

Iron reduction in the metal-rich guts of wood-feeding termites

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ABSTRACT

Termites play important roles in lignocellulose and humus turnover in diverse terrestrial ecosystems, and are significant sources of global atmospheric methane and carbon dioxide. All known termite species engage in obligate, complex nutritional symbioses with their gut microbes to carry out such processes. Several hundred microbial species, representing a broad phylogenetic and physiological diversity, are found within the well-bounded, microliter-in-scale gut ecosystem of a given termite. However, most of these species have never been obtained in laboratory culture, and little can be said about their functional roles in the gut community or symbiosis. Herein, an unappreciated facet of the gut chemistry and microbiology of wood-feeding termites is revealed: the redox metabolism of iron. Gut fluids from field-collected termites contained millimolar amounts of ferrous iron and other heavy metals. When iron(III) hydroxides were amended to a filter paper diet of *Zootermopsis nevadensis*, a dampwood termite collected in the San Gabriel Mountains of Southern California, the specimens accumulated high levels of iron(II) in their guts. Additionally, iron was reduced at rapid initial rates in anoxic gut homogenates prepared from field-collected *Z. nevadensis* specimens. A *Clostridium* sp. and a *Desulfovibrio* sp. were isolated from dilution-to-extinction enrichments of *Z. nevadensis* gut contents and were found to reduce iron(III), as did the termite gut spirochete *Treponema primitia*. The iron in the guts of wood-feeding termites may influence the pathways of carbon- and electron-flow, as well as microbial community composition in these tiny ecosystems of global importance.

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INTRODUCTION

Termites are one of our planet's most diverse and abundant animal groups. There are *c.* 2600 termite species (Kambhampati & Eggleton, 2000). Estimates suggest that there are between 10^{15} and 10^{17} termite individuals, reaching biomass levels ranging from 5 to 60 g m⁻² in the *c.* 10^{13} m² of tropical forest (Sugimoto *et al.*, 2000). These animals are important players in numerous geochemical cycles. For example, by virtue of their keen ability to metabolize plant lignocellulose and humus rich soils, termites are thought to account for as much as 2% of global CO₂ emissions and 4% of global CH₄ emissions (Sugimoto *et al.*, 2000).

Termite guts contain a distinct morphological and genetic microbial diversity as yet found nowhere else on Earth as judged by past microscopic studies and gene inventories (Lilburn *et al.*, 1999; Friedrich *et al.*, 2001; Schmitt-Wagner *et al.*, 2003b). Ribosomal RNA gene inventories have confirmed that a single termite gut, ~one µL in volume, may contain in excess of 200

microbial species (Hongoh *et al.*, 2003) and that the composition of the gut community varies markedly with the species of termite examined (Lilburn *et al.*, 1999). As early as 80 years ago, anaerobic flagellate protozoa (*Eucarya*) became recognized for the key roles they play in the fermentation of cellulose and other plant-derived polysaccharides in termites that contain them. Such protozoa are found only in the 'lower' termite families. 'Higher' termites don't have these single-celled eukaryotes (Cleveland, 1923; Yamin, 1978).

All known termites contain a diversity of bacterial and archaeal species (Breznak, 1975; Breznak, 1982; Leadbetter & Breznak, 1996; Brauman *et al.*, 2001; Friedrich *et al.*, 2001; Hongoh *et al.*, 2003); however, it had not been until the last quarter century that key roles of prokaryotes in the mutualism became well established. Bacteria are now implicated as major sources of one of the termite's key energy nutrients, acetate (Odelson & Breznak, 1983). CO₂-reducing homoacetogenic bacteria process H₂ and lactate (Breznak, 1994; Tholen & Brune, 2000),

key intermediates of the cellulose fermentation, providing their host with as much as a third of its major energy substrate, acetate. It has been postulated that termite gut homoacetogenic bacteria outcompete gut methanoarchaea for H₂, thereby reducing the potential contribution of termites to the global methane budget (Breznak & Switzer, 1986; Brauman *et al.*, 1992). Alternatively, it has been proposed that these two physiological groups are actually tapping different electron donor pools *in situ*, thus are not really in direct competition at all (Tholen & Brune, 2000). Bacteria are also recognized to play critical roles in the N metabolism of their host, both via N₂-fixation (Breznak *et al.*, 1973; Lilburn *et al.*, 2001) and the fermentation of the insect's nitrogenous waste product, uric acid, into reusable nutrients (Potrikus & Breznak, 1980; Potrikus & Breznak, 1981). Both activities serve to alleviate the nutritional stresses encumbered by the insect's N-poor diet, ultimately allowing termites to, for example, grow and process more lignocellulose than would otherwise be possible.

Despite the discoveries of these important functions, we give sketchy appreciation for the full repertoire of microbe-mediated processes occurring in the guts of termites. Recent examples of this are the discoveries of iron reduction by unknown microbes in the less well studied termites that feed exclusively on iron rich soils (Kappler & Brune, 2002), and of a small but possibly significant S(IV)-(S0)-(S-II) cycle occurring in a diversity of termites (Brauman *et al.*, 1990; Trinkerl *et al.*, 1990; Kuhnigk *et al.*, 1996; Frohlich *et al.*, 1999).

In summary, the majority of the morphological and phylogenetic microbial diversity present in termite guts have yet to be recovered in laboratory culture. With the aim of better characterizing the chemistry of the habitat where these diverse microbes live, and in the hope of designing more effective cultivation media, we performed an inductively coupled plasma mass spectrometry (ICP-MS) analysis of gut fluids collected from two termite species, *Zootermopsis nevadensis* and *Incisitermes minor*. The results underscore how much we can continue to expect to learn about many key details of the termite gut ecosystem, and how the new insights into such details can help guide laboratory experiments aimed at identifying and reaching a better understanding of previously uncultivated gut microbiota.

MATERIALS AND METHODS

ICP-MS analysis of gut fluid

Zootermopsis nevadensis and *Incisitermes minor* specimens were collected in the San Gabriel National Forest, California, USA. Guts were extracted from chilled specimens using Teflon-coated forceps, placed on squares of clean Parafilm, and processed immediately. The hindgut compartment was ruptured gently with the forceps, and *c.* 5 µL of gut fluid from *Z. nevadensis*, or *c.* 1 µL from *Incisitermes minor*, was extracted using a 5 µL capillary micropipette. Fluids collected from about

Table 1 ICP-MS analysis of termite hindgut fluids

Element	<i>Zootermopsis nevadensis</i> gut fluid with contents*		Clarified gut fluid†	<i>Incisitermes minor</i> gut fluid with contents*
	mM	mg (g dry wt) ⁻¹	mM	mM
K	76	11.3	99	150
P	41	4.84	42	not determined
Na	19	1.66	25	145
Mg	5.7	0.52	5.5	40
Ca	2.4	0.37	1.4	11
Fe	1.7	0.36	0.2	1.1
Zn	0.55	0.14	0.01	0.40
Al	0.54	0.06	0.04	1.2
Mn	0.09	0.02	0.04	0.35
Ba	0.09	0.05	0.01	0.03
Cu	0.07	0.02	0.02	0.09

Background contamination was less than 1–10% for all elements analysed, and all measurements were made with 20% or better precision.

