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Roles of Oxygen and the Intestinal Microflora in the Metabolism of Lignin-Derived Phenylpropanoids and Other Monoaromatic Compounds by Termites

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Prompted by our limited understanding of the degradation of lignin and lignin-derived aromatic metabolites in termites, we studied the metabolism of monoaromatic model compounds by termites and their gut microflora. Feeding trials performed with [*ring*-U-^{14}C]benzoic acid and [*ring*-U-^{14}C]cinnamic acid revealed the general ability of termites of the major feeding guilds (wood and soil feeders and fungus cultivators) to mineralize the aromatic nucleus. Up to 70% of the radioactive label was released as $^{14}CO_2$; the remainder was more or less equally distributed among termite bodies, gut contents, and feces. Gut homogenates of the wood-feeding termites *Nasutitermes lujae* (Wasmann) and *Reticulitermes flavipes* (Kollar) mineralized ring-labeled benzoic or cinnamic acid only if oxygen was present. In the absence of oxygen, benzoate was not attacked, and cinnamate was only reduced to phenylpropionate. Similar results were obtained with other, nonlabeled lignin-related phenylpropanoids (ferulic, 3,4-dihydroxycinnamic, and 4-hydroxycinnamic acids), whose ring moieties underwent degradation only if oxygen was present. Under anoxic conditions, the substrates were merely modified (by side chain reduction and demethylation), and this modification occurred at the same time as a net accumulation of phenylpropanoids formed endogenously in the gut homogenate, a phenomenon not observed under oxic conditions. Enumeration by the most-probable-number technique revealed that each *N. lujae* gut contained about 10^5 bacteria that were capable of completely mineralizing aromatic substrates in the presence of oxygen (about 10^8 bacteria per ml). In the absence of oxygen, small numbers of ring-modifying microorganisms were found (<50 bacteria per gut), but none of these microorganisms were capable of ring cleavage. Similar results were obtained with gut homogenates of *R. flavipes*, except that a larger number of anaerobic ring-modifying microorganisms was present (>5 × 10^3 bacteria per gut). Neither inclusion of potential cosubstrates (H_2, pyruvate, lactate) nor inclusion of hydrogenotrophic partner organisms resulted in anoxic ring cleavage in most-probable-number tubes prepared with gut homogenates of either termite. The oxygen dependence of aromatic ring cleavage by the termite gut microbiota is consistent with the presence, and uptake by microbes, of O_2 in the peripheral region of otherwise anoxic gut lumina (as reported in the accompanying paper [A. Brune, D. Emerson, and J. A. Breznak, Appl. Environ. Microbiol. 61:2681–2687, 1995]). Taken together, our results indicate that microbial degradation of plant aromatic compounds can occur in termite guts and may contribute to the carbon and energy requirement of the host.

Lignin is the second most abundant component of wood and one of the most abundant biopolymers on earth. However, because of its subunit structure consisting of nonrepetitive phenylpropane subunits randomly linked by various C—C and ether bonds, as well as its intimate association and chemical cross-linking with the carbohydrate fraction of plant cell walls, lignin is difficult to degrade enzymatically, and its presence in plant tissue also significantly impedes cellulose degradation (14). Except for a few lignin-solubilizing and/or -degrading actinomycetes (11, 20, 25), lignin degradation by microorganisms is largely the domain of aerobic fungi.

Certain terrestrial animals exploit lignin-rich plant materials as nutrient resources. Among the most important of these are termites, which, together with their gut and external microflora, contribute significantly to the mineralization of lignocellulosic plant residues and material derived from it (for example, humus) (8, 19). Although the mechanisms of cellulose digestion in termites have become much clearer over the past two decades, our understanding of lignin degradation remains incomplete and is surrounded by ambiguities (for a critical review see reference 4). Despite various reports and speculation concerning the ability of termites to degrade lignin, there is still no convincing evidence that the polymeric backbone of lignin is depolymerized during passage through the termite gut. Moreover, virtually nothing is known about the fate of the aromatic compounds that might constitute potential depolymerization products of lignin or that are present in relatively large amounts extraneous to the lignocellulosic cell wall, such as monomeric plant phenolics, lignans, depsides, and flavonoids.

