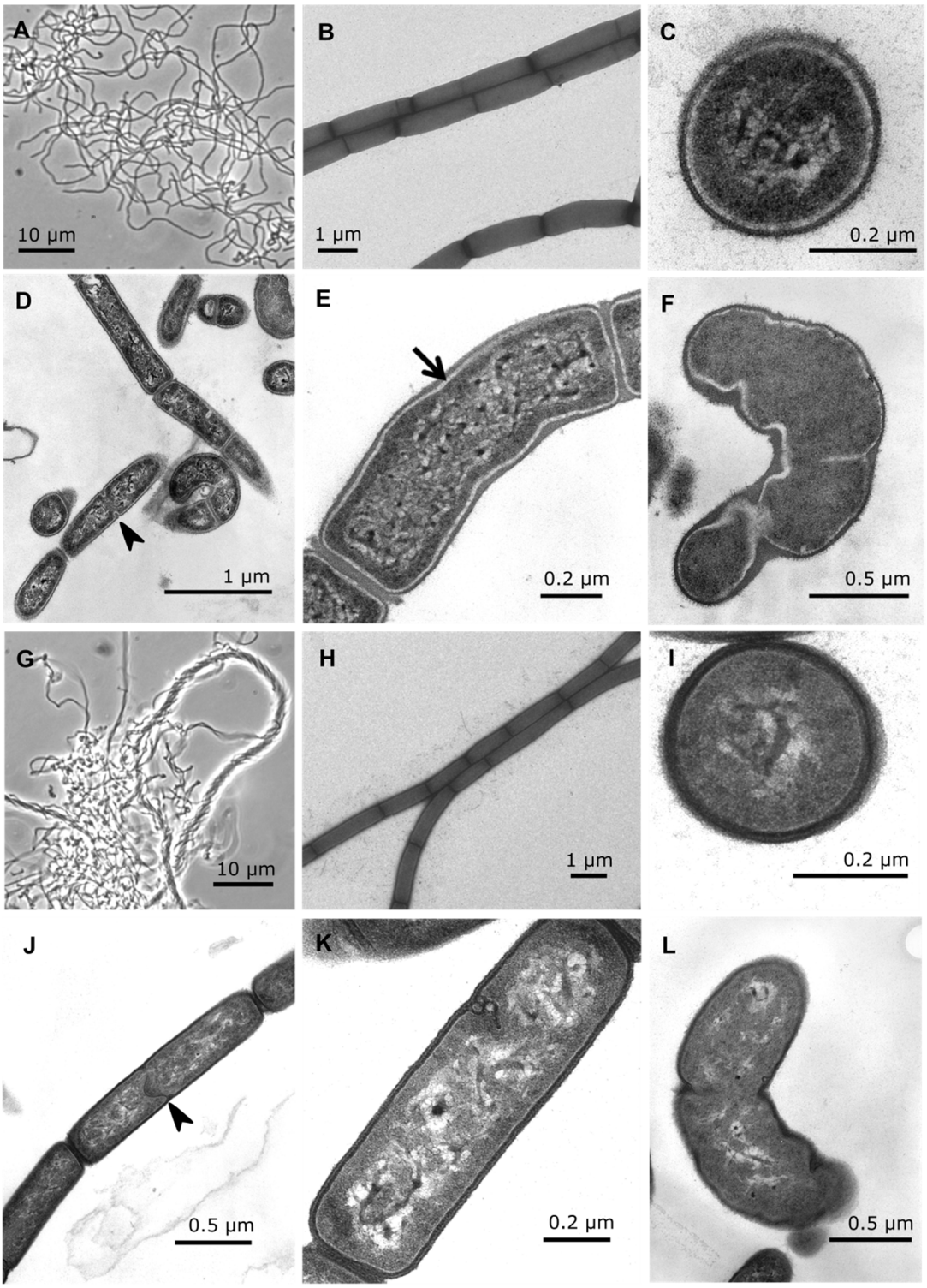

Figure 4.1. Morphology and ultrastructure of *Breznakia blatticola* strain ErySL (A–F) and *Breznakia pachnodae* strain Pei061 (G–L). Images show phase-contrast micrographs (A, G), transmission electron micrographs of negatively stained preparations (B, H), and ultra-thin sections. Ultra-thin sections represent cross sections (C, I), dividing cells (D, J), and longitudinal sections (E, K), and details of the pleomorphic cells formed by both strains (F, L). The arrow points to the electron-lucent space between the cytoplasmic membrane and the cell wall observed only in strain ErySL. Arrowheads point to the septum of dividing cells. Cultures were sampled in the late exponential phase.

Physiology of growth

Strains ErySL and Pei061 both grew fermentatively on glucose and a variety of other carbohydrates. In basal medium with glucose, strain Pei061 grew faster and reached higher cell yields than strain ErySL (Figure 4.2); the doubling times were 2.0 h (strain ErySL) and 0.93 h (strain Pei061). At low substrate concentration, both strains fermented glucose to formate, ethanol, and acetate; hydrogen was not produced (Table 4.1). While strain ErySL dissimilated 1 glucose to 1.5 formate, 1.1 ethanol, and 0.8 acetate, strain Pei061 formed also small amounts of lactate (1.5 formate, 1.2 ethanol, 0.9 acetate, and 0.1 lactate per glucose). Carbon and electron recoveries were almost identical and close to unity, which indicated that all products were detected. The fermentation product spectrum of both strains changed with the glucose concentration in the medium. With increasing glucose concentration, both strains formed increasing amounts of lactate; the effect was more pronounced with strain Pei061 (Table 4.2). Growth rates on D-fructose, D-maltose, D-mannitol, salicin, D-trehalose, D-cellobiose, and *N*-acetyl-glucosamine were similar to those on glucose. Both strains grew also on D-ribose (at lower growth rates), on D-melibiose (with an extended lag phase), and on D-galactose (here, strain Pei061 grew much slower than strain ErySL); only strain Pei061 grew on D-gluconate. Neither strain grew on D-raffinose, L-lactate, or L-malate.

The elemental compositions of the strains were similar (strain ErySL: 41.1% C, 37.7% O, 7.1% H, 11.3% N, 2.3% P, 0.50% S; strain Pei061: 37.8% C, 42.1% O, 7.6% H, 10.1% N, 2.2% P, 0.22% S). In contrast to strain Pei061, strain ErySL grew (albeit more slowly) on glucose also in the absence of yeast extract and Casamino acids (OD_{578} = 0.23 vs. 0.30). Both strains grew slightly (OD_{578} = 0.04) in basal medium without glucose; an increase in the yeast extract concentration (0.5%) led to a proportional increase in growth yield, but Casamino acids (0.5%) were not fermented.

Both strains grew within a temperature range from 15 to 40 °C; no growth occurred at 10 or 45 °C. Growth yields were highest at 25 °C, but growth rates were highest at 30 °C. For both strains, growth occurred at a pH range from 6 to 11, with a broad optimum between pH 7 and 10. Cultures survived at least six months without transfer to fresh medium.

Table 4.1 Fermentation products, electron and carbon balance, and growth yield of strains ErySL and Pei061. Values are means of triplicate analyses (less than 10% deviation) of 1-Liter cultures.

Strain	Glucose consumed	Cell mass	Glucose assimilated[a]	Glucose dissimilated[b]	Products (mM)[c]				Carbon recovery	Electron recovery	Growth yield (Y_S)[d]
	(mM)	(g/L)	(mM)	(mM)	Formate	Ethanol	Acetate	Lactate	(%)	(%)	(g mol^{-1})
ErySL	4.5	0.10	0.58	3.92	6.0	4.4	3.0	0.0	95	95	22
Pei061	4.5	0.27	1.49	3.01	4.6	3.5	2.7	0.3	107	105	59

[a] Based on the elemental composition of the cells and the dry weight of the cultures at OD_{578} = 0.1 (strain ErySL: 40 mg l^{-1}, $C_{4.0}H_{8.4}O_{2.8}N_{0.9}$; strain Pei061: 47 mg l^{-1}, $C_{4.0}H_{9.5}O_{3.3}N_{0.9}$).

[b] Difference between the amount of glucose consumed and that assimilated into biomass.

[c] Products formed on basal medium without glucose were subtracted.

[d] Based on consumed glucose.

Table 4.2 Influence of glucose concentration on the fermentation products of strains ErySL and Pei061. Cultures were grown in 5 ml basal medium with different glucose concentrations under anoxic conditions. Values are means of results obtained with duplicate cultures (less than 10% deviation).

Glucose (mM)	Products (mol per mol glucose)[a]			
	Formate	Ethanol	Acetate	Lactate
Strain ErySL				
5	1.40	0.87	0.77	–[b]
10	1.56	0.92	0.78	0.09
15[c]	1.34	0.77	0.58	0.20
Strain Pei061				
5	1.05	0.66	0.54	0.04
10	0.83	0.62	0.45	0.41
15	0.53	0.47	0.24	0.89

[a] Products formed on basal medium without glucose were subtracted.

[b] Below the detection limit (0.02 mM).

[c] Only 12.3 mM glucose was consumed.

Response to oxygen

Neither strain ErySL nor strain Pei061 required a reduced medium to initiate growth. In contrast to strain ErySL, which was completely inhibited by the presence of oxygen, strain Pei061 still grew on glucose at an initial headspace concentration of 1% oxygen, albeit with a pronounced lag phase, and reached almost the same cell density as under anoxic conditions (Figure 4.2).

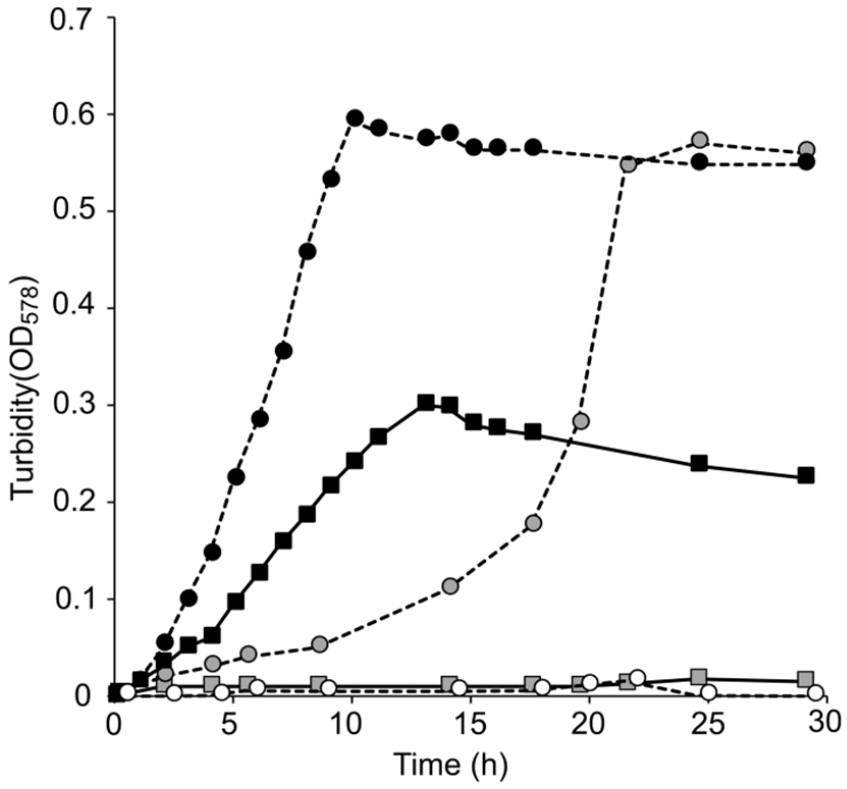

Figure 4.2. Growth curves of strain ErySL (squares) and strain Pei061 (circles) under anoxic conditions (black) and with 1% oxygen (gray) and 2% oxygen (white) added to the headspace. Values are means of duplicate cultures grown in basal medium under N_2/CO_2 (80:20, v/v) with 5 mM glucose; mean deviations were smaller than the symbols. Oxygen concentrations in the headspace are the initial values.

Fermentation products shifted from formate and ethanol towards acetate and lactate. Although growth yields were not significantly affected, the electron recovery at the end of growth was slightly lower than under anoxic conditions (Table 4.3). The color change of the redox indicator indicated that oxygen was completely consumed already during the early growth phase. At 2% oxygen in the headspace, however, also the growth of strain Pei061 was completely inhibited, and the medium remained oxidized. Both strains were catalase negative and oxidase negative.

The metabolism of strain Pei061 and its response to oxygen resembled that of *Erysipelothrix inopinata*. Both strains formed the same fermentation products under anoxic conditions, and increasing oxygen concentrations caused a substantial shift from formate and ethanol towards lactate production. In contrast to strain Pei061, the electron recovery in *E. inopinata* remained almost unaffected even in cultures containing 4% oxygen in the headspace (Table 4.3). Although growth yields of *E. inopinata* decreased with increasing oxygen concentration in the headspace, the strain grew slightly (OD_{578} = 0.06) even under air. At 4% oxygen in the headspace, the medium remained oxidized throughout the incubation.

The growth of *Faecalicoccus pleomorphus* was completely inhibited already by 1% oxygen in the headspace. In contrast to the other strains, *F. pleomorphus* grew under a headspace of N_2/CO_2 only if the medium contained a reducing agent. The fermentation products from glucose were distinctly different from those of the other strains, particularly with respect to the formation of lactate and butyrate and the absence of ethanol.

Table 4.3 Influence of oxygen on growth and fermentation products of strains ErySL and Pei061, *Faecalicoccus pleomorphus* strain DSM 20574, and *Erysipelothrix inopinata* strain DSM 15511. Cultures were grown in basal medium with glucose. Values are means obtained with duplicate cultures (less than 10% deviation).

Strain and O_2 concentration [a]	Turbidity (OD_{578})	Glucose assim. (mM)	Glucose dissim. (mM)	Products (mM)[b]						Electron recovery (%)
				Formate	Ethanol	Acetate	Lactate	Butyrate	Malate	
Strain ErySL										
0%	0.25	0.58[c]	3.92	6.31	3.93	3.45	–	–	–	93
Strain Pei061										
0%	0.60	1.57[c]	2.93	4.73	2.95	2.43	0.16	–	–	94
1%	0.57	1.49[c]	3.01	3.27	2.12	2.79	0.32	–	–	80
F. pleomorphus										
0%	0.42	0.98[d]	3.52	3.50	–	0.60	4.50	0.90	0.50	106
E. inopinata										
0%	0.21	0.49[d]	3.43	7.13	3.28	3.10	0.34	–	0.04	101
1%	0.19	0.44[d]	3.54	6.01	3.12	3.48	0.33	–	0.11	97
4%	0.13	0.30[d]	3.89	4.31	2.36	3.00	2.11	–	0.24	96

[a] Headspace concentration (v/v); initial values.

[b] Products formed on basal medium without glucose were subtracted. –, below the detection limit (< 0.02 mM)

[c] Estimated using the turbidity of the culture with the experimentally determined OD-to-dry-weight ratio and the elemental composition of the respective strains (Table 4.1).

[d] Estimated using the OD-to-dry-weight ratio and the elemental composition of strain ErySL.

Phylogenetic analysis

Based on their 16S rRNA gene sequences, strains ErySL and Pei061 are clearly embedded in the radiation of other members of the family *Erysipelotrichaceae*. They occupy a basal position in the clade representing the former *Clostridium* cluster XVI (Collins et al., 1994), which contains the species *Clostridium innocuum*, *Faecalicoccus* (*Streptococcus*) *pleomorphus*, and *Holdemanella biformis* (*Eubacterium biforme*), but also many other formerly misplaced *Firmicutes* that have been reclassified or await reclassification (Figure 4.3). Closest relatives are *Eubacterium tortuosum*, *Eubacterium dolichum,* and *C. innocuum* (87–90% sequence similarity).

The entire cluster is phylogenetically distinct from the genus *Erysipelothrix* and several other lineages of *Erysipelotrichaceae* (Figure 4.3). Furthermore, it is clearly separated from the members of the former clostridial clusters XVII [*Eggerthia* (*Lactobacillus*) *catenaformis* and *Kandleria vitulina* (*Lactobacillus vitulinus*)] and XVIII (*Clostridium ramosum* and *Clostridium spiroforme*) recognized by Collins et al. (1994) and other species that were originally included in the family *Erysipelotrichaceae* (Stackebrandt, 2009) but recently reclassified in new families (*Coprobacillaceae* and *Turicibacteraceae*; Verbarg et al., 2014).

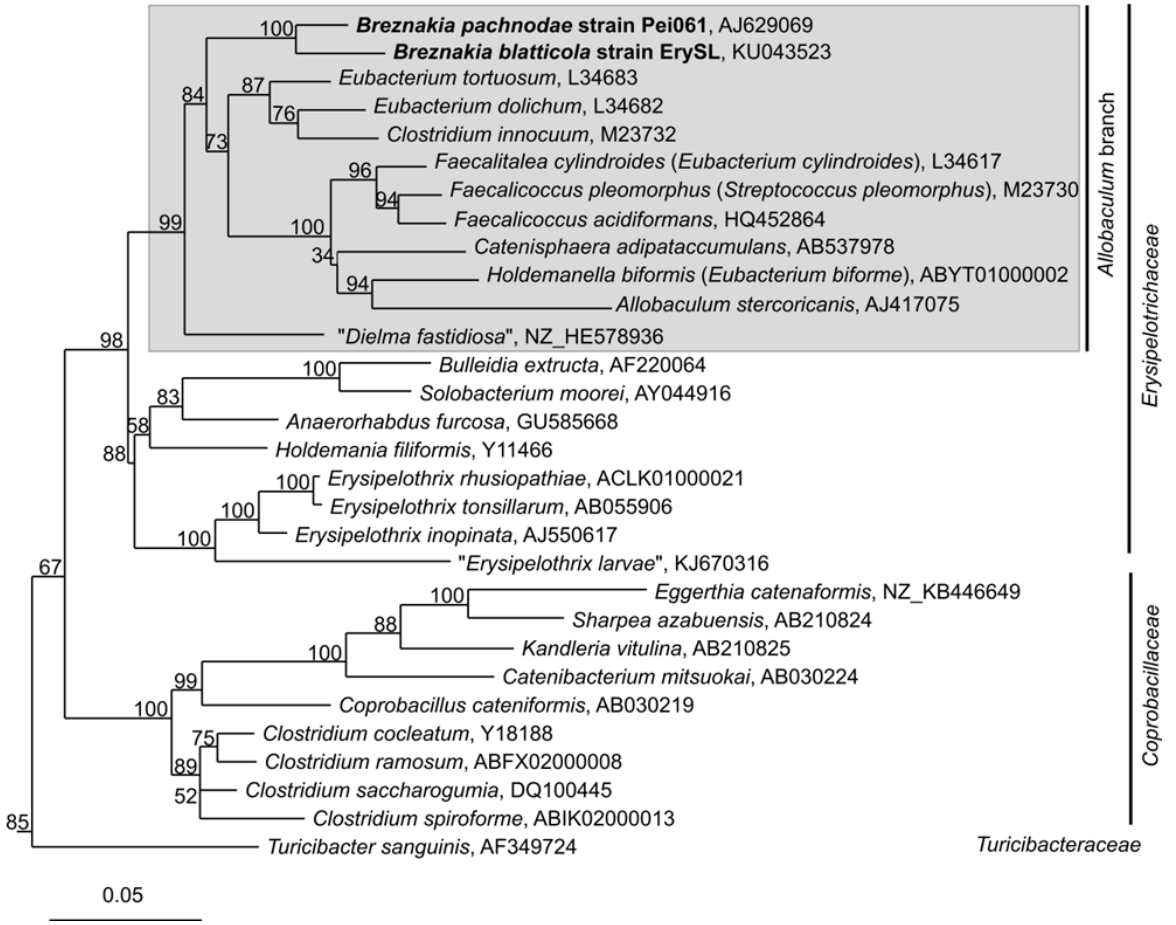

Figure 4.3. Maximum-likelihood tree showing the relationship of strains ErySL and Pei061 to all other species of the family *Erysipelotrichaceae* and their closest relatives. The phylogenetic analysis is based on an unambiguous alignment (1463 sequence positions) of the 16S rRNA gene sequences of the respective type strains; GenBank accession numbers are included. Numbers at nodes are bootstrap values, based on 100 replications. The tree was rooted using various members of *Tenericutes* as outgroup.

Chemotaxonomic analysis

The predominant fatty acids (and fatty acid derivatives) of strain ErySL were $C_{16:0}$ dimethyl acetal (DMA) (28.6%), $C_{18:1}$ *cis*-9 (13.1%), $C_{16:1}$ *cis*-9 (13.0%) and $C_{16:0}$ (12.5%) (for details, see Table S4.1). The fatty acid pattern shows highest similarity to that of the *Clostridium innocuum*-GC subgroup A (match in BHIBLA library 3.80). The predominant component of strain ErySL ($C_{16:0}$ DMA) is also a major component in *Eubacterium tortuosum*, *Clostridium innocuum, Faecalicoccus pleomorphus*, and several other family members but absent in the genus *Erysipelothrix* (Verbarg et al., 2014). This is in agreement with the more distant phylogenetic relationship between the species in the *Allobaculum* branch and the type genus.

Discussion

The results of this study clearly identify the isolates as a new genus-level lineage of *Erysipelotrichaceae*. The ultrastructural analysis of the new strains is the first highly resolved description of representatives of this family. The comparative analysis of the metabolic properties of the new strains and their oxygen relationships with selected representatives of *Erysipelotrichaceae* strongly indicates that the description of the family requires revision.

