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The gut microflora of *Reticulitermes flavipes*, its relation to oxygen, and evidence for oxygen-dependent acetogenesis by the most abundant *Enterococcus* sp.

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Abstract

We previously demonstrated a potentially deep penetration of oxygen into the hindgut lumen of wood-feeding termites, high oxygen consumption rates of the symbiotic gut microbiota, and an indispensable role of oxygen in the mineralization of aromatic compounds. Here we characterize the gut microflora of the wood-feeding termite *Reticulitermes flavipes*, focusing on metabolic capacities and relation to oxygen of the most prevalent physiotypes. The carbohydrate-utilizing isolates, enumerated on liquid and solid growth media (3.1×10^5 cells per gut; gut liquid volume $\sim$0.3 μl), consisted of aerotolerant lactic acid bacteria (58%) and surprisingly large numbers of facultatively aerobic (20%) and even strictly aerobic bacteria (22%). The second largest group of cultivable gut bacteria were hydrogen-oxidizing methanogens (3.0×10^5 cells per gut), which were cultivated only on liquid media. Together, these two groups represented 10% of the total microscopic counts obtained by DAPI staining. The large number of lactic acid bacteria agreed with the metabolic product profiles in anoxic serial dilutions of gut homogenates in which lactate was the main product from glucose in the highest dilutions. Other physiological groups, e.g. propionate and butyrate producers, were much less abundant. Lactate was not, however, a major metabolite in the hindgut fluid, a phenomenon hitherto explained by a rapid turnover of lactate by other gut bacteria. In view of the relatively small number of lactate-oxidizing and lactate-fermenting bacteria, this explanation is not sufficient. We show that the isolate *Enterococcus* strain RfL6, representing the most abundant physiotype among the carbohydrate-utilizing gut bacteria, was not purely fermentative, but consumed oxygen during growth on glucose, accompanied by a complete shift in the product spectrum from lactate to acetate, and was able to oxidize lactate to acetate when oxygen was present. This agrees with the observation that acetate is the major product of the hindgut metabolism of *R. flavipes* ($\sim$70 mM in the hindgut fluid). Together with previous findings, these results underline the obvious but so far neglected importance of oxygen and contribute to the emerging concept that the termite hindguts are far from simple, anoxic fermenters, but axially and radially structured, heterogeneous systems characterized by steep gradients of metabolites.

Keywords: Wood-feeding termite; Insect; Intestinal microflora; Carbohydrate fermentation; Oxygen; Lactic acid bacteria

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Dedicated to Prof. Dr. Achim Kröger on the occasion of his 60th birthday.

1. Introduction

Since the early studies of Cleveland [1], Trager [2] and Hungate [3], the hindgut of lower termites has

been considered an anoxic habitat where anaerobic microorganisms ferment wood polysaccharides to short-chain fatty acids, which in turn serve as the sole carbon and energy source for the host, conferring on the symbiotic relationship the metabolic capacity to digest lignocellulosic matter (for review, see [4]). Initially, investigations focused on the rich assemblage of protozoa found in the hindguts of the (phylogenetically) lower termites, where a variety of unique, anaerobic flagellates are the key agents of cellulose depolymerization. But the gut microbiota of lower termites also comprises an array of diverse bacteria which completely replace the eukaryotic symbionts in virtually all (phylogenetically) higher termites. While carbohydrate-fermenting bacteria seem to have little importance for the depolymerization of cellulose [5], the metabolic activities of strictly anaerobic, homoacetogenic and methanogenic bacteria play an important role in the carbon and electron flow in both lower and higher termites [6,7].

However, this concept of the termite hindgut as an anoxic, purely fermentative system has several serious shortcomings. Firstly, an earlier, quantitative study of the gut microflora of the lower, wood-feeding termite *Reticulitermes flavipes* by Schultz and Breznak [8] revealed the presence of large numbers of aerotolerant and facultatively aerobic bacteria, whereas strictly anaerobic isolates were less frequent. Eutick at al. [9] completely failed to isolate strictly anaerobic bacteria from the guts of several lower and higher wood-feeding termites, but again reported large numbers of strictly and facultatively aerobic isolates.

Secondly, a fermentative model lacks a mechanistic explanation for the degradation of lignin and other polyphenolic substrates, which to date cannot be conceived to occur in the absence of oxygen. Brune et al. [10] recently showed an apparently indispensable role of oxygen in the mineralization even of monoaromatic compounds in both lower and higher wood-feeding termites. While the importance of intestinal lignin degradation especially in the case of the lower wood-feeding termites may be argued [4], it cannot be ignored that lignin-derived humic compounds represent the major dietary component of all soil-feeding higher termites, which are globally far more abundant than the wood feeders [13].

Thirdly, and most importantly, a purely fermentative model does not take into account that a microhabitat as small as a termite hindgut can, from a physico-chemical viewpoint, only maintain its anoxic status within its oxic surroundings if the oxygen entering by diffusion is rapidly and continuously removed, which again should have a considerable impact on the intestinal carbon and electron flow. In a recent study employing microsensor techniques, Brune et al. [14] demonstrated that termite guts in fact exhibit high oxygen uptake rates, and that oxygen penetrates significantly into the hindgut lumen, creating a structured habitat with a micro-oxic periphery around an anoxic center.

To investigate the consequences of this scenario, we re-evaluated the composition of the gut microflora in *R. flavipes*, focusing on metabolic capacities and the relation to oxygen of the most prevalent physiotypes.