*Corresponds to the elemental composition of the particulate-laden luminal contents (including gut fluids) at the time of harvest from the hindgut.

†Corresponds to the elemental composition of the gut fluid that had been cleared of particles via centrifugation after harvest from the hindgut.

200 specimens of each species were pooled in 1.5 mL centrifuge tubes. For analysis of the total elemental composition of luminal contents, particulate-laden suspensions were amended with double distilled HNO₃ to a final acid concentration of 40% v/v, and heated at 95 °C for 2 h. In other analyses, samples were clarified via centrifugation prior to acid digestion (i.e. to remove microbial cells, wood particles, and other undissolved particulates present in the gut fluid). Data reported in Table 1 reflect the elemental composition of the original particulate-laden luminal contents (including gut fluids) vs. the original gut fluid that had been cleared of particles via centrifugation. After acid digestion, all solutions were further clarified via centrifugation at *c.* 15 294 g and diluted with deionized water to a final concentration of 1% v/v HNO₃. Serial 10-fold dilutions of samples were made in 1% v/v HNO₃ in order to obtain concentration ranges suitable for accurate ICP-MS analysis. Samples were analysed using both Hewlett Packard 4500 and Finigan Element ICP-MS instruments; conventional ICP-MS standards were employed. To evaluate the extent of metal contamination occurring during experimental manipulations, 5 µL volumes of deionized water were handled with the same utensils, exposed to the similar surfaces, pooled, and processed in otherwise identical fashion to the gut fluids. Background contamination was less than 1–10% for all elements analysed, and measurements were made with 20% or better precision.

Analysis of reduced iron

The ferrozine assay (Stookey, 1970) with minor modifications was used for the colourimetric determination of reduced and total iron present in the hindguts of freshly collected *Z.*

nevadensis specimens as well as those placed on defined diets in the laboratory. For the latter, groups of 10–15 worker larvae were placed on a diet of Whatman no. 1 filter paper impregnated with either 300 mM poorly crystalline iron hydroxides (Schwertmann & Cornell, 2001) or with water alone, and incubated at 23 °C in the dark. After 48 h, specimens were processed for iron and microscopic analyses. Immediately upon extraction, 10 individual guts were placed in 1 mL of 1 M HCl and disrupted in an acid-washed, ground-glass homogenizer. After passing the homogenate through a 0.22 µm filter, the solution was processed using the standard ferrozine reagent (Stokey, 1970). To complete the back calculation for gut iron: the fresh and dry weights of hindguts extracted from representative, *Z. nevadensis* specimens (average = 38.5 mg fresh weight, $n = 6$) were determined to be 10.3 mg and 2.7 mg, respectively, indicating the volume of fluid in the gut and gut-tissues of analysed specimens to be 7.6 µL.

To determine the potential rates of iron reduction from gut contents: 5 guts were extracted; aspirated into a 1 mL tuberculin syringe with 4YACo medium [identical in composition to 2YACo medium (Leadbetter *et al.*, 1999) except 4% v/v yeast autolysate was used]; and ruptured upon their passage through a 22-gauge syringe needle into 5 mL of the iron(III) hydroxide amended, 4YACo medium with a H₂/CO₂ (80%/20% v/v) headspace. Duplicate samples were removed from reaction vessels every 15–30 min during the first 5 h of incubation and analysed using ferrozine reagents. For analysis of mineral products formed during metabolism of pure cultures: XRD was performed using a Scintag Pad V X-ray Powder Diffractometer and Raman spectroscopy using a Renishaw M1000 Micro Raman Spectrometer System; authentic mineral standards were obtained from the Division of Geology & Planetary Science at Caltech.

Strain enrichment and isolation

The 4YACo medium containing 50 mM bromoethane sulphonate (to inhibit methanogenesis) was prepared under an anoxic, H₂/CO₂ (80/20, v/v) headspace as previously described for 2YACo media (Leadbetter *et al.*, 1999) and amended with 2.5 mM poorly crystalline iron(III) hydroxides (Lovley & Phillips, 1986a). Dilution-to-extinction enrichment and strain isolation procedures have been described (Leadbetter & Breznak, 1996; Leadbetter *et al.*, 1999). *Clostridium* ZIRB-1 and *Treponema* ZAS-2 are available from the German Culture collection as DSM 15498 and DSM 12427, respectively.

Phylogenetic characterization of isolates

Details of the methods employed during the amplification and cloning of near-full-length rRNA-encoding genes have been summarized elsewhere (Leadbetter & Greenberg, 2000; Flagan *et al.*, 2003; Salmassi & Leadbetter, 2003). Sequencing was performed at the DNA Sequencing Core Facility at the Beckman

Institute of Caltech using the dideoxy chain termination method, Sequenase (United States Biochemical), and a Perkin-Elmer ABI 373 A automatic sequencer. Sequence reads were assembled and edited using SEQUENCHER software for Windows (Genecodes). Multiple sequence alignments and phylogenetic analyses were performed using the ARB freeware package (www.arb-home.de/) running within the Linux environment (Ludwig *et al.*, 2004). Trees were constructed using PUZZLE-MAP 5.0 maximum likelihood analyses (Schmidt *et al.*, 2002). For strain ZIRB-1 1145 unambiguous, aligned nucleotide positions were used in a 10 000 puzzling step analysis; for strain ZIRB-2 1004 unambiguous, aligned nucleotide positions were used.

Other sequences included in tree construction were selected on the basis of their close similarity or close clustering with the isolates (i.e. after performing preliminary analyses using NCBI BLAST, the RDP-II sequence match function), and treeing algorithms within ARB. Phylogenetic tree layout-editing was performed using TREEVIEW 1.6.6 for Windows (Page, 1996). The sequences of the rDNA encoding genes for strains ZIRB-1 and ZIRB-2 have been submitted to GenBank as AY532163 and AY532164, respectively.