Morphology and ultrastructure

The rod-shaped morphology of the new strains and the tendency of the cells to remain connected in chains are commonly encountered among current members of *Erysipelotrichaceae* (e.g. Seeliger, 1957; Moore and Holdeman, 1974; Moore et al., 1976; Willems et al., 1997). For unknown reasons, later descriptions of the family refer to the formation of filaments (Jones, 1986; Verbarg et al., 2004; Stackebrandt, 2009) but at least in strains ErySL and Pei061, the septa between individual cells are clearly articulated, which indicates that their morphotype results from an incomplete separation of the daughter cells and not from the absence of cell division, as in true filaments (Burdett and Murray, 1974; Justice et al., 2008; Young et al., 2006). However, rod-shaped cells and growth in chains are not traits characteristic of the entire family (cf. *Faecalicoccus pleomorphus* or *Clostridium innocuum*) (Table 4.4). Also the absence of endospore formation is not shared with all family members; it has been proposed that the loss of the ability to form endospores occurred independently in several lineages of *Erysipelotrichaceae* and is related to the extent of genome reduction (Davis et al., 2013).

So far, the only ultrastructural analyses available for members of *Erysipelotrichaceae* are for *Bulleidia extructa* (Downes et al., 2000) and "*Dielma fastidiosa*" (Ramasamy et al., 2013), a species that has not been validly published. In both cases, the images are of poor resolution, and particularly the purportedly Gram-negative cell wall structure of "*Dielma fastidiosa*" (Ramasamy et al., 2013) is highly questionable. Our detailed ultrastructural analysis of strains ErySL and Pei061 indicates that both possess a Gram-positive cell envelope. Although the lipid bilayer of the cytoplasmic membrane was not preserved, it is likely bordering the electron-lucent region below the thick, electron-dense peptidoglycan layer, which was particularly pronounced in strain ErySL. It is possible that this layer is a true periplasmic space, as previously documented for several other Gram-positive bacteria (Matias and Beveridge,

2005; Zuber et al., 2006), but because of the chemical fixation of the cells, an artifact cannot be fully excluded. It is noteworthy that also the TEM of an ultra-thin section of *Bulleidia extructa* shows a similar electron-lucent zone (see Figure 1 of Downes et al., 2000).

Fermentative metabolism

With the exception of the genus *Erysipelothrix*, all members of *Erysipelotrichaceae* have been described as anaerobic bacteria (see below). Like other members of the family, strains ErySL and Pei061 grow fermentatively on a variety of carbohydrates, including hexoses, pentoses, and disaccharides; Casamino acids are not fermented, but unknown components of yeast extract are required as growth factors.

The fermentation products of glucose were typical for a mixed-acid fermentation and resembled those formed by the distantly related *Erysipelothrix inopinata* but differed from those formed by their closest relatives, the members of the *Allobaculum* branch. All of these strains have been reported to produce butyrate; some of them, e.g. *Clostridium innocuum,* also produce large amounts of hydrogen (Rainey et al., 2009; Table 4.4); and *Faecalicoccus pleomorphus* and *Catenisphaera adipataccumulans* showed homolactic fermentation when grown at high glucose concentrations (equivalent to 28 mM or 56 mM) (Barnes et al., 1977; Kanno et al., 2015). Interestingly, *F. pleomorphus* produced also substantial amounts of formate and butyrate at low glucose concentration (this study, Table 4.3). A similar effect was observed for the new strains, where the fermentation products shifted towards lactate production at higher glucose concentrations, particularly in strain Pei061. Such shifts have also been described for chemostat cultures of *Lactobacillus casei* (De Vries et al., 1970), *Klebsiella aerogenes* (Teixeira de Mattos and Tempest, 1983), and several *Streptococcus* spp. (Thomas et al., 1979; Condon, 1987).

Table 4.4 Comparison of the phenotypic properties of strains Pei061 and ErySL with the type strains of the *Allobaculum* branch of *Erysipelotrichaceae*.

Property	**Strain ErySL**[a]	**Strain Pei061**[a]	***Clostridium innocuum***[b]	***Eubacterium tortuosum***[c]	***Eubacterium dolichum***[d]	***Faecalicoccus pleomorphus***[e]	***Faecalitalea cylindroides***[f]	***Catenisphaera adipataccumulans***[g]	***Holdemanella biformis***[h]	***Allobaculum stercoricanis***[i]	***Erysipelothrix inopinata***[j]
Cell morphology	Rods, chains	Rods, long chains	Rods, single or pairs	Rods, long chains	Rods, long chains	Coccoid rods, pleomorphic	Rods, single or chains	Cocci, chains	Cocci, pairs or chains	Rods	Rods, chains
G+C content (mol%)	34.1[k]	ND	43–44	ND	ND	39.4	31	47.7	32	37.9	37.5
Gram stain	+	+	+	ND	+	+	+	+	+	+	+
Endospores	–	–	+	ND	–	–	–	–	–	–	–
Motility	–	–	–	–	–	ND	–	–	–	–	–
Catalase	–	–	ND	ND	ND	–	ND	–	ND	–	–
Oxidase	–	–	ND	ND	ND	ND	ND	–	ND	ND	–
pH optimum	7–9	7–10	ND	ND	ND	ND	ND	5.5–8.5	ND	ND	8
Growth temp. (C°)	15–40	15–40	25–45	37–41	30–45	37–45	37-45	30–50	37	30–40	20–40
Growth under air	–	–	–	ND	–	–	–	–	–	–	+
Aerobic growth (respiration)	–	–	ND	ND	ND	–[a]	ND	ND	ND	ND	–[a]
Anaerobic growth	+	+	+	+	+	+	+	+	+	+	+
Fermentation products[l]	F, E, A, (l)[m]	F, E, A, l, (L)[m]	B, L, A, f, s	L, B, s, a	B, L	L, F, B, a, m[a]	L,B, a,f	L, a, b, f	L, B, c	L, B, e	F, E, A, l, m[a]
H_2 formation	–	–	+	+	(+)	–[a]	–	+	+	ND	–[a]

ND, not determined; (+) trace amounts were sometimes detected.
[a] This study.
[b] Smith and King, 1961; Rainey et al., 2009.
[c] Moore and Holdeman Moore, 1986.
[d] Moore et al., 1976.
[e] Barnes et al., 1977; De Maesschalck et al., 2014.
[f] Cato et al., 1974; De Maesschalck et al., 2014.
[g] Kanno et al., 2015.
[h] Moore and Holdeman, 1974; Moore and Holdeman Moore, 1986.
[I] Greetham et al., 2004.
[j] Verbarg et al., 2004.
[k] Based on draft genome (unpublished results).
[l] A, acetate; B, butyrate; E, ethanol; F, formate; L, lactate; P, propionate; M, malate; S, succinate C, caproate; ranked by decreasing abundance. Lower case letters denote minor amounts.
[m] Only at higher glucose concentrations.

Relationship to oxygen

The new strains differed in their tolerance to the presence of oxygen. While strain ErySL is a strict anaerobe and does not grow in the presence of oxygen, strain Pei061 can initiate growth in media with 1% oxygen in the headspace. Also the majority of the species of *Erysipelotrichaceae* are described as obligately anaerobic, e.g. *Faecalicoccus pleomorphus* (Barnes et al., 1977), *Allobaculum stercoricanis* (Greetham et al., 2004), *Eubacterium dolichum* (Moore et al., 1976), *Eubacterium cylindroides* (Cato et al., 1974), *Solobacterium moorei* (Kageyama und Benno, 2000), and *Clostridium innocuum* (Smith and King, 1961).

A notable exception are members of the genus *Erysipelothrix*, which have been described as facultatively anaerobic (Stackebrandt, 2009) or microaerophilic (Verbarg et al., 2014). However, this seems to be based on a misinterpretation of the original observations of *Erysipelothrix rhusiopathiae* (Jones et al., 1986) and *Erysipelothrix inopinata* (Verbarg et al., 2004), which have been reported to grow under "aerobic" and "anaerobic" conditions (i.e., the presence and absence of oxygen). A careful survey of the literature did not provide any evidence that supports the claim of a respiratory metabolism in the genus *Erysipelothrix* or any other members of the family, and also the basis for the classification as "weakly fermentative" (Jones, 1986; Stackebrandt, 2009) did not become apparent. Both *Erysipelothrix rhusiopathiae* (Robertson and McCullough, 1968) and *E. inopinata* (this study) show robust, fermentative growth on glucose. In *E. inopinata*, the presence of oxygen did not stimulate growth yields nor did it affect the electron recovery in the fermentation products (this study). Together with the absence of cytochromes and isoprenoid quinones in *E. inopinata* and all other family members investigated in that respect (Collins et al., 1979; Verbarg et al., 2004; Verbarg et al., 2014), these results are a clear indication that *Erysipelotrichaceae* are neither "microaerophilic" (Verbarg et al., 2014) nor "aerobic to facultatively anaerobic" (Verbarg et al., 2004; Stackebrandt, 2009). These terms are reserved for microorganisms capable of aerobic respiration that either favor low oxygen concentrations (microaerophilic) or switch to an alternative metabolism (anaerobic respiration or fermentation) when oxygen is absent (Madigan et al., 2012). Rather, *E. inopinata* is an anaerobe with a purely fermentative metabolism but a much higher oxygen tolerance than other family members, which allows it to grow not only under hypoxic conditions (like strain Pei061) but also at atmospheric oxygen concentrations (albeit with a severely reduced growth yield).

Ecology

Strains ErySL and Pei061 grow on glucose and a variety of other sugars, which are present in the insect gut due to the hydrolytic breakdown of polymers like starch, cellulose, and hemicelluloses (Chapman, 2013). Their substrate spectrum is somewhat broader than that of other family members (Verbarg et al., 2014), but the requirement for yeast extract matches the requirements for organic growth factors typical of other members of *Erysipelotrichaceae*, which mostly colonize the intestinal tracts of animals (Verbarg et al., 2014). Although the fermentation products of both strains are similar, the growth yield of strain Pei061 is considerably higher than that of strain ErySL. Since there are hardly any differences in the elemental composition of the two strains and structures resembling storage materials have not been detected in the ultrastructural analysis, an accumulation of glycogen, lipids, or phosphate granules can be excluded. Rather, the higher growth yield of strain Pei061 appear to be based on a more efficient assimilation of yeast extract and Casamino acids into cell mass.

The isolation of strains ErySL and Pei061 from highly diluted gut homogenates suggests that they are numerically abundant members of the gut microbiota of their respective hosts. This is supported by the results of previous, cultivation-independent studies, which documented that the phylotypes corresponding to the respective isolates (~99% sequence similarity) represented about 2% of the clones in libraries obtained from the hindgut of *Shelfordella lateralis* (GenBank accession numbers JN680636–37; Schauer et al., 2012) and the midgut of *Pachnoda ephippiata* (GenBank accession number AJ576412; Egert et al., 2003). Also in pyrosequencing libraries of the colon of *S. lateralis* (Schauer et al., 2014), up to 1.2% of the reads (depending on the particular diet) were classified to the corresponding 'uncultured_3' lineage. The absence of this lineage from pyrosequencing libraries of *P. ephippiata* hindguts (Dietrich et al., 2014) suggests that in the case of this scarab beetle larva, its members are restricted to the midgut. The colonization of this highly alkaline compartment (up to pH 10; Lemke et al., 2003) is in agreement with the broad pH optimum of strain Pei061 (pH 7 to 10).

The pleomorphic cells with their thick cell envelope may be resting stages that allow survival under adverse conditions (e.g. desiccation or the exposure to oxygen) during environmental transmission to other hosts.

Taxonomy

According to the 16S rRNA gene tree, the family *Erysipelotrichaceae* consists of two sister branches, which are defined by *Erysipelothrix* and *Allobaculum* and their respective relatives.

16S rRNA gene sequence analysis of strains ErySL and Pei061 identified them as a separate line of descent in the radiation of the family *Erysipelotrichaceae*. Due to the well-supported monophyletic status of the clade comprising strains ErySL and Pei061 and their phylogenetic distance to other members of the family (83–90% sequence similarity), the classification of the new isolates requires the creation of a new taxon at the genus level.

Fatty acid analysis of strain ErySL confirmed highest similarities to *Clostridium innocuum*, which is in agreement with the basal position in the *Allobaculum* branch (harboring *C. innocuum*) in the 16S rRNA-based phylogeny (Figure 4.3). The phenotypic differences between the two strains, together with the substantial dissimilarity of their 16S rRNA gene sequences (>93%) justifies their classification as separate species (Stackebrandt and Goebel, 1994).

The purely fermentative growth of *E. inopinata* documented in this study and the absence of evidence for microaerophilic growth in any study of members of the family *Erysipelotrichaceae* call for an emended description of *Erysipelothrix inopinata* and the whole family *Erysipelotrichaceae*.

Emended description of *Erysipelothrix inopinata*

The description is as given for *Erysipelothrix inopinata* by Verbarg et al. (2004) with the following additions:

Aerotolerant anaerobic. Growth occurs best under anoxic conditions. Metabolism is fermentative. Major end products on 5 mM glucose are formate, acetate, ethanol, and lactate.

Emended description of the family *Erysipelotrichaceae*

The description is as given for *Erysipelotrichaceae* by Verbarg et al. (2014) with the following corrections:

Some strains have a tendency to form chains. Strictly anaerobic or aerotolerant. Metabolism is fermentative.

Description of *Breznakia* gen. nov.

Breznakia (Brez.na'ki.a. N. L. fem. n. *Breznakia*, named after the contemporary American microbiologist John A. Breznak, in recognition of his important contributions to insect gut microbiology).

Gram-positive staining, non-motile, non-spore-forming rods; occur in chains; pleomorphic in older cultures. Strictly anaerobic to slightly aerotolerant, catalase- and oxidase-negative. Fermentative metabolism. Major end products on 5 mM glucose are formate, acetate, and ethanol.

Habitat: Representatives of this genus have been isolated so far only from the intestinal tract of insects.

Type species: *Breznakia blatticola* gen. nov., sp. nov.

Description of *Breznakia blatticola* sp. nov.

Breznakia blatticola (blat.ti'co.la. L. fem. n. *blatta* cockroach; L. suff.-*cola*, inhabitant of; N. L. n. *blatticola* inhabitant of the cockroach). Cells are rod-shaped, with a variable length of 0.8–2.5 µm and a diameter of 0.3–0.4 µm. Cells often occur in chains. Growth on D-fructose, D-galactose, D-glucose, D-ribose, D-cellobiose, D-maltose, D-melibiose, D-trehalose, *N*-acetyl-D-glucosamine, D-mannitol, and salicin. Growth occurs only in the absence of oxygen in the headspace. Metabolism is obligately fermentative. Major end products on 5 mM glucose are formate, acetate, and ethanol. At higher glucose concentrations, additionally lactate is produced in minor amounts. Temperature range of growth 15–40 °C, optimum between 15 and 30 °C. pH range of growth 6–11; optimum at pH 7–10.

The major fatty acids or fatty acid derivatives are $C_{16:0}$ dimethyl acetal (DMA) (28.6%), $C_{18:1}$ *cis*-9 (13.1%), $C_{16:1}$ *cis*-9 (13.0%) and $C_{16:0}$ (12.5%). The DNA G+C content is 34.1 mol%.

Habitat: So far isolated only from the intestinal tract of the cockroach *Shelfordella lateralis*.

Type strain: ErySL^T (= DSM 28867^T = JCM 30190^T).

GenBank accession number: KU043523.

Description of *Breznakia pachnodae* sp. nov.

Breznakia pachnodae (pach.no'dae. L. gen. n. *pachnodae*, of *Pachnoda*, a genus of scarab beetles, referring to the origin of the isolate). Cells are rod-shaped, with a variable length of 0.8–2.5 µm and a diameter of 0.3–0.4 µm. Cells often occur in long chains with twisted and

rope-like structures. Growth on D-fructose, D-galactose, D-glucose, D-ribose, D-cellobiose, D-maltose, D-melibiose, D-trehalose, *N*-acetyl-D-glucosamine, D-mannitol, salicin, and D-gluconate. Growth occurs best in the absence of oxygen and is completely inhibited by 2% oxygen in the headspace. Metabolism is fermentative. Major end products on 5 mM glucose are formate, acetate, and ethanol. At a higher glucose concentration, additionally lactate is produced. Temperature range of growth of 15–40 °C, optimum between 20 and 30 °C. pH range of growth of 6–11; optimum at pH 7–10.

Habitat: So far isolated only from the intestinal tract of the larva of *Pachnoda ephippiata*. Type strain: Pei061^T (= DSM 16784^T = JCM 30191^T).

GenBank accession number: AJ629069.

Acknowledgements

This work was supported within the LOEWE program of the State of Hessen (Center for Synthetic Microbiology) and by the Max Planck Society. We thank Uwe Maier and his group at the Philipps-Universität Marburg for offering electron microscopy of negatively stained specimens and Karen A. Brune for linguistic comments on the manuscript.

References

1. **Alcaide M, Messina E, Richter M, Bargiela R, Peplies J, Huws SA, Newbold CJ, Golyshin PN, Simón MA, López G, et al.** 2012. Gene sets for utilization of primary and secondary nutrition supplies in the distal gut of endangered Iberian lynx. PLoS ONE **7:**e51521, http://doi.org/10.1371/journal.pone.0051521.
2. **Andert J, Marten A, Brandl R, Brune A.** 2010. Inter- and intraspecific comparison of the bacterial assemblages in the hindgut of humivorous scarab beetle larvae (*Pachnoda* spp.). FEMS Microbiol Ecol **74:**439–449.
3. **Atlas, RM.** 2010. Handbook of microbiological media, p 2036. 4th ed. Taylor and Francis. Boca Raton, Florida.
4. **Barnes EM, Impey CS, Stevens BJH, Peel JL.** 1977. *Streptococcus pleomorphus* sp. nov.: An anaerobic *Streptococcus* isolated mainly from caeca of birds. J Gen Microbiol **102:**45–53.

5. **Boga HI, Brune A.** 2003. Hydrogen-dependent oxygen reduction by homoacetogenic bacteria isolated from termite guts. Appl Environ Microbiol **69:**779–786.
6. **Burdett IDJ, Murray RGE.** 1974. Septum formation in *Escherichia coli*: characterization of septal structure and the effects of antibiotics on cell division. J Bacteriol **119:**303–324.
7. **Cato EP, Salmon CW, Holdeman IV.** 1974. *Eubacterium cylindroides* (Rocchi) Holdeman and Moore: Emended description and designation of neotype strain. Int J Syst Evol Microbiol **24:**256–259.
8. **Chapman RF.** 2013. The alimentary canal, digestion and absorption, p 46–79. *In* Simpson SJ, Douglas AE (ed), The insects: Structure and Function, 5th ed, Cambridge Univ. Press, Cambridge, UK.
9. **Chen W, Liu F, Ling Z, Tong X, Xiang C.** 2012. Human intestinal lumen and mucosa-associated microbiota in patients with colorectal cancer. PLoS ONE **7:**e39743, http://doi.org/10.1371/journal.pone.0039743.
10. **Collins MD, Jones D, Goodfellow M, Minnikin DE.** 1979. Isoprenoid quinone composition as a guide to the classification of *Listeria*, *Brochothrix*, *Erysipelothrix* and *Caryophanon*. J Gen Microbiol **111:**453–457.
11. **Collins MD, Lawson PA, Willems A, Cordoba JJ, Fernandez-Garayzabal J, Garcia P, Cai J, Hippe H, Farrow JAE.** 1994. The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. Int J Syst Bacteriol **44:**812–826.
12. **Condon S.** 1987. Responses of lactic acid bacteria to oxygen. FEMS Microbiol Rev **46:**269–280.
13. **Davis JJ, Xia F, Overbeek RA, Olsen GJ.** 2013. Genomes of the class *Erysipelotrichia* clarify the firmicute origin of the class *Mollicutes*. Int J Syst Evol Microbiol **63:**2727–2741.
14. **De Maesschalck C, Van Immerseel F, Eeckhaut V, De Baere S, Cnockaert M, Croubels S, Haesebrouck F, Ducatelle R, Vandamme P.** 2014. *Faecalicoccus acidiformans* gen. nov., sp. nov., isolated from the chicken caecum, and reclassification of *Streptococcus pleomorphus* (Barnes et al 1977), *Eubacterium biforme* (Eggerth 1935) and *Eubacterium cylindroides* (Cato et al. 1974) as *Faecalicoccus pleomorphus* comb. nov., *Holdemanella biformis* gen. nov., comb. nov. and *Faecalitalea cylindroides* gen.

nov., comb. nov., respectively, within the family *Erysipelotrichaceae*. Int J Syst Evol Microbiol **64:**3877–3884.

15. **De Vries W, Kapteijn WMC, van der Beek EG, Stouthamer AH.** 1970. Molar growth yields and fermentation balances of *Lactobacillus casei* L3 in batch cultures and in continuous cultures. J Gen Microbiol **63:**333–345.
16. **Dietrich C, Köhler T, Brune A.** 2014. The cockroach origin of the termite gut microbiota: patterns in bacterial community structure reflect major evolutionary events. Appl Environ Microbiol **80:**2261–9.
17. **Dinh DM, Volpe GE, Duffalo C, Bhalchandra S, Tai AK, Kane AV, Wanke CA, Ward HD.** 2015. Intestinal microbiota, microbial translocation, and systemic inflammation in chronic HIV infection. J Infect Dis **211:**19–27.
18. **Downes J, Olsvik B, Hiom SJ, Spratt DA, Cheeseman SL, Olsen I, Weightman AJ, Wade WG.** 2000. *Bulleidia extructa* gen. nov., sp. nov., isolated from the oral cavity. Int J Syst Evol Microbiol **50:**979–983.
19. **Eichler B, Pfennig N.** 1986. Characterization of a new platelet-forming purple sulfur bacterium, *Amoebobacter pedioformis* sp. nov. Arch Microbiol **146:**295–300.
20. **Egert M, Wagner B, Lemke T, Brune A, Friedrich MW.** 2003. Microbial community structure in midgut and hindgut of the humus-feeding larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae). Appl Environ Microbiol **69:**6659–6668.
21. **Fleissner CK, Huebel N, Abd El-Bary MM, Loh G, Klaus S, Blaut M.** 2010. Absence of intestinal microbiota does not protect mice from diet-induced obesity. Br J Nutr **104:**919–929.
22. **Greetham HL, Gibson GR, Giffard C, Hippe H, Merkhoffer B, Steiner U, Falsen E, Collins MD.** 2004. *Allobaculum stercoricanis* gen. nov., sp. nov., isolated from canine feces. Anaerobe **10:**301–307.
23. **Greiner T, Bäckhed F.** 2011. Effects of the gut microbiota on obesity and glucose homeostasis. Trends Endocrinol Metab **22:**117–123.
24. **Han I Congeevaram, S Ki, DW Oh, BT Park, J.** 2010. Bacterial community analysis of swine manure treated with autothermal thermophilic aerobic digestion. Appl Microbiol Biotechnol **89:**835–842.
25. **Jones D.** 1986. Genus *Erysipelothrix*, p 1245–1249. *In* Sneath PHA, Mair NS, Sharpe ME, Holt JG (ed), Bergey's Manual of Systematic Bacteriology, Williams & Wilkins, Baltimore.

