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Oxygen Affects Gut Bacterial Colonization and Metabolic Activities in a Gnotobiotic Cockroach Model

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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 germfree nymphs with two bacterial strains isolated from the guts of conventional cockroaches. Fluorescence microscopy revealed that both strains specifically colonized the germfree 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. The pure cultures formed the typical products of mixed-acid or butyrate fermentation, whereas the guts of gnotobiotic cockroaches accumulated mostly lactate and acetate. Similar shifts toward more-oxidized products were observed when the pure cultures were exposed to oxygen, which corroborated the strong effects of oxygen on the metabolic fluxes previously observed in termite guts. Oxygen microsensor profiles of the guts of germfree, gnotobiotic, and conventional cockroaches indicated that both gut tissue and microbiota contribute to oxygen consumption and suggest that the oxygen status influences the colonization success.

Many insects, particularly those feeding on a fiber-rich diet, possess a dense and complex microbiota. The most prominent examples 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 tracts (1, 2). During recent years, the microbial community structure of many termites has been studied in detail, and the evolutionary patterns in the gut microbiota of termites and their closest phylogenetic relatives, the cockroaches, are slowly emerging (3). In particular, the application of high-throughput sequencing techniques provides sufficient resolution and sampling depth to distinguish the phylogenetic and environmental drivers of the community structure (4, 5, 6).

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 the first insights into the functional potentials of the gut community (7, 8, 9), 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 (10), 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 the 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 the influx of oxygen, whose importance increases inversely proportionally with the radius of a gut compartment (11), strongly affects the fermentative processes in the entire hindgut community (12), but this remains to be investigated with pure cultures. Early colonization of the (presumably oxic) gut and the 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 (13, 14), a tempting hypothesis that awaits experimental testing.

Experiments with germfree animals inoculated with one or more strains of defined gut microorganisms may provide excellent opportunities to approach these questions. Such gnotobiotic animals (15) can be used to characterize the responses 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 so far have been restricted mostly to gnotobiotic mammals, particularly rats, mice, and piglets (16, 17, 18, 19).

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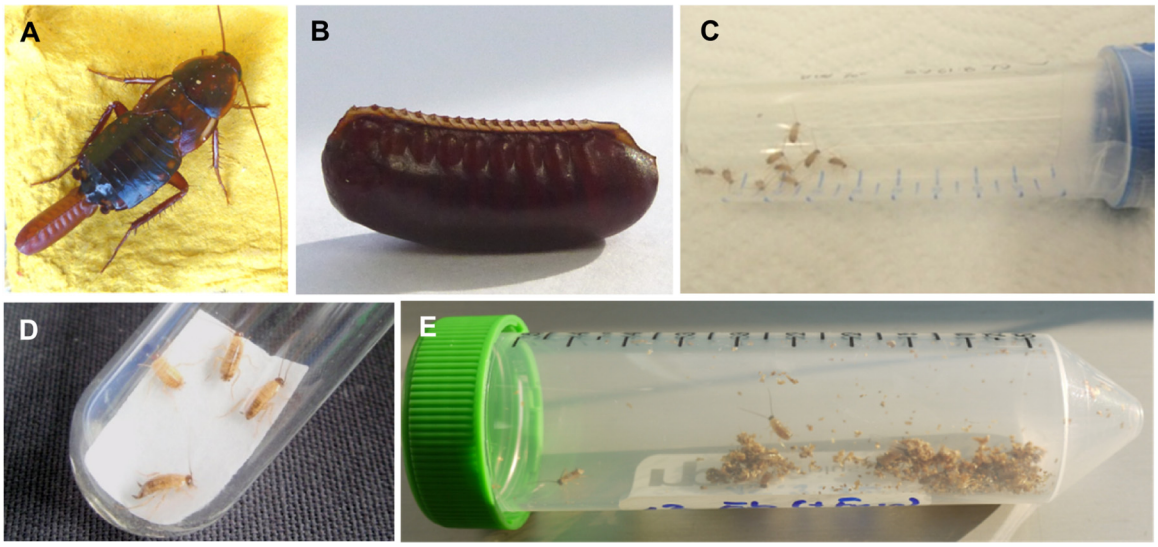

FIG 1 *Shelfordella lateralis* as a gnotobiotic model system. (A) Adult female with ootheca; (B) ootheca sterilized with 2% peracetic acid; (C) germfree hatchlings; (D) germfree cockroaches on filter paper strips soaked with bacterial culture; (E) gnotobiotic cockroaches in containers with autoclaved wheat bran.