2. Materials and methods

2.1. Media

Anaerobic cultures were grown in a bicarbonate-buffered mineral medium (AM 4) under N_2/CO_2 atmosphere (80:20, v/v), supplemented with yeast extract and casamino acids (each 1 g/l; Difco, Detroit, MI, USA) [10]. The medium for aerobic cultivation (MM 4) was similar, but was buffered with sodium phosphate (20 mM), and contained less yeast extract and casamino acids (each 0.5 g/l) [10]. AM 4 contained resazurin (10 mg/l) and was usually pre-reduced with Na_2S (1 mM). If hydrogen was added to the headspace as a reductant (0.5 bar H_2), Na_2S was omitted and a palladium catalyst was added to the medium (Pd on activated carbon (5% Pd), Aldrich, Steinheim, Germany; final catalyst concentration 50 mg/l). For anoxic deep-agar dilution series and for oxic spread plates, media were solidified with agar (1% or 2% w/v, respectively) washed three times with distilled water. For pure cultures of lactic acid bacteria and for enumeration of hydrogen-oxidizing bacteria, AM 4 was used, but reducing agent and resazurin were omitted.

Substrates were added to the media from sterile stock solutions before dispensing into MPN tubes. Substrate concentrations for aerobic liquid cultures

were (in mM): D-glucose, D-xylose (2); D-cellobiose (1); L-lactate, succinate (4); acetate, ethanol, 2,3-butanediol (5); formate (10); aromatic compounds (2). For anaerobic cultures, the amount of substrate was doubled. For enumerations on solid media, a substrate mixture containing glucose, xylose, cellobiose, lactate, succinate, and acetate was used (all concentrations as above). All organic acids were added as sodium salts. All cultures were incubated at 30°C in the dark. The tubes of aerobic cultures were slanted and gently agitated by shaking at 100 rpm.

2.2. Preparation of gut homogenates and enumeration of bacteria

R. flavipes (Kollar) (Rhinotermitidae) were collected near Dansville, MI, USA, and maintained in the laboratory on a diet of European white birch (*Betula pendula* Roth) and water. For all enumeration procedures, termites were degutted, and the extracted guts were pooled and thoroughly homogenized with a glass homogenizer in sterile, anoxic buffered saline solution (BSS; in g/l: K_2HPO_4 2, KH_2PO_4 1, KCl 1.5, NaCl 1.5). The homogenates (10 guts/ml) were serially diluted (1:10) in substrate-free medium (triplicate series). Homogenates were prepared in a glovebox under N_2 (2–5% H_2); all cultivation steps strictly followed generally accepted anaerobic procedures [11]. If Pd/H_2 was used as reductant, it was verified that the media were properly reduced by the time of inoculation.

For three-tube most-probable-number determinations (MPNs) and metabolic product profiles, each tube from the serial dilutions was inoculated into homologous liquid media containing the test substrates. Inoculated tubes were scored as positive if substrate degradation was confirmed by the results of HPLC analysis of the culture supernatant after 2 weeks (oxic) or 4 weeks (anoxic) of incubation. For MPNs of homoacetogens, methanogens, and of lactate-, propionate-, and butyrate-producing bacteria, production of the respective products was used as indicator. Additionally, the presence of methanogens was confirmed by testing for cofactor F_{420} autofluorescence with epifluorescence microscopy [12], that of aerobic hydrogen oxidizers by testing hydrogen utilization using gas chromatography.

For viable counts on solid media, 0.1-ml aliquots of each dilution (triplicate series) were spread on agar plates (MM 4) or were diluted in anoxic deep-agar tubes (AM 4). Viable counts were calculated from the average number of colonies developing on plates or in deep-agar tubes within 2 or 4 weeks of incubation, respectively.

The total number of bacterial cells per gut was determined with the following procedure (modified after [15]): formaldehyde-fixed gut homogenates (0.1 gut per ml BSS; 2% formalin) were stained with 4,6-diamino-2-phenylindole (DAPI; 1.0 μg/ml) at 4°C in the dark for 20 min; 10-ml aliquots were filtered onto pre-stained polycarbonate membrane filters (0.2 μm pore size; Nuclepore, Pleasanton, CA, USA) and rinsed with 3 ml BSS. All solutions were pre-filtered to exclude contaminating bacteria and particles. Bacteria were counted on the filter by epifluorescence microscopy using an ocular grid and randomly selected fields (100 μm^2) until either 20 fields or 400 bacteria were counted; cells were assumed to be Poisson distributed [15].

The major metabolites in the hindgut fluid were determined as follows: 20 guts were homogenized with a small glass rod in a polypropylene vial in 50 μl BSS containing 5 mM Na_2-malonate as internal standard. After centrifugation (15 min at $14000 \times g$), the supernatant was clarified by centrifugation (5 min at $14000 \times g$) through a PVDF centrifuge filter for 1.5-ml polypropylene vials (Whatman, Maidstone, UK) and analyzed by HPLC.

2.3. Isolation and characterization of strains

Representatives of the most abundant morphotypes both from the solid media and from the highest positive MPN tubes were isolated and characterized. The isolates were first grouped by their macroscopic (colony types) and microscopic characters, which were corroborated by Gram staining, oxidase and catalase tests. To ensure that the groups were homogeneous, 5–10 representatives per group were subjected to a more detailed physiological characterization, involving growth tests on various substrates, product spectra, and the commercial identification system API 20 A (bioMérieux, Nürtingen, Germany). Preliminary classification of strains followed the criteria of Bergey's Manual [16], but was not

intended to be exhaustive, since we focused mainly on their metabolic capacities.

2.4. Influence of oxygen on fermentation product patterns

Cultures were grown in butyl rubber-stoppered 160-ml serum vials containing 20 ml AM 4 medium without reducing agent, 2 mM D-glucose, and, when indicated, bovine liver catalase (1 U/ml; Sigma, Deisenhofen, Germany) and were gently agitated by shaking the vials at 100 rpm in a horizontal position to achieve optimal gas exchange between medium and headspace. The headspace gas was N_2/CO_2 (80:20, v/v), and varying amounts of air were added. Culture supernatants were analyzed by HPLC.