26. **Justice SS, Hunstad DA, Cegelski L, Hultgren SJ.** 2008. Morphological plasticity as a bacterial survival strategy. Nat Rev Microbiol **6:**162–168.
27. **Kaakoush NO.** 2015. Insights into the role of *Erysipelotrichaceae* in the human host. Front Cell Infect Microbiol **5:**84, http://doi.org/10.3389/fcimb.2015.00084.
28. **Kageyama A, Benno Y.** 2000. Phylogenic and phenotypic characterization of some *Eubacterium*-like isolates from human feces: Description of *Solobacterium moorei* gen. nov., sp. nov. Microbiol Immunol **44:**223–227.
29. **Kanno M, Katayama T, Morita N, Tamaki H, Hanada S, Kamagata Y.** 2015. *Catenisphaera adipataccumulans* gen. nov., sp. nov., a member of the family *Erysipelotrichaceae* isolated from an anaerobic digester. Int J Syst Evol Microbiol **65:**805–810.
30. **Köhler T, Dietrich C, Scheffrahn RH, Brune A.** 2012. High-resolution analysis of gut environment and bacterial microbiota reveals functional compartmentation of the gut in wood-feeding higher termites *Nasutitermes* spp. Appl Environ Microbiol **78:**4691–4701.
31. **Kuykendall LD, Roy MA, O'Neill JJ, Devine TE.** 1988. Fatty acids, antibiotic resistance, and deoxyribonucleic acid homology groups of *Bradyrhizobium japonicum*. Int J Syst Bacteriol **38:**358–361.
32. **Lechner U.** 2009. Genus VII. *Sedimentibacter*, p 1137–1141. *In:* De Vos P, Garrity GM, Jones D, Krieg NR, Ludwig W, Rainey FA, Schleifer KH, Whitman WB. (ed), Bergey's Manual of Systematic Bacteriology. 2nd ed, Springer, New York.
33. **Lemke T, Stingl U, Egert M, Friedrich MW, Brune A.** 2003. Physicochemical conditions and microbial activities in the highly alkaline gut of the humus-feeding larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae). Appl Environ Microbiol **69:**6650–6658.
34. **Ludwig W, Schleifer KH, Whitman WB.** 2009. Revised road map to the phylum *Firmicutes*, p 1–14. *In* De Vos P, Garrity GM, Jones D, Krieg NR, Ludwig W, Rainey FA, Schleifer KH, Whitman WB (ed), Bergey's Manual of Systematic Bacteriology, 2nd ed, Springer, New York.
35. **Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar, Buchner A, Lai T, Steppi S, Jobb G, et al.** 2004. ARB: a software environment for sequence data. Nucleic Acids Res **32:**1363–1371.
36. **Madigan MT, Martinko JM, Stahl D, Clark DP.** 2012. Metabolism and Growth, p 118–149. *In* Brock Biology of Microorganisms Thirteenth Edition, Pearson, San Francisco.

37. **Matias VRF, Beveridge TJ.** 2005. Cryo-electron microscopy reveals native polymeric cell wall structure in *Bacillus subtilis* and the existence of a periplasmic space. Mol Microbiol **56:**240–251.
38. **Mikaelyan A, Köhler T, Lampert N, Rohland J, Boga H, Meuser K, Brune A.** 2015. Classifying the bacterial gut microbiota of termites and cockroaches: a curated phylogenetic reference database (DictDb). Syst Appl Microbiol **38:**472–482.
39. **Miller LT.** 1982. A single derivatization method for bacterial fatty acid methyl esters including hydroxy acids. J Clin Microbiol **16:**584–586.
40. **Moore WEC, Holdeman LV.** 1974. Human fecal flora: the normal flora of 20 Japanese-Hawaiians. Appl Microbiol **27:**961–979.
41. **Moore WEC, Holdeman Moore LV.** 1986. The Genus *Eubacterium* Prévot 1938, p 1353–1373. *In* Sneath PHA, Mair NS, Sharpe ME, Holt JG (ed), Bergey's Manual of Systematic Bacteriology, Williams & Wilkins, Baltimore.
42. **Moore WEC, Johnson JL, Holdeman LV.** 1976. Emendation of *Bacteroidaceae* and *Butyrivibrio* and description of *Desulfomonas* gen. nov. and ten new species in the genera *Desulfomonas, Butyrivibrio, Eubacterium, Clostridium,* and *Ruminococcus*. Int J Syst Bacteriol **26:**238–252.
43. **Murray RGE, Doetsch RN, Fobinow CF.** 1994. Determinative and cytological light microscopy, p 21–41. *In* Gerhardt P, Murray RGE, Wood WA, Krieg NR (ed), Methods for General and Molecular Bacteriology, American Society for Microbiology, Washington, DC.
44. **Pfennig N.** 1978. *Rhodocyclus purpureus* gen. nov. and sp. nov., a ring-shaped, vitamin B_{12}-requiring member of the family *Rhodospirillaceae*. Int J Syst Bacteriol **28:**283–288.
45. **Pfennig N, Trüper HG.** 1981. Isolation of members of the families *Chromatiaceae* and *Chlorobiaceae*, p 279–289. *In* Starr MP, Stolp H, Trüper HG, Balows A, Schlegel HG (ed), The Prokaryotes, Springer, Berlin.
46. **Rainey FA, Hollen BJ, Small A.** 2009. Genus *Clostridium*, pp. 738–828. *In* De Vos P, Garrity GM, Jones D, Krieg NR, Ludwig W, Rainey FA, Schleifer KH, Whitman WB (ed), Bergey's Manual of Systematic Bacteriology. 2nd ed, Springer, New York.
47. **Ramasamy D, Lagier JC, Nguyen TT, Raoult D, Fournier PE.** 2013. Non contiguous-finished genome sequence and description of *Dielma fastidiosa* gen. nov., sp. nov., a new member of the family *Erysipelotrichaceae*. Stand Genomic Sci **8:**336 351.

48. **Robertson DC, McCullough WG.** 1968. Glucose catabolism of *Erysipelothrix rhusiopathiae*. J Bacteriol **95:**2112–2116.

49. **Schauer C, Thompson C, Brune A.** 2014. Pyrotag sequencing of the gut microbiota of the cockroach *Shelfordella lateralis* reveals a highly dynamic core but only limited effects of diet on community structure. PLoS ONE **9:**e85861, http://doi.org/10.1371/journal.pone.0085861.

50. **Schauer C, Thompson CL, Brune A.** 2012. The bacterial community in the gut of the cockroach *Shelfordella lateralis* reflects the close evolutionary relatedness of cockroaches and termites. Appl Environ Microbiol **78:**2758–2767.

51. **Schuler S, Conrad R.** 1990. Soils contain two different activities for oxidation of hydrogen. FEMS Microbiol Ecol **73:**77–84.

52. **Seeliger HPR.** 1957. *Erysipelothrix*, p 597–598. *In* Breed RS, Murray EGD, Smith NR (ed), Bergey's Manual of Determinative Bacteriology. 7th ed, Williams & Wilkins, Baltimore.

53. **Smith LD, King E.** 1962. *Clostridium innocuum*, sp. n., a sporeforming anaerobe isolated from human infections. J Bacteriol **83:**938–939.

54. **Stackebrandt E.** 2009. Genus I. *Erysipelothrix*, p 1299–1306. *In* De Vos P, Garrity GM, Jones D, Krieg NR, Ludwig W, Rainey FA, Schleifer KH, Whitman WB (ed), Bergey's Manual of Systematic Bacteriology. 2nd ed, Springer, New York.

55. **Stackebrandt E, Goebel BM.** 1994. A place for DNA-DNA reassociation and 16S rRNA sequence-analysis in the present species definition in bacteriology. Int J Syst Evol Bacteriol **44:**846–849.

56. **Strassert JF, Desai MS, Radek R, Brune, A.** 2010. Identification and localization of the multiple bacterial symbionts of the termite gut flagellate *Joenia annectens.* Microbiology **156:** 2068–2079.

57. **Teixeira de Mattos MJ, Tempest DW.** 1983. Metabolic and energetic aspects of the growth of *Klebsiella aerogenes* NCTC 418 on glucose in anaerobic chemostat culture. Arch Microbiol **134:**80–85.

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

59. **Thomas TD, Ellwood DC, Longyear MV.** 1979. Change from homo- to heterolactic fermentation by *Streptococcus lactis* resulting from glucose limitation in anaerobic chemostat cultures. J Bacteriol **138:**109–117.

60. **Turnbaugh PJ, Bäckhed F, Fulton L, Gordon JI.** 2008. Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. Cell Host Microbe **3:**213–223.

61. **Verbarg S, Göker M, Scheuner S, Schumann P, Stackebrandt E.** 2014. The families *Erysipelotrichaceae* emend., *Coprobacillaceae* fam. nov., and *Turicibacteraceae* fam. nov., p 79–105. *In* Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F (ed), The Prokaryotes. 4th ed, Springer, Berlin.

62. **Verbarg S, Rheims H, Emus S, Frühling A, Kroppenstedt RM, Stackebrandt E, Schumann P.** 2004. *Erysipelothrix inopinata* sp. nov., isolated in the course of sterile filtration of vegetable peptone broth, and description of *Erysipelotrichaceae*. Int J Syst Evol Microbiol **54:**221–225.

63. **Weisburg WG, Tully JG, Rose DL, Petzel JP, Oyaizu H, Yang D, Mandelco L, Sechrest J, Lawrence TG, Van Etten J, et al.** 1989. A phylogenetic analysis of the mycoplasmas: basis for their classification. J Bacteriol **171:**6455–6467.

64. **Willems A, Moore WE, Weiss N, Collins MD.** 1997. Phenotypic and phylogenetic characterization of some *Eubacterium*-like isolates containing a novel type B wall murein from human feces: description of *Holdemania filiformis* gen. nov., sp. nov. Int J Syst Bacteriol **47:**1201–1204.

65. **Widdel F, Pfennig N.** 1981. Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. I. Isolation of new sulfate-reducing bacteria enriched with acetate from saline environments. Description of *Desulfobacter postgatei* gen. nov., sp. nov. Arch Microbiol **129:**395–400.

66. **Yarza P, Richter M, Peplies JR, Euzeby J, Amann R, Schleifer KH, Ludwig W, Glöckner FO, Rosselló-Móra R.** 2008. "The All-Species Living Tree project: A 16S rRNA-based phylogenetic tree of all sequenced type strains". Syst Appl Microbiol **31:**241–250.

67. **Young KD.** 2006. The selective value of bacterial shape. Microbiol Mol Biol Rev **70:**660–703.

68. **Zheng H, Dietrich C, Radek R, Brune A.** 2016. *Endomicrobium proavitum*, the first isolate of *Endomicrobia* class nov. (phylum *Elusimicrobia*) – an ultramicrobacterium with an unusual cell cycle that fixes nitrogen with a group IV nitrogenase. Environ Microbiol **18:**191–204.
69. **Zuber B, Haenni M, Ribeiro T, Minnig K, Lopes F, Moreillon P, Dubochet J.** 2006. Granular layer in the periplasmic space of gram-positive bacteria and fine structures of *Enterococcus gallinarum* and *Streptococcus gordonii* septa revealed by cryo-electron microscopy of vitreous sections. J Bacteriol **188:**6652–6660.

Supplementary Material

Table S4.1 Fatty acid composition of members of the *Erysipelotrichaceae* (values <0.1% of total were omitted): 1, Strain ErySL; 2, *Clostridium innocuum* DSM 22910^T 3, *Eubacterium tortuosum* DSM 3987^T; 4, *Faecalicoccus pleomorphus* DSM 20574^T; 5, *Allobaculum stercoricanis* DSM 13633^T; 6, *Solobacterium moorei* DSM 22971^T; 7, *Bulleidia extructa* DSM 13220^T; 8, *Holdemania filiformis* DSM 12042^T; 9, *E. rhusiopathiae* DSM 5055^T; 10, *E. tonsillarum* DSM 14972^T; and 11, *E. inopinata* DSM 15511^T. Values for strain ErySL were from this study; all others were taken from Verbarg et al., 2014 (The Prokaryotes, 4th edn., Springer, Berlin, pp. 79-105).

Fatty acid	1	2	3	4	5	6	7	8	9	10	11
C12:0	0.22	2.34	2.46	–	–	–	–	–	–	–	–
C14:0	3.83	7.58	10.88	8.93	1.09	1.14	1.50	2.18	1.84	1.80	2.76
C16:0 ALDE	5.71	1.68	2.39	1.26	–	3.97	–	2.47	–	–	–
C15:0	–	–	1.72	–	–	–	–	–	–	–	–
C16:1 *cis*-7	5.08	3.11	1.21	–	–	–	–	–	–	–	–
C16:1 *cis*-9	13.02	4.15	–	3.31	–	–	1.19	1.34	1.57	1.17	1.26
C16:0	12.54	26.59	28.66	21.62	35.27	11.33	31.46	21.67	33.53	34.25	44.61
Feature 6	–		1.03	–	–	–	–	–	–	–	–
C16:0 DMA	28.63	9.87	12.59	4.69	0.62	20.08	–	11.68	–	–	–
C18:0 ALDE	1.58	1.26	–	2.92	–	2.08	–	–	–	–	–
C17:0 DMA	–	–	–	–	–	1.02	–	–	–	–	–
C17:0	–	–	1.29	–	–	–	1.82	–	–	1.00	–
C18:2 *cis*-9,12	–	11.14	5.84	7.28	18.20	8.20	19.48	21.77	22.44	22.85	12.05
C18:1 *cis*-9	13.12	14.86	7.79	21.54	23.01	18.62	20.74	17.68	19.93	21.48	26.95
Feature 10	4.45	2.78	2.19	4.26	4.21	–	6.20	3.42	4.75	4.57	2.81
C18:1 t11?	–	–	–	–	–	6.62	–	–	–	–	–
C18:0	3.41	10.33	3.20	12.47	15.06	7.76	15.13	6.22	11.42	10.46	5.36
Feature 11	–	–	1.65	–	–	4.41	–	–	–	–	–
C18:1 *cis*-9 DMA	0.96	–	4.92	–	–	2.78	–	1.08	–	–	–
C18:1 *cis*-11 DMA	0.45	–	0.95	0.36	–	–	–	1.09	0.24	0.22	–
C18:0 DMA	4.04	2.67	1.74	4.55	–	7.31	–	3.23	–	–	–
19cyc 9,10/:1	–	–	1.59	–	–	–	–	–	–	–	–
19cyc 9,10 DMA	–	–	1.28	–	–	–	–	–	–	–	–

Feature 10 (C18:1c11/t9/t6 or UN 17.834), Feature 11 (C18:2 DMA or C17:0 iso 3OH)

Chapter 5

Manuscript in preparation

Ereboglobus luteus gen. nov. sp. nov., a novel propionigenic verrucomicrobium isolated from the gut of the cockroach *Shelfordella lateralis*, and emended description of the genus *Diplosphaera*

Dorothee Tegtmeier[1#], Alexandra Belitz[1#], Renate Radek[2], Thomas Heimerl[3] and Andreas Brune[1]

[1] Department of Biogeochemistry, Max Planck Institute for Terrestrial Microbiology, Karl-von-Frisch-Straße 10, 35043 Marburg, Germany

[2] Institute of Biology/Zoology, Free University of Berlin, Königin-Luise-Strasse 1–3, 14195 Berlin, Germany

[3] Laboratory for Cell Biology, Philipps-Universität Marburg, Karl-von-Frisch-Str. 8, 35043 Marburg, Germany

[#]These authors contributed equally to this work

Contributions

D.T. planned and designed the study, isolated strain Ho45, supervised the bachelor project of Alexandra Belitz, analyzed fermentation products, performed phylogenetic analysis, discussed results, evaluated and visualized data, and wrote the manuscript.

A.Be. planned and designed the study, performed growth tests, analyzed fermentation products and physiological properties of strain Ho45, discussed results, evaluated and visualized data.*

R.R. performed ultra-thin sections and electron microscopy.

T.H. performed negative staining, freeze substitution and embedding of strain Ho45.

A.Br. planned and designed the study, supervised the work and discussed results.

*These results have been reported already in the BSc thesis of Alexandra Belitz (Universität Marburg, 2015)

Abstract

A novel, Gram-negative, yellow-pigmented, coccus-shaped, motile bacterium (designated strain Ho45) of *Verrucomicrobia*, was isolated from the intestinal tract of the cockroach *Shelfordella lateralis*. Strain Ho45 belongs to the family *Opitutaceae* that currently contains only three validly described species, which show less than 93% sequence similarity with the new isolate. Ultrastructural analysis revealed a Gram-negative cell envelope with an outer membrane and a periplasmic space. Detailed metabolic analysis showed that strain Ho45 has a purely fermentative metabolism with propionate and acetate formed in a 2:1 ratio. It tolerated low amounts of oxygen in the medium, but cell yield and product formation decreased with increasing oxygen concentration. However, contrary to its close relative *Diplosphaera colitermitum*, which was isolated from the termite *Reticulitermes flavipes*, it did not grow under atmospheric oxygen concentrations. Moreover, our results show that *D. colitermitum* is not obligately aerobic as previously described but showed also a fermentative metabolism under anoxic conditions, similar to that of strain Ho45 and the closely related *Opitutus terrae.* Based on phenotypic and phylogenetic evidence, we propose that strain Ho45 is classified as member of a novel genus, *Ereboglobus luteus* gen. nov. sp. nov. with strain Ho45 as type strain and provide an emended description of the genus *Diplosphaera*.

Introduction

The phylum *Verrucomicrobia* (Hedlund et al., 1997) belongs to the PVC superphylum, which also harbors the phyla *Planctomycetes* and *Chlamydia* and the sister groups *Poribacteria* and *Lentisphaera* (Wagner and Horn, 2006). *Verrucomicrobia* occur worldwide in soils (e.g. Hugenholtz et al., 1998; Hackl et al., 2004; Sangwan et al., 2005) and based on their abundance are considered to be of ecological importance (Bergmann et al., 2011). High-throughput sequencing recently revealed that up to 23% of all sequences obtained from soil belong to the phylum *Verrucomicrobia* (Bergmann et al., 2011). Furthermore, they are present in the human intestinal tract (Derrien et al., 2004; Dubourg et al., 2013), as well as in the guts of wood-feeding termites (Hongoh et al., 2003; Stevenson et al., 2004; Köhler et al., 2012; Wertz et al., 2012) and omnivorous cockroaches (Schauer et al., 2014). Some isolates of *Verrucomicrobia* are capable of nitrogen fixation and methane oxidation (Dunfield et al., 2007; Hou et al., 2008; Islam et al., 2008), which corroborates the ecological relevance of this phylum. Despite their huge abundance in diverse ecosystems, the majority of *Verrucomicrobia* is uncultivated so far.

Especially, little is known about the family *Opitutaceae* (Choo et al., 2007) due to the lack of isolates. Members of *Opitutaceae* are Gram-negative, non-spore-forming cocci with a relatively high G+C-content of 60.5– 65.8 mol %. However, only three isolates have been described so far: *Alterococcus agarolyticus*, *Opitutus terrae* and *Diplosphaera colitermitum*, all three showing fundamental differences in their metabolism and physiology. *Alterococcus agarolyticus* is a facultatively anaerobic, halophilic, thermophilic bacterium that was isolated from a hot spring (Shieh and Jean, 1998). *O. terrae* was isolated from rice field-soil and was described as obligately anaerobic propionigenic bacterium (Chin et al., 2001), whereas the closely related *D. colitermitum* was described as microaerophilic and non-fermenting bacterium with optimal growth rates between 2–8% oxygen (Wertz et al., 2012; Isanapong et al., 2013). *D. colitermitum* was isolated from the gut of the termite *Reticulitermes flavipes* and is capable of nitrogen-fixation (Wertz et al., 2012), which is an important aspect of symbiosis in the termite gut (Benemann, 1973; Breznak et al., 1973; Wertz et al., 2012). Besides *D. colitermitum* (TAV2) a second strain of *Opitutaceae*, designated TAV1, was isolated from the same termite species (Stevenson et al., 2004). Both genomes (TAV1 and TAV2) have been sequenced (Isanapong et al., 2012; Wertz et al., 2012), though strain TAV1 has not been validly described yet.

We isolated a novel member of *Opitutaceae* (strain Ho45) from the hindgut of the cockroach *Shelfordella lateralis* that does not match the properties of its closest relatives *D. colitermitum* (TAV2) and TAV1. Here, we report the characterization of strain Ho45, including a detailed ultrastructural and metabolic analysis, which is so far scarce for the entire family. Furthermore, we provide new data on the energy metabolism of *D. colitermitum*. Based on phenotypic and phylogenetic evidence, we propose that strain Ho45 is classified as a species of a novel genus, *Ereboglobus luteus* sp. nov. gen. nov., and provide an emended description of the genus *Diplosphaera*.

Materials and Methods

Isolation and cultivation

Shelfordella lateralis was obtained from a commercial breeder and maintained as previously described (Schauer et al., 2012). An adult female cockroach was dissected and the hindgut was placed in a 16-ml culture tube with 1 ml AM5 medium (Tegtmeier et al., 2016) amended with 1 mM dithiothreitol (DTT) and glass beads. The tube was gassed with N_2/CO_2 (80:20, v/v) and closed with a rubber stopper. After vortexing the tube for 10 min, the resulting gut homogenate was serial diluted in AM5 medium. Pure cultures from high dilutions of *S. lateralis* hindgut homogenate were isolated by two consecutive agar dilution series (Pfennig and Trüper, 1981) in basal medium amended with Casamino acids and yeast extract (0.1% w/v each) and 1mM cysteine, incubated under a headspace of H_2/CO_2 (80:20, v/v) at 30 °C.