The issue of lignin degradation in termites is particularly intriguing in that the hindgut, which is the largest and most heavily colonized portion of the termite gut, is generally considered to be an anoxic environment. To date, however, there is no known mechanism that allows depolymerization of lignin in the absence of oxygen. In fact, even degradation of small lignin-related molecules, typically 3,4-substituted benzenoids or phenylpropanoids, is among the slowest and mechanistically most challenging processes in the anaerobic degradation of

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TABLE 1. Mineralization of [*ring*-U-^{14}C]benzoate by termites belonging to different feeding guilds[a]

Species	Food source[b]	Distribution of ^{14}C (%)[c]				
		$^{14}CO_2$	Termite bodies	Residual food	Feces	Total
Wood-feeding termites						
Nasutitermes lujae	FD	23.7	13.5	32.8	0.9	70.9
	CA	43.0	8.8	12.3	1.2	65.3
Microcerotermes parvus	FD	43.5	2.7	16.0	4.9	67.1
	CA	42.1	3.6	13.2	0.8	59.7
Reticulitermes flavipes	FD	52.9	10.9	13.7	6.4	83.9
Soil-feeding termites						
Cubitermes sp.	FD	15.1	11.0	16.2	4.0	46.3
	SA	44.4	9.9	3.2	4.8	62.3
Cubitermes speciosus	FD	35.1	1.1	8.8	2.5	47.5
	SA	40.5	2.6	—[d]	3.7	46.8
Thoracotermes macrothorax	FD	24.7	8.3	15.5	7.2	55.7
	SA	47.7	2.8	—[d]	2.8	53.3
Crenetermes albotarsalis	FD	33.1	5.3	3.2	3.1	44.7
	SA	46.5	1.4	—[d]	10.5	58.4
Fungus-cultivating termite	FD	2.6	1.2	74.0	0.3	78.1
Macrotermes nobilis	CA	39.7	2.1	20.4	0.3	62.5

[a] Termites were tested within 48 h of collection by using feeding vials like those shown in Fig. 1A and a 5-day, fixed-time assay.
[b] FD, filter disk; CA, cellulose agar; SA, soil agar.
[c] Amounts of radioactivity were determined after material was extracted overnight with scintillation fluid ($^{14}CO_2$ and residual food) or was treated with tissue solubilizer (termite bodies and feces) (see Materials and Methods).
[d] Residual food could not be separated from feces, with which it was mixed and plastered onto the inside wall of the vial.

naturally occurring aromatic compounds (27). Therefore, in an effort to extend our limited understanding of the degradation of plant-derived aromatic compounds by termites, we assessed the general ability of termites from different feeding guilds (i.e., wood feeders, soil feeders, and fungus cultivators) to metabolize aromatic compounds and the relevance of gut microbes to the dissimilatory process. Our results, which demonstrated the importance of oxygen for cleavage of the aromatic nucleus, prompted a follow-up study (6) of the oxygen gradients that exist in termite guts and the importance of gut microbes as an oxygen sink.

MATERIALS AND METHODS

Termites. The species which were collected in the Mayombe rain forest in the Republic of Congo included *Crenetermes albotarsalis* (Sjöstedt), *Cubitermes speciosus* Sjöstedt, an unidentified *Cubitermes* species, and *Thoracotermes macrothorax* (Sjöstedt) (all Termitinae); *Macrotermes nobilis* (Sjöstedt) (Macrotermitinae); *Microcerotermes parvus* (Haviland) (Amitermitinae); and *Nasutitermes lujae* (Wasmann) (Nasutitermitinae). *Nasutitermes nigriceps* (Haldeman) (Termitinae) was collected by M. Collins, Washington, D.C., on the Cayman Islands. *Nasutitermes costalis* (Holmgren) was a gift from J. Traniello, Boston, Mass. All of these insects are so-called "higher termites" (family Termitidae), and most of them were used within 48 h of collection. However, *N. lujae*, *N. costalis*, and *N. nigriceps* were also obtained from laboratory cultures maintained at ambient temperature on a diet of white birch (*Betula papyrifera* Marsh.) and water. The lower termite *Reticulitermes flavipes* (Kollar) (Rhinotermitidae) was collected near Dansville, Mich., and was used within 48 h of collection or after various periods of maintenance in a laboratory as described previously (23). The feeding guilds to which most of these termites belong are shown in Table 1; *N. nigriceps* and *N. costalis* are both wood feeders.