2.5. Oxygen uptake of cell suspensions

Glucose-grown cells of *Enterococcus* strain RfL6 were harvested by centrifugation (20 min at $6000 \times g$) in the late exponential growth phase. Cells were washed once with 100 mM potassium phosphate buffer (pH 7.0) and resuspended in the same buffer; all procedures were performed at 4°C. Uptake rates of dissolved oxygen by cell suspensions were measured under rapid mixing in the absence of a gas headspace in the 10-ml cell of a Model 53 Clark-type oxygen meter (YSI, Yellow Springs, OH, USA) at 30°C. The cell suspensions (0.25–1.2 mg (dry wt.) ml^{-1}) were diluted with aerated buffer, and oxygen consumption was recorded before and after addition of various substrates. When indicated, catalase (see above) was added to a final concentration of 1 U/ml.

2.6. Analytical methods

Fermentation products of anaerobic bacteria were analyzed by ion-exclusion chromatography on an Aminex HPX-87H column (300×7.8 mm; BioRad, Hercules, CA, USA) at 40°C, using a mobile phase of 5 mM H_2SO_4 (0.6 ml min^{-1}) and an ERC-7512 refractive index detector (Erma, Tokyo, Japan).

Aromatic compounds were quantitated by reversed-phase HPLC as previously described [17]; peak identity was verified using external standards. Hydrogen and methane were determined by analysis of the headspace using gas chromatography [18,19]. The presence of sulfide was tested using the method of Cline [20]. H_2O_2 was determined with horseradish peroxidase and *o*-dianisidin as cosubstrate [21].

2.7. Calculation of electron recovery

To ensure that we accounted for all major products formed in the respective metabolic reactions, all anoxic MPNs and all pure culture studies were subjected to a balance of the electrons contained in the products vs. the consumed substrate. All metabolites were formally oxidized to CO_2, and the electrons theoretically released from the respective amounts of products were compared with those of the amount of substrate consumed. Expressed on a percent basis, this calculation yielded the electron recovery. In the graphical presentation of the results, this parameter has the advantage that the importance of the individual products with respect to their electron content is directly evident. Yeast extract and casamino acids present in the media led to additional products which did not stem from the main substrate, therefore electron recovery sometimes exceeded 100%.

2.8. Statistical methods

MPNs were computed using the universal equation for MPN calculation [22]. When average values were calculated, confidence intervals are given for a 95% confidence level.

3. Results

3.1. Enumeration of gut bacteria in liquid serial dilutions

Triplicate serial dilutions of *R. flavipes* gut homogenates were inoculated and incubated under both oxic and anoxic conditions. Table 1 compares the MPNs of bacteria utilizing various substrates. The largest MPN was obtained for carbohydrate-fermenting bacteria (1.9×10^5 per gut), but carbohydrate-oxidizing bacteria were present in a similar concentration (8.6×10^4 per gut). Bacteria capable of aerobically oxidizing the products of carbohydrate-fermenting bacteria were slightly less abun-

Table 1
Three-tube MPN estimates of the bacteria in the *R. flavipes* gut capable of degrading various substrates under oxic or anoxic conditions

Substrate	MPN (cells gut^{-1})	
	Oxic conditions	Anoxic conditions
Carbohydrates		
D-Glucose	8.6×10^4	1.9×10^5
D-Cellobiose	8.6×10^4	8.6×10^4
D-Xylose	4.8×10^4	1.9×10^5
Intermediates[a]		
L-Lactate	1.9×10^4	1.5×10^4
Acetate	4.8×10^4	<10
Formate	1.9×10^4	<10
Ethanol	4.8×10^2	–[c]
Succinate	3.0×10^4	–[c]
Aromatic compounds[b]		
Cinnamate	1.5×10^4	4.8×10^3
Vanillin	1.5×10^4	4.8×10^3
Ferulate	<10	4.8×10^3

[a]All major or minor products of the carbohydrate-fermenting isolates.
[b]Aromatic compounds were mineralized only in the presence of oxygen. In the absence of oxygen, only side-chain modifications occurred (see text).
[c]Not tested.

dant; of such compounds, only lactate was fermented in the absence of oxygen. Bacteria that aerobically mineralize the aromatic nucleus of cinnamic acid and vanillin occurred at a similar frequency (1.5×10^4 per gut). Under anoxic conditions, however, metabolism of aromatic substrates occurred only in lower dilutions, and the compounds were not degraded but only modified in their aromatic ring substituents: cinnamic acid was converted to phenylpropanoic acid, vanillin yielded vanillic acid and protocatechuic acid, and ferulic acid gave the respective saturated and/or demethylated phenylpropanoids.

Table 2 compares the MPNs obtained for various metabolic groups of carbohydrate-fermenting and hydrogen-utilizing bacteria. The most abundant carbohydrate-fermenting bacteria formed lactate as the major product from glucose and other sugars. Propionate- and butyrate-producing bacteria were present in lower numbers; it remains to be established whether these products were formed directly from the carbohydrates or after their fermentation to lactate by the numerically prevalent lactogenic bacteria. The methanogens enriched with H_2/CO_2 were non-motile rods forming short chains and were found in higher numbers ($MPN = 3.0\times10^5$ cells per gut) if Pd/H_2-reduced medium was used. Both homoacetogenic bacteria and aerobic, lithoautotrophic hydrogen oxidizers were enriched only in lower dilutions. MPNs of homoacetogens did not increase if the reducing agent was omitted or substituted by Pd/H_2, or if formate, methanol, 3,4,5-trimethoxybenzoate or 2,3-butanediol was used as an alternative substrate. No cellulolytic bacteria were enriched in oxic or anoxic medium in MPNs on microcrystalline cellulose (Avicel, Sigma) or with filter paper as carbon and energy source. No sulfate-reducing bacteria were enriched when sulfate was added to the medium in MPNs on lactate.