For further experiments, the AM5 medium was routinely amended with yeast extract and Casamino acids (0.1% each), cysteine and DTT as reducing agents (1 mM each) and resazurin (0.8 mg/L) as redox indicator and kept under a headspace of N_2/CO_2 (80:20, v/v). This “basal medium” routinely received glucose (5 mM) as substrate and was dispensed (4.5 ml) into 16-ml rubber-stoppered culture tubes, which were inoculated with overnight cultures (0.5 ml) and incubated at 30 °C.

Opitutus terrae (DSM 11246) was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), (Braunschweig, Germany). *Diplosphaera colitermitum* TAV2 was kindly provided by J.L.M Rodrigues, Department of Biology, of the University of Texas, Arlington (Texas, USA).

Growth and physiology

Growth was tested on D-fructose, D-galactose, D-maltose, D-ribose, D-melibiose, D-trehalose, D-cellobiose, D-sucrose, D-xylose, cellulose, starch, galacturonic acid, mannitol, L-lactate, L-malate, pyruvate, succinate, fumarate, glycerol, glutamate, alanine (each 10 mM) and pectin (0.5%). Growth on acetate (10 mM) was tested under oxic, microoxic (2% oxygen in the headspace) and anoxic conditions. Additionally, growth on glucose was tested with different concentrations of yeast extract (0.05; 0.1; 0.2 and 0.5%) and Casamino acids (0.05 and 0.1%).

The temperature range for growth was determined at 10, 15, 20, 25, 30, 35, 37, 40 and 45 °C. The pH range of growth was determined in MM5 medium (Tegtmeier et al., 2016) that received the same amendments as AM5 "basal medium", and was incubated under a N_2 headspace; the pH of the medium was adjusted by adding different buffer systems (sodium phosphate buffer, pH 2.0, 6.0, 7.0 and 12.0; 4-(2-hydroxyethyl)-1 piperazineethanesulfonic acid (HEPES), pH 8.0; sodium acetate buffer, pH 4.0 and sodium carbonate buffer, pH 10.0; each at a final concentration of 20 mM).

Oxygen tolerance was tested in non-reduced medium that received different amounts of sterile-filtered (0.2 µm pore size) oxygen in the headspace, followed by incubation on a roller mixer (60 rpm). Salt tolerance was tested by adding different amounts of NaCl (0–4% at interval of 0.5%) to the medium. Growth was measured photometrically by following the increase in optical density at 578 nm (OD_{578}) using a culture tube photometer (Spectronic 20^+, Milton Roy; path length ca. 1.3 cm).

For dry weight determination, strain Ho45 was grown (in duplicate) in 500 ml AM5 medium with the same amendments as described above. The cells were harvested by centrifugation (14,000 × *g*; 10 min), washed with ammonium acetate solution (20 mM), and dried overnight at 80 °C.

Gram reaction was tested by Gram staining according to Murray et al. (1994) and by the KOH method (Gregersen, 1978). *Bacillus subtilis* JH642 and *Escherichia coli* DH5α were used as controls. Motility was tested in stab tubes containing AM5 basal medium amended with 5 mM glucose and 1% agar under a headspace of N_2/CO_2 (80:20, v/v).

Oxidase activity was tested with liquid cultures using oxidase test strips (Bactident, Merck, Darmstadt) and *E. coli* and *B. subtilis* as controls. Presence of catalase was assayed by observing gas formation upon adding a drop of H_2O_2 (3%) on bacterial cells, harvested by centrifugation; *E. coli* and *Clostridium mangenotii* (DSM 1289) were used as controls.

Test for utilization of alternative electron acceptors was performed in basal AM5 medium amended with 5mM glucose and 5 mM sulfate or nitrate. Nitrite was analyzed using test strips (Quantofix, Macherey-Nagel, Düren). Sulfide was assayed colorimetrically with zinc acetate (1 mM), ADMA-Reagent (4-amino-*N*,*N* dimethylaniline sulfate, 8.5 mM in 20% sulfuric acid) and iron(III)-solution (2 mM).

Metabolic products

Fully grown cultures were centrifuged, the cell-free supernatants were acidified with H_2SO_4 (50 mM final concentration) and analyzed by HPLC with an ion-exclusion column (Resin H+, IEX, 8 µm, Grom, Rottenburg, Germany) and a refractive index detector (Schauer et al., 2012). Hydrogen concentrations in the culture headspace were analyzed by gas chromatography using a thermal conductivity detector (Schuler and Conrad, 1990). Products formed in controls without glucose were subtracted. Since the bicarbonate buffer did not allow the analysis of CO_2 formation, the calculation of carbon balances involved the assumption that acetate production was accompanied by the formation of one CO_2 and succinate production by the consumption of one CO_2.

For computation of electron balances, all metabolites were formally oxidized to CO_2, and the number of electrons theoretically released from the respective amounts of products was compared with that of the amount of substrate dissimilated (Tholen et al., 1997). These calculations yielded the electrons of dissimilated glucose recovered in fermentation products expressed on a per cent basis (electron recovery).

Light and electron microscopy

Light microscopy was conducted with an Axiophot photomicroscope (Zeiss, Oberkochen, Germany). Non-stained cultures were routinely examined using phase contrast illumination (100 × objective). For transmission electron microscopy (TEM), cells were fixed with glutardialdehyde (1.25 % final concentration). For negative staining, the fixed cells (5 µl) were pipetted onto a carbon-coated copper grid, washed twice with water and contrasted with uranyl acetate. Cells were examined with a JEOL JEM-2100 transmission electron microscope (JEOL, Tokyo, Japan) equipped with an LaB6 cathode and operated at 120 kV. For ultrastructural analysis, 2 µl of concentrated cells were frozen under high pressure, freeze substituted and embedded in Epon resin as described previously (Peschke et al., 2013). Freeze substitution was performed with HUGA (0.5% uranylacetate, 0.5% glutardialdehyde, 5% H_2O

in acetone). Ultrathin sections were cut with a microtome equipped with a diamond blade and contrasted with uranylacetate and lead citrate. The sections were examined with a Philips EM 208 transmission electron microscope.

Nitrogen fixation

The *nifH*-Gene was amplified using the primer IGK (5'ATAGGATCCAARGG NGGNATHGGNAA-3') and YAA (5' GACCTGCAGATRTTRTTNGCNGCRTA-3') as previously described (Ohkuma et al., 1996). Active nitrogen fixation was tested in nitrogen-free AM5 (without ammonium chloride, Casamino acids and yeast extract) amended with 10 mM glucose under N_2/CO_2 (80:20, v/v) headspace.

Spectral analysis

To test for the presence of cytochromes, strain Ho45 was grown in 10 mM glucose, harvested in the late exponential growth phase by centrifugation (10.000 × g for 10 min) and resuspended in phosphate buffer (0.1 M, pH 7). Cells were chilled on ice and disrupted with a French press at 138 MPa in repeated cycles. Cell debris was removed by centrifugation (30.000 × *g*, 20 min). The cell free supernatant was separated into a membrane fraction and a soluble fraction via ultracentrifugation (12.600 × *g*, 1 h). The membrane fraction was recovered in phosphate buffer (0.1 M, pH 7) with 0.2% *t*-octylphenoxypolyethoxyethanol (Triton X-100). Difference spectra of sodium dithionite-reduced and air-oxidized preparations were obtained for both fractions. In order to test the presence of carotenoids, cells were harvested by centrifugation, resuspended in ethanol (70%), centrifuged again and spectra were measured from the clear supernatant directly.

Phylogenetic analysis

16S rRNA genes were amplified with *Bacteria*-specific primers and sequenced by Sanger sequencing (Strassert et al., 2010). The sequences were imported into the DictDb reference database (version 3.0; Mikaelyan et al., 2015), which contained all type strains included in the Living Tree Project (Yarza et al., 2008), and aligned against the existing alignment using the *fast aligner* included in the ARB software package (version 5.5; Ludwig et al., 2004). The alignment was checked manually and corrected where necessary. Highly variable base positions were excluded from the alignment using a frequency-based filter (minimum similarity 50%) calculated for all high-quality full-length sequences of *Opitutae* (1358

sequence positions). A maximum-likelihood tree was constructed using fastDNAml algorithm included in ARB (1000 bootstraps).

The 16S rRNA gene sequences of strain Ho45 (KU670677) has been submitted to GenBank (http://www.ncbi.nlm.nih.gov).

Results

Isolation and morphological characterization

Strain Ho45 was obtained from a serially diluted hindgut homogenate of *Shelfordella lateralis*. The culture derived from a single colony picked from the ultimate dilution step that yielded bacterial colonies within two weeks of incubation. To ensure clonality, the culture was subjected to a further deep-agar dilution series. In sloppy agar, strain Ho45 formed yellow, round, convex colonies of a diameter of 1–3 mm with well-defined edges. The cells were motile cocci with a diameter of 0.3–0.4 µm (Figure 5.1A) and stained Gram-negative.

Transmission electron microscopy showed that strain Ho45 contains (from outside to inside) a layer with two electron-dense parts and one electron-lucent layer in between (trilamellar structure), which resembles most likely the outer membrane (9.5 nm), followed by a large electron-lucent layer, which is likely the periplasmic space (38–52 nm) and a second layer with trilamellar structure, which resembles probably the lipid bilayer of the cytoplasmic membrane (5 nm). A cell wall was not visible, which is in agreement with the negative result of the Gram staining.

The cytoplasm was granular in the periphery and condensed in the center of the cells (Figure 5.1B). Endospores were never observed. Negatively stained specimens showed dividing cells with septum formation and a wrinkled surface structure (Figure 5.1C). A single filamentous cell appendage was occasionally observed (20 nm; Figure 5.1D).

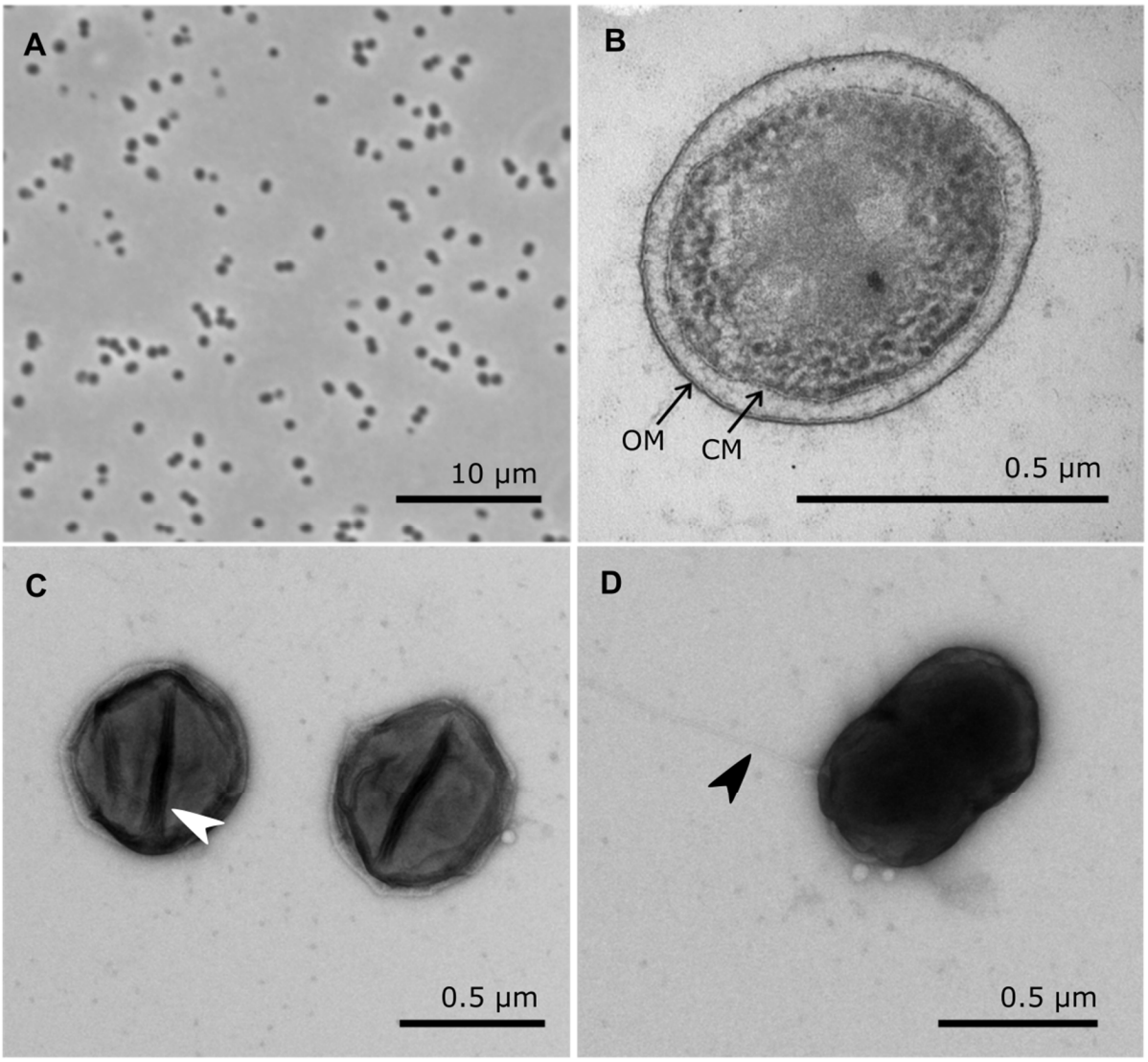

Figure 5.1. Morphology and ultrastructure of *Ereboglobus luteus* strain Ho45. Image A shows a phase-contrast micrograph of cells from a growing culture. Image B shows a transmission electron micrograph of a cell from an ultra-thin section, showing the outer membrane (OM), followed by a periplasmic space and a cytoplasmic membrane (CM). Images C and D show negatively stained preparations of cells in an early (C) or late stage of division (D). The white arrowhead points to the septum of dividing cells. The black arrowhead indicates a filamentous appendage, probably a flagellum. All samples were taken in the late exponential phase.

Growth and physiology

Strain Ho45 grew fermentatively on glucose and a variety of other carbohydrates, including D-fructose, D-galactose, D-ribose, D-cellobiose, D-maltose, D-melibiose, D-trehalose, D-sucrose, galacturonic acid and pectin, but not on D-xylose, D-mannitol, glycerol, L-lactate, pyruvate, L-malate, fumarate, succinate, L-alanine, glutamate, cellulose and starch. No growth was observed on acetate, neither under oxic, microoxic or anoxic conditions. Neither nitrate not sulfate were reduced with glucose as electron donor. Cells did not grow in nitrogen-free medium, and no *nifH* genes were detected by PCR.

The doubling time during growth on glucose was 11.9 h. Robust growth on glucose required yeast extract (minimum concentration 0.05%). Growth was further enhanced by the addition of 0.1% yeast extract, whereas 0.5% yeast extract lead to a decrease of growth. In the presence of yeast extract, growth on glucose was not further enhanced when Casamino acids (0.05%) were added. Slight growth occurred also on glucose without yeast extract and Casamino acids (OD 0.15). Glucose-free controls showed that growth on basal medium was negligible (OD < 0.03). Cultures survived for six months without transfer to fresh medium.

The temperature for growth ranged from 15 to 37 °C. No growth occurred at 10 or 40 °C. Highest cell yields were obtained in a range from 15 to 30 °C, whereas highest growth rate was at 35 °C. Growth occurred at a pH range from 6 to 9, with an optimum at pH 7. Salt tolerance was up to 2% NaCl, but cells formed strong aggregations with 2% NaCl.

Strain Ho45 showed highest cell yield and shortest doubling time under completely anoxic conditions. The strain grew also with up to 4% oxygen in the headspace. However, cell yields decreased and the lag-phase extended, with increasing oxygen concentrations (Figure 5.2). The reduced status of the redox indicator resazurin at the end of the incubation indicated that oxygen was completely consumed during growth at 1 and 2% oxygen. With 4 % oxygen in the headspace the medium remained oxidized and with 8% oxygen in the headspace, growth was completely inhibited. No catalase and no oxidase activity were found.

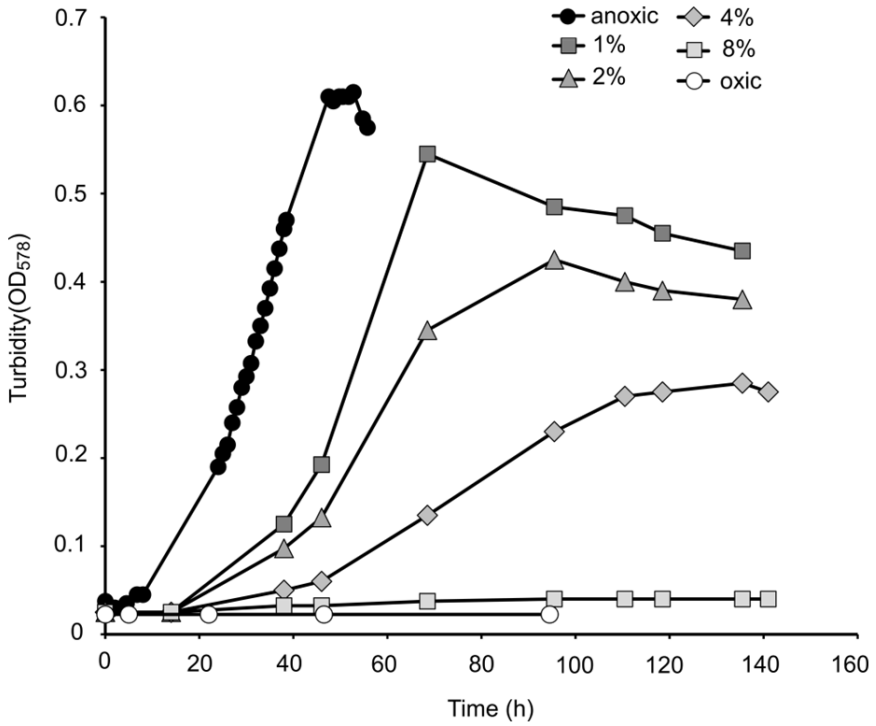

Figure 5.2. Growth curves of strain Ho45 cultivated on glucose (5 mM) under anoxic conditions and with different oxygen concentrations in the headspace. Values for anoxic cultures are indicated by black symbols and values for cultures with oxygen in the headspace are indicated by grey and white symbols (see legend across the figure). Values are means of duplicate cultures; mean deviations were smaller than the symbols. Oxygen concentrations in the headspace are the initial values.

Metabolite profiles

Under anoxic conditions, strain Ho45 fermented glucose to propionate, acetate and succinate; hydrogen was not produced. The reaction stoichiometry on glucose was 1.37 mol propionate, 0.74 mol acetate and 0.14 mol succinate per mol dissimilated glucose, which results in a (propionate + succinate) to acetate ratio of 2:1. Carbon and electron recoveries were almost identical, which confirms the assumption that one CO_2 was formed per acetate and one CO_2 was consumed per succinate (Table 5.1).

With increasing oxygen concentration in the headspace, cell yield decreased and less fermentation products were formed, resulting in a decrease of electron- and carbon recovery. The (propionate + succinate) to acetate ratio did not change with up to 2 % oxygen in the headspace. However, with 4 % oxygen in the headspace less acetate, but larger amounts of malate were formed and the propionate-to-acetate ratio changed slightly from 2:1 to 2:0.5 (Table 5.1). Growth and fermentation were completely inhibited by 8% oxygen.

Table 5.1 Fermentation products, electron and carbon balance, and growth yield of strain Ho45 cultivated with different oxygen concentrations in the headspace. Values are means of results obtained with duplicate cultures (less than 10% deviation).

Oxygen conc.[a]	Glucose consumed	Cell mass[b]	Glucose assimilated[c]	Glucose dissimilated[d]	Products (mM)[e]				Electron recovery[f]	Carbon recovery[g]	Growth yield (Y_S)
	(mM)	(mg/L)	(mM)	(mM)	Propionate	Acetate	Succinate	Malate	(%)	(%)	(g mol^{-1})
anoxic	4.80	239.9	1.65	3.15	4.41	2.32	0.45	0.00	115	114	50.0
1%	4.50	222.3	1.53	2.97	3.32	1.95	0.44	0.11	98	99	49.4
2%	4.50	183.3	1.26	3.24	2.67	1.51	0.62	0.13	77	77	40.7
4%	3.98	115.1	0.79	3.19	2.31	0.72	0.49	0.70	70	70	28.9

[a] Headspace concentration (v/v); initial values.

[b] Estimated using the turbidity of the culture with the experimentally determined dry weight of 39 mg at OD = 0.1.

[c] Estimated using the cell mass of strain Ho45 and an elemental composition for *Pseudomonas* sp. cells of $C_4H_8O_2N$ (Mayberry et al., 1968), which corresponds to 6.9 mmol glucose per g cell mass.

[d] Difference between the amount of glucose consumed and assimilated into biomass.

[e] Products formed on basal medium without glucose were subtracted.

[f] Electrons of dissimilated glucose recovered in fermentation products.

[g] Carbon of dissimilated glucose recovered in fermentation products.

Product formation of strain Ho45 increased with increasing glucose concentration. Up to 14.5 mM glucose were consumed; at higher concentrations product formation did not further increase. With higher glucose concentrations (7.5 to 20 mM), additionally formate was produced, and the (propionate + succinate) to acetate ratio shifted slightly from 2:1 to 2.4:1 (Table 5.2).

Table 5.2 Influence of glucose concentration on the fermentation products of strain Ho45. Cultures were grown in 5 ml basal medium with different glucose concentrations under anoxic conditions. Values are means of results obtained with duplicate cultures (less than 10% deviation).