Feeding experiments. Groups of 12 live termites (10 worker larvae and 2 soldiers) were placed in butyl rubber-stoppered 8-ml serum vials, each of which contained a food source amended with a ^{14}C-labeled compound or with antibiotics. The food source was either a 6-mm-diameter disk of Whatman no. 1 filter paper (plain or, for *R. flavipes*, coated with 20 μl of 1% agar) or a 125-mm^3 nugget of 2% (wt/vol) agar containing 2% (wt/vol) ball-milled cellulose particles or, for soil-feeding species, topsoil from the vicinity of the nest from which the insects were collected. ^{14}C-labeled aromatic acids were applied as 10-μl aqueous solutions of their sodium salts (0.1 μmol; 40 to 100 kBq μmol^{-1}); [U-^{14}C]cellulose was applied as 5 μl of an aqueous suspension (10 mg ml^{-1}; 7.25 kBq mg^{-1}); antibiotics were applied as 5 μl of an NaOH-neutralized aqueous mixture (tetracycline, penicillin G, and chloramphenicol; 5 μg each). The amendments were allowed to soak into the food sources before the food sources were added to the vials.

In fixed-time assays (which were convenient for field experiments in the Republic of Congo), each vial contained a filter paper wick (6 cm^2; Whatman no. 3) that was suspended from a needle inserted into the base of the stopper and was impregnated with 0.2 ml of 1 N NaOH to trap any $^{14}CO_2$ evolved (Fig. 1A). Such vials were opened for about 30 s daily to allow for replenishment of O_2 and were resealed with the same stoppers. In time course assays (which were more easily conducted in the laboratory), the vials did not contain NaOH-impregnated filter wicks, but instead the headspace gas was flushed daily with humidified air for 10 min (10 ml min^{-1}) into a $^{14}CO_2$ trap consisting of two consecutive 7-ml scintillation vials filled with 0.5 ml of β-phenethylamine in 4 ml of scintillation fluid

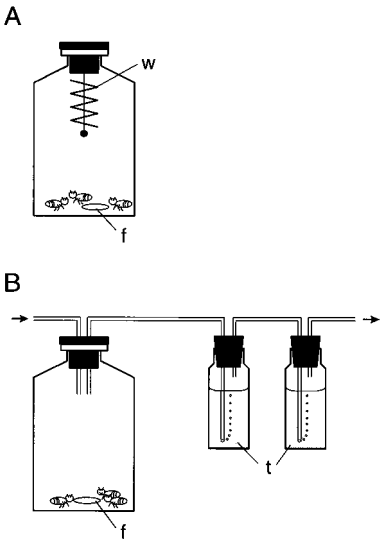

FIG. 1. Incubation vials used for fixed-time (A) and time course (B) measurements of degradation by live termites of ^{14}C-labeled aromatic compounds supplied with a food source (f). $^{14}CO_2$ released into the gas phase was either trapped on an NaOH-impregnated filter wick (w) (A) or periodically swept out and into a $^{14}CO_2$ trap (t) by using humidified air (B). For details see Materials and Methods.

(Fig. 1B). The levels of trapping efficiency for $^{14}CO_2$ were >99.5% with the phenethylamine traps and >99.9% with the NaOH wicks. After 5 to 7 days, the vials were opened, and the distribution of radioactivity was determined (see below). All feeding experiments were performed at room temperature (22 to 25°C).