RESULTS

Termite gut fluids contain millimolar amounts of ferrous iron and high µmolar amounts of several other heavy metals

Gut fluids from several hundred specimens of *Zootermopsis nevadensis*, a dampwood termite, and *Incisitermes minor*, a drywood termite, were collected and analysed using ICP-MS. Fluids contained four elements (sodium, potassium, magnesium, and phosphorous) that we had expected to encounter in high concentrations based on a preliminary characterization of *Zootermopsis angusticollis* gut-fluid reported 25 years ago (Yamin, 1978). However, because many animal-microbe symbioses are typified by conditions of iron limitation (Graf & Ruby, 2000), we were surprised that the gut contents from freshly collected specimens of *Z. nevadensis* and *I. minor* contained 1.7 and 1.1 millimolar total iron, respectively, and notable amounts of other heavy metals (Table 1). Colorimetric analysis of *Z. nevadensis* gut contents yielded a similar value for iron (1.3 mM), 92% of it present as the reduced, ferrous form. Gut fluid, obtained by centrifugation of the gut contents to remove microbial cells and wood or other particulates prior to acid digestion, contained substantially less iron (0.2 mM, Table 1), suggesting that the metal is present either as an insoluble form in the gut fluid, or is sequestered within microbial cells, or is a component of not-yet-digested-dietary particles. As many forms of iron(III) serve as electron sinks in the physiology of diverse bacteria (Arnold *et al.*, 1986; Lovley & Phillips, 1986b; Straub *et al.*, 2001; Schröder *et al.*, 2003), we were interested in whether iron reducing microbes might occur and be active in the hindgut of *Z. nevadensis*.

Ferric iron oxides become reduced in the gut of *Zootermopsis nevadensis* termites

To explore whether iron is reduced upon its introduction into the hindgut, we amended poorly crystalline iron(III) hydroxides to the filter paper diet of laboratory-maintained *Z. nevadensis* specimens. Within 48–72 h, termites fed with iron-supplemented diet developed an obvious darkening in their abdomens compared to those fed with only filter paper (Fig. 1A). Upon extraction, the hindguts of iron-fed specimens appeared chalky and opaque, speckled with small dark zones approximately 50 μm in diameter (Fig. 1B); guts from the control group remained tan-coloured, translucent, and without any dark mottling. Close examination of the epithelium of iron-fed specimens revealed each dark zone to be comprised of a dark material containing numerous bacillus-like and spiral forms measuring *c.* 1.5–3 by 1 μm in dimension (Fig. 1C). Ferrozine analysis revealed that the gut of iron-fed specimens had increased 10-fold over that of the controls, reaching 15.9 ± 6.6 mmol total iron ($n = 2$ homogenates each comprised of 10 guts). Strikingly, 73% (11.6 ± 3.7 mmol) of the accumulated gut iron occurred as iron(II). In complementary experiments using specimens freshly collected from the field, we examined the rate at which iron was reduced in *Zootermopsis*-gut homogenates. When disrupted gut contents were incubated in 4YACo medium, iron(III) was reduced at initial rates ranging from 4.1 to 8.8 $\mu\text{moles iron(II) generated} \cdot \text{h}^{-1} \cdot (\text{g fresh weight termite})^{-1}$ [$n = 3$ 5-gut homogenates]. The reaction mixture used during this analysis contained complex organic carbon and was H_2 -replete, however, the nature of the electron donor(s) used during the process was not further examined. Iron(III) was not reduced in gut-free controls. Such rates of reduction compared well with those previously reported (Brauman *et al.*, 1992) for two electron sink reactions of importance in the *Zootermopsis*-gut ecosystem: CO_2 -reductive methanogenesis [$1.33 \mu\text{moles CO}_2$ -derived methane generated $\cdot \text{h}^{-1} \cdot (\text{g fresh weight termite})^{-1}$] and CO_2 -reductive acetogenesis [$0.33 \mu\text{moles CO}_2$ -derived acetate generated $\cdot \text{h}^{-1} \cdot (\text{g fresh weight termite})^{-1}$]. Iron(III)/iron(II) redox potentials vary as a function of the nature and concentration of the iron minerals in question, but generally are more positive than -100 mV under conditions encountered in many anaerobic ecosystems (Thamdrup, 2000; Straub *et al.*, 2001). Because of this, iron has the potential to serve as a sink for lignocellulose-derived electrons in the guts of *Z. nevadensis*, although it would not necessarily be in competition for the same exact electron donor(s) used during CO_2 -reductive acetogenesis and methanogenesis.

The iron(II) mineral vivianite is generated during the *in vitro* cultivation of isolated termite gut microbes

To begin examining whether microbes might play roles in gut iron reduction, dilution-to-extinction anaerobic enrichment cultures were initiated by inoculating and serially diluting

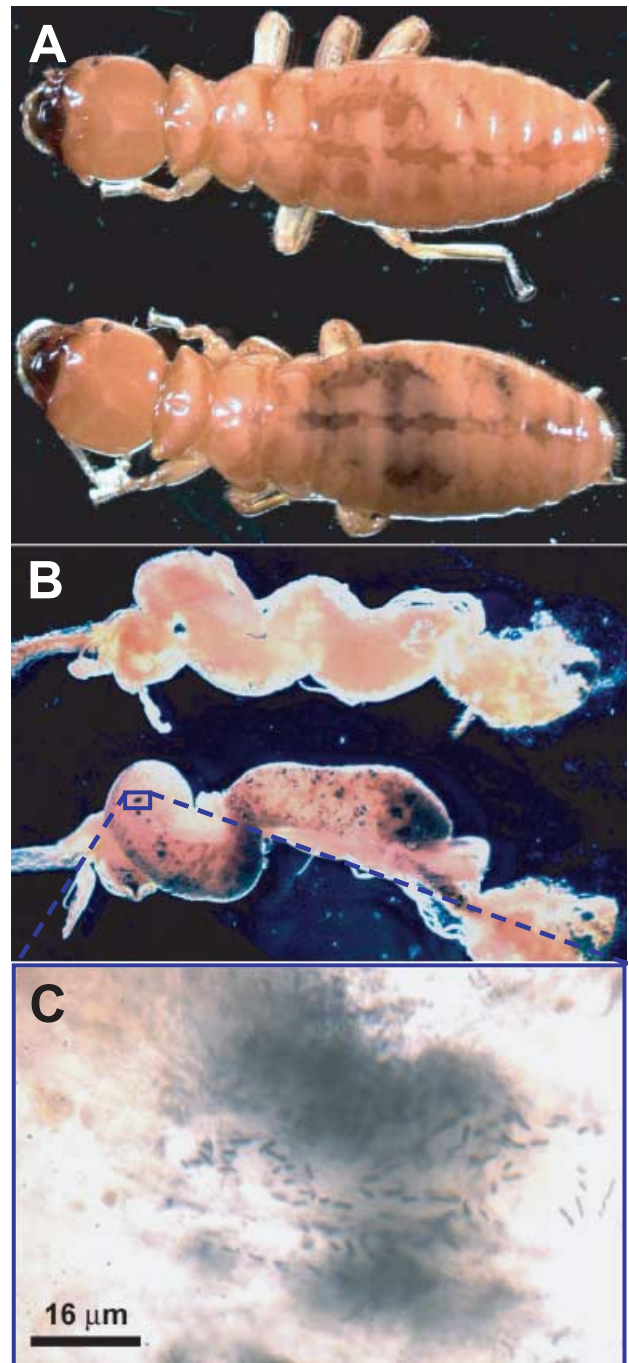


Fig. 1 Ingested iron hydroxides reduced in the *Zootermopsis nevadensis* gut. (A) Specimens fed with a filter paper plus water diet alone (top) or with orange-coloured iron(III) hydroxides (below). Within 48 h, the abdomen of iron-fed specimens darkened considerably. (B) Gut extracted from a specimen fed with a control diet (top) vs. one amended with orange-coloured iron(III) hydroxides (below). Note the chalky opacity and dark spots associated with the gut from the iron-fed specimen. (C) Light micrograph magnifying a representative dark spot on the gut epithelium of an iron-fed specimen. Note the numerous bacillus-like forms associated with the dark material.