Glucose (mM)	Products (mol per mol glucose)[a]			
	Propionate	Acetate	Succinate	Formate
2.5	1.36	0.74	0.14	0.00
5.0	1.05	0.53	0.13	0.02
7.5	0.91	0.46	0.14	0.08
10.0	0.92	0.46	0.11	0.08
12.5	0.86	0.42	0.12	0.08
15.0[b]	0.97	0.45	0.13	0.11
17.5[c]	1.00	0.47	0.12	0.12
20.0[d]	0.86	0.41	0.12	0.11

[a] Products formed on basal medium without glucose were subtracted.

[b] Only 13.4 mM glucose were consumed.

[c] Only 12.9 mM glucose were consumed.

[d] Only 14.5 mM glucose were consumed.

Comparison with *Opitutus terrae* and *Diplosphaera colitermitum*

Under anoxic conditions, *Diplosphaera colitermitum* and *Opitutus terrae* formed the same fermentation products than strain Ho45, with the typical 2:1 rate of propionate to acetate (Table 5.3). *O. terrae* additionally produced low amounts of hydrogen (2 mmol/ mol glucose). Growth and fermentation of *O. terrae* were already completely inhibited with 4% oxygen in the headspace, whereas *D. colitermitum* was able to grow at atmospheric oxygen concentrations. Similarly to strain Ho45, the electron recovery of *O. terrae* decreased with the addition of oxygen, but the effect was less pronounced. However, cell yield was not influenced by 1 or 2 % oxygen in the headspace (Table 5.3). Like strain Ho45, neither *Opitutus terrae* nor *Diplosphaera colitermitum* reduced nitrate or sulfate. In contrast to strain Ho45, *D. colitermitum* grew in nitrogen-free medium. Confirmatory the *NifH* gene was detected by PCR only for *D. colitermitum* but not for strain Ho45 and *O. terrae*.

Table 5.3 Fermentation products of *Opitutus terrae* (DSM 11246) and *Diplosphaera colitermitum* (TAV2). Data for strain Ho45 were taken from Table 5.1 for comparison. Cultures were grown in 5 ml basal medium with 4.8 mM glucose. Values are means of results obtained with duplicate cultures (less than 10% deviation).

Strain and oxygen conc. [a]	Turbidity (OD_{578})	Glucose assimilated (mM)[b]	Glucose dissimilated (mM)[c]	Products (mM)[d]			Electron recovery (%)[e]
				Propionate	Acetate	Succinate	
Opitutus terrae							
0%	0.57	1.53	3.27	4.91	3.15	0.61	130
1%	0.58	1.55	3.25	4.30	2.86	0.58	117
2%	0.58	1.55	3.25	4.36	2.98	0.53	118
Diplosphaera colitermitum							
0%	0.49	1.31	3.49	4.42	2.15	0.48	102
Strain Ho45							
0%	0.62	1.65	3.15	4.41	2.32	0.45	115

[a] Headspace concentration (v/v); initial values.

[b] Estimated using the turbidity of the culture with the experimentally determined OD-to-dry-weight ratio of strain Ho45 and an elemental composition of $C_4H_8O_2N$ (Table 5.1).

[c] Difference between the amount of glucose consumed and assimilated into biomass.

[d] Products formed on basal medium without glucose were subtracted.

[e] Electron recoveries in fermentation products, based on dissimilated glucose.

Spectral analysis

The presence of cytochromes was tested by recording difference spectra of sodium dithionite-reduced and air-oxidized preparations. The spectrum of the membrane fraction of strain Ho45 showed an absorption maximum at 439 nm. There was no shift of the absorption maximum after the reduction with sodium dithionite, which indicates the absence of redox active components like cytochromes. No peaks were detected in the soluble fraction. Analysis of ethanolic extracts of the cell pellets yielded spectra that showed three absorption maxima at 402, 420 and 442 nm, which are characteristic for carotenoids (Figure 5.3) and likely responsible for the yellow pigmentation of strain Ho45.

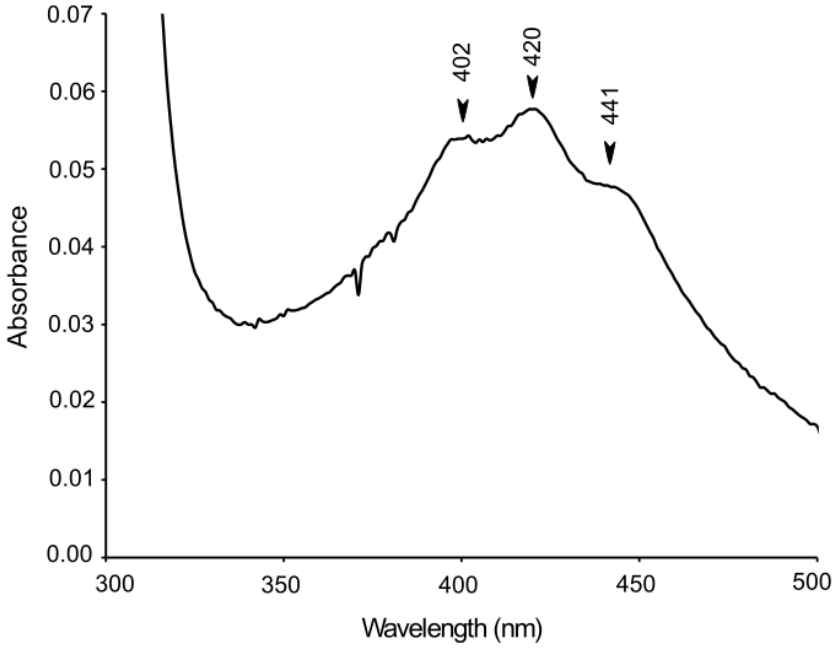

Figure 5.3. Absorption spectrum of ethanol extracts of Ho45 cells showing a three-modal absorption maximum in the range of 400 to 450 nm. Absorption maxima are indicated by arrowheads.

Phylogenetic analysis

Strain Ho45 belongs to the family *Opitutaceae* within the phylum *Verrucomicrobia*. Strain TAV1, which was isolated from the gut of the termite *Reticulitermes flavipes* (Wertz et al., 2012), was identified as the closest cultivated relative (94% sequence similarity). The closest described relatives are *Diplosphaera colitermitum* TAV2 (Wertz et al., 2012), which was also isolated from *Reticulitermes flavipes*, and *Opitutus terrae* PB90-1 (Chin et al., 2001), which

was isolated from rice field soil (91% and 92% sequence similarity, respectively). *Alterococcus agarolyticus* (Shieh and Jean, 1998) is more distantly related to strain Ho45 and has a basal position to the other cultivated representatives of the family *Opitutaceae*. Furthermore the phylogenetic tree shows that the family *Puniceicoccaceae*, which harbors isolates from marine and aquatic environments (Choo et al., 2007), is a sister group of *Opitutaceae* (Figure 5.4).

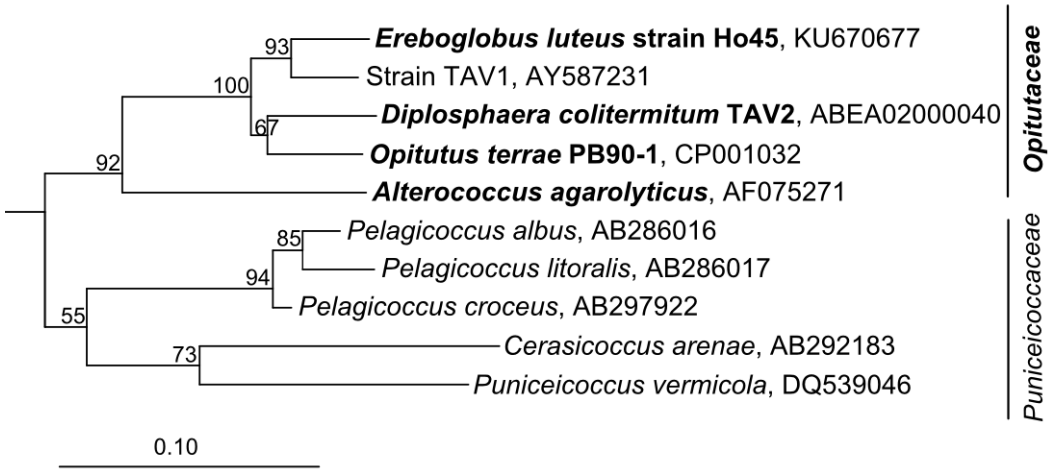

Figure 5.4. Maximum-likelihood tree showing the relationship of strain Ho45 to all other species of the family *Opitutaceae* and their closest relatives. The phylogenetic analysis is based on an unambiguous alignment (1358 sequence positions) of 16S rRNA gene sequences of the respective type strains; GenBank accession numbers are included. Numbers at nodes indicate bootstrap values, based on 1000 replications. The tree was rooted using *Lentisphaera araneosa* as outgroup.

Discussion

The results of this study clearly identify strain Ho45 as a new genus-level lineage of *Opitutaceae*. We provide a detailed ultrastructural analysis of strain Ho45 and a comparative analysis of its metabolic properties with the closest related described species *Opitutus terrae* (Chin et al., 2001) and *Diplosphaera colitermitum* (Wertz et al., 2012). Our results showed that *D. colitermitum* has a fermentative energy metabolism, which contradicts the previous description given by Wertz et al. (2012), and indicates that the description of the genus *Diplosphaera* requires revision.

Morphology and ultrastructure

The yellow color of strain Ho45 cells is a unique feature as none of the closest relatives showed colored colonies (Table 5.4). Our spectral analysis has shown that the yellow color of strain Ho45 does not originate from the presence of cytochromes but likely arises from membrane-bound carotenoids. The three-modal absorption maximum in the range of 400 to 450 nm resembles that of lutein and other xanthophylles (Stahl and Sies, 2003). Carotenoids have also been reported for *Puniceicoccus vermicola* (Choo et al., 2007) and *Cerasicoccus arenae* (Yoon et al., 2007), which belong to the family *Puniceicoccaceae*, a sister group of *Opitutaceae* (Figure 5.4). Although *D. colitermitum* and strain TAV1 possess cytochromes, the cells appear colorless (Table 5.4).

The morphology of strain Ho45 is similar to that of *Opitutus terrae* and *Diplosphaera colitermitum*, as all three strains are cocci. However, motility was only found for strain Ho45 and *O. terrae* but not for *D. colitermitum* (Table 5.4). Motility of strain Ho45 was further supported by the presence of a filamentous appendage, which resembles probably a flagellum. Our detailed ultrastructural analysis showed that the cells of strain Ho45 contain a Gram-negative cell envelope with an outer membrane and a wide periplasmic space. Although wide periplasmic spaces have been reported also for other *Verrucomicrobia* (Lindsay et al., 2001; Speth et al., 2012), we cannot fully exclude a potential artifact from the preparation due to potential osmotic stress. The granular areas at the periphery of the cytoplasm are likely ribosomes and the condensed area in the center of the cytoplasm indicates the nucleoid. A wrinkled surface structure like observed in the negatively stained specimens of strain Ho45, has also been reported for *D. colitermitum* (Wertz et al., 2012).

Growth and fermentative metabolism

Similar to *Opitutus terrae* and *Diplosphaera colitermitum*, strain Ho45 grew on a variety of carbohydrates, including hexoses, pentoses and disaccharides, which are present in the guts of insects due to the hydrolytic breakdown of polymers like starch, cellulose and hemicellulose (Chapman, 2013). Moreover, strain Ho45 and *O. terrae* degraded pectin (Table 5.4), which is a common plant polymer and also present in the guts of cockroaches (Sprenger et al., 2007). Strain Ho45 showed a typical propionic acid fermentation with a propionate to acetate ratio of 2:1, which was described for many other propionigenic bacteria (e.g. Schuppert et al., 1992; Chin et al., 2001; Boga et al., 2007). However, in contrast to other propionigenic bacteria (De Vries et al., 1972), we can exclude propionic acid fermentation via the acrylate pathway

(Prabhu et al., 2012), as lactate was utilized neither by strain Ho45 nor by *O. terrae* (Table 5.4) and further suggest a lack of the enzyme lactate dehydrogenase, which catalyzes the reversible reaction from lactate to pyruvate (Allen et al., 1964).

The high electron recovery of strain Ho45 under anoxic conditions may result from additional products derived from yeast extract and Casamino acids. Although products on basal medium (0.1% Casamino acids and yeast extract) were only formed in minor amounts and subtracted from the analysis, we cannot exclude a possible utilization of these compounds only or more effectively when glucose is present.

O. terrae showed a similar fermentation pattern than strain Ho45. However, we did not find a production of ethanol but confirmed the production of low amounts of hydrogen like already described by Chin et al. (2001), which distinguishes *O. terrae* from strain Ho45 and *D. colitermitum* (Table 5.4).

Although Wertz et al. (2012) described *D. colitermitum* as microaerophilic and non-fermentative and reported that it was not able to grow under anoxic conditions, our results showed that the strain grows fermentatively under complete anoxia. Like strain Ho45, *D. colitermitum* fermented glucose completely to propionate (+ succinate) and acetate in the typical 2:1 ratio. These new findings necessitate an emended description of the genus *Diplosphaera.*

Table 5.4 Comparison of phenotypic properties of strain Ho45 with the closest relatives from the family *Opitutaceae*.

Property	**Strain Ho45**	***Opitutus terrae***[a]	***Diplosphaera colitermitum***[b]	**TAV1**[b]
Cell morphology	Cocci	Cocci	Diplococci	Diplococci
Color	yellow	colorless	colorless	colorless
G+C content (mol%)	59.7[c]	73.7	60.5	63.2[d]
Gram stain	–	–	–	–
Endospores	–	–	–	–
Motility	+	+	–	–
Catalase	–	–	–	–
Oxidase	–	–	–	–
Cytochromes	–	ND	+	+
pH optimum	6.0–9.0	5.5–9.0	5.5–7.5	ND
Growth temp. (C°)	15–37	10–37	15–35	ND
Growth under air	–	–	+	–
Anaerobic growth	+	+	+[e]	+
Respiration (aerobic)	–	–	+	+
Fermentation products[f]	P, A, S	P, A, E	P, A, S[e]	ND
H_2 formation	–	+	–[e]	ND
Substrate utilization				
D-Glucose	+	+	+	+
D-Galactose	+	+	+	+
D-Maltose	+	+	+	+
D-Cellobiose	+	+	+	+
D-Xylose	–	–	–	+
D-Fructose	+	+	–	ND
D-Ribose	+	–	–	ND
D-Melibiose	+	+	ND	ND
D-Trehalose	+	ND	–	ND
Starch	–	+	+	+
Pectin	+	+	ND	ND
L-Lactate	–	–	ND	ND
L-Malate	–	–	ND	ND

ND, not determined.

[a] Chin et al., 2001.

[b] Wertz et al., 2012.

[c] Based on draft genome (unpublished results).

[d] Isanapong et al., 2012.

[e] This study.

[f] P, propionate; A, acetate; E, ethanol; S, succinate; ranked by decreasing abundance.

Relationship to oxygen

Although all three strains showed a very similar fermentative metabolism, they showed differences in their relationship to oxygen. *Diplosphaera colitermitum* grows respiratory under microaerophilic or atmospheric oxygen concentrations (Wertz et al., 2012), whereas *Opitutus terrae* has been described as obligately anaerobic (Chin et al., 2001). Like *Opitutus terrae*, strain Ho45 is an anaerobe with a slight oxygen tolerance and a purely fermentative metabolism.

With increasing oxygen concentration in the headspace strain Ho45 showed a strong decrease in fermentation products, which is likely a result from the inhibitory effects of oxygen on the fermentation process. Such an effect was also documented for other propionigenic bacteria and was accompanied with pyruvate accumulation (De Vries et al., 1972; Pan and Imlay et al., 2001). However, strain Ho45 did not accumulate pyruvate. Since the amount of dissimilated glucose was not affected by up to 4% oxygen, but fewer products were found, electron and carbon recovery decreased with increasing oxygen concentrations. A decrease in electron recovery similar to strain Ho45 was also found with *Sporotalea propionica* (a propionigenic bacterium isolated from the termite gut), and indicated that oxygen served as electron acceptor (Boga et al., 2007). However, we can exclude respiratory activity of strain Ho45, as the lag-phase increased and cell yield strongly decreased already with low oxygen concentrations in the headspace. Also an oxidation of acetate to CO_2 is unlikely, as no growth occurred on acetate under oxic or microoxic conditions.

Nevertheless, the reduced status of the redox indicator at the end of the incubation under microoxic conditions indicated that strain Ho45 is able to reduce small amounts of oxygen. The removal of oxygen by non-respiratory activities is a common phenomenon in anaerobes (e.g. De Vries et al., 1978; Chen et al., 1993; Karnholz et al., 2002; Riebe et al., 2009) and even many strict anaerobes possess enzymes that detoxify oxygen or oxygen radicals (Jenney et al., 1999; Brioukhanov and Netrusov, 2007).

In contrast to *Sporotalea propionica* (Boga et. al. 2007), fermentation of strain Ho45 did not shift towards acetate, when grown with low oxygen concentrations in the headspace. The propionate to acetate ratio remained 2:1 at 2 % oxygen and even changed to 2:0.5 at 4 % oxygen. The accumulation of malate at 4% oxygen likely results from a decreased turnover to fumarate due to an inhibition of the fumarase by oxygen, like observed for the propionigenic bacterium *Bacteroides thetaiotaomicron* (Pan and Imlay et al., 2001). Likewise, in *E. coli* the

fumarase showed highest activity at anoxic conditions (Park and Gunsalus, 1995; Tseng, 1997; Tseng et al., 2001).

Opitutus terrae, which was described as obligately anaerobic (Chin et al., 2001), also tolerated low amounts of oxygen in the medium. Since cell yield of *O. terrae* (in contrast to strain Ho45) was not influenced by the addition of 1 or 2% oxygen, but growth was completely inhibited by higher oxygen concentrations, we can likely exclude respiratory activity for the closest described relative of strain Ho45.

The obligately fermentative metabolism of strain Ho45 and its status as anaerobe was further confirmed, since no evidence for the existence of cytochromes or catalase was found. Nonetheless, the close relative *D. colitermitum* possesses a cytochrome cbb_3 oxidase (Wertz et al., 2012), which indicates a capability of respiration under oxic or microoxic conditions. Moreover, the ability of nitrogen fixation, which was found only for *D. colitermitum* (Wertz et al., 2012; this study) but not for strain Ho45, requires the expression of a cytochrome cbb_3 oxidase. The cbb_3 oxidase causes a low oxygen partial pressure and ensures that the oxygen sensitive nitrogenase is not compromised, when nitrogen-fixing bacteria are grown under microoxic conditions (Preisig et al., 1993). Since *D. colitermitum* grows also fermentatively under anoxic conditions (this study), we propose to describe the strain as facultatively anaerobic.

Ecological relevance

The isolation of strain Ho45 from a highly diluted gut homogenate suggests that it is a numerically abundant member of the gut microbiota of *S. lateralis*. Due to the ability to reduce oxygen, strain Ho45 may contribute to a reduced environment in the gut and encourage the growth of strict anaerobes, which require a negative redox potential for growth. The production of acetate by strain Ho45 and *D. colitermitum* is an important feature, as acetate is the most important energy source for the host (Breznak and Kane, 1990).

Since nitrogen fixation was shown for *Diplosphaera colitermitum* (Wertz et al., 2012; this study), this strain may play an important role in nitrogen fixation in termites, which is one of the most crucial aspects of symbiosis, as the diet of wood-feeding termites is low in nitrogen (Benemann, 1973; Breznak et al., 1973; Yamada et al., 2007). Our findings that strain Ho45 is not able to fix nitrogen, matches the assumption that, contrary to the termite gut, nitrogen fixation is not an essential process in the cockroach gut (Cruden and Markovetz, 1987). Cockroaches do not depend on nitrogen fixation, as they are mostly omnivorous and also

contain an intracellular *Blattabacterium* sp. in their fatbody, which plays an essential role in nitrogen recycling, allowing them to subsist also on nitrogen-poor diets (Sabree et al., 2009).

Taxonomy

The phenotypic differences and the phylogenetic distance of strain Ho45 to other species in the family *Opitutaceae* strongly suggest that the classification of the new isolate requires the creation of a new taxon at the genus level. The classification of strain Ho45 as a new genus of *Opitutaceae* is further supported by the low sequence similarity (92%) of the 16S rRNA gene with the closest related described species of this family, which lies below the taxonomic threshold at genus level (Yarza et al., 2014). Furthermore, our new findings about the metabolism of *Diplosphaera colitermitum*, call for an emended description of the type species.

Emended description of *Diplosphaera*

The genus description is, at present, the same as for the type species, *Diplosphaera colitermitum* given by Wertz et al. (2012) with the following additions and corrections.

Facultatively anaerobic. Grows best under anoxic and microoxic conditions. Metabolism is respiratory and fermentative. Major end products on 5 mM glucose are propionate, acetate and succinate.

Description of *Ereboglobus* gen. nov.

Ereboglobus (E're.bo.glo.bus. G. masc. nom. Erebos, the darkness; L. masc. nom. globus, the globe. N.L. masc. nom. Ereboglobus, globular bacterium from the darkness).

Gram-negative staining, motile, non-spore forming, cocci. Obligately anaerobic, slightly aerotolerant, catalase and oxidase negative. Heterotrophic, purely fermentative metabolism. Major end products on 5 mM glucose are propionate, acetate and succinate.

Type species: *Ereboglobus luteus* gen. nov., sp. nov.

Description of *Ereboglobus luteus* sp. nov.

Ereboglobus luteus (lu'te.us. L. masc. adj. luteus, yellow; *Ereboglobus luteus*, yellow, globular bacterium from the darkness).

Cells are coccus-shaped, with a diameter of 0.5–0.6 μm. Growth on D-fructose, D-galactose, D-sucrose, D-maltose, D-cellobiose, D-trehalose, D-ribose, D-melibiose, galacturonic acid and pectin. Growth occurs best in the absence of oxygen and is completely inhibited with an initial

concentration of 8% oxygen in the headspace. Metabolism is purely fermentative. Major end products on 5 mM glucose are propionate, acetate and succinate. Temperature range of growth 15–37 °C. pH range of growth 6–9 (optimum pH7). The DNA G+C content is 59.7 mol%.