Metabolism of ^{14}C-labeled substrates by termite gut homogenates. Termites were introduced into an anoxic glove box and degutted by using aseptic procedures and fine-tipped forceps (23). The extracted guts were pooled (40 guts ml^{-1}) in a glass tissue homogenizer containing an anoxic buffered salts solution (0.2 g of K_2HPO_4 per liter, 0.1 g of KH_2PO_4 per liter, 0.15 g of KCl per liter, and 0.15 g of NaCl per liter) and homogenized thoroughly (5). Portions (450 μl) of homogenate were then distributed into 5-ml serum vials, each of which contained a small magnetic stirrer bar and was stoppered with a butyl rubber septum. After the vials were removed from the glove box, the headspace of each vial was exchanged immediately with either 100% N_2 or air. Reactions were initiated by adding an anoxic ^{14}C-substrate solution (50 μl; 0.5 mM; 16 to 24 kBq ml^{-1}). The reaction mixtures were incubated at 30°C on a magnetic stirrer for 24 h and then acidified with 100 μl of 1 N HCl to stop the reaction. While each mixture was being stirred constantly, the headspace gas was swept for 20 min at a rate of 10 ml min^{-1} into a $^{14}CO_2$ trap, as described above for the feeding experiments. The acidified reaction mixture was then centrifuged at 13,000 × *g* for 10 min, and the supernatant was fractionated by high-performance liquid chromatography (HPLC). Samples were removed at all steps and analyzed for radioactivity.

Determination of radioactivity. The amount of $^{14}CO_2$ swept out of the feeding and reaction vials was measured directly in the scintillation vials used as traps. NaOH-impregnated filter wicks containing trapped $^{14}CO_2$, as well as residual (uneaten) food sources, were placed in scintillation vials containing 10 ml of scintillation cocktail, and the amount of radioactivity was determined after overnight incubation at room temperature. Extracted termite guts and degutted termite bodies were pooled separately and macerated in 0.1 ml of tissue solubilizer (0.5% Triton X-100 and 1% nitrilotriacetic acid in 1 N NaOH, incubated for 1 h at 100°C). Solubilized tissue samples were transferred quantitatively into a scintillation vial together with a rinse solution consisting of 0.1 ml of 1 N HCl, and then 4 ml of scintillation cocktail was added. Any feces deposited during feeding experiments were solubilized in the feeding vials as just described, except that all volumes were increased fivefold and each preparation was incubated for 15 min in an ultrasonic water bath prior to heating.

Since the recovery of ^{14}C radioactivity from insoluble material was incomplete when this method was used, we also used a wet combustion method for most laboratory (i.e., nonfield) feeding experiments, as follows (modified from the method of Coughtrey et al. [10]). Termite bodies, guts, and food particles (<5 mg [dry weight]) were transferred to 17-ml screw-cap culture tubes, whereas feces were allowed to remain in the feeding vials. To each vial and tube we added 100 mg of $K_2Cr_2O_4$ and 2 ml of a mixture of concentrated H_2SO_4 and 85% H_3PO_4 (3:2, vol/vol). Each vessel was closed immediately with a Teflon-covered rubber septum and autoclaved for 1 h at 120°C. $^{14}CO_2$ was trapped by flushing the headspace gas into CO_2 traps as described above. Autoclaving was repeated twice, after which no additional $^{14}CO_2$ was released; by that time, there was no residual radioactivity in the combustion solution.

Radioactivity was quantified with a Delta 300 liquid scintillation counter (Searle, Des Plaines, Ill.), and values were quench corrected by using an internal standard. Bio-Safe II liquid scintillation cocktail (RPI, Mount Prospect, Ill.) was used throughout these experiments.

Microbiological media. Organisms were cultivated anaerobically by using bicarbonate-buffered mineral medium AM-4 under an N_2-CO_2 (9:1, vol/vol) atmosphere. Basal medium AM-4 contained (per liter) 1 g of NaCl, 0.5 g of KCl, 0.4 g of $MgCl_2 \cdot 6H_2O$, 0.1 g of $CaCl_2 \cdot 2H_2O$, 0.3 g of NH_4Cl, 0.2 g of KH_2PO_4, 0.15 g of Na_2SO_4, 0.5 g of Bacto Yeast Extract (Difco Laboratories, Detroit, Mich.), and 0.5 g of Casamino Acids (Difco). $NaHCO_3$ (final concentration, 30 mM) trace element solution SL 10 (28), a selenite-tungstate solution (28), a seven-vitamin solution (29), and other supplements (see below) were added to the autoclaved medium from sterile stock solutions as described by Widdel and Pfennig (29). The reducing agent incorporated into the medium was either a mixture of dithiothreitol and cysteine (1 mM each), Na_2S (1 mM), or 0.01% powdered $PdCl_2$ coupled with headspace gas containing 0.5 atm (ca. 50 kPa) of H_2. The pH was adjusted to 7.2 with HCl.