While germfree mammals can be obtained only by Cesarean section, germfree insects are easily generated by chemical surface sterilization of eggs (20, 21, 22, 23). Unfortunately, termites are intractable as gnotobiotic models due to their eusociality and obligate dependence on their gut microbiota for food digestion. In contrast, their closest relatives, the nonsocial 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 that against the potentially detrimental effects of the sterilization process (20, 21). 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 (24). 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 provision of nutrients (25).

We selected the cockroach *Shelfordella lateralis*, an omnivorous member of the cockroach family *Blattidae*, the sister group of termites (26), 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 (27, 28) and also comprise methanogenic archaea (29, 30) and ciliate protists (31). 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 (32). The gut environment of cockroaches resembles that encountered in many termites (27, 28), which explains why the gut microbiotas of termites and cockroaches share several common lineages (4, 27) and may allow the use of cockroach guts as a surrogate environment for studying the termite gut microbiota.

With this gnotobiotic cockroach model (Fig. 1), we studied the colonization of the germfree 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 effects 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 germfree cockroaches. *Shelfordella lateralis* was obtained from a commercial breeder and maintained as previously described (27). 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. (20) with several modifications. After a 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; Kellogg's, 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 (33); amplicons were purified and sequenced as described earlier (34).

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, the gut contents of individual germfree 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 medium. Facultatively anaerobic bacteria were obtained on nutrient agar plates (Difco; Becton, Dickinson, Franklin Lakes, NJ, USA) and incubated under air at 30°C. Obligately anaerobic bacteria were isolated on fastidious-anaerobe agar (35) that had been incubated in an anoxic jar under an atmosphere of N_2-CO_2 (80:20 [vol/vol]) 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 by following the protocol of Sharma and Schimke (36). 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 of 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 the 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 *Escherichia 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 germfree cockroaches. Strain EbSL and strain FuSL were routinely grown in AM5 medium (37) containing 5 mM glucose, 0.2% yeast extract, 0.4% Casamino Acids, 2 mM cysteine, and 1 mM dithiothreitol (DTT) (but no ampicillin) and kept under a headspace of N_2-CO_2 (80:20 [vol/vol]). 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 (optical density at 578 nm [OD_{578}] of 0.4 to 0.45) were applied onto sterile filter paper strips, which were immediately placed into sterile 50-ml tubes with five newly hatched germfree 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-labeled strain EbSL was grown aerobically on LB medium with ampicillin overnight to allow maturation of the GFP fluorophore and inoculated into germfree 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 (FISH) with the *Bacteria*-specific probe EUB338 (38) at 46°C as previously described (39).

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) 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 (40) using primers specific for *Enterobacteriaceae* (5′-ATGGCTGTCGTCAGCTCGT-3′ and 5′-CCTACTTCTTTTGCAACCCACTC-3′) (41) and for *Fusobacteriaceae* (5′-GGATTTATTGGGCGTAAAGC-3′ and 5′-GGCATTCCTACAAAT ATCTACGAA-3′) (42) 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′-C CGTCAATTCMTTTRAGTT-3′) (33) (primer 519fc as modified by Stubner [40]).

Each sample was analyzed in duplicate with at least three independent determinations, which typically showed a 10 to 30% deviation. All calibration curves were linear over a range of at least 6 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) (rRNA database [43]).

Fermentation products of pure cultures *in vitro*. The 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 the reducing agent, and 0.8 mg/liter resazurin as the redox indicator. Tubes were inoculated with 4% preculture and incubated at 30°C. The influence of oxygen on the growth and fermentation products was tested in nonreduced medium by the addition of different amounts of sterile oxygen to the headspace of cultures incubated on a rotary shaker.

Growth was determined photometrically by following the increase in OD_{578} using a culture tube photometer (Spectronic 20+, path length of ca. 1.3 cm; Milton Roy); optical densities of >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 high-performance liquid chromatography (HPLC) with an ion-exclusion column (Resin H^+ IEX column, 8 μm; Grom, Rottenburg, Germany) and a refractive index detector (27). The hydrogen concentration in the culture headspaces was analyzed by gas chromatography with a thermal conductivity detector (44).