3.2. Enumeration of bacteria on solid media and characterization of isolates

As a second approach, we enumerated the gut bacteria by colony counts, using spread plates for oxic and deep-agar dilution series for anoxic counts, and a mixture of substrates (see Section 2). Subsequent isolation and general characterization of the

Table 2
Three-tube MPN estimates of various metabolic groups of fermenting and hydrogen-oxidizing bacteria in the *R. flavipes* gut

Metabolic group	Substrate	MPN (cells gut^{-1})
Anoxic conditions		
Lactate producers	D-Glucose[a]	1.9×10^5
Propionate producers	D-Glucose[a]	3.0×10^4
	L-Lactate	1.5×10^4
Butyrate producers	D-Glucose[a]	4.8×10^3
Methanogens	H_2/CO_2[b]	3.0×10^5
Homoacetogens	H_2/CO_2[b]	5.0×10^2
	3,4,5-Trimethoxy-benzoate[b]	<10
	2,3-Butanediol[b]	<10
Oxic conditions		
Aerobic H_2-oxidizing bacteria	H_2/O_2[c]	4.8×10^3

[a]Similar results were obtained with D-xylose and D-cellobiose.
[b]Reducing agent: Pd/H_2.
[c]Headspace: air plus 0.5 bar H_2.

Table 3
Enumeration of bacteria in the *R. flavipes* gut by direct dilution on solid media under oxic and anoxic conditions

Relation to oxygen	Colony count (CFUs gut^{-1})[a]		Number of morphotypes	Classification of isolates (see text)	Glucose fermentation products[b]
	Oxic conditions ($n = 9$)	Anoxic conditions ($n = 7$)			
Aerotolerant anaerobes	$7.2 \pm 1.3 \times 10^4$	$1.8 \pm 0.4 \times 10^5$	1	*Enterococcus* sp.	L, A, F, E
Facultative anaerobes	$2.9 \pm 1.0 \times 10^4$	$6.3 \pm 2.2 \times 10^4$	2	Enterobacteriaceae	A, F, e, l, s
Strict aerobes	$1.9 \pm 1.2 \times 10^4$	–	2[c]	–[d]	–

The grouping of isolates obtained from the highest dilutions was preceded by a general characterization, including a fermentation product analysis (see text).
[a]For solid media, a substrate mixture was used (see Section 2).
[b]Upper- and lower-case letters refer to major and minor (< 10 mol%) amounts of the following products: (L, l) lactate; (A) acetate; (F) formate; (E, e) ethanol; (s) succinate.
[c]An additional morphotype was isolated from liquid dilutions only with an MPN of 4.8×10^4 cells per gut.
[d]Taxonomic affiliation was not determined.

numerically dominant colonies obtained in the highest dilutions on solid media by the criteria described in Section 2.3 made it possible to group the isolates by physiotypes, and to assign them to major taxonomic entities. The relative distribution of the different physiological groups among the total colonies counted was similar with both cultivation methods, except that strictly aerobic bacteria were obtained only on plates (Table 3). Aerotolerant lactic acid bacteria predominated, accounting for 74% of all colonies in deep-agar tubes and 60% of all colonies on agar plates. The colonies consisted of non-motile coccoid cells (0.6–1 × 1–1.3 μm), which stained Gram-positive, and occurred singly or in short chains; all strains tested were aerotolerant, but catalase- and oxidase-negative. They fermented glucose, cellobiose, and xylose, forming lactate as the major product in addition to smaller amounts of ethanol, formate, and acetate. When yeast extract and casamino acids were omitted from the medium, the isolates grew only sparsely. Only minute colonies formed on plates incubated at ambient oxygen partial pressure, and the cocci often failed to grow in liquid culture under air if the tubes were agitated. It was later found that growth was much more robust when the yeast extract concentration in the medium

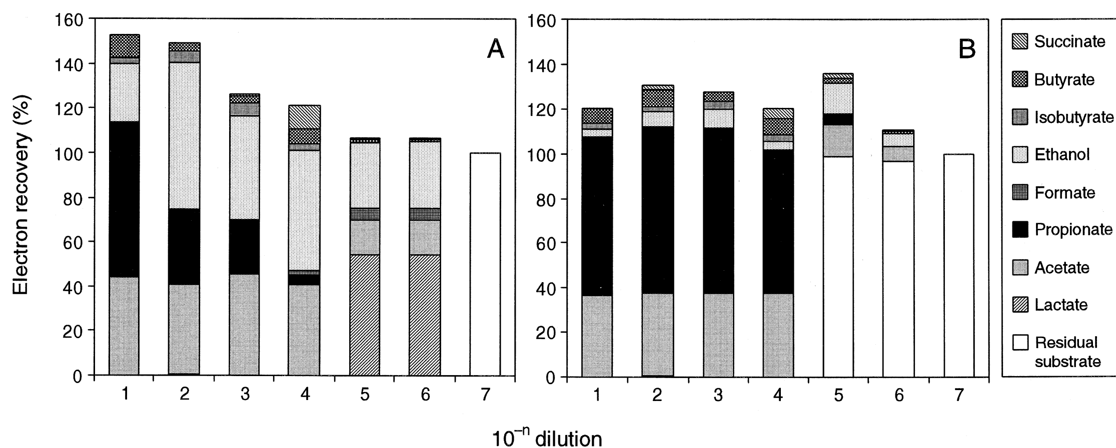

Fig. 1. Metabolic product profiles of anoxic serial dilutions of *R. flavipes* gut homogenates with 4 mM glucose (A) or 8 mM lactate (B) as substrate. The electron recovery was calculated as described in Section 2.7. Excess electrons stem from the background of the medium which was not subtracted, but partially also indicate homoacetogenic activity, since Pd/H_2-reduced medium was used. To facilitate reading of the figure, observe that the shadings in the columns are stacked in the same order as in the legend on the right.