Basal medium MM-4 was used for aerobic cultivation. This medium was identical to basal medium AM-4 except that $NaHCO_3$ was replaced by sodium phosphate buffer (pH 7.2; final concentration, 20 mM; autoclaved separately), the final concentrations of $MgCl_2 \cdot 6H_2O$ and $CaCl_2 \cdot 2H_2O$ were decreased to 0.1 and 0.015 g $liter^{-1}$, respectively, and trace element solution SL 10 was replaced by trace element solution SL 11 (12).

All of the media received the following additional supplements, which were added from concentrated stock solutions (final concentrations): folic acid and riboflavin, 50 μg/liter each; isobutyric, 2-methylbutyric, *n*-valeric, and isovaleric acids, 25 μM each; phenylacetic and phenylpropionic acids, 5 μM each; naphthoquinone, 2.5 μM; and lipoic acid, 1 μM.

Substrates were added to media from NaOH-neutralized sterile stock solutions to final concentrations of 2.5 mM (anoxic media) and 1 mM (oxic media). To stimulate syntrophic or mixotrophic processes, either H_2 (0.5 atm), formate (20 mM), fructose (2.5 mM), or an H_2-CO_2-grown culture of *Clostridium mayombei* (15) (10%, vol/vol) was added to anaerobic cultures where indicated below.

All cultures were incubated at 30°C in the dark. Aerobic cultures were agitated by rotary or reciprocal shaking.

MPN determinations. For most-probable-number (MPN) determinations under oxic culture conditions, we prepared serial 10-fold dilutions of gut homogenates (10 guts ml^{-1}) (see above) (in triplicate) in substrate-free basal medium MM-4. These dilutions were used to inoculate standard culture tubes containing medium supplemented with individual substrates. To determine MPNs of anaerobic bacteria, similar procedures were performed, except that the gut homogenates were prepared and diluted in an anoxic glove box in substrate-free basal medium AM-4 and the resulting dilutions were inoculated into homologous medium containing the test substrate under an N_2-CO_2 (9:1, vol/vol) atmosphere.

Inoculated tubes were considered positive when substrate degradation was confirmed by the results of an HPLC analysis of the culture supernatant after 2 weeks (oxic) or 4 weeks (anoxic) of incubation. Numerical data were computed by using the universal equation for calculating MPN (17).

Analytical methods. Aromatic compounds were quantified by HPLC by using a Waters gradient system with UV detection (Millipore, Milford, Mass.) equipped with a Brownlee Spheri-5 RP-18 column (100 by 4.6 mm; Applied Biosystems, Foster City, Calif.). Separation was achieved by using a 20 mM ammonium phosphate buffer–methanol gradient (pH 2.6) at a rate of 1.3 ml min^{-1}. Peak identities were verified by cochromatography of standards and by UV spectroscopy and comparison of the A_{212}/A_{254} ratios of eluted compounds.

To measure the distribution of ^{14}C radioactivity in HPLC chromatograms, 0.5-ml fractions of eluted material were collected in scintillation vials and mixed with 4-ml portions of scintillation cocktail, and the amounts of ^{14}C were determined as described above.