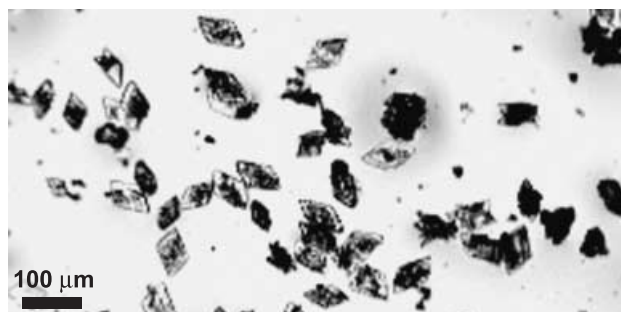


Fig. 2 Representative vivianite [iron(II) phosphate] crystals formed after the reductive metabolism of poorly crystalline, iron(III) hydroxides by any of the three iron metabolizing isolates representing the bacterial phyla *Firmicutes*, *Proteobacteria*, and *Spirocheates*.

disrupted guts of freshly collected *Z. nevadensis* specimens into 4YACo medium amended with 2.5 mM poorly crystalline iron(III) hydroxides. Over an 8-week incubation period, the iron hydroxides in the six dilution-enrichment tubes containing a range from 1 to $1 \cdot 10^{-5}$ gut equivalents had either darkened considerably or had been transformed into a white crystalline product. Colorimetric analyses indicated that the iron in such cultures had been fully reduced, whereas it remained as iron(III) oxide in both uninoculated controls and culture tubes in which the viable inoculum had been diluted to extinction (i.e. containing $\leq 1 \cdot 10^{-6}$ gut equivalents). The results imply that upwards of 10^7 iron reducing bacteria (ml *Zootermopsis*-gut fluid) $^{-1}$ can be cultivated with relative ease using existing media formulations.

Two distinct bacterial strains were isolated from dilution-to-extinction cultures and were demonstrated to catalyse the reduction of poorly crystalline iron(III) hydroxides *in vitro*. The first, strain ZIRB-1, was isolated from a dilution indicating its abundance to be on the order of 10^4 cells · (ml of gut fluid) $^{-1}$. The isolate reduced poorly crystalline iron(III) hydroxide and iron(III) phosphate precipitates to iron(II), which ultimately precipitated as a white, crystalline product (Fig. 2). The possible reduction of iron(III) citrate was not examined. The culture did not reduce either EDTA- or NTA-chelated iron(III). The reasons for this are unknown, but the results were surprising inasmuch as many microbes preferentially reduce iron(III) chelates over the less soluble forms (Straub & Schink, 2004). X-ray diffraction (XRD) and Raman spectroscopic analyses revealed that the white crystals were the mineral vivianite, iron(II) phosphate. Strain ZIRB-1 also grew robustly in 4YACo medium under fermentative conditions (i.e. without any added iron). When such cultures were pasteurized after growth and thereafter amended with iron, the iron was not reduced, suggesting that active cells, not chemically reactive metabolic products, were necessary for iron reduction. Cells of strain ZIRB-1 were slowly motile rods measuring 3.2 by 1.5 μm in dimension (Fig. 3A). The SSU rRNA from ZIRB-1 gathers within the *Peptostreptococcaceae*,

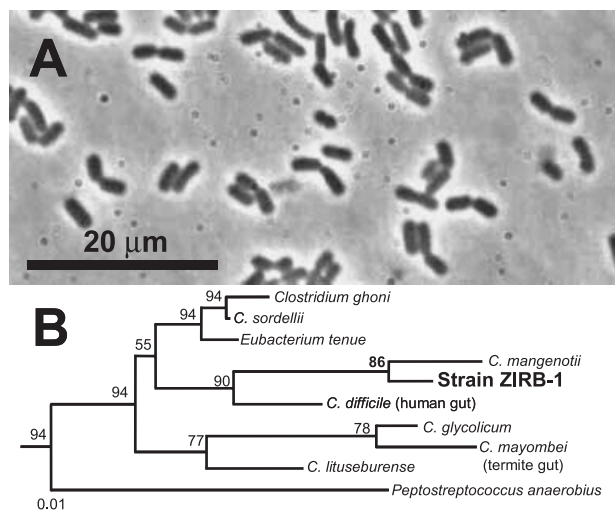


Fig. 3 Morphology and phylogeny of Strain ZIRB-1. (A) Phase-contrast micrograph of vegetative cells. (B) SSU rRNA-based maximum-likelihood phylogeny of the isolate and other selected species of *Clostridia* (*Firmicutes*). Bar represents the evolutionary distance as changes per nucleotide position, determined by measuring the lengths of the horizontal lines connecting the species.

and was most similar, sharing 99.1% sequence identity with a 639 residue partial rRNA gene obtained from *Clostridium* sp. MH8, a nitrate-reducing earthworm gut isolate (Ihssen *et al.*, 2003). Among full-length genes, the strain ZIRB-1 gene shared 97.5% sequence identity with the SSU rRNA gene from *Clostridium manganotii* (Fig. 3B), a little-studied organism isolated from Ivory Coast soils (Prévot & Zimmès-Chaveron, 1947). The gene also shared 96.3% to 96.6% identity with three short (~300 residue) rDNA clones ('UN36', 'UN84', and 'UN109') amplified from the gut contents of a Japanese termite, *Reticulitermes speratus* (Ohkuma & Kudo, 1996). Non-full length gene sequences (i.e. from earthworm- and *Reticulitermes*-guts) were not included in construction of Fig. 3(B). Cells of strain ZIRB-1 were not observed to form endospores or to be resistant to pasteurization.