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 from the amount of substrate consumed (45). The amount of glucose assimilated into the biomass was estimated using the turbidity of the culture (OD_{578} of 0.1 corresponds to a dry weight of 30 mg $liter^{-1}$) and an elemental composition of $C_4H_8O_2N$ for bacterial cells (46), 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, 9 days after inoculation) were dissected under a stereomicroscope using sterile forceps. The fat body surrounding each hindgut was carefully removed, and 10 hindguts were pooled and homogenized in 150 μl water by sonication

(ultrasonic processor UP50H). Samples were prepared and analyzed by HPLC as previously described in Schauer et al. (27).

Hydrogen emission by living cockroaches (first-instar nymphs, 7 days after inoculation) was measured by gas chromatography with a packed Mol Sieve 5A column (80/100 mesh; 70 cm by 6.35 mm) and a reduction gas detector (RGD2; Trace Analytical, Menlo Park, CA, USA). For the measurement, pools of ca. 10 cockroaches were placed in 15-ml glass vials closed with a rubber stopper. With the respiratory activity of *S. lateralis* (47), it was estimated that the oxygen concentration in the vials decreased by 0.3 to 1.1% per hour of incubation. Hydrogen production rates were determined from the linear increase of the 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, the 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 (48).

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 (20, 21, 22), we tested several biocides for their applicability in the surface sterilization of *S. lateralis* oothecae. The 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 ootheca 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 the untreated controls (11 ± 3.1; $n = 20$). However, surface sterilization significantly influenced the number of oothecae that yielded healthy hatchlings (36% of treated oothecae versus 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 to 6 μm long and 1 to 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.

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 medium. We selected one facultatively anaerobic strain 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 to 97% sequence similarity to that of the species described 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 medium (49).

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

Fluorescence microscopy of monocolonized guts showed that both strains preferentially colonized the hindgut (Fig. 2A to C); 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 (Fig. 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 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 1).

***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 germfree 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 the gut homogenates of germfree and gnotobiotic cockroaches but lower in those 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 small amounts of ethanol and succinate. Gut homogenates of conventional cockroaches contained acetate and lactate, but lactate accumu-

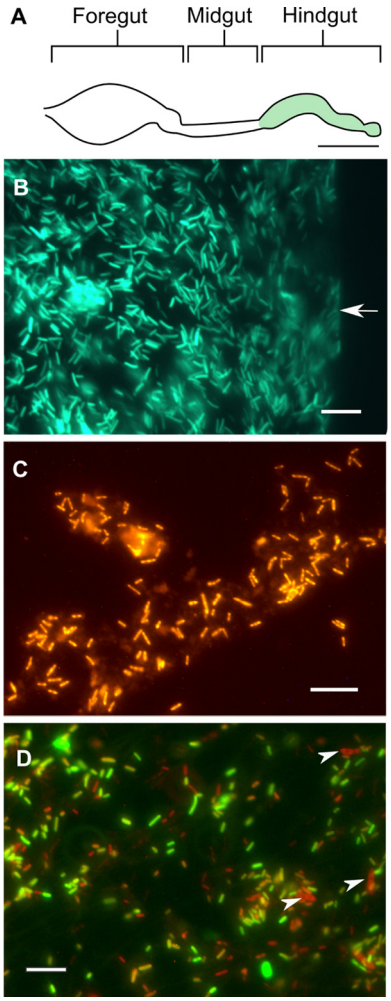

FIG 2 Epifluorescence micrographs of hindguts colonized with strains EbSL and FuSL. (A) Scheme illustrating the structure of the intestinal tract of *Shelfordella lateralis* and the exclusive location of fluorescent cells throughout the hindgut. (B) Periphery of an intact hindgut colonized by GFP-labeled 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 the GFP fluorescence and FISH signal. Cells of *Blattabacterium* sp. are indicated by arrowheads. Bars, ca. 2 mm (A) and 10 μm (B to D).

lated in smaller amounts. In gut homogenates of germfree cockroaches, only small amounts of lactate were detected (Table 2).

The 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 2). The hydrogen emission rates of monoassociated cockroaches were in the same range as the rates of 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, germfree cockroaches did not emit any hydrogen.

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), their fermentation products differed substantially from those produced *in situ* in the guts of monoassociated cockroaches.

The major fermentation products of the strictly anaerobic strain FuSL in pure culture were butyrate, acetate, and hydrogen. With only 1% oxygen in the headspace, the growth rate, cell yield, and butyrate production decreased significantly, whereas acetate production slightly increased (Table 3). Oxygen was completely consumed in the 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) 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 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 yield of 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). 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 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.