Habitat: So far isolated only from the intestinal tract of the cockroach *Shelfordella lateralis*. Type strain: Ho45^T DSM (102184^T) and JCM (currently awaiting accession number).

GenBank accession number: KU670677.

References

1. **Allen SH, Kellermeyer RW, Stjernholm RL, Wood HG.** 1964. Purification and properties of enzymes involved in the propionic acid fermentation. J Bacteriol **87:**171 187.
2. **Benemann JR.** 1973. Nitrogen fixation in termites. Science **181:**164–165.
3. **Bergmann GT, Bates ST, Eilers KG, Lauber CL, Caporaso JG, Walters WA, Knight R, Fierer N.** 2011. The under-recognized dominance of *Verrucomicrobia* in soil bacterial communities. Soil Biol Biochem **43:**1450–1455.
4. **Boga HI, Ji R, Ludwig W, Brune A.** 2007. *Sporotalea propionica* gen. nov. sp. nov., a hydrogen-oxidizing, oxygen-reducing, propionigenic firmicute from the intestinal tract of a soil-feeding termite. Arch Microbiol **187:**15–27.
5. **Breznak JA, Kane MD.** 1990. Microbial H_2/CO_2 acetogenesis in animal guts: nature and nutritional significance. FEMS Microbiol. Rev. **87:**309–314.
6. **Breznak JA, Brill WJ, Mertins JW, Coppel HC.** 1973. Nitrogen fixation in termites. Nature **244:**577–580.
7. **Brioukhanov AL, Netrusov AI.** 2007. Aerotolerance of strictly anaerobic microorganisms and factors of defense against oxidative stress: a review. Appl Biochem Microbiol **43:**567–582.
8. **Chapman RF.** 2013. The alimentary canal, digestion and absorption, p. 46–79. *In* Simpson SJ, Douglas AE (ed), The Insects: Structure and Function. 5th ed, Cambridge Univ. Press, Cambridge, UK.
9. **Chen L, Liu MY, Legall J, Fareleira P, Santos H, Xavier AV.** 1993. Purification and characterization of an NADH-rubredoxin oxidoreductase involved in the utilization of oxygen by *Desulfovibrio gigas*. Eur J Biochem **216:**443–448.

10. **Chin KJ, Liesack W, Janssen PH.** 2001. *Opitutus terrae* gen. nov., sp. nov., to accommodate novel strains of the division "Verrucomicrobia" isolated from rice paddy soil. Int J Syst Evol Microbiol **51:**1965–1968.
11. **Choo Y-J, Lee K, Song J, Cho J-C.** 2007. *Puniceicoccus vermicola* gen. nov., sp. nov., a novel marine bacterium, and description of *Puniceicoccaceae* fam. nov., *Puniceicoccales* ord. nov., *Opitutaceae* fam. nov., *Opitutales* ord. nov. and *Opitutae* classis nov. in the phylum "*Verrucomicrobia*." Int J Syst Evol Microbiol **57:**532–537.
12. **Cruden DL, Markovetz AJ.** 1987. Microbial ecology of the cockroach gut. Annu Rev Microbiol **41:**617–643.
13. **De Vries W, van Wijck-Kapteijn WMC, Stouthamer AH.** 1972. Influence of oxygen on growth, cytochrome synthesis and fermentation pattern in propionic acid bacteria. J Gen Microbiol **71:**515–524.
14. **De Vries W, Donkers C, Boellaard M, Stouthamer AH.** 1978. Oxygen metabolism by the anaerobic bacterium *Veillonella alcalescens.* Arch Microbiol **119:**167–174.
15. **Derrien M, Vaughan EE, Plugge CM, De Vos WM.** 2004. *Akkermansia muciniphila* gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. Int J Syst Evol Microbiol **54:**1469–1476.
16. **Dubourg G, Lagier JC, Armougom F, Robert C, Audoly G, Papazian L, Raoult D.** 2013. High-level colonisation of the human gut by Verrucomicrobia following broad-spectrum antibiotic treatment. Int J Antimicrob Agents **41:**149–155.
17. **Dunfield PF, Yuryev A, Senin P, Smirnova AV, Stott MB, Hou S, Ly B, Saw JH, Zhou Z, Ren Y, et al..** 2007. Methane oxidation by an extremely acidophilic bacterium of the phylum *Verrucomicrobia*. Nature **450:**879–882.
18. **Gregersen T.** 1978. Rapid method for distinction of Gramnegative from Gram positive bacteria. Eur J Appl Microbiol Biotechnol **5:**123–127.
19. **Hackl E, Zechmeister-Boltenstern S, Bodrossy L, Sessitsch A.** 2004. Comparison of diversities and compositions of bacterial populations inhabiting natural forest soils. Appl Environ Microbiol **70:**5057–5065.
20. **Hedlund BP, Gosink JJ, Staley JT.** 1997. *Verrucomicrobia* div. nov., a new division of the bacteria containing three new species of *Prosthecobacter*. Antonie Van Leeuwenhoek **72:**29–38.

21. **Hongoh Y, Ohkuma M, Kudo T.** 2003. Molecular analysis of bacterial microbiota in the gut of the termite *Reticulitermes speratus* (Isoptera; Rhinotermitidae). FEMS Microbiol Ecol **44:**231–242.
22. **Hou S, Makarova KS, Saw JH, Senin P, Ly BV, Zhou Z, Ren Y, Wang J, Galperin MY, Omelchenko MV, et al.** 2008. Complete genome sequence of the extremely acidophilic methanotroph isolate V4, *Methylacidiphilum infernorum*, a representative of the bacterial phylum *Verrucomicrobia*. Biol Direct **3:**26.
23. **Hugenholtz P, Goebel BM, Pace NR.** 1998. Impact of culture independent studies on the emerging phylogenetic view of bacterial diversity. J Bacteriol **180:**4765–4774.
24. **Isanapong J, Goodwin L, Bruce D, Chen A, Detter C, Han J, Han CS, Held B, Huntemann M, Ivanova N, et al.** 2012. High-quality draft genome sequence of the *Opitutaceae* bacterium strain TAV1, a symbiont of the wood-feeding termite *Reticulitermes flavipes*. J Bacteriol **194:**2744–2745.
25. **Isanapong J, Sealy Hambright W, Willis AG, Boonmee A, Callister SJ, Burnum KE, Paša-Tolić L, Nicora CD, Wertz JT, Schmidt TM, Rodrigues JLM.** 2013. Development of an ecophysiological model for *Diplosphaera colotermitum* TAV2, a termite hindgut Verrucomicrobium. ISME J **7:**1803–1813.
26. **Islam T, Jensen S, Reigstad LJ, Larsen O, Birkeland NK.** 2008. Methane oxidation at 55 degrees C and pH 2 by a thermoacidophilic bacterium belonging to the *Verrucomicrobia* phylum. Proc Natl Acad Sci USA **105:**300–304.
27. **Jenney FE Jr., Verhagen MFJM, Cui X, Adams MWW.** 1999. Anaerobic microbes: oxygen detoxification without superoxide dismutase. Science **286:**306–309.
28. **Karnholz A, Küsel K, Gössner A, Schramm A, Drake HL.** 2002. Tolerance and metabolic response of acetogenic bacteria toward oxygen. Appl Environ Microbiol **68:**1005–1009.
29. **Köhler T, Dietrich C, Scheffrahn RH, Brune A.** 2012. High-resolution analysis of gut environment and bacterial microbiota reveals functional compartmentation of the gut in wood-feeding higher termites *Nasutitermes* spp. Appl Environ Microbiol **78:**4691–4701.
30. **Lindsay MR, Webb RI, Strous M, Jetten MS, Butler MK, Forde RJ, Fuerst JA.** 2001. Cell compartmentalisation in planctomycetes: novel types of structural organisation for the bacterial cell. Arch Microbiol **175:**413–429.

31. **Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar, Buchner A, Lai T, Steppi S, Jobb G, et al.** 2004. ARB: a software environment for sequence data. Nucleic Acids Res **32:**1363–1371.
32. **Mayberry WR, Prochazka GJ, Payne WJ.** 1968. Factors derived from studies of aerobic growth in minimal media. J Bacteriol **96:**1424–1426.
33. **Mikaelyan A, Köhler T, Lampert N, Rohland J, Boga H, Meuser K, Brune A.** 2015. Classifying the bacterial gut microbiota of termites and cockroaches: a curated phylogenetic reference database (DictDb). Syst Appl Microbiol **38:**472–482.
34. **Murray RGE, Doetsch RN, Fobinow CF.** 1994. Determinative and cytological light microscopy, p 21–41. *In* Gerhardt P, Murray RGE, Wood WA, Krieg NR (ed), Methods for General and Molecular Bacteriology. American Society for Microbiology, Washington, DC.
35. **Ohkuma M, Noda S, Usami R, Horikoshi K, Kudo T.** 1996. Diversity of nitrogen fixation genes in the symbiotic intestinal microflora of the termite *Reticulitermes speratus*. Appl Environ Microbiol **62:**2747–2752.
36. **Pan N, Imlay JA.** 2001. How does oxygen inhibit central metabolism in the obligate anaerobe *Bacteroides thetaiotaomicron*? Mol Microbiol **39:**1562–71.
37. **Park SJ, Gunsalus RP.** 1995. Oxygen, iron, carbon, and superoxide control of the fumarase *fumA* and *fumC* genes of *Escherichia coli*: role of the arcA, fnr, and soxR gene products. J Bacteriol **177:**6255–6262.
38. **Prabhu R, Altman E, Eiteman MA.** 2012. Lactate and acrylate metabolism by *Megasphaera elsdenii* under batch and steadystate conditions. Appl Environ Microbiol **78:**8564–8570.
39. **Preisig O, Anthamatten D, Hennecke H.** 1993. Genes for a microaerobically induced oxidase complex in *Bradyrhizobium japonicum* are essential for a nitrogen-fixing endosymbiosis. Proc Natl Acad Sci USA **90:**3309–3313.
40. **Peschke M, Moog D, Klingl A, Maier UG, Hempel F.** 2013. Evidence for glycoprotein transport into complex plastids. Proc Natl Acad Sci USA **110:**10860–10865.
41. **Pfennig N, Trüper HG.** 1981. Isolation of members of the families *Chromatiaceae* and *Chlorobiaceae*, p 279–289. *In* Starr MP, Stolp H, Trüper HG, Balows A, Schlegel HG (ed), The Prokaryotes, Springer, Berlin.
42. **Riebe O, Fischer RJ, Wampler DA, Kurtz DM Jr, Bahl H.** 2009. Pathway for H_2O_2 and O_2 detoxification in *Clostridium acetobutylicum*. Microbiology **155:** 16–24.

43. **Sabree ZL, Kambhampati S, Moran NA.** 2009. Nitrogen recycling and nutritional provisioning by *Blattabacterium*, the cockroach endosymbiont. Proc Natl Acad Sci USA **106:**19521–19526.
44. **Sangwan P, Kovac S, Davis KER, Sait M, Janssen PH.** 2005. Detection and cultivation of soil Verrucomicrobia. Appl Environ Microbiol **71:**8402–8410.
45. **Schauer C, Thompson C, Brune A.** 2014. Pyrotag sequencing of the gut microbiota of the cockroach *Shelfordella lateralis* reveals a highly dynamic core but only limited effects of diet on community structure. PLoS One **9:**e85861. doi:10.1371/journal.pone.0085861.
46. **Schauer C, Thompson CL, Brune A.** 2012. The bacterial community in the gut of the cockroach *Shelfordella lateralis* reflects the close evolutionary relatedness of cockroaches and termites. Appl Environ Microbiol **78:**2758–2767.
47. **Schuler S, Conrad R.** 1990. Soils contain two different activities for oxidation of hydrogen. FEMS Microbiol EcoI **73:**77–84.
48. **Schuppert B, Schink B, Trösch W.** 1992. Batch and continuous production of propionic acid from whey permeate by *Propionibacterium acidi-propionici* in a three-electrode amperometric culture system. Appl Microbiol Biotechnol **7:**549–553.
49. **Shieh WY, Jean WD.** 1998. *Alterococcus agarolyticus*, gen. nov., sp. nov., a halophilic thermophilic bacterium capable of agar degradation. Can J Microbiol **44:**637–645.
50. **Speth DR, van Teeseling MCF, Jetten MSM.** 2012. Genomic analysis indicates the presence of an asymmetric bilayer outer membrane in *Planctomycetes* and *Verrucomicrobia*. Front Microbiol **3:**304.
51. **Sprenger WW, Hackstein JHP, Keltjens JT.** 2007. The competitive success of *Methanomicrococcus blatticola*, a dominant methylotrophic methanogen in the cockroach hindgut, is supported by high substrate affinities and favorable thermodynamics. FEMS Microbiol Ecol **60:**266–275.
52. **Stahl W, Sies H.** 2003. Antioxidant activity of carotenoids. Mol Aspects Med **24:**345–351.
53. **Stevenson BS, Eichorst SA, Wertz JT, Schmidt TM, Breznak JA.** 2004. New strategies for cultivation and detection of previously uncultured microbes. Appl Environ Microbiol **70:**4748–4755.
54. **Strassert JF, Desai MS, Radek R, Brune A.** 2010. Identification and localization of the multiple bacterial symbionts of the termite gut flagellate *Joenia annectens.* Microbiology **156:**2068–2079.

55. **Tegtmeier D, Riese C, Geissinger O, Radek R, Brune A.** 2016. *Breznakia blatticola* gen. nov. sp. nov. and *Breznakia pachnodae* sp. nov., two fermenting bacteria isolated from insect guts, and emended description of the family *Erysipelotrichaceae*. Syst Appl Microbiol (in press).

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

57. **Tseng CP.** 1997. Regulation of fumarase (*fumB*) gene expression in *Escherichia coli* in response to oxygen, iron and heme availability: role of the arcA, fur, and hemA gene products. FEMS Microbiol Lett **157:**67–72.

58. **Tseng C-P, Yu C-C, Lin H-H, Chang C-Y, Kuo J-T.** 2001. Oxygen- and growth rate dependent regulation of *Escherichia coli* fumarase (FumA, FumB, and FumC) activity. J Bacteriol **183:**461–467.

59. **Wagner M, Horn M.** 2006. The *Planctomycetes, Verrucomicrobia, Chlamydiae* and sister phyla comprise a superphylum with biotechnological and medical relevance. Curr Opin Biotechnol **17:**241–249.

60. **Wertz JT, Kim E, Breznak JA, Schmidt TM, Rodrigues JLM.** 2012. Genomic and physiological characterization of the *Verrucomicrobia* isolate *Diplosphaera colitermitum* gen. nov., sp. nov., reveals microaerophily and nitrogen fixation genes. Appl Environ Microbiol **78:**1544–1555.

61. **Yamada A, Inoue T, Noda S, Hongoh Y, Ohkuma M.** 2007. Evolutionary trend of phylogenetic diversity of nitrogen fixation genes in the gut community of wood-feeding termites. Mol Ecol **16:**3768–3777.

62. **Yarza P, Richter M, Peplies JR, Euzéby J, Amann R, Schleifer KH, Ludwig W, Glöckner FO, Rosselló-Móra R.** 2008. The All-Species Living Tree project: A 16S rRNA-based phylogenetic tree of all sequenced type strains. Syst Appl Microbiol **31:**241–250.

63. **Yarza P, Yilmaz P, Pruesse E, Glöckner FO, Ludwig W, Schleifer KH, Whitman WB, Euzéby J, Amann R, Rosselló-Móra R.** 2014. Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences. Nat Rev Microbiol **12:**635–645.

64. **Yoon J, Matsuo Y, Matsuda S, Adachi K, Kasai H, Yokota A.** 2007. *Cerasicoccus arenae* gen. nov., sp. nov., a carotenoid-producing marine representative of the family *Puniceicoccaceae* within the phylum '*Verrucomicrobia*', isolated from marine sand. Int J Syst Evol Microbiol **57:**2067–2072.

Chapter 6

General Discussion

This work contains a detailed characterization of the gut microbiota and metabolic processes in different developmental stages of the cockroach *Shelfordella lateralis*, which gives first insights into primary colonization and succession of the insect gut microbiota. I also studied the primary colonization of germ-free cockroach nymphs by facultatively and obligately anaerobic bacterial strains and analyzed their interactions within this synthetic community. Moreover, this study provides a comprehensive metabolic analysis of the facultatively and obligately anaerobic bacteria in gnotobiotic cockroaches (*in situ*) and in pure culture (*in vitro*). Additionally, novel bacterial isolates that represent new genera were obtained from insect guts and provided deeper insights into the metabolism of gut bacteria and their relationship to oxygen.

The following section presents a summary and detailed discussion of the major findings obtained in this thesis, including the elucidation of mechanisms and biotic and abiotic factors that govern the assembly of microbial communities and metabolic networks in insect guts. The relevance of gnotobiotic model systems in microbial ecology and possible future applications are discussed.

Bacterial community succession is linked to changes in gut environment

In the course of the life cycle of *Shelfordella lateralis*, the gut microbiota underwent successive changes, which were most pronounced in the early development of the cockroach. Especially the microbial community structure and metabolic processes in the guts of first instar nymphs were distinct from that of all other instars and adults and showed also strongest variations. Cockroaches of the first instar typically showed a strong dominance of groups that comprise facultatively anaerobic and oxygen-tolerant anaerobic bacteria, e.g. *Pseudomonadaceae*, *Enterobacteriaceae*, *Enterococcaceae* and *Lactobacillaceae*. The latter three groups are known to produce lactate (Schleifer, 2009; Lory, 2014; Tegtmeier et al., 2016, chapter 3), and explain the high lactate concentrations only found in the guts of first instar cockroaches. By contrast, cockroaches from second instar until adulthood shared a more similar microbial

community structure, consisting of abundant groups like *Porphyromonadaceae, Ruminococcaceae*, *Lachnospiraceae* and *Rikenellaceae*. These groups comprise mostly obligately anaerobic bacteria, and are typically found in the anoxic guts of adult cockroaches (Schauer et al., 2012; Schauer et al.; 2014, Bauer et al., 2015; Mikaelyan et al., 2016).

I found substantial variations in community composition even among pools of the first instar, which suggests that following hatching, the germ-free gut is colonized at random with microbes available in the environment, including the food source, the surface of the ootheca and feces of other colony members (Figure 6.1). Since cockroaches reared in isolation showed a similar bacterial gut microbiota than conventional ones, the most likely explanation is that the majority of the bacteria is acquired from the environment following stochastic events and not by vertical inheritance. Even pools of first instar guts were often dominated by one or two bacterial groups, whereas the guts of the other instars and adults showed a more even distribution of the taxa. Therefore, I propose that those groups that are picked up from the environment by the freshly hatched nymphs and are favored by the conditions in the gut, colonize the gut initially in high density. This is possible, because microorganisms that compete for space and nutrients are not yet established, and would imply that initial colonization follows proverbially the rule “first come first served”.

Oxygen is an important environmental factor in guts of the first instar and presumably favors a rapid and dense colonization by fast growing aerobic or oxygen-tolerant taxa. The early colonizers disappear from the gut already in the second instar, likely because limiting oxygen supply leads to an increase in anoxic microhabitats, which creates favorable conditions for colonization by anaerobic microorganisms. Despite the obvious effect of oxygen, also other environmental factors like gut structure, pH, redox potential, nutrient availability, digestive enzymes and the immune system of the insect may also play a role in shaping the community structure (Dillon and Dillon, 2004; Engel and Moran, 2013) (Figure 6.1). However, an effect of dietary shifts on the gut microbiota of *S. lateralis* was not clearly observable due to a high variability among individuals (Schauer et al., 2014).

Also, the shedding of the exoskeletal lining of crop and hindgut during the molt is a serious event, which results in a disruption of the gut microbiota (Engel and Moran 2013, see Figure 6.1) and likely explains variations and dynamics in the gut microbiota among pools of cockroaches from the second instar to adulthood. The reinoculation with microorganisms from a possibly large reservoir after each molt introduces another stochastic element in community

assembly and may further explain strong variations in the gut microbiota among individual adult cockroaches found by Schauer et al. (2014).

In conclusion, oxygen is an important driver of community composition, which explains the strong changes in the gut microbiota from first to second instar. Furthermore, stochastic events and the selection of microorganisms from the environment that are favored by the conditions in the gut or are functionally adapted to the gut environment play an important role in community assembly.

Acquisition and succession of methanogens

The acquisition of methanogens required contact to other cockroaches that harbor methanogens in their intestinal tracts. The ingestion of methanogens occurs most likely via coprophagy and starts already in the early nymphal development, but a methanogenic community needs up to fourteen days (until the second instar) to develop to such density that methane emission is detectable. The presence of oxygen in the guts of first instar cockroaches likely does not allow a proper colonization by strictly anaerobic methanogens. Furthermore, methane emission was very irregular and low, when compared to adults, which is probably caused by a disruption of the gut microbiota due to frequent molts of the cockroach until adulthood. The absence of ciliates in the early instars may also contribute to the low methane emission rates, as methane emission was found to be positively correlated with the amount of intestinal ciliates, which harbor endosymbiotic methanogens (Gijzen et al., 1991).

By contrast, hydrogen producing microbial communities were already established in the first instar. During the development of the cockroach, hydrogen emission decreased constantly, indicating lower formation rates or a consumption of hydrogen. Especially in adults methanogenic archaea substantially contributed to hydrogen consumption. However, in the younger instars hydrogen accumulated, likely because hydrogen consuming microorganisms were poorly established (Figure 6.1). The decrease of the microbial density with the age of the cockroach further indicates that the decrease in hydrogen emission results at least partially from a lower abundance of fermenting bacteria. However, also the consumption by other hydrogen consuming microorganisms cannot be excluded. However, homoacetogenesis is only a minor metabolic process in adults of the closely related cockroach *Periplaneta americana*, as most of the CO_2 and hydrogen was incorporated in methane rather than acetate (Breznak and Switzer, 1986; Kane and Breznak, 1991). In order to get further insights on the

fate of hydrogen, investigations into the homoacetogenic activity and the methanogenic community composition in the guts of the different nymphal stages of *S. lateralis* are required.