Sources of chemicals. [*ring*-U^{14}C]cinnamic acid (97%) was obtained from Amersham, Arlington Heights, Ill., and [U-^{14}C]cellulose was obtained from ICN, Irvine, Calif. [*ring*-U-^{14}C]benzoic acid (>98%), nonlabeled aromatic compounds, and antibiotics were obtained from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Feeding experiments. Freshly collected termites representing three major feeding guilds (wood feeders, soil feeders, and fungus cultivators) degraded [*ring*-U-^{14}C]benzoate during a 5-day, fixed-time feeding experiment. With all species, $^{14}CO_2$ accounted for a significant fraction of the ^{14}C label that was ingested (i.e., that disappeared from the food source), indicating that some or all of the aromatic ring of benzoate was susceptible to complete mineralization (Table 1). Although a ^{14}C inventory revealed incomplete recovery of the radioactivity initially added as benzoate, this was attributable to suboptimal extraction and quantification of radioactivity associated with residual food material, feces, and termite bodies (see below).

In follow-up experiments performed with an expanded roster of wood-feeding species and an additional aromatic substrate ([*ring*-U-^{14}C]cinnamate), the time course of degradation was examined more closely by collecting respired $^{14}CO_2$ at daily intervals. Representative results obtained with four species are shown in Fig. 2, and ^{14}C inventories for all of the experiments are shown in Table 2. From 39 to 73% of the aromatic ring carbon was respired to CO_2 over an 8-day period, depending on the termite species and the substrate, and in general benzoate was mineralized to a greater extent than cinnamate. That the aromatic compounds were degraded within the termite bodies (and not externally on the filter disk or within voided feces) was shown by the fact that $^{14}CO_2$ evolution ceased immediately when termites were removed from the feeding vials (Fig. 2A); the removed termites continued to evolve $^{14}CO_2$ for a short time after they were transferred to fresh, substrate-free vials (data not shown).

When termites were first exposed to the labeled aromatic compounds, a lag period of 1 to 2 days occurred before the maximum rates of $^{14}CO_2$ evolution were measured (Fig. 2C). In contrast, no lag in $^{14}CO_2$ evolution was observed when *R. flavipes* was exposed to [U-^{14}C]cellulose (Fig. 2B). The observed lag in $^{14}CO_2$ evolution from the aromatic substrates may reflect a transient deterrence of feeding caused by the presence of the free aromatic compounds on the food source

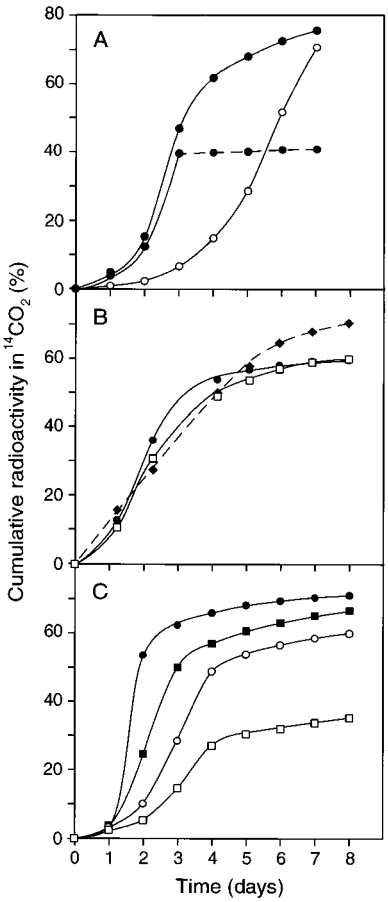

FIG. 2. Time course emission of $^{14}CO_2$ by live termites feeding on ^{14}C-labeled substrates. (A) *N. costalis* feeding on [*ring*-U-^{14}C]benzoate without antibiotics (●) or after prefeeding with a mixture of antibiotics (○). Accumulation of $^{14}CO_2$ in vials ceased immediately after termites were removed (– – –). (B) *R. flavipes* feeding on [*ring*-U-^{14}C]benzoate (●), [*ring*-U-^{14}C]cinnamate (□), or [U-^{14}C]cellulose (◆). (C) *Microcerotermes parvus* (● and ■) and *N. lujae* (○ and □) feeding on [*ring*-U-^{14}C]benzoate (● and ○) or [*ring*-U-^{14}C]cinnamate (■ and □).