A second isolate, strain ZIRB-2, was obtained from a 10^{-5} dilution of gut contents, implying its *in situ* population to be on the order of 10^7 cells per ml of gut fluid. The SSU rRNA-encoding gene from this highly motile, 3.0 by 0.7 μm spirillum shared 92.6% and 91.0% sequence identity, respectively, with a rDNA genes cloned from the gut contents of the termite *Reticulitermes speratus* (Hongoh *et al.*, 2003) and from *Desulfovibrio cuneatus* strain STL1, a psychrotolerant, sulphate-reducing isolate from freshwater sediments (Sass *et al.*, 1998) (Figs 4A,B). Despite its affiliation with a genus typified by species exhibiting robust, sulfidogenic growth using lactate plus sulphate, this isolate grew only slowly and to low cell yields when the growth medium was supplemented with these two (or any other tested) substrates. In cultures of strain ZIRB-24 grown in YACo medium amended with poorly crystalline

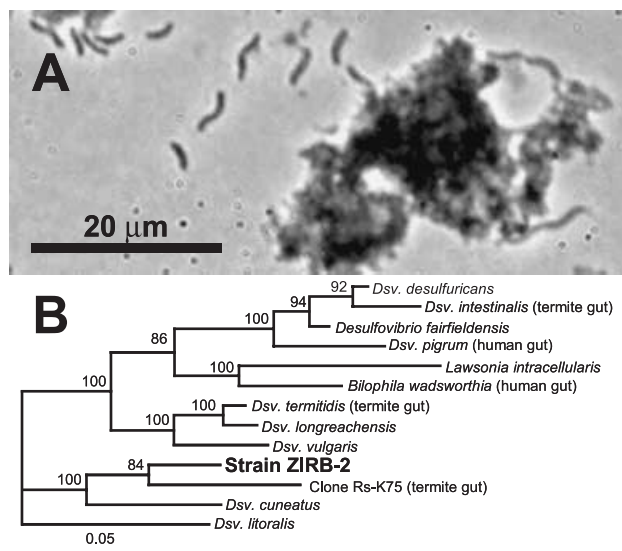


Fig. 4 Morphology and phylogeny of Strain ZIRB-2. (A) Phase contrast micrograph of cells loosely associated with partially reduced iron hydroxides. (B) SSU rRNA-based maximum-likelihood phylogeny of strain ZIRB-2 and other selected members of the supergenus *Desulfovibrio* (δ -Proteobacteria).

iron(III) hydroxide, vivianite and an uncharacterized brown, magnetic precipitate accumulated. Strain ZIRB-2 did not grow in the medium without the addition of iron or other electron acceptors.

A previously characterized *Zootermopsis*-gut spirochetal isolate, *Treponema primitia* ZAS-2 (Leadbetter *et al.*, 1999; Graber *et al.*, 2004) was examined for its ability to reduce iron, a property not previously reported for any member of the phylum *Spirochaetes*. *Treponema primitia*, a strain known to be capable of good growth and CO₂-reductive homoacetogenesis in the test medium, exhibited the property of slowly reducing poorly crystalline iron hydroxides (forming vivianite) over several weeks. The rate of iron reduction by this spirochete was markedly accelerated by adding anthraquinone disulphonate (AQDS), a soluble, model compound for the quinone moieties of humic acids and also a widely examined extracellular electron shuttle between bacteria and iron minerals (Lovley *et al.*, 1996). All three isolates reduced AQDS to its corresponding hydroquinone in the absence of added iron. When cultures of *T. primitia* ZAS-2 were grown in media without added iron, followed by pasteurization after the end of growth, iron added to culture fluids post heat-treatment was not reduced. This suggested that active, growing cells, and not chemically reactive metabolic products, were necessary for iron reduction.

The nature and full spectrum of electron donors utilized, and other products formed during the reduction of iron by the three strains was not determined. Whether energy is conserved by cells during the process (i.e. in a respiration) or whether iron stimulates fermentative growth has not yet been estab-

lished for any of the three strains. In a 'facilitated fermentation', the transfer of reducing equivalents to an inorganic electron such as what iron does would not be coupled to energy conservation via electron transport processes in the membrane, but could stimulate substrate level phosphorylation(s) occurring as a result of oxidation reactions. Such physiological possibilities await further investigation.

DISCUSSION

These findings establish that the hindgut ecosystem of the wood-feeding, 'lower' dampwood termite *Zootermopsis nevadensis* contains (1) significant concentrations of iron(II) and other heavy metals; (2) a potent iron-reducing capacity; and (3) a diversity of microbes capable of reducing iron *in vitro*. These findings were initially surprising to us, as most discussions on the terminal electron acceptors for anaerobic processes occurring in the guts of wood-feeding termites have focused on CO₂ reduction to acetate or methane (Breznak & Brune, 1994). Many host-microbe associations are typified by conditions of extreme iron limitation (Graf & Ruby, 2000; Bina *et al.*, 2003), and elevated iron is actually known to cause gastrointestinal stress to the point of killing several invertebrates. For example, iron(III) is used as the key ingredient in over-the-counter biocides used to control certain garden pests such as molluscs. But perhaps, the results are not as surprising when the iron content of the wood and bark is considered. A study on the iron content of six species of tree (Misra *et al.*, 1993), including *Pinus ponderosa* (the diet of the field-collected *Zootermopsis* specimens used in this study), reported that wood generally contained 0.008–0.016 mg Fe · (g dry wt wood)⁻¹, and bark 0.002–0.047 mg Fe · (g dry wt bark)⁻¹. This would mean that *Z. nevadensis* would only need to enrich iron by *c.* 10–200 fold over its dietary material to reach the observed 0.36 mg Fe · (g dry wt gut contents)⁻¹ (Table 1). It remains unclear how the other heavy metals in the termite gut are acquired or maintained in the termite-gut ecosystem, or how the insect and its microbes protect themselves from the possible toxic effects of these elements. It may also be possible that in addition to feeding on wood, *Z. nevadensis* and *Incisitermes minor* might ingest at least small amounts of soil, but to our knowledge such behaviour has not been reported for these species.

Termites have been reported to be a source of biological magnetite particles, but the details of the biosynthesis and location of them within the insect has not been discussed (Maher, 1998). A report on soil-feeding termites has indicated that iron present in the ingested soil is reduced upon its passage through the gut (Kappler & Brune, 2002), almost certainly by a diverse gut microbiota that remain to be elucidated. The concentration of gut iron was not reported in that study, but it likely closely mirrors that of the ingested soil, which was reported to range from 0.9 to 9 mg Fe · (g dry wt soil)⁻¹. In comparison, the field collected specimens of *Z. nevadensis*

examined in this study contained $0.36 \text{ mg Fe} \cdot (\text{g dry wt gut contents})^{-1}$; when fed with diet rich in iron(III), specimens accumulated gut iron to *c.* 10-fold higher levels. Because *Z. nevadensis* is amenable to laboratory cultivation and can be placed on defined diets (e.g. cellulose filter paper with and without characterized preparations of iron or other metals), it becomes an excellent candidate for performing studies aimed at reaching a better understanding of iron metabolism in other termite species or other feeding guilds. In that regard, it should be noted that geophagy is the *dedicated* nutritional lifestyle of roughly half of all termite species (Brauman *et al.*, 2000). Termites that are exclusively soil-feeding are extremely difficult to maintain in laboratory settings, can not be placed on defined diets at this time, and are only found restricted to 'exotic' locales, so even less is known about the details of their gut microbiology than of wood-feeders (Schmitt-Wagner & Brune, 1999; Tholen & Brune, 1999; Friedrich *et al.*, 2001; Schmitt-Wagner *et al.*, 2003a).