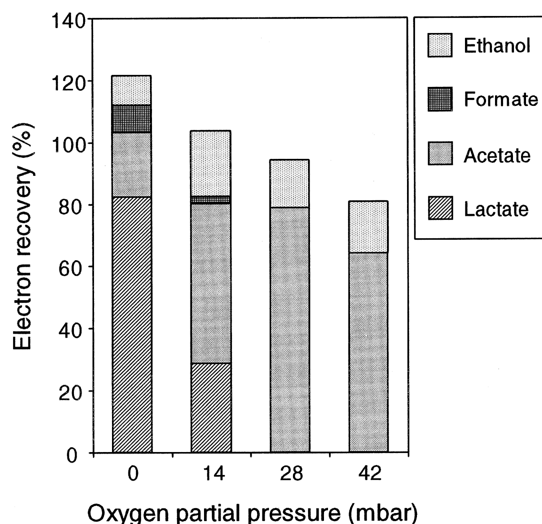

Fig. 2. Influence of oxygen partial pressure in the culture headspace on the product pattern of glucose fermentation by *Enterococcus* strain RfL6. Substrate concentration was 2 mM; the electron recovery was calculated as described in Section 2.7. Excess electrons stem from the background of the medium which was not subtracted.

was increased to 0.2%. According to all standard bacteriological tests, this group consisted of 'lactic' or 'fecal streptococci' [16,23,24]; they are currently the subject of a more detailed study. A representative strain, RfL6, was assigned to the genus *Enterococcus* on the basis of a 16S rDNA sequence analysis (Tholen, Bauer, Overmann and Brune, in preparation).

Another colony type, found both in anoxic deep-agar tubes and on oxic plates (26% or 24% of the total colonies, respectively), consisted of two very similar morphotypes which were not numerically differentiated. They were Gram-negative, facultatively anaerobic motile rods (0.6–0.7×1–1.5 μm and 0.6–0.7×1–1.3 μm, respectively); both were catalase-positive and oxidase-negative, and were tentatively classified as Enterobacteriaceae. In the absence of oxygen, they formed acetate and formate, and smaller amounts of ethanol, lactate, and succinate from glucose. The two groups differed slightly from each other only in the results of the API 20A test. The strictly aerobic isolates (16% of all colonies) comprised two morphotypes: a shorter (0.5×1–1.5 μm) and a slightly longer (0.5×2–2.5 μm), non-motile rod; both were Gram-negative, and oxidase- and catalase-positive. In the presence of oxygen, all facultatively anaerobic and strictly aerobic isolates completely oxidized various sugars (glucose, cellobiose, xylose) and also some of the fermentation products formed by the anaerobic isolates (acetate, succinate, and lactate), with the following exceptions: of the strictly aerobic strains, the shorter rod did not utilize cellobiose, and the longer rod did not utilize lactate, acetate, or succinate. No isolate oxidized formate or grew on or transformed any of the aromatic compounds tested (Table 1) under oxic or anoxic conditions.

All isolates obtained from the highest positive liquid dilutions resembled those strains isolated by direct dilution in solid medium in all characters tested, except that no lactic acid bacteria were isolated from the oxic series; since they grew only poorly in the presence of air (see above), they were probably outgrown by the aerobic strains. An additional physiotype was observed in liquid dilution series on cellobiose (MPN = 4.8×10^4 cells per gut), and

Table 4
Oxygen-dependent oxidation of fermentation products by *Enterococcus* strain RfL6 pre-grown anaerobically on glucose

	Time (h)	Electron recovery (%)				
		Lactate	Acetate	Ethanol	Formate	Total
Anoxic phase[a]	15	52.3 ± 3.1	15.8 ± 1.7	51.7 ± 4.0	8.2 ± 0.5	128.0 ± 8.3
Oxic phase[b]	52	35.7 ± 2.1	30.3 ± 1.0	22.8 ± 4.1	8.2 ± 0.1	97.0 ± 3.1
Control (anoxic)[c]	52	50.0	22.8	48.0	9.8	130.6
Control (oxic)[c]	52	25.1	45.2	20.6	4.9	95.8

Products are compared as electron recovery (average ± S.D.; $n = 2$) from the substrate (2 mM). Excess electrons stem from the background of the medium, which was not subtracted.

[a]Sample taken before addition of oxygen, after the anaerobic culture had entered stationary phase.

[b]After addition of oxygen (38 mbar; 187 μmol, 7.5 mM based on medium volume) to the headspace, cultures were incubated for 36 h before sampling.

[c]Controls incubated for the full time period in the absence or presence of oxygen.