(filter disks) or the need for induction of aromatic compound-degrading activity, because no lag period was observed when termites were adapted to (i.e., prefed) homologous unlabeled substrates (data not shown). Involvement of gut microbes in the catabolism of the ^{14}C-labeled aromatic substrates was suggested by an extended lag in $^{14}CO_2$ evolution when termites were pretreated with antibacterial drugs, although the amount of $^{14}CO_2$ eventually evolved after 7 days was nearly the same as the amount evolved in untreated controls (Fig. 2A).

Table 2 summarizes the results obtained with all wood feeders and includes a ^{14}C-inventory based in part on wet combustion of nongaseous components. Assuming that each C atom in a given substrate was equally susceptible to mineralization and that each C atom was oxidized to CO_2 at the same rate, the rates of mineralization of the aromatic substrates (estimated from the rates of $^{14}CO_2$ evolution) ranged from 1 to 3 nmol of substrate day^{-1} termite^{-1}. In contrast, the rate of mineralization of [U-^{14}C]cellulose by *R. flavipes* was about 100-fold faster. As in the fixed-time assays (see above), the bulk of the ingested radioactivity was recovered as $^{14}CO_2$, and, with the exception of *N. costalis* fed cinnamate, only about 5% of the radioactivity was present in feces. This suggested that most of the aromatic ring C that was ingested was completely mineralized. In addition, wet combustion of insoluble materials (food, feces, and termite bodies) yielded more complete recovery of ^{14}C than the recovery obtained in the fixed-time feeding experiment (see above), although the level of recovery was still generally less than 100%. Some of the unrecovered aromatic ^{14}C may have been evolved by termites as a volatile compound(s) not trapped by alkaline phenethylamine (e.g., by CH_4 formation [2]), or it may have been incorporated into materials (e.g., cuticular polymers [1], fecal components) that were relatively refractory to wet combustion.

Metabolism of aromatic compounds by gut homogenates. To determine whether mineralization of monoaromatic compounds by live termites might be attributable to their guts or to the resident gut microflora, gut homogenates from *N. lujae* and *R. flavipes* were incubated with [*ring*-U-^{14}C]benzoate or [*ring*-U-^{14}C]cinnamate for 24 h, and the transformation of these substrates was assessed (Table 3). Under anoxic conditions,

TABLE 2. Mineralization of ring-U-^{14}C-labeled aromatic compounds and [U-^{14}C]cellulose by wood-feeding termites

Species	Substrate[a]	Rate of mineralization[b]	Distribution of ^{14}C (%)[c]					*n*
			$^{14}CO_2$	Termite bodies	Food (filter disk)	Feces	Total	
Nasutitermes nigriceps	Benzoate	1.98 ± 0.27	55.0 ± 14.6	18.3 ± 7.3	18.7 ± 0.9	3.1 ± 0.4	113 ± 1.3	2
Nasutitermes costalis	Benzoate	3.10 ± 0.75	72.0 ± 5.2	8.1 ± 5.8	16.2 ± 4.4	5.0 ± 1.0	106 ± 10.3	6
	Cinnamate	1.60 ± 0.21	53.8 ± 0.5	2.9 ± 0.1	2.2 ± 0.3	19.7 ± 2.2	78.6 ± 2.0	2
Nasutitermes arborum	Benzoate	1.99 ± 0.31	66.3 ± 0.0	5.9 ± 1.2	5.2 ± 0.8	7.7 ± 0.3	85.2 ± 2.3	2
Nasutitermes lujae	Benzoate	2.83 ± 0.54	63.4 ± 3.5	11.1 ± 2.6	2.1 ± 0.4	8.7 ± 0.0	85.3 ± 1.3	2
	Cinnamate	2.33 ± 0.18	52.2 ± 7.4	8.3 ± 2.3	12.2 ± 0.8	4.5 ± 1.5	77.2 ± 11.9	2
Microcerotermes parvus	Benzoate	3.42 ± 0.78	72.6 ± 2.0	4.1 ± 0.1	3.7 ± 0.1	7.4 ± 1.3	87.8 ± 0.7	2
	Cinnamate	1.82 ± 0.28	57.4 ± 8.8	4.9 ± 0.1	3.5 ± 0.9	8.4 ± 1.0	74.3 ± 8.7	2
Reticulitermes flavipes	Benzoate	2.54 ± 0.36	59.2 ± 7.8	16.9 ± 10.8	4.0 ± 4.1	5.0 ± 1.7	86.9 ± 11.8	4
	Cinnamate	1.19 ± 0.88	39.0 ± 16.9	21.9 ± 15.2	1.1 ± 1.6	5.1 ± 1.4	67.1 ± 11.2	6
	Cellulose	290.0 ± 76.0	84.1 ± 19.3	19.0 ± 7.2	0.0 ± 0.0	5.5 ± 5.2	108 ± 16.3	4