Termites may be significant sites of microbial reduction of iron globally. The potential (unrestricted) rates of iron reduction observed in *Z. nevadensis* gut homogenates during this study corresponds to $15.3\text{--}32.8 \mu\text{moles iron(II) generated} \cdot \text{h}^{-1} \cdot (\text{g dry weight})^{-1}$. Such compare quite strongly with potential rates observed in other anaerobic environments [e.g. at the surface of the roots of marine and freshwater macrophytes (King & Garey, 1999)], reported to be $0.5\text{--}15 \mu\text{moles iron(II) generated} \cdot \text{h}^{-1} \cdot (\text{g dry weight})^{-1}$. Because there are so many uncertainties about the concentrations of gut iron and rates of its reduction in others of the *c.* 2600 species of termite found worldwide, it is difficult to extrapolate or predict with any certainty how important termites might be to iron cycling processes globally. However, it should be noted that *conservative* estimates place termite biomass abundances at $\geq 5 \text{ g m}^{-2}$ for *c.* 10^{13} m^{-2} of the Earth's tropical forests (Sugimoto *et al.*, 2000), opening the possibility that Tg amounts of iron might be reductively processed by termite gut microbes every year.

Iron(III) is considered to be superior to CO_2 for use as an electron sink during anaerobic microbial metabolism (Lovley & Phillips, 1986b). For example, iron reducing bacteria exhibit lower thresholds for H_2 than do hydrogenotrophic methanarchaea (Lovley & Goodwin, 1988). Curiously, despite the high iron contents in their diets and gut contents, soil-feeding termites and *Zootermopsis* sp. emit methane at some of the highest rates observed in termites in general. Indeed, even the addition of elevated iron(III) to the diet of *Zootermopsis* did not depress gut methanogenesis by these insects in the least (unpublished results), even though iron accumulates its reduced form to high levels in the guts of termites fed with such a diet. This suggests that different pools of electron donors may be being oxidized in what would be essentially noncompeting processes. As an alternative to H_2 and lactate, which are known or postulated to serve as the electron donors for CO_2 -reductive methanogenesis and or

acetogenesis, acetate or lignin aromatic monomers might be utilized during gut iron reduction. Both are known to be metabolized by several iron reducing bacteria (Coates *et al.*, 2001). Such will have to be further evaluated in future studies on isolates strains and gut homogenates.

The isolation of a novel, abundant iron reducing *Desulfovibrio* sp. during the course of this study raises several intriguing issues, and extends the possible roles for members in this genus in the hindgut ecophysiology of termites. Several *Desulfovibrio* strains, including two described as new species, have previously been isolated from the guts of diverse soil- and wood-feeding termites on the basis of their ability to reduce sulphate to sulphide (Brauman *et al.*, 1990; Trinkerl *et al.*, 1990; Frohlich *et al.*, 1999). A small but significant cycling of low concentrations of S in the guts of termites has been proposed (Kuhnigk *et al.*, 1996). The presence of *Desulfovibrio*-like organisms and other closely related δ -*Proteobacteria* in termite guts has also been deduced via rRNA gene inventories. Thus, it is very possible that S and Fe cycles in the gut may be closely coupled, in some instances mediated by the same organisms. As an interesting side note, the presence of iron may also serve to bind and so detoxify sulphide generated in the hindgut. Breznak and coworkers have previously noted that many termite gut isolates are inhibited by even low concentrations of sulphide, which is often employed as an O_2 scavenging agent or S source during the cultivation of strict anaerobes (Breznak & Switzer, 1986; Breznak *et al.*, 1988). The possibility exists then that free sulphide occurs only rarely in these gut ecosystems, becoming bound to iron as soon as it is generated.

The iron(III) reduced in the guts of termites also leads to questions about the fate of the iron(II) that is generated in the process. The peripheral regions of termite hindguts are known to be microoxic in all termite species that have been examined using O_2 -microelectrodes (Brune *et al.*, 1995; Brune & Friedrich, 2000). Thus, iron(II) generated by the anaerobic microbiota likely becomes subject to a rapid re-oxidation at the surface of the gut epithelium (i.e. either by iron oxidizing bacteria (Emerson & Moyer, 1997) or by Fenton reactions involving hydrogen peroxide). Fenton chemistry is well established to be employed by lignin-degrading terrestrial fungi (Kirk & Farrell, 1987). While the generation of reactive oxygen radicals would likely pose an immediate stress to the gut microbiota, it might also serve as a potent chemical catalyst in the partial release of utilizable nutrient from recalcitrant lignin fractions in the termite's foodstuff.

In summary, iron and other heavy metals occur in abundance in the guts of wood-feeding termites and might be expected to influence the microbial community and processes of the gut ecosystem in a number of different ways. We extend both the number of environments in which iron reduction is known to occur, as well as the roster of recognized bacterial phyla found capable of performing such reactions. After more than a century and a half of study, we have much to learn, and

can expect to continue to be surprised by the full diversity of the key processes and microbiota at play in the hindguts of termites.