Taken together, methanogens are acquired via coprophagy. Oxygen in the guts of first instar nymphs inhibits the colonization by methanogens and they can establish properly only in adults, where they contribute significantly to hydrogen consumption.

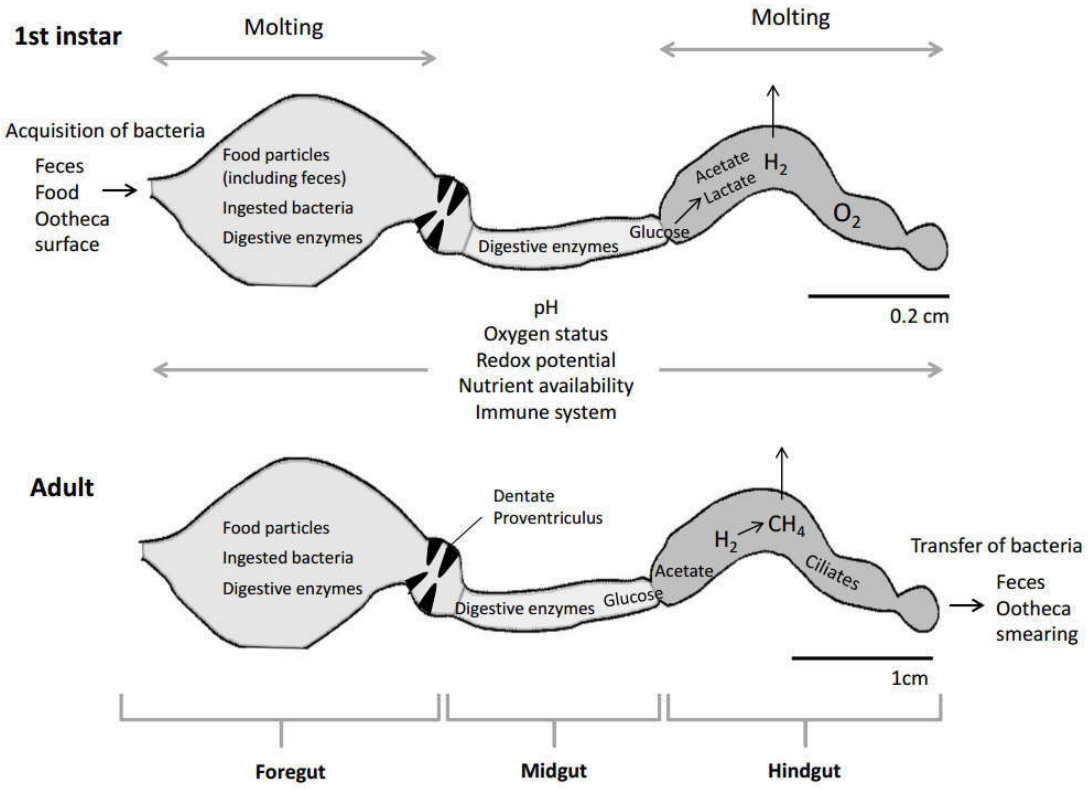

Figure 6.1. Hypothetical scheme of biotic and abiotic factors shaping microbial community assembly in the guts of first instar nymphs and adult *Shelfordella lateralis* based on the scheme by Engel and Moran (2013), including the findings of the present study. Major gut metabolites are indicated in the respective gut section. Gray shades indicate the proportional bacterial density in the respective gut compartments (bacterial density ranked by saturation of gray shades). Proportions of the bacterial density in each gut section in adults are from Schauer et al. (2012) and assumed to be similar in first instar nymphs.

Colonization of germ-free *Shelfordella lateralis* by facultatively and obligately anaerobic model strains

In order to study the primary colonization of the gut and the metabolism and interactions of selected bacteria in their natural environment, we established the cockroach *Shelfordella*

lateralis as gnotobiotic model system. The autochthonous bacterial model strains EbSL and FuSL were chosen due to their differences in their relationship to oxygen (facultative anaerobe vs. obligate anaerobe) and their fermentative metabolism. Both strains were suitable for experiments in gnotobiotic cockroaches, as they colonized the hindgut in high density in monoassociation, which allowed the analysis of metabolites of the strains *in situ*.

Strains EbSL and FuSL exclusively colonized the germ-free hindgut. Also in conventional cockroaches, the hindgut showed the highest density and diversity of bacteria when compared to the other gut compartments (Cruden and Markovetz, 1984; Schauer et al., 2012), which suggests that the hindgut provides favorable conditions for microbial colonization. Since both strains were originally also isolated from the hindgut of *S. lateralis*, this compartment is likely their natural habitat to which they are best adapted. A colonization of foregut and midgut may also be suppressed by high activities of digestive enzymes in these compartments (Bignell, 1981; Terra and Ferreira, 1994; Chapman, 2013). High density populations in monoassociation were likely possible due to the preference (in the case of strain EbSL) or slight tolerance (in the case of strain FuSL) towards oxygen, which is present in the guts of germ-free cockroaches. However, population densities of strain FuSL were always lower than that of strain EbSL, possibly due to reduced cell yields of strain FuSL in oxygen containing parts of the hindgut.

Also studies about the assembly of gut communities in the mammalian gut showed that first gut colonizers are typically facultative anaerobes, which create a reduced environment favorable for the colonization of obligate anaerobes that later constitute the majority of the climax community (Favier et al., 2002; Gillilland et al., 2012). However, our studies with cockroaches diassociated with strains EbSL and FuSL, could not confirm this hypothesis. Although colonization by the facultatively anaerobic strain EbSL created an anoxic environment in the hindgut, precolonization with this strain did not enhance the colonization success of the obligately anaerobic strain (FuSL) but completely suppressed it. Therefore, I suggest that the obligate anaerobe was outcompeted by the facultative anaerobe, possibly due to better colonization success of the facultative anaerobe in the oxic zones of the gut.

However, also antagonistic effects should be considered. Since strains EbSL and FuSL colonize the same microhabitat, they might compete for space and nutrients. Ushijima and Ozaki (1988) found antagonistic effects of *E. coli* on *Staphylococcus aureus* and proposed that motile *E. coli* cells can achieve a more effective absorption of substrates and may outcompete non-motile strains. This might also explain the colonization success of strain EbSL, since the

cells are motile (data not shown) like many other *Enterobacteriaceae* (Octavia and Lan, 2014), and strain FuSL is non-motile (data not shown), like *Fusobacterium varium* from cockroach guts (Foglesong et al., 1984). However, glucose, which is an important substrate for both strains, was not a limiting factor in the hindguts of gnotobiotic cockroaches. Furthermore, digestive enzymes form the midgut and their degradation products are a potential substrate for strain FuSL and other *Fusobacterium* sp., which are known to ferment amino acids (e.g. Buckel and Barker, 1974; Resmer and White, 2011). Nevertheless, also other nutrients might be a limiting factor for growth of strain FuSL. Also a potential production of anti-microbial substances by strain EbSL might contribute to this phenomenon.

Acid production of strains EbSL and FuSL is unlikely to cause a strong inhibition of growth, since the midgut and hindgut fluid of cockroaches have buffering capacity. The buffering capacity of the gut fluid is partly attributed to ammonia and amino acids, which are part of the secretions from the Malpighian tubules (Bignell, 1981; Cruden and Markovetz, 1987). In agreement, the pH in the hindgut of conventional adult cockroaches, which showed similar fermentation products as gnotobiotic cockroaches, was almost neutral (Schauer et al., 2012).

In conclusion, both strains showed hindgut-specific colonization. Strain FuSL was outcompeted by strain EbSL, which implied that in this case precolonization by a facultative anaerobe did not favor the colonization by an obligate anaerobe. Further facultatively and obligately anaerobic isolates, which are able to colonize the germ-free cockroach gut and long term studies are necessary to answer this ecological question.

Oxygen status of the cockroach gut

While the guts of adult cockroaches are anoxic in all compartments (e.g. Ritter, 1961; Warhurst, 1964; Schauer et al., 2012), first instar *S. lateralis* nymphs showed oxygen partial pressures around 3 kPa in the posterior part of the hindgut. Conversely, oxygen partial pressures in the midgut and anterior hindgut were surprisingly low (<1 kPa), not only in conventional first instar nymphs but also in germ-free nymphs. This is an interesting finding, since no bacteria that consume oxygen are present in germ-free cockroaches. Therefore, I conclude that oxygen is consumed by respiratory activity of the gut epithelium. The midgut tissue of cockroaches possesses large columnar epithelial cells with associated mitochondria and is thicker than the hindgut tissue (cells of the midgut epithelium show a height of 100 µm and cells of the hindgut epithelium show a height of only 15-25 µm) (Bignell, 1981).

Accordingly, the midgut tissue must contribute significantly to oxygen consumption. Furthermore, I assume that oxygen consumption by the gut tissue is also responsible for the production of low amounts of lactate in the guts of germ-free nymphs, which is probably caused by a switch to anaerobic metabolism in the gut tissue due to a limiting oxygen supply.

Influx of oxygen into the gut lumen is also dependent on the surface-to-volume ratio of the gut (Brune, 1998) and penetration depth of oxygen into the gut lumen is likely dependent on the thickness of the gut tissue. The greater thickness of the midgut tissue compared to the other gut sections (Bignell, 1981), would additionally explain the lower oxygen partial pressure in the midgut lumen. In conventional cockroaches the hindgut is an enlarged fermentation chamber and shows a larger volume than the midgut (Cruden and Markovetz, 1987), which probably leads to a larger anoxic fraction of the hindgut lumen as compared to the midgut lumen. However, since germ-free cockroaches do not show the typical hindgut enlargement (data not shown), likely due to the absence of a microbiota, these cockroaches show a larger oxic fraction of the hindgut lumen than conventional cockroaches.

Ritter (1961) postulated that anaerobic conditions in the guts of conventional cockroaches are caused by the presence of glutathione, which is a strong reducing agent. Glutathione is present in the Malpighian tubules of *Periplaneta americana* (Metcalf, 1943) and may contribute, besides the consumption of oxygen by the gut tissue and the bacterial microbiota, to low oxygen partial pressures and low redox potentials in the midgut and anterior colon.

All these findings are in agreement with studies by Schauer et al. (2012), who showed that the redox potential was lowest in the midgut of adult *S. lateralis* (Figure 1.2), although the hindgut exhibits a larger volume and a higher bacterial density than the midgut.

Metabolic activities of pure cultures *in vitro* and in their activities in their natural environment

I found strong differences between fermentation products of strains EbSL and FuSL in anoxic pure culture (*in vitro*) and in association with the gut of gnotobiotic cockroaches (*in situ*), which indicated that the metabolism of both strains was strongly affected by microenvironmental factors in the gut habitat. While strains EbSL and FuSL showed typical products of a mixed-acid fermentation (formate, ethanol and acetate) or butyrate fermentation

(butyrate and acetate) in anoxic pure culture, their fermentation pattern shifted in both cases to acetate and lactate, when associated with the cockroach gut (Figure 6.2).

Oxygen is an important factor that influences fermentative processes in the termite gut. Studies by Tholen and Brune (2000) showed that hindgut fermentations shifted to more oxidized products when intact guts were incubated under oxic conditions. The influence of inflowing oxygen provides an explanation for the absence of butyrate and the strong accumulation of acetate in cockroaches colonized by strain FuSL. A shift from butyrate to acetate was confirmed *in vitro*, when cultures of strain FuSL were exposed to microoxic conditions. The strong influence of oxygen on hindgut metabolism was further supported by the absence of formate and the low concentrations of ethanol observed in cockroaches colonized with strain EbSL and the corresponding effects of oxygen *in vitro*. The decreased formate production probably results from the inhibitory effects of oxygen on pyruvate-formate lyase (Abbe et al., 1982; Yamada et al., 1985; Takahashi et al., 1987).

The strong lactate accumulation in the guts of cockroaches colonized by strain EbSL or FuSL likely results from elevated glucose levels in the guts of germ-free and gnotobiotic cockroaches (up to 36 mM). When cultivated with low glucose concentrations (5 mM), neither strain EbSL nor strain FuSL produced significant amounts of lactate, but at higher glucose concentrations (10 and 15 mM) lactate also strongly accumulated *in vitro* (especially with strain EbSL). Similar shifts towards lactate at high glucose concentrations were observed also for *Fusobacterium varium* (Resmer and White, 2011) and for chemostat-cultures of *Klebsiella aerogenes* (Teixeira de Mattos and Tempest, 1983) and several *Streptococcus* spp. (Thomas et al., 1979; Condon, 1987). Taken together, oxygen and glucose concentrations were the most important factors that influence the metabolic activities of strains EbSL and FuSL in the gut environment.

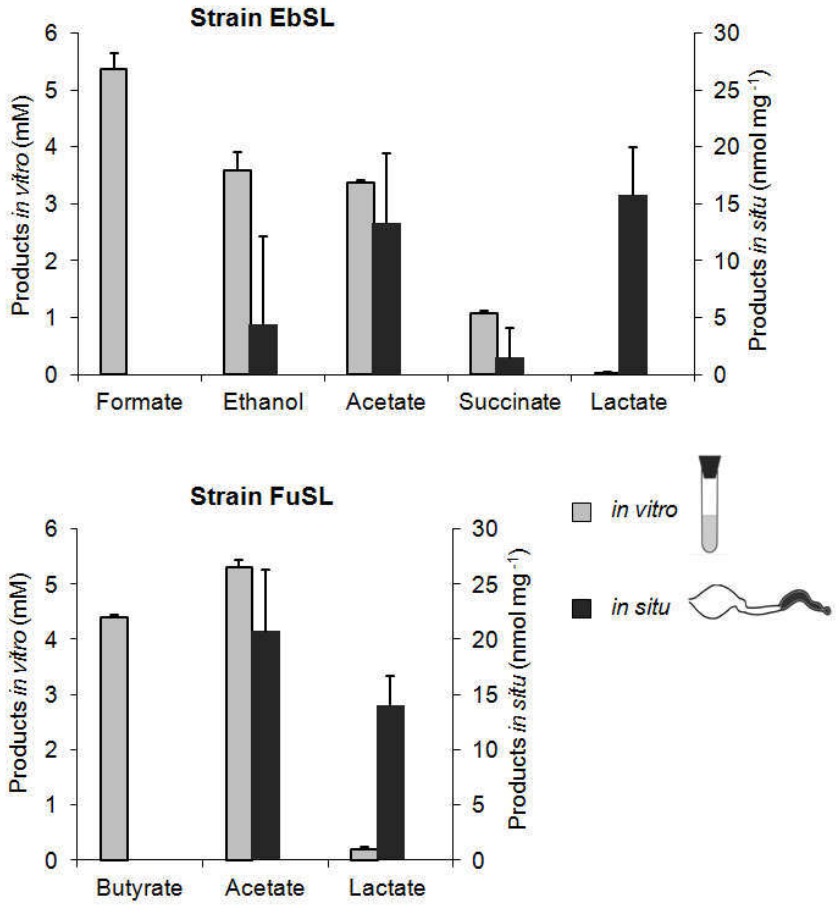

Figure 6.2. Fermentation products of strains EbSL and FuSL in anoxic pure culture with 5mM glucose (*in vitro*) compared to products found in gnotobiotic cockroach guts, colonized with strain EbSL or FuSL (*in situ*). Data are from Tegtmeier et al., 2016 (Chapter 3).

S. lateralis as gnotobiotic model to study complex metabolic networks

Gnotobiotic cockroaches with defined microbial communities constitute a powerful tool to study a variety of metabolic processes *in situ*. *S. lateralis* has proven to be a suitable model system for gnotobiotic studies with defined populations of one or two autochthonous (indigenous) model strains (Chapter 3). Gnotobiotic cockroaches that were colonized by strains EbSL or FuSL accumulated lactate and acetate in the hindgut and emitted hydrogen, which underscores a dense colonization and the prevalence of fermentative processes in the hindgut. The production of lactate provides a possibility to couple primary fermentations with secondary fermentations, e.g. propionic acid fermentation, and to study lactate turnover in insect guts. The production of hydrogen is the prerequisite to couple fermentative processes with hydrogenotrophic processes and may allow the creation of complex synthetic microbial

networks, e.g. methanogenic or homoacetogenic communities. In order to create such communities additional isolates from the gut of *S. lateralis* are required, since autochthonous strains likely have a better chance to colonize the gut than foreign ones (see Chapter 1).

The creation of synthetic methanogenic communities is a challenging task, since methanogenesis in the intestinal tract requires a more complex microbial community structure, which provides important substrates and growth factors. A colonization of the gut by *Methanimicrococcus blatticola*, which is so far the only described methanogenic isolate from the cockroach gut, is dependent on the provision of methanol and hydrogen (Sprenger et al., 2000). Furthermore, *M. blatticola* is strictly dependent on the presence of complex organic nutrients including acetate, yeast extract and even the methanogenic coenzyme M (Sprenger et al., 2005). Therefore, a colonization of the gut by *M. blatticola* probably requires the presence of another bacterium that provides the important substrates and nutrients and a second methanogen that is able to synthesize coenzyme M. A hydrogenotrophic methanogen, e.g. a *Methanobrevibacter* species, would be a more promising candidate to create such a methanogenic network, since hydrogenotrophic methanogens do not depend on the provision of methanol and usually can synthesize coenzyme M (Balch and Wolfe, 1979). Due to the oxygen sensitivity of methanogenic archaea, the creation of synthetic methanogenic communities would warrant an elaborate method that allows the inoculation of germ-free cockroaches without exposing the inoculums to oxygen. Furthermore, the conditions in the gnotobiotic gut must be anoxic, which requires a precolonization with a bacterium that is capable to create such a reduced environment, e.g. strain EbSL. The creation of synthetic methanogenic communities would be useful to identify factors limiting the colonization of the intestinal tract by methanogens. A better understanding of the ecology of methanogenic communities may contribute in future to develop strategies to reduce methanogenesis also in ruminants.

Germ-free cockroaches as model system and future applications

Subsequent studies have shown that *S. lateralis* is also a suitable model to study the assembly of complex microbial communities. Mikaelyan et al. (2016) inoculated germ-free *S. lateralis* with the gut microbiota of termites and mice and could show that community assembly in cockroach guts is deterministic. Termite-specific taxa, like *Endomicrobia* or *Spirochaetes* were unable to colonize germ-free cockroaches, whereas taxa that are abundant in

conventional cockroaches and also present in the foreign inocula (albeit in lower abundance), were selectively enriched in xenobiotic cockroaches. The exposure of xenobiotic cockroaches to conventional adults restored their normal microbiota, which indicated that autochthonous bacteria outcompete foreign strains and that the habitat of cockroaches plays an important role in the selection of bacterial lineages.

Gnotobiotic cockroaches would also provide a useful tool to study host-microbe interactions and possible benefits of the gut microbiota for the host. Dillon et al. (2005) used gnotobiotic locusts to study the impact of the gut microbiota against pathogen invasion and could show that a more diverse gut microbiota significantly protects against invasion by the pathogen *Serratia marcescens*. In the context of our studies on acquisition and succession of the gut microbiota (Chapter 2), it might be worth mentioning that following hatching the initially germ-free guts of conventional first instar *S. lateralis* nymphs were occasionally colonized by dominant populations of *Serratia* sp. Therefore, I suggest that cockroaches are more prone to infections after hatching as a stable gut microbiota is not established yet. Using gnotobiotic cockroaches, we may identify certain bacteria that protect against colonization by pathogens.

Gnotobiotic model systems could also find an application in pest control. Recent studies with germ-free *Blatella germanica* cockroaches (a common urban pest species) have shown that the gut microbiota plays an important role in mediating insect-insect communication. Gut bacteria produce fecal aggregation pheromones consisting of volatile carboxylic acids. These compounds are emitted by the feces, which attract conspecifics and mediate aggregation (Wada-Katsumata et al., 2015). Earlier studies by Dillon et al. (2002) using germ-free desert locusts have shown that the gut microbiota is involved in the production of cohesion pheromones, which mediates swarming of the insects and causes severe agricultural damage.

Germ-free models have also been used to study the influence of gut bacteria on development and immune system of insects. Such studies focused mostly on *Drosophila* (e.g. Muyskens and Guillemin, 2008; Shin et al., 2011; Newell and Douglas, 2014), since *Drosophila* research benefits from an easily manipulated genome, a fantastic resource of transgenic tools, and a large amount of well-established techniques to study innate immunity (Neyen et al., 2014). However, since the gut microbiota of *Drosophila* is low in diversity and density (Wong et al., 2011; Broderick et al., 2014), immunity research of insects with a more complex and abundant gut microbiota like cockroaches would be an interesting perspective.

Novel isolates of anaerobic bacteria from insect guts and their response to oxygen

The isolation of novel strains of so far uncultured lineages of *Erysipelotrichaceae* and *Opitutaceae* from the guts of *Shelfordella lateralis* and *Pachnoda ephippiata* considerably enhances our knowledge about the underexplored family *Opitutaceae* and the partly inaccurate described family *Erysipelotrichaceae*.

Strains ErySL and Pei061 represent a new genus of obligately anaerobic *Erysipelotrichaceae* and showed a typical mixed-acid fermentation (formate, ethanol and acetate). With the exception of the genus *Erysipelothrix*, also all other members of *Erysipelotrichaceae* have been described as anaerobic bacteria (e.g. Smith and King, 1961; Cato et al., 1974; Moore et al., 1976; Barnes et al., 1977; Kageyama and Benno, 2000; Greetham et al., 2004). Contradictory, members of the genus *Erysipelothrix* have been described as facultatively anaerobic (Jones et al., 1986; Stackebrandt, 2009; Bang et al., 2015) or microaerophilic (Verbarg et al., 2014). Based on the generic description of *Erysipelothrix* also the whole family *Erysipelotrichaceae* was described as aerobic to facultatively anaerobic with a respiratory and weakly fermentative metabolism (Verbarg et al., 2004; Stackebrandt, 2009; Verbarg et al., 2014). However, in our study *Erysipelothrix inopinata* grew fermentatively on glucose and showed the same fermentation products as strains ErySL and Pei061.