Oxygen status of the gut. Microsensor measurements revealed strong differences in the oxygen status in the different regions of the agarose-embedded guts of conventional, germfree, and gnotobiotic cockroaches (Fig. 3). In all cockroaches, oxygen partial pressures were generally low in the foregut and midgut compartments, often under the detection limit of the sensor (ca. 0.15 kPa). In germfree and conventional guts, the axial oxygen profiles were more varied than in gnotobiotic guts. The highest partial pressures were encountered in the posterior hindgut, where the oxygen levels in the germfree cockroaches surpassed those in the gnotobiotic and conventional cockroaches. Guts colonized with strain EbSL consistently exhibited only very low oxygen concentrations in all compartments.

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 con-

TABLE 1 Quantification of strains EbSL and FuSL in the guts of gnotobiotic cockroaches via qPCR with family-specific primers

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

[a] Values are means (± standard deviations [SD]) from three replicate experiments with five hindguts each. Results from conventional cockroaches are shown for comparison.
[b] Estimated using the rRNA gene copy number of seven for *Enterobacter* spp. and five for *Fusobacterium nucleatum*.
[c] Cockroaches monoassociated with either strain EbSL or strain FuSL.
[d] Cockroaches diassociated with strains EbSL and FuSL, inoculated in the given order (the second strain 2 days after the first).
[e] Conventional cockroaches (first instar) from two replicate experiments. The total number of bacterial 16S rRNA genes was between 8.2 and 24.8 × 10^7 per mg gut.

firmed 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 levels of colonization success are most likely due to their different responses to oxygen.

Model organisms specifically colonize the germfree hindgut. GFP labeling is a useful tool to localize bacterial strains in the gut. Husseneder and Grace (50) 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 from gut tissue or food particles (Fig. 2B).

The exclusive colonization of the germfree 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 of conventional cockroaches, where the hindgut shows the highest density and diversity of bacteria among all gut compartments (27, 51). It is likely that the colonization of foregut and midgut is suppressed by the high activities of digestive enzymes in these compartments (52, 53, 54).

The colonization densities of strains EbSL and FuSL in monoassociation and diassociation are much higher than those of all *Enterobacteriaceae* and *Fusobacteriaceae* in the hindguts of conventional cockroaches and, in the case of strain EbSL, even surpassed the total cell counts in the hindguts of conventional adults (2.2 [±1.6] × 10^7 cells mg^{-1} gut) (27). The high colonization densities in gnotobiotic cockroaches are 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 (31).

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 early colonization with a facultative anaerobe does not necessarily favor colonization by obligate anaerobes. On the contrary, the 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 might be explained by its capacity for respiration. However, glucose does not appear to be a limiting factor in the gnotobiotic gut, so that antagonistic effects resulting in the suppression of strain FuSL also cannot be excluded. Similarly, in disassociated gnotobiotic rats, the relative abundance of *Fusobacterium varium* was almost an order of magnitude lower than that of *Bacteroides thetaiotaomicron* (16).

In view of the complete growth inhibition of the obligately anaerobic strain FuSL at only 2% oxygen in the headspace, its

TABLE 2 Gut metabolites and hydrogen emission rates of gnotobiotic *S. lateralis* monoassociated with strain EbSL or strain FuSL and germfree and conventional cockroaches of the same age group

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

[a] Values are means (± SD) from four replicate experiments, using homogenates of 10 hindguts with an average fresh weight of 0.2 ± 0.06 mg per gut. —, below the detection limit (<1 nmol mg^{-1}).
[b] Values are means (± SD) from three to four replicate experiments with pools of ca. 10 cockroaches, each with a fresh weight of 8.0 ± 2.3 mg per cockroach.
[c] —, below the detection limit (<1 pmol mg^{-1} h^{-1}).