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REFERENCES

- Arnold RG, Olson TM, Hoffmann MR (1986) Kinetics and mechanism of dissimilative Fe (III) reduction by *Pseudomonas* sp. 200. *Biotechnology and Bioengineering* **28** (11), 1657–1671.
- Bina J, Zhu J, Dziejman M, Faruque S, Calderwood S, Mekalanos J (2003) ToxR regulon of *Vibrio cholerae* and its expression in vibrios shed by cholera patients. *Proceedings of the National Academy of Sciences of the United States of America* **100** (5), 2801–2806.
- Brauman A, Doré J, Eggleton P, Bignell D, Breznak JA, Kane MD (2001) Molecular phylogenetic profiling of prokaryotic communities in guts of termites with different feeding habits. *FEMS Microbiology Ecology* **35** (1), 27–36.
- Brauman A, Kane MD, Labat M, Breznak JA (1992) Genesis of acetate and methane by gut bacteria of nutritionally diverse termites. *Science* **257** (5075), 1384–1387.
- Brauman A, Koenig JF, Dutreix J, Garcia JL (1990) Characterization of 2-sulfate-reducing bacteria from the gut of the soil-feeding termite, *Cubitermes speciosus*. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **58** (4), 271–275.
- Brauman A, Bignell DE, Tayasu I (2000) Soil-feeding termites: biology, microbial associations, and digestive mechanisms. In: *Termites: Evolution, Sociality, Symbioses, Ecology* (eds Abe T, Bignell DE, Higashi M). Kluwer Academic Publishers, Dordrecht/Boston/London, pp. 233–260.
- Breznak JA (1975) Symbiotic relationships between termites and their intestinal microbiota. *Symposium of the Society for Experimental Biology* **29**, 559–580.
- Breznak JA (1982) Intestinal microbiota of termites and other xylophagous insects. *Annual Reviews of Microbiology* **36**, 323–343.
- Breznak JA (1994) Acetogenesis from carbon dioxide in termite guts. In: *Acetogenesis* (ed. Drake HL). Chapman & Hall, New York, pp. 303–330.
- Breznak JA, Brill WJ, Mertins JW, Coppel HC (1973) Nitrogen fixation in termites. *Nature* **244** (5418), 577–580.
- Breznak JA, Brune A (1994) Role of microorganisms in the digestion of lignocellulose in termites. *Annual Reviews of Entomology* **39**, 453–487.
- Breznak JA, Switzer JM (1986) Acetate synthesis from H₂ plus CO₂ by termite gut microbes. *Applied and Environmental Microbiology* **52**, 623–630.
- Breznak JA, Switzer JM, Seitz HJ (1988) *Sporomusa termitida* sp. nov., an H₂/CO₂-utilizing acetogen isolated from termites. *Archives of Microbiology* **150** (3), 282–288.
- Brune A, Emerson D, Breznak JA (1995) The termite gut microflora as an oxygen sink: microelectrode determination of oxygen and pH gradients in guts of lower and higher termites. *Applied and Environmental Microbiology* **61** (7), 2681–2687.
- Brune A, Friedrich M (2000) Microecology of the termite gut: structure and function on a microscale. *Current Opinions in Microbiology* **3**, 263–269.
- Cleveland L (1923) Symbiosis between termites and their intestinal protozoa. *Proceedings of the National Academy of Sciences of the United States of America* **9**, 424–428.
- Coates JD, Bhupathiraju VK, Achenbach LA, McInerney MJ, Lovley DR (2001) *Geobacter hydrogenophilus*, *Geobacter chapellei* and *Geobacter grbiciae*, three new, strictly anaerobic, dissimilatory Fe (III)-reducers. *International Journal of Systematic and Evolutionary Microbiology* **51** (2), 581–588.
- Emerson D, Moyer C (1997) Isolation and characterization of novel iron-oxidizing bacteria that grow at circumneutral pH. *Applied and Environmental Microbiology* **63** (12), 4784–4792.
- Flagan S, Ching W-K, Leadbetter JR (2003) *Arthrobacter* strain VAI-A utilizes acyl-homoserine lactone inactivation products and stimulates quorum signal biodegradation by *Variovorax paradoxus*. *Applied and Environmental Microbiology* **69** (2), 909–916.
- Friedrich MW, Schmitt-Wagner D, Lueders T, Brune A (2001) Axial differences in community structure of *Crenarchaeota* and *Euryarchaeota* in the highly compartmentalized gut of the soil-feeding termite *Cubitermes orthognathus*. *Applied and Environmental Microbiology* **67** (10), 4880–4890.
- Frohlich J, Sass H, Babenzien HD, Kuhnigk T, Varma A, Saxena S, Nalepa C, Pfeiffer P, König H (1999) Isolation of *Desulfovibrio intestinalis* sp. nov. from the hindgut of the lower termite *Mastotermes darwiniensis*. *Canadian Journal of Microbiology* **45** (2), 145–152.
- Graber JR, Leadbetter JR, Breznak JA (2004) Description of *Treponema azotonutricium* sp. nov. & *Treponema primitia* sp. nov., the first spirochetes isolated from termite guts. *Applied and Environmental Microbiology* **70** (3), 1315–1320.
- Graf J, Ruby EG (2000) Novel effects of a transposon insertion in the *Vibrio fischeri* glnD gene: defects in iron uptake and symbiotic persistence in addition to nitrogen utilization. *Molecular Microbiology* **37** (1), 168–179.
- Hongoh Y, Ohkuma M, Kudo T (2003) Molecular analysis of bacterial microbiota in the gut of the termite *Reticulitermes speratus* (*Isoptera*; *Rhinotermitidae*). *FEMS Microbiology Ecology* **44** (2), 231–242.
- Ihssen J, Horn MA, Matthies C, Gossner A, Schramm A, Drake HL (2003) N₂O-producing microorganisms in the gut of the earthworm *Aporrectodea caliginosa* are indicative of ingested soil bacteria. *Applied and Environmental Microbiology* **69** (3), 1655–1661.
- Kambhampati S, Eggleton P (2000) Chapter 1 – Taxonomy and phylogeny of termites. In: *Termites: Evolution, Sociality, Symbioses, Ecology* (eds Abe T, Bignell DE, Higashi M). Kluwer Academic Publishers, Dordrecht, Boston, London, pp. 1–24.
- Kappler A, Brune A (2002) Dynamics of redox potential and changes in redox state of iron and humic acids during gut passage in soil-feeding termites (*Cubitermes* spp.). *Soil Biology and Biochemistry* **34**, 221–227.
- King GM, Garey MA (1999) Ferric iron reduction by bacteria associated with the roots of freshwater and marine macrophytes. *Applied and Environmental Microbiology* **65** (10), 4393–4398.
- Kirk TK, Farrell RL (1987) Enzymatic ‘combustion’: the microbial degradation of lignin. *Annual Reviews of Microbiology* **41**, 465–505.
- Kuhnigk T, Branke J, Krekeler D, Cypionka H, König H (1996) A feasible role of sulfate-reducing bacteria in the termite gut. *Systematic and Applied Microbiology* **19** (2), 139–149.
- Leadbetter JR, Breznak JA (1996) Physiological ecology of *Methanobrevibacter cuticularis* sp. nov. & *Methanobrevibacter curvatus* sp. nov., isolated from the hindgut of the termite