Table 5
Oxygen uptake of cell suspensions of *Enterococcus* strain RfL6 pre-grown on glucose, under oxic (210 mbar O_2) or anoxic conditions

Substrate[a]	Growth conditions	Substrate consumed (nmol)	Oxygen consumed (nmol)	Ratio of oxygen to substrate	Oxygen consumption rate (nmol min^{-1} (mg dry wt.)$^{-1}$)	*n*
D-Glucose	anoxic	315	530 ± 41	1.7 ± 0.1	69.1 ± 5.7	10
	oxic	315	648 ± 34	2.1 ± 0.2	25.3 ± 4.3	10
D-Cellobiose	anoxic	150	641 ± 52	4.3 ± 0.4	26.2 ± 5.9	5
	oxic	150	526 ± 41	3.5 ± 0.2	12.9 ± 1.7	5
Lactate	anoxic	100	69 ± 8	0.7 ± 0.1	6.1 ± 1.6	5
	oxic	100	92 ± 9	0.9 ± 0.1	4.0 ± 0.7	5
Ethanol	anoxic	n.d.[c]	n.d.	–	< 1	5
	oxic	n.d.	n.d.	–	< 1	5
Control[b]	anoxic	–	–	–	13.3 ± 2.4	5
	oxic	–	–	–	15.1 ± 1.6	5

All values are background-corrected and resemble the oxygen consumption caused by addition of the specific substrate.
[a]No increase in oxygen consumption with D-xylose, pyruvate, acetate or ethanol.
[b]Uncorrected oxygen consumption of washed cell suspensions prior to addition of external substrate.
[c]Not detectable.

was found to grow only poorly on solid media. It was a Gram-positive, short, non-motile rod (0.5 × 0.7–1.0 μm), catalase-positive and oxidase-negative, which oxidized cellobiose and glucose, but not lactate.

From the results obtained by direct dilution (Table 3), including the morphotype that was obtained only from liquid dilution series on cellobiose, a total number of carbohydrate-utilizing bacteria of 3.1×10^5 per gut was calculated. Including the almost equally high MPN of the methanogens, the total number of gut bacteria characterized in this study amounted to 9.7% of the total microscopic counts, which was determined with 6.3 (± 0.9)$\times 10^6$ bacteria per gut ($n = 4$) after DAPI staining.

3.3. Metabolic product profiles

In order to identify the prevalent physiological groups in the individual liquid dilutions, all anoxic dilution series were analyzed for fermentation products. Fig. 1 shows two typical metabolic product profiles, obtained with different substrates. Lactate was the main product from glucose in the highest dilutions (Fig. 1A), which is in agreement with the abundance of lactic acid bacteria observed in the direct dilutions on solid media. In lower dilutions, propionate was formed instead of lactate, indicating lactate fermentation by a smaller population of propionigenic bacteria. This agrees with the product profiles of the dilution series on lactate (Fig. 1B). Both profiles show the presence of small populations of bacteria producing butyrate, isobutyrate, and succinate in lower dilutions, which were not isolated using solid media, probably due to their low abundance. Significant amounts of these products were also present in substrate-free controls, stemming from the supplementary yeast extract and casamino acids included in the medium, and are responsible for the excess electron recovery especially in the lower dilutions.

Only traces of CH_4 were formed in the series on glucose and lactate. The presence of homoacetogens in dilution series on hydrogen (data not shown) was also reflected in the slightly increased electron recovery in the series on glucose (Fig. 1A, the first two dilutions), but was not observed in the respective parallels on lactate. When Na_2SO_4 (10 mM) was included in serial dilutions on lactate, no sulfide was formed and product profiles remained unchanged, indicating that lactate-oxidizing sulfate-reducing bacteria were absent.

3.4. Shift in fermentation product pattern

Both the enumerations on solid media and the metabolic product profiles of liquid serial dilutions indicated that lactic acid bacteria were the major group of cultivable carbohydrate-fermenting gut bacteria, whereas lactate-utilizing bacteria were present

in much lower numbers. A determination of the metabolites in the hindgut fluid, however, gave acetate as the major fermentation product (19.5 ± 5.4 nmol per gut; n=7), whereas only traces of lactate were found in two of seven separate homogenates (<1 nmol per gut).

Since we have good reasons to assume that the periphery of the *R. flavipes* hindgut is at least micro-oxic [14], we chose *Enterococcus* strain RfL6, representing the most abundant physiotype among the carbohydrate-degrading gut microflora (Table 3), to determine whether the presence of oxygen might influence their fermentation balance. Fig. 2 shows the results of an experiment where *Enterococcus* strain RfL6 was grown in batch culture on glucose at various oxygen partial pressures. In the absence of oxygen, glucose was fermented mainly to lactate, and smaller amounts of acetate, ethanol, and formate. In the presence of oxygen, this pattern shifted towards acetate formation at the expense of lactate and formate production, until at a headspace partial pressure of 28 mbar O_2, no lactate, but only acetate and small amounts of ethanol were produced. In addition, the electron recovery in the fermentation products decreased with increasing oxygen partial pressure, indicating that oxygen served as an electron acceptor. In a separate experiment, a small volume of air (equivalent to 38 mbar O_2) was added to the cultures after growth had reached the stationary phase. Upon continued incubation, the concentrations of lactate and ethanol, which were formed during fermentative growth, decreased again, whereas only acetate concentration increased further (Table 4); evidence for a direct oxidation of fermentation products. H_2O_2 was below the detection limit (<0.1 mM) in supernatants of cultures grown in the presence of oxygen (2 mM or 5 mM glucose) and analyzed in the early stationary phase. Addition of catalase to the medium did not affect growth rates or molar growth yields on glucose.

3.5. *Oxygen uptake of pure cultures in cell suspensions*

Cell suspensions of glucose-grown *Enterococcus* strain RfL6 consumed oxygen when glucose or cellobiose was added (Table 5). Lactate was oxidized at lower rates. Xylose, ethanol, pyruvate or acetate did not give rise to oxygen consumption, irrespective of the cultivation conditions, nor did heat-inactivated controls. Addition of catalase to the assay mixture did not change the oxygen consumption rates. Oxygen-to-glucose stoichiometries were close to 2:1, indicating a complete oxidation of glucose to acetate and CO_2. The rates of glucose-dependent oxygen uptake of cell suspensions were highest with anoxically grown cells (69.1 nmol min^{-1} (mg dry wt.)$^{-1}$), which is about 30% of the glucose consumption rate estimated for growing cultures, assuming an oxygen-to-glucose stoichiometry of 2:1. If cells were precultivated aerobically, oxygen consumption rates were consistently lower.