When treated with oxygen, formate production of strain Pei061 and *E. inopinata* strongly decreased, like previously also observed for strain EbSL *in vitro* and in gnotobiotic cockroaches (Tegtmeier et al., 2016, see chapter 3). Formate decline is likely caused by an inhibition of the oxygen-sensitive pyruvate-formate lyase (Abbe et al., 1982; Yamada et al., 1985; Takahashi et al., 1987). The presence of oxygen did not affect the electron recovery in the fermentation products but strongly reduced growth yields of *E. inopinata*, which indicates that the strain is neither facultatively anaerobic nor microaerophilic. This is further supported by the absence of cytochromes and isoprenoid quinones in *E. inopinata* and all other family members investigated in that respect (Collins et al., 1979; Verbarg et al., 2004; Verbarg et al., 2014). Also, a careful survey of the literature did not provide any evidence that supports the claim of a respiratory metabolism in the genus *Erysipelothrix* or any other member of the family. However, *E. inopinata* grew robustly on solid media but poorly in liquid culture at atmospheric oxygen concentrations. Therefore, I propose that *E. inopinata* is an aerotolerant

anaerobe. Based on our findings we corrected and emended the description of *E. inopinata* and the family *Erysipelotrichaceae*.

Strain Ho45 represents a new genus of *Opitutaceae*, a bacterial family that is little explored due to the lack of isolates. Strain Ho45 is also an anaerobic bacterium and showed a typical propionic acid fermentation with a propionate to acetate ratio of 2:1, which was described for many other propionigenic bacteria (e.g. Schuppert et al., 1992; Chin et al., 2001; Boga et al., 2007). It showed no respiratory activity as the lag-phase increased and cell yield strongly decreased with increasing oxygen concentrations in the headspace. Growth was completely inhibited by 8% oxygen in the headspace. By contrast, its closest relative *Diplosphaera colitermitum* was described as microaerophilic and non-fermentative, not able to grow under anoxic conditions (Wertz et al., 2012; Isanapong et al., 2013). Surprisingly, *D. colitermitum* grew not only under microoxic or oxic conditions but also under completely anoxic conditions, and clearly fermented glucose to the same products as strain Ho45. However, *D. colitermitum* possesses a cytochrome cbb_3 oxidase, which indicates the capability of respiration under oxic or microoxic conditions (Wertz et al., 2012). As *D. colitermitum* grew also fermentatively under anoxic conditions in this study, I propose to describe the strain as facultatively anaerobic.

Taken together, most of the *Erysipelotrichaceae* and *Opitutaceae*, which were often isolated from gut environments, are anaerobic with a fermentative metabolism. Oxygen strongly influenced growth yields and metabolic product profiles of many of these gut bacteria. Some of the strains showed oxygen-tolerance (to different extend), which often resulted in a misinterpretation regarding their relationship to oxygen. Consequently, some members of these families were mistakenly described as aerobic or microaerophilic in the literature, which required revision of the original descriptions.

Concluding remarks

This study has shown that oxygen is an important environmental driver of microbial community structure and strongly influences colonization success and microbial metabolism in cockroach guts. Despite a strong stochastic element in community assembly, especially in the guts of first instar cockroaches, the microbiota seems to be shaped by the selection of microorganisms from the environment that are functionally adapted to the gut environment.

Shelfordella lateralis as gnotobiotic model system offered an excellent opportunity to study the metabolism and interactions of individual bacteria in their natural environment. Oxygen

and glucose concentrations, which are the major factors that influence microbial activities in gnotobiotic cockroaches, also strongly influenced growth and metabolism of the novel anaerobic isolates that were obtained in this study from insect guts.

The gnotobiotic cockroach model will provide a valuable tool in "synthetic microbial ecology" and will likely improve our understanding of community ecology. It could be also applied for future studies with synthetic methanogenic communities or to study host-microbe interactions.

References

1. **Abbe K, Takahashi S, Yamada T.** 1982. Involvement of oxygen sensitive pyruvate formate-lyase in mixed-acid fermentation by *Streptococcus mutans* under strictly anaerobic conditions. J Bacteriol **152:**175–182.
2. **Balch WE, Wolfe RS. 1979.** Specificity and biological distribution of coenzyme M (2-mercaptoethanesulfonic acid). J Bacteriol **137:**256–263.
3. **Bang B-H, Rhee M-S, Chang D-H, Park D-S, Kim B-C.** 2015. *Erysipelothrix larvae* sp. nov., isolated from the larval gut of the rhinoceros beetle, *Trypoxylus dichotomus* (Coleoptera: Scarabaeidae). Antonie Van Leeuwenhoek **107:**443–451.
4. **Barnes EM, Impey CS, Stevens BJH, Peel JL.** 1977. *Streptococcus pleomorphus* sp. nov.: An anaerobic *Streptococcus* isolated mainly from caeca of birds. J Gen Microbiol **102:**45–53.
5. **Bauer E, Lampert N, Mikaelyan A, Köhler T, Maekawa K, Brune A.** 2015. Physicochemical conditions, metabolites, and community structure of the bacterial microbiota in the gut of wood-feeding cockroaches (Blaberidae : Panesthiinae). FEMS Microbiol Ecol **91:**1–14.
6. **Bignell DE.** 1981. Nutrition and digestion, p 57–86. *In* Bell WJ, Adiyodi KG (ed), The American cockroach, Chapman & Hall, London.
7. **Boga HI, Ji R, Ludwig W, Brune A.** 2007. *Sporotalea propionica* gen. nov. sp. nov., a hydrogen-oxidizing, oxygen-reducing, propionigenic firmicute from the intestinal tract of a soilfeeding termite. Arch Microbiol **187:**15–27.
8. **Breznak, JA, Switzer JM.** 1986. Acetate synthesis from H_2 plus CO_2 by termite gut microbes. Appl Environ Microbiol **52:**623–630.
9. **Broderick NA, Buchon N, Lemaitre B.** 2014. Microbiota-induced changes in *Drosophila melanogaster* host gene expression and gut morphology. mBio **5:**e01117–14.

10. **Brune A.** 1998. Termite guts: the world's smallest bioreactors. Trends Biotechnol **16:**16–21.
11. **Buckel W, Barker HA** 1974. Two pathways of glutamate fermentation by anaerobic bacteria. J Bacteriol **117:**1248-1260.
12. **Cato EP, Salmon CW, Holdeman IV.** 1974. *Eubacterium cylindroides* (Rocchi) Holdeman and Moore: Emended description and designation of neotype strain. Int J Syst Evol Microbiol **24:**256–259.
13. **Chapman RF.** 2013. The alimentary canal, digestion and absorption, p 46–79. *In* Simpson SJ, Douglas AE (ed), The insects: structure and function, 5th ed, Cambridge Univ. Press, Cambridge, UK.
14. **Chin KJ, Liesack W, Janssen PH.** 2001. *Opitutus terrae* gen. nov., sp. nov., to accommodate novel strains of the division "Verrucomicrobia" isolated from rice paddy soil. Int J Syst Evol Microbiol **51:**1965–1968.
15. **Collins MD, Jones D, Goodfellow M, Minnikin DE.** 1979. Isoprenoid quinone composition as a guide to the classification of *Listeria*, *Brochothrix*, *Erysipelothrix* and *Caryophanon*. J Gen Microbiol **111:**453–457.
16. **Condon S.** 1987. Responses of lactic acid bacteria to oxygen. FEMS Microbiol Rev **46:**269–280.
17. **Cruden DL, Markovetz AJ.** 1984. Microbial aspects of the cockroach hindgut. Arch Microbiol **138:**131–139.
18. **Cruden DL, Markovetz AJ.** 1987. Microbial ecology of the cockroach gut. Annu. Rev. Microbiol. **41:**617–643.
19. **Dillon RJ, Dillon VM.** 2004. The gut bacteria of insects: nonpathogenic interactions. Annu Rev Entomol **49:**71–92.
20. **Dillon RJ, Vennard CT, Charnley AK.** 2002. A note: Gut bacteria produce components of a locust cohesion pheromone. J Appl Microbiol **92:**759–63.
21. **Dillon, RJ, Vennard CT, Buckling A, Charnley AK.** 2005. Diversity of locust gut bacteria protects against pathogen invasion. Ecology Letters. **8:**1291–1298.
22. **Engel P, Moran NA.** 2013. The gut microbiota of insects – diversity in structure and function. FEMS Microbiol Rev **37:**699–735.
23. **Favier CF, Vaughan EE, De Vos WM, Akkermans ADL.** 2002. Molecular monitoring of succession of bacterial communities in human neonates. Appl Environ Microbiol **68:**119–226.

24. **Foglesong MA, Cruden DL, Markovetz AJ.** 1984. Pleomorphism of Fusobacteria isolated from the cockroach hindgut. J Bacteriol **158:**474–480.
25. **Gijzen HJ, Broers CA, Barugahare M, Stumm CK.** 1991. Methanogenic bacteria as endosymbionts of the ciliate *Nyctotherus ovalis* in the cockroach hindgut. Appl Environ Microbiol **57:**1630–1634.
26. **Gillilland MG, Erb-Downward JR, Bassis CM, Shen MC, Toews GB, Young VB, Huffnagle GB.** 2012. Ecological succession of bacterial communities during conventionalization of germ-free mice. Appl Environ Microbiol **78:**2359–2366.
27. **Greetham HL, Gibson GR, Giffard C, Hippe H, Merkhoffer B, Steiner U, Falsen E, Collins MD.** 2004. *Allobaculum stercoricanis* gen. nov., sp. nov., isolated from canine feces. Anaerobe **10:**301–307.
28. **Isanapong J, Sealy Hambright W, Willis AG, Boonmee A, Callister SJ, Burnum KE, Paša-Tolić L, Nicora CD, Wertz JT, Schmidt TM, Rodrigues JL**. 2013. Development of an ecophysiological model for *Diplosphaera colotermitum* TAV2, a termite hindgut Verrucomicrobium. ISME J **7:**1803–1813.
29. **Jones D.** 1986. Genus *Erysipelothrix*, p 1245–1249. *In* Sneath PHA, Mair NS, Sharpe ME, Holt JG (ed), Bergey's Manual of Systematic Bacteriology, Williams & Wilkins, Baltimore.
30. **Kageyama A, Benno Y.** 2000. Phylogenic and phenotypic characterization of some *Eubacterium*-like isolates from human feces: Description of *Solobacterium moorei* gen. nov., sp. nov. Microbiol Immunol **44:**223–227.
31. **Kane MD, Breznak JA.** 1991. Effect of host diet on production of organic acids and methane by cockroach gut bacteria. Appl Environ Microbiol **57:**2628–2634.
32. **Lory S.** 2014. The Family *Enterococcaceae*, p 75–77. *In* Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F (ed), The Prokaryotes. 4th ed, Springer, Berlin.
33. **Metcalf, RL.** 1943. The storage and interaction of water soluble vitamins in the Malpighian system of *Periplaneta americana* (L.). Arch Biochem **2:**55.
34. **Mikaelyan A, Thompson CL, Hofer MJ, Brune A.** 2016. Deterministic assembly of complex bacterial communities in guts of germ-free cockroaches. Appl Environ Microbiol **82:**1256–1263.

35. **Moore WEC, Johnson JL, Holdeman LV.** 1976. Emendation of *Bacteroidaceae* and *Butyrivibrio* and description of *Desulfomonas* gen. nov. and ten new species in the genera *Desulfomonas, Butyrivibrio, Eubacterium, Clostridium,* and *Ruminococcus*. Int J Syst Bacteriol **26:**238–252.
36. **Muyskens JB, Guillemin K.** 2008. Bugs inside bugs: what the fruit fly can teach us about immune and microbial balance in the gut. Cell Host Micr **3:**118.
37. **Newell PD, Douglas AE.** 2014. Among-species interactions determine the impact of gut microbiota on nutrient allocation in *Drosophila melanogaster*. Appl Environ Microbiol **80:**788–796.
38. **Neyen C, Bretscher AJ, Binggeli O, Lemaitre B.** 2014. Methods to study *Drosophila* immunity. Methods **68:**116–128.
39. **Octavia S, Lan R.** 2014. The Family *Enterobacteriaceae*, p 225–286. *In* Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F (ed), The Prokaryotes. 4th ed, Springer, Berlin.
40. **Resmer KL, White RL.** 2011. Metabolic footprinting of the anaerobic bacterium *Fusobacterium varium* using 1H NMR spectroscopy. Mol Biosyst **7:**2220–2227.
41. **Ritter, H.** 1961. Glutathione-controlled anaerobiosis in *Cryptocercus*, and its detection by polarography. Biol Bull **121:**330–346.
42. **Schauer C, Thompson C, Brune A.** 2014. Pyrotag sequencing of the gut microbiota of the cockroach *Shelfordella lateralis* reveals a highly dynamic core but only limited effects of diet on community structure. PLoS One **9:**e85861. doi:10.1371/journal.pone.0085861.
43. **Schauer C, Thompson CL, Brune A**. 2012. The bacterial community in the gut of the cockroach *Shelfordella lateralis* reflects the close evolutionary relatedness of cockroaches and termites. Appl Environ Microbiol **78:**2758–2767.
44. **Schleifer K-H**, 2009. Family I. *Lactobacillaceae*, p 465. *In* De Vos P, Garrity GM, Jones D, Krieg NR, Ludwig W, Rainey FA, Schleifer KH, Whitman WB (ed), Bergey's Manual of Systematic Bacteriology. 2nd ed, Springer, New York.
45. **Schuppert B, Schink B, Trosch W.** 1992. Batch and continuous production of propionic acid from whey fermeate by *Propionibacterium acidipropionici* in a three-electrode amperometric culture system. Appl Microbiol Biotechnol **7:**549–553.
46. **Shin SC, Kim S-H, You H, Kim B, Kim AC, Lee K-A, Yoon JH, Ryu JH, Lee WJ, et al.** 2011. *Drosophila* microbiome modulates host developmental and metabolic homeostasis via insulin signaling. Science **334:**670–674.

47. **Smith LD, King E.** 1962. *Clostridium innocuum*, sp. n., a sporeforming anaerobe isolated from human infections. J Bacteriol **83:**938–939.
48. **Sprenger WW, Hackstein JHP, Keltjens JT.** 2005. The energy metabolism of *Methanomicrococcus blatticola*: physiological and biochemical aspects. Antonie v. Leeuwenhoek **87:**289–299.
49. **Sprenger WW, van Belzen MC, Rosenberg J, Hackstein JHP, Keltjens JT.** 2000. *Methanomicrococcus blatticola* gen. nov., sp., nov., a methanol- and methylamine-reducing methanogen from the hindgut of the cockroach *Periplaneta americana*. Int J Syst Evol Microbiol **50:**1989–1999.
50. **Stackebrandt E.** 2009. Genus I. *Erysipelothrix*, p 1299–1306. *In* De Vos P, Garrity GM, Jones D, Krieg NR, Ludwig W, Rainey FA, Schleifer KH, Whitman WB (ed), Bergey's Manual of Systematic Bacteriology. 2nd ed, Springer, New York.
51. **Takahashi N, Abbe K, Takahashi-Abbe S, Yamada T.** 1987. Oxygen sensitivity of sugar metabolism and interconversion of pyruvate formate lyase in intact cells of *Streptococcus mutans* and *Streptococcus sanguis*. Infect Immun **55:**652–656.
52. **Tegtmeier D, Thompson CL, Schauer C, Brune A.** 2016. Oxygen affects gut bacterial colonization and metabolic activities in a gnotobiotic cockroach model. Appl Environ Microbiol **82:**1080–1089.
53. **Teixeira de Mattos MJ, Tempest DW**. 1983. Metabolic and energetic aspects of the growth of *Klebsiella aerogenes* NCTC 418 on glucose in anaerobic chemostat culture. Arch Microbiol **134:**80–85.
54. **Terra WR, Ferreira C.** 1994. Insect digestive enzymes: properties, compartmentalization and function. Comp Biochem Physiol B **109:**1–62.
55. **Tholen A, Brune A.** 2000. Impact of oxygen on metabolic fluxes and *in situ* rates of reductive acetogenesis in the hindgut of the wood-feeding termite *Reticulitermes flavipes*. Environ Microbiol **2:**436–449.
56. **Thomas TD, Ellwood DC, Longyear MV.** 1979. Change from homo- to heterolactic fermentation by *Streptococcus lactis* resulting from glucose limitation in anaerobic chemostat cultures. J Bacteriol **138:**109–117.
57. **Ushijima T, Ozaki Y.** 1988. Factors influencing potent antagonistic effects of *Escherichia coli* and *Bacteroides ovatus* on *Staphylococcus aureus* in anaerobic continuous flow cultures. Can J Microbiol **34:**645–650.

58. **Verbarg S, Göker M, Scheuner S, Schumann P, Stackebrandt E.** 2014. The families *Erysipelotrichaceae* emend., *Coprobacillaceae* fam. nov., and *Turicibacteraceae* fam. nov., p 79–105. *In* Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F (ed), The Prokaryotes. 4th ed, Springer, Berlin.

59. **Verbarg S, Rheims H, Emus S, Frühling A, Kroppenstedt RM, Stackebrandt E, Schumann, P.** 2004. *Erysipelothrix inopinata* sp. nov., isolated in the course of sterile filtration of vegetable peptone broth, and description of *Erysipelotrichaceae*. Int J Syst Evol Microbiol **54:**221–225.

60. **Wada-Katsumata A, Zurek L, Nalyanya G, Roelofs WL, Zhang A, Schal C.** 2015. Gut bacteria mediate aggregation in the German cockroach. Proc Natl Acad Sci USA **112:**15578–15683.

61. **Warhurst DC.** 1964. Growth and survival, *in vitro* and *in vivo* of *Endolimax blattae*, an entozoic amoeba of cockroaches. PhD Thesis. University of Leicester, UK.

62. **Wertz JT, Kim E, Breznak JA, Schmidt TM, Rodrigues JLM.** 2012. Genomic and physiological characterization of the *Verrucomicrobia* isolate *Diplosphaera colitermitum* gen. nov., sp. nov., reveals microaerophily and nitrogen fixation genes. Appl Environ Microbiol **78:**1544–1555.

63. **Wong ACN, Ng P, Douglas AE.** 2011. Low diversity bacterial community in the gut of the fruitfly *Drosophila melanogaster*. Environ Microbiol **13:**1889–1900.

64. **Yamada T, Takahashi-Abbe S, Abbe.** 1985. Effects of oxygen on pyruvate formate-lyase in situ and sugar metabolism of *Streptococcus mutans* and *Streptococcus sanguis*. Infect Immun **47:**129–134.

Danksagung

An erster Stelle möchte ich mich ganz herzlich bei Prof. Dr. Andreas Brune für die Bereitstellung des spannenden Themas, die lehrreichen Diskussionen, die vielseitige Unterstützung während meiner Arbeit sowie für die Übernahme des Erstgutachtens bedanken.

Außerdem danke ich Prof. Dr. Wolfgang Buckel und Prof. Dr. Ralf Conrad für ihre Beteiligung an meinem „Thesis Advisory Committee“ sowie Prof. Dr. Roland Brandl und Prof. Dr. Susanne Önel für ihre Beteiligung an meinem Prüfungskomitee.

Prof. Dr. Wolfgang Buckel danke ich außerdem herzlich für die Übernahme des Zweitgutachtens.

Ein besonders großer Dank gilt allen aktuellen und ehemaligen Mitgliedern der „Termitengruppe“ für die entspannte und fröhliche Arbeitsatmosphäre, die lustigen und interessanten Gespräche in der Mensa, die Hilfsbereitschaft und die vielen ertragreichen Diskussionen.

Besonders möchte ich mich bei Kristina Lang und Katja Meuser für die unzähligen Ratschläge und die Hilfestellung bei zahlreichen Techniken im Labor und für die vielen netten Gespräche bedanken. Ein besonderer Dank gilt auch Kathrina Klomann, die mich in der Endphase der Doktorarbeit sehr unterstützt und aufgebaut hat.

Weiterhin möchte ich mich sehr bei Cornelius Riese und Alexandra Belitz, die durch ihre Bachelorarbeiten ebenfalls zu dieser Arbeit beigetragen haben, bedanken.

Insbesondere bedanke ich mich bei Alexandra Belitz, Karen Rossmassler und Wanyang Wang für die vielen guten Gespräche und lustigen Momente während meiner Zeit in der Arbeitsgruppe.

Ein besonderer Dank gilt auch Niclas Lampert und Aram Mikaelyan für ihre Unterstützung bei zahlreichen bioinformatischen Fragestellungen und die guten Diskussionen im „Cockroach-Meeting“. Aram Mikaelyan und Kristina Lang danke ich auch sehr herzlich für die hilfreichen Anmerkungen und Tipps für meine Arbeit.

Des Weiteren danke ich allen Mitarbeitern der Abteilung „Biogeochemie“ für ihre hilfreichen Ratschläge.

Von ganzen Herzen danke ich meinem Freund Martin Wittich, der mich während der ganzen Zeit sehr unterstützt hat, mich stets zum Lachen gebracht hat und immer für mich da war.

Weiterhin danke ich sehr meiner Familie und meinen besten Freunden Irene und Elisabet und deren Familie für die schönen Stunden in der Heimat, die traumhafte Zeit in Griechenland und die vielen aufbauenden und erheiternden Gespräche am Telefon.

Ein besonderer Dank gilt auch meinen lieben Freunden Claudia, Michael, Alex, Sabine, Vanessa und Hannah für die vielen guten Gespräche, Unternehmungen und Ablenkung vom oftmals stressigen Alltag.

Erklärung der Eigenständigkeit

Ich versichere, dass ich meine Dissertation

„The gut microbiota of the cockroach *Shelfordella lateralis*: Primary colonization, succession, and metabolic response to microenvironmental conditions"

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, Mai 2016