- Reticulitermes flavipes*. *Applied and Environmental Microbiology* **62** (10), 3620–3631.
- Leadbetter JR, Greenberg EP (2000) Metabolism of acyl-homoserine lactone quorum-sensing signals by *Variovorax paradoxus*. *Journal of Bacteriology* **182** (24), 6921–6926.
- Leadbetter JR, Schmidt TM, Graber JR, Breznak JA (1999) Acetogenesis from H₂ plus CO₂ by spirochetes from termite guts. *Science* **283** (5402), 686–689.
- Lilburn TG, Kim KS, Ostrom NE, Byzek KR, Leadbetter JR, Breznak JA (2001) Nitrogen fixation by symbiotic and free-living spirochetes. *Science* **292** (5526), 2495–2498.
- Lilburn TG, Schmidt TM, Breznak JA (1999) Phylogenetic diversity of termite gut spirochaetes. *Environmental Microbiology* **1** (4), 331–345.
- Lovley DR, Coates JD, Blunt-Harris EL, Phillips EJP, Woodward JC (1996) Humic substances as electron acceptors for microbial respiration. *Nature* **382**, 445–448.
- Lovley DR, Goodwin S (1988) Hydrogen concentrations as an indicator of the predominant terminal electron accepting reactions in aquatic sediments. *Geochimica et Cosmochimica Acta* **52**, 2993–3003.
- Lovley DR, Phillips EJP (1986a) Availability of ferric iron for microbial reduction in bottom sediments of the freshwater tidal Potomac River. *Applied and Environmental Microbiology* **52** (4), 751–757.
- Lovley DR, Phillips EJP (1986b) Organic matter mineralization with reduction of ferric iron in anaerobic sediments. *Applied and Environmental Microbiology* **51** (4), 683–689.
- Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadlukumar Buchner A, Lai T, Steppi S, Jobb G, Forster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grumann S, Hermann S, Jost R, König A, Liss T, Lussmann R, May M, Nonhoff B, Reichel B, Strehlow R, Stamatakis A, Stuckmann N, Vilbig A, Lenke M, Ludwig T, Bode A, Schleifer KH (2004) ARB: a software environment for sequence data. *Nucleic Acids Research* **32** (4), 1363–1371.
- Maher BA (1998) Magnetite biomineralization in termites. *Proceedings of the Royal Society: Biology Sciences* **265** (1397), 733–737.
- Misra MK, Ragland KW, Baker AJ (1993) Wood ash composition as a function of furnace temperature. *Biomass and Bioenergy* **4** (2), 103–116.
- Odelson DA, Breznak JA (1983) Volatile fatty acid production by the hindgut microbiota of xylophagous termites. *Applied and Environmental Microbiology* **45** (5), 1602–1613.
- Ohkuma M, Kudo T (1996) Phylogenetic diversity of the intestinal bacterial community in the termite *Reticulitermes speratus*. *Applied and Environmental Microbiology* **62** (2), 461–468.
- Page RDM (1996) TREEVIEW: an application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* **12**, 357–358.
- Potrikus CJ, Breznak JA (1980) Anaerobic degradation of uric acid by gut bacteria of termites. *Applied and Environmental Microbiology* **40** (1), 125–132.
- Potrikus CJ, Breznak JA (1981) Gut bacteria recycle uric acid nitrogen in termites: a strategy for nutrient conservation. *Proceedings of the National Academy of Sciences of the United States of America* **78** (7), 4601–4605.
- Prévot AR, Zimmès-Chaveron J (1947) Étude d'une nouvelle espèce anaérobie de Côte d'Ivoire *Inflabilis mangenoti* n. sp. *Annales de l'Institut Pasteur (Paris)* **73**, 602–604.
- Salmassi TM, Leadbetter JR (2003) Analysis of genes of tetrahydrofolate-dependent metabolism from cultivated spirochaetes and the gut community of the termite *Zootermopsis angusticollis*. *Microbiology* **149**, 2529–2537.
- Sass H, Berchtold M, Branke J, König H, Cypionka H, Babenzien HD (1998) Psychrotolerant sulfate-reducing bacteria from anoxic freshwater sediment, description of *Desulfovibrio cuneatus* sp. nov. & *Desulfovibrio litoralis* sp. nov. *Systematic and Applied Microbiology* **21** (2), 212–219.
- Schmidt HA, Strimmer K, Vingron M, von Haeseler A (2002) TREE-PUZZLE: maximum likelihood phylogenetic analysis using quartets and parallel computing. *Bioinformatics* **18** (3), 502–504.
- Schmitt-Wagner D, Brune A (1999) Hydrogen profiles and localization of methanogenic activities in the highly compartmentalized hindgut of soil-feeding higher termites (*Cubitermes* spp.). *Applied and Environmental Microbiology* **65** (10), 4490–4496.
- Schmitt-Wagner D, Friedrich MW, Wagner B, Brune A (2003a) Axial dynamics, stability, and interspecies similarity of bacterial community structure in the highly compartmentalized gut of soil-feeding termites (*Cubitermes* spp.). *Applied and Environmental Microbiology* **69** (10), 6018–6024.
- Schmitt-Wagner D, Friedrich MW, Wagner B, Brune A (2003b) Phylogenetic diversity, abundance, and axial distribution of bacteria in the intestinal tract of two soil-feeding termites (*Cubitermes* spp.). *Applied and Environmental Microbiology* **69** (10), 6007–6017.
- Schröder I, Johnson E, de Vries S (2003) Microbial ferric iron reductases. *FEMS Microbiology Reviews* **27** (2–3), 427–447.
- Schwertmann U, Cornell RM (2001) Iron oxides in the laboratory: preparation and characterization, 2nd Completely Revised and Enlarged Edition. Jossey-Bass.
- Stookey LL (1970) Ferrozine: a new spectrophotometric reagent for iron. *Analytical Chemistry* **42** (7), 779–781.
- Straub K, Benz M, Schink B (2001) Iron metabolism in anoxic environments at near neutral pH. *FEMS Microbiology Ecology* **34**, 181–186.
- Straub KL, Schink B (2004) Ferrihydrite reduction by *Geobacter* species is stimulated by secondary bacteria. *Archives of Microbiology* **182** (2–3), 175–181.
- Sugimoto A, Bignell DE, Macdonald JA (2000) Chapter 19 – Global impact of termites on the carbon cycle and atmospheric trace gases. In: *Termites: Evolution, Sociality, Symbioses, Ecology* (eds Abe T, Bignell DE, Higashi M). Kluwer Academic Publishers, Dordrecht, Boston, London, pp. 409–435.
- Thamdrup B (2000) Bacterial manganese and iron reduction in aquatic sediments. In: *Advances in Microbial Ecology* (ed. Schink B). Kluwer Academic, New York, pp. 41–84.
- Tholen A, Brune A (1999) Localization and *in situ* activities of homoacetogenic bacteria in the highly compartmentalized hindgut of soil-feeding higher termites (*Cubitermes* spp.). *Applied and Environmental Microbiology* **65** (10), 4497–4505.
- Tholen A, Brune A (2000) Impact of oxygen on metabolic fluxes and *in situ* rates of reductive acetogenesis in the hindgut of the wood-feeding termite *Reticulitermes flavipes*. *Environmental Microbiology* **2** (4), 436–449.
- Trinkerl M, Breunig A, Schauder R, König H (1990) *Desulfovibrio termitidis* sp. nov., a carbohydrate-degrading sulfate-reducing bacterium from the hindgut of a termite. *Systematic and Applied Microbiology* **13** (4), 372–377.
- Yamin M (1978) Axenic cultivation of the cellulolytic flagellate *Trichomitopsis termopsidis* (Cleveland) from the termite *Zootermopsis*. *Journal of Protozoology* **25**, 535–538.