The capacity for lactate oxidation, however, was much lower (Table 5). Ethanol oxidation was not detectable in cell suspensions, but is apparent from the results of the experiment in Table 4, where, due to the long incubation time, the observed disappearance of lactate and ethanol can be explained by minimum rates of oxygen consumption in the range of 1 nmol min^{-1} (mg dry wt.)$^{-1}$ (calculations not shown). This value is around the estimated detection limit of the oxygen consumption assay, which was caused by the relatively high background activity already present in the controls, probably due to endogenous substrates.

4. Discussion

Our results show that the oxygen status of the *R. flavipes* hindgut is clearly reflected in the metabolic traits of major metabolic groups constituting the cultivable bacterial community, and in their relation to oxygen. Almost one-third of the bacteria cultivated on solid media consisted of strictly aerobic and facultatively anaerobic strains, and more than two-thirds were aerotolerant lactic acid bacteria, tentatively assigned to the genus *Enterococcus* (Table 3). These results are in agreement with earlier reports on the composition of the gut microflora of wood-feeding termites. Schultz and Breznak [8] found two-thirds of randomly isolated colonies from dilutions of *R. flavipes* gut homogenates to be *Streptococcus* (now *Lactococcus*) *lactis*; all other isolates were either facultatively and strictly anaerobic bacteria, characterized as members of Enterobacteriaceae

and as *Bacteroides* sp. Eutick et al. [9] also reported a prevalence of streptococci and enterobacteria in the guts of various lower and higher wood-feeding termites, and were unsuccessful at isolating any strictly anaerobic bacteria. Other studies of termite gut microorganisms reported the presence of similar bacteria, but were not quantitative (for reviews, see [4,25]). The failure of Martin and Mundt to find any lactic acid bacteria in *R. flavipes* guts [26] might lie in the medium used, which included azide to select for fecal streptococci; a compound known to inhibit growth of lactococci [27] but also certain enterococci [28]. Occurrence of lactic acid bacteria in intestinal tracts of insects is not restricted to termites; streptococci are also found in the guts of, for example, desert locusts [30] and crickets [31]. Much in contrast to the mammalian large intestine or the rumen, which are dominated by strictly anaerobic, fermenting bacteria [32,33], the small intestinal tracts of termites, and maybe also those of other insects, seem to harbor a bacterial flora which consists in its majority of aerotolerant or even aerobic types.

Despite the obvious abundance of lactic acid bacteria in the *R. flavipes* gut, acetate and not lactate is the major metabolite in the hindgut fluid. The average concentration of acetate amounts to 71.4 mM when based on a total hindgut liquid volume of 0.27 mm^3 [29], whereas we found only traces of lactate. This is in good agreement with the results of Odelson and Breznak [34]. Acetate is the major fermentation product also in the guts of other lower termites, and again lactate, if present at all, is found only in small amounts [3,35]. Schultz and Breznak [29] suggested that an inter-species lactate transfer between lactate-producing *Streptococcus* (now *Lactococcus*) *lactis* and a lactate-consuming *Bacteroides* sp. might explain this discrepancy. However, we found that the total abundance of lactate-fermenting anaerobic and lactate-oxidizing aerobic bacteria (Table 1) is almost one order of magnitude lower than that of the lactate-producing, anaerobic bacteria (Tables 2 and 3), which is also evident from the metabolic product profiles obtained on glucose and lactate (Fig. 1).

However, glucose-grown *Enterococcus* strain RfL6 completely shifted its product spectrum from lactate to acetate formation already at low oxygen partial pressures (Fig. 2) and is capable of an oxygen-dependent oxidation of lactate and ethanol to acetate (Tables 4 and 5). Furthermore, cell suspensions showed oxygen uptake rates which were almost 30% of the respiratory rates reported for *Escherichia coli* K-12 growing on glucose (250 nmol (mg dry wt.)$^{-1}$ min^{-1} [36]), and the oxygen-to-glucose stoichiometries indicate the capacity to completely oxidize glucose to acetate and CO_2 (Table 5). Such phenomena also occur in *Lactobacillus plantarum* [37] and *Streptococcus* (now *Enterococcus*) *faecium* [38], where they are caused by the combined activities of oxidases and peroxidases and by an oxygen-dependent inhibition of pyruvate-formate lyase (for review see [39]).

These findings and the numerical prevalence of lactic acid bacteria among the carbohydrate-utilizing isolates suggest strongly that in situ, lactate formation is decreased in favor of acetate by such oxygen-dependent reactions. Due to the stratified nature of the hindgut habitat, however, oxygen-dependent processes can occur only in its periphery [14]. Any lactate formed in the gut center will be either consumed by strictly anaerobic bacteria, or will diffuse into the micro-oxic periphery, where it can be oxidized by the lactic acid bacteria or by aerobic lactate oxidizers. Given the abundance of lactic acid bacteria (Table 3) and the specific oxygen consumption rate of the isolate *Enterococcus* strain RfL6 (Table 5), we can estimate a maximum rate of oxygen consumption by the lactic acid bacteria in the *R. flavipes* hindgut of 3.0 pmol min^{-1} termite^{-1}, which would account for about 1.7% of the oxygen consumption rate of the isolated hindgut, estimated from microelectrode profiles [14], and for about 0.3% of the total respiratory activity of this termite [34]. However, the actual contribution of oxygen-dependent acetogenesis from lactate versus the amount of metabolites oxidized by aerobic bacteria will depend on the oxygen status of the hindgut and the spatial arrangement of the individual physiological groups of bacteria involved in the process.