TABLE 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[a]

Strain and oxygen concn (%)[b]	Turbidity (OD_{578})[c]	Dissimilated glucose (mM)[d]	Concn (mM) of indicated product[e]							Electron recovery (%)[f]
			Formate	Ethanol	Succinate	Acetate	Hydrogen	Butyrate	Lactate	
FuSL										
0	0.65	3.7	—	—	—	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
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] Values are means of results from duplicate cultures (typically <10% deviation).
[b] Initial values.
[c] Values include cell mass formed on basal medium (0.1% Casamino Acids and 0.1% yeast extract).
[d] Dissimilated glucose accounts for the amount of glucose assimilated into the biomass.
[e] Values include the products formed on basal medium (0.1% Casamino Acids and 0.1% yeast extract). —, below the detection limit (<0.02 mM).
[f] Electron recoveries in fermentation products, based on dissimilated glucose.
[g] Corrected for the large amount of cell mass formed on basal medium under oxic conditions (OD_{578} of 0.93).

capacity to robustly colonize the hindguts of germfree cockroaches in monoassociation is remarkable. The decreased oxygen partial pressure in hindguts colonized with strain FuSL in comparison to that in germfree 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 (55) and can grow in dense cultures in a chemostat even under atmospheric oxygen partial pressure (56).

The colonization of the germfree hindgut with strain FuSL is probably facilitated by the low oxygen partial pressure in the anterior hindgut, which is <1 kPa even in germfree 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 small amounts of lactate in the hindguts of germfree nymphs, which is likely caused by a switch to anaerobic metabolism in the gut tissue due to the limit in the oxygen supply.

The further reduction of the 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 nonrespiratory activities is a common phenomenon in anaerobes (57, 58, 59, 60) and has been documented for lactic acid bacteria, homoacetogens, and methanogens isolated from the intestinal tracts of termites (37, 45, 61, 62, 63); many obligate anaerobes possess enzymes that detoxify oxygen or oxygen radicals (64, 65).

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 (12). Conversely, metabolites such as butyrate and formate, which do not accumulate in gnotobiotic cockroaches, lack the concentration gradients required for efficient diffusive transport toward the epithelium.

TABLE 4 Metabolic products of strains EbSL and FuSL cultivated with different glucose concentrations under anoxic conditions[a]

Strain and glucose concn (mM)	Concn (mol per mol glucose) of indicated product[b]					
	Formate	Ethanol	Succinate	Acetate	Butyrate	Lactate
FuSL						
5	—	—	—	0.47	0.60	0.03
10	—	—	—	0.22	0.56	0.10
15[c]	—	—	—	0.20	0.53	0.11
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] The hydrogen concentration was not determined. Values are means of results from duplicate cultures (<10% deviation).
[b] Calculated from consumed glucose, after subtraction of products formed on basal medium (0.1% Casamino Acids and 0.1% yeast extract). —, below the detection limit (<0.02 mM).
[c] Only 12.3 mM glucose was consumed.

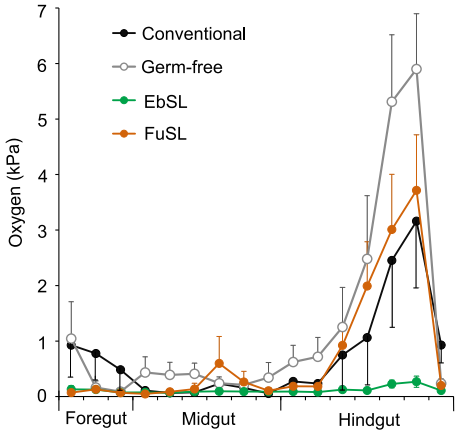

FIG 3 Oxygen partial pressure in the guts of gnotobiotic, germfree, and conventional first-instar cockroaches. The axial profiles were measured with microsensors at the gut center. The symbols are means (± standard errors of the mean [SEM]) from replicate measurements with eight different guts. Error bars are shown in one direction only for clarity.

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 (11, 66). 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 (12).

This effect explains 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 also been described for the more oxygen-tolerant *Fusobacterium nucleatum* when incubated under oxic conditions (56). 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* (67, 68, 69). 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 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 small 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 effects. Such shifts toward increased lactate formation in the presence of nonlimiting concentrations of glucose have also been described for chemostat cultures of *Klebsiella aerogenes* (70) and several *Streptococcus* spp. (71, 72) and are in agreement with the production of lactate by *Fusobacterium varium* grown at high glucose concentrations (73). 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 (12).

Despite the obvious effects of oxygen on the metabolic processes of both strains, the hydrogen emissions of the gnotobiotic cockroaches underscore 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 also opens interesting perspectives for future studies, because production of hydrogen allows coupling of 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 (30, 74).

Last, the gnotobiotic cockroach model might provide a valuable tool in "synthetic microbial ecology" by helping to identify factors governing community assembly in cockroaches. It will likely also improve 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 prohibit gnotobiotic studies.

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