[a] Aromatic acids were applied to filter disks as their Na^+ salts.

[b] Rate of substrate mineralization, expressed in nanomoles per day per termite (assuming that all of the ^{14}C atoms of the substrate could be oxidized to $^{14}CO_2$ with equal facility).

[c] Data are expressed as the percentages of the original ^{14}C label present on the filter disk and were determined after 6 to 8 days. Values are the means ± standard deviations of *n* separate trials.

TABLE 3. Metabolism of [*ring*-U-^{14}C]benzoate and [*ring*-U-^{14}C]cinnamate by gut homogenates of *N. lujae* and *R. flavipes*[a]

Species	Substrate	Gas phase	Distribution of ^{14}C (%)[b]				
			$^{14}CO_2$	Homogenate	Soluble fraction[c]	Particulate fraction[c,d]	Total
N. lujae	Benzoate	N_2	0.1	93.6	89.3	4.3	93.7
		Air	33.8	33.4	6.5	26.9	67.2
	Cinnamate	N_2	0.0	90.6	85.7	4.9	90.7
		Air	14.7	77.9	55.7	22.1	92.6
R. flavipes	Benzoate	N_2	0.0	101.6	96.4	5.3	101.7
		Air	44.9	44.9	5.2	39.7	89.8
	Cinnamate	N_2	0.0	99.0	88.8	10.1	99.0
		Air	13.5	84.0	56.6	27.4	97.6

[a] Substrates (final concentration, 0.05 mM) were added to homogenates containing 40 gut equivalents per ml.
[b] Percentages of the original ^{14}C label added to the homogenates, determined after 24 h of incubation.
[c] Soluble and particulate (i.e., sedimentable) fractions were obtained after homogenates were centrifuged for 10 min at 13,000 × *g*.
[d] Values were calculated by determining the difference between the amount of radioactivity in the homogenate and the amount of radioactivity in the soluble fraction.

little or no radioactivity was released as $^{14}CO_2$, and most of the ^{14}C remaining in the reaction mixture was present in the soluble (i.e., supernatant) fraction after centrifugation. In contrast, in the presence of oxygen, 34 and 45% of the benzoate ring was respired to $^{14}CO_2$ by gut homogenates of *N. lujae* and *R. flavipes*, respectively. Most of the benzoate ^{14}C remaining in the homogenates was associated with the particulate fraction, presumably having been incorporated into microbial cells. Oxidation of cinnamate also required the presence of oxygen, although the amount of ring-^{14}C respired to $^{14}CO_2$ (ca. 15% by gut homogenates from either species) was less than the amount formed from benzoate, and most of the ^{14}C remaining in the homogenates was present in the soluble fraction.

The nature of the ^{14}C label remaining in soluble fractions was examined by performing an HPLC analysis (Fig. 3). Under anoxic incubation conditions with gut homogenates from either species, the radioactivity originally present as [*ring*-U-^{14}C]benzoate (eluting in fractions 5 and 6) remained essentially unchanged (Fig. 3A and C). In the presence of air, however, virtually all of the benzoate disappeared within 24 h, and only traces of radioactivity remained in the soluble fraction (Fig. 3E and G). In contrast, significant amounts of [*ring*-U-^{14}C]cinnamate (fractions 9 and 10) disappeared when preparations were incubated with gut homogenates under either anoxic or oxic conditions (Fig. 3B, D, F, and H). However, under anoxic conditions, the cinnamate that disappeared was