In a recent publication, Kuhnigk et al. [40] reported the presence of sulfate-reducing bacteria in a number of termites, and the ability of several isolates (*Desulfovibrio* spp.) to use oxygen as electron acceptor in the oxidation of hydrogen or lactate. Such activities would certainly contribute to the oxygen consumption and to oxygen-dependent acetogen-

esis in the gut periphery. The highest number of sulfate-reducing bacteria was reported for *Reticulitermes santonensis* (2×10^7 per ml paunch content). Using the maximum specific rate of oxygen uptake for the isolate KRS1 obtained from this termite species ([40], their Table 6) we estimated an oxygen consumption rate of 0.4 pmol min^{-1} $termite^{-1}$, which is about one-tenth of the estimated rate of oxygen consumption by the lactic acid bacteria in the *R. flavipes* gut (see above). *R. flavipes* (this study), in contrast to *R. santonensis*, but also several other termite species studied by Kuhnigk et al. [40], did not contain any or only small numbers of sulfate reducers in their guts.

The numbers of bacteria degrading simple aromatic compounds were relatively low, and ring degradation was observed only under oxic conditions (Table 1). This agrees with Kuhnigk et al. [41], who found no anaerobic bacteria among the 53 species of bacteria mineralizing aromatic compounds that were isolated randomly from various lower and higher termites, and also with our previous finding that the presence of oxygen is a prerequisite for an oxidation of benzoic and cinnamic acid to CO_2 by *R. flavipes* gut homogenates [10].

The total number of bacterial cells in the *R. flavipes* gut, determined by DAPI staining (6.3×10^6 bacteria per gut), is twice as high as the number reported earlier by Schultz and Breznak [8] using acridine orange. Based on a liquid volume of 0.27 mm^3 [29], this translates to 2.3×10^{10} bacteria per ml, which is still significantly lower than the bacterial densities in mammalian intestinal tracts or in the rumen [32,33]. However, such values have to be interpreted with great caution, since all cell counts in particle-rich environments (e.g., gut contents or soil) are prone to large methodological errors, related to non-specific dye binding to debris or dead cells, to autofluorescence of wood and soil particles, to the dye concentrations used, and to any individual bias involved when differentiating cells from particles.

Nevertheless, the bacterial numbers obtained by cultivation in this and earlier studies [8,9,29] represent only a fraction of the total bacteria present. The two largest groups were the carbohydrate-utilizing isolates (3.1×10^5 cells per gut, almost half of them aerobic) and the methanogenic bacteria observed in the liquid dilution series (3.0×10^5 cells per gut). Together, they amount to about 10% of our total cell counts. Only recently, Leadbetter and Breznak [42] succeeded in isolating several *Methanobrevibacter* spp. from *R. flavipes* hindguts, with a reported frequency of about 10^6 cells per gut, which were associated with the hindgut epithelium. However, it is frustrating that numerous other conspicuous morphotypes in the *R. flavipes* hindgut, as documented in detail by an electron microscopic study by Breznak and Pankratz [43], such as the large array of different spirochetes, still escape cultivation. Also the homoacetogenic bacteria, a physiological group which exhibits high activities of H_2/CO_2-dependent homoacetogenesis in *R. flavipes* gut homogenates [34], were enriched only in our lowest dilutions.

The most important flux of carbon and electrons in the *R. flavipes* hindgut is undoubtedly that comprised by the acetogenic pathways. The strictly anaerobic, cellulolytic flagellates in the hindgut microbiota of *R. flavipes* and all other lower termites [44] should be responsible for the most significant portion of the carbohydrate turnover, forming acetate and hydrogen as main fermentation products, but unfortunately only very few species have been studied in pure culture [4]. While acetate is an important nutritional basis for the termite, hydrogen is assumed to support the acetogenic activity in *R. flavipes* [34,46] and the methanogenic population residing in the hindgut epithelium [42]. Besides the 'microfauna', also the carbohydrate-fermenting microflora contributes to acetate formation, but carbon and electron flow in the gut periphery will be strongly influenced by the vast and continuous diffusive influx of oxygen, which is driven by the steep concentration gradients between the oxic hindgut epithelium and the anoxic center. Nothing is known about the effect of low oxygen partial pressures on the hindgut flagellates, but it may be worth mentioning that a study of oxygen-sensitive rumen ciliates showed that, if supplied at low partial pressure, oxygen may indeed function as an electron acceptor also for anaerobic protozoa [45], causing a shift in the fermentation pattern quite similar to the phenomena reported in this study.

After all, it is the oxygen consumption of the aerobic and facultatively anaerobic members of the microbial community residing in the gut periphery, fu-

eled mainly by the electron donors provided by the fermentative processes in the gut center, that allows an overall fermentative hindgut metabolism to be maintained. If the oxic shell is extended by exposure of the termites to hyperbaric oxygen, the anaerobic protozoa and the spirochetes are killed, and the hindgut redox potential rises to positive values [4]. It was shown that the re-establishment of the low redox potential after a hyperbaric oxygen treatment of wood-feeding termites is correlated with an increase of viable counts of aerobic bacteria [47].

Taking into account the accumulated evidence of this study and of previous findings [10,14,48], we have to conclude that the termite hindgut is far from being a simple, anoxic fermenter, but that host and microbial activities render it an axially and radially structured, heterogeneous system characterized by steep gradients of metabolites. Such gradients should again govern the spatial arrangement of individual members of gut microbiota, and might be the key to the growth requirements of many so far uncultivated species. To arrive at a final model of carbon and electron flow in the hindgut of *R. flavipes* and other wood-feeding termites, it will be important to determine both the location and the in situ activities of the various physiological groups of bacteria whose presence, relative number, and potential activities were demonstrated in this study, and to clarify the nature and function of the large proportion of hindgut bacteria which still escape cultivation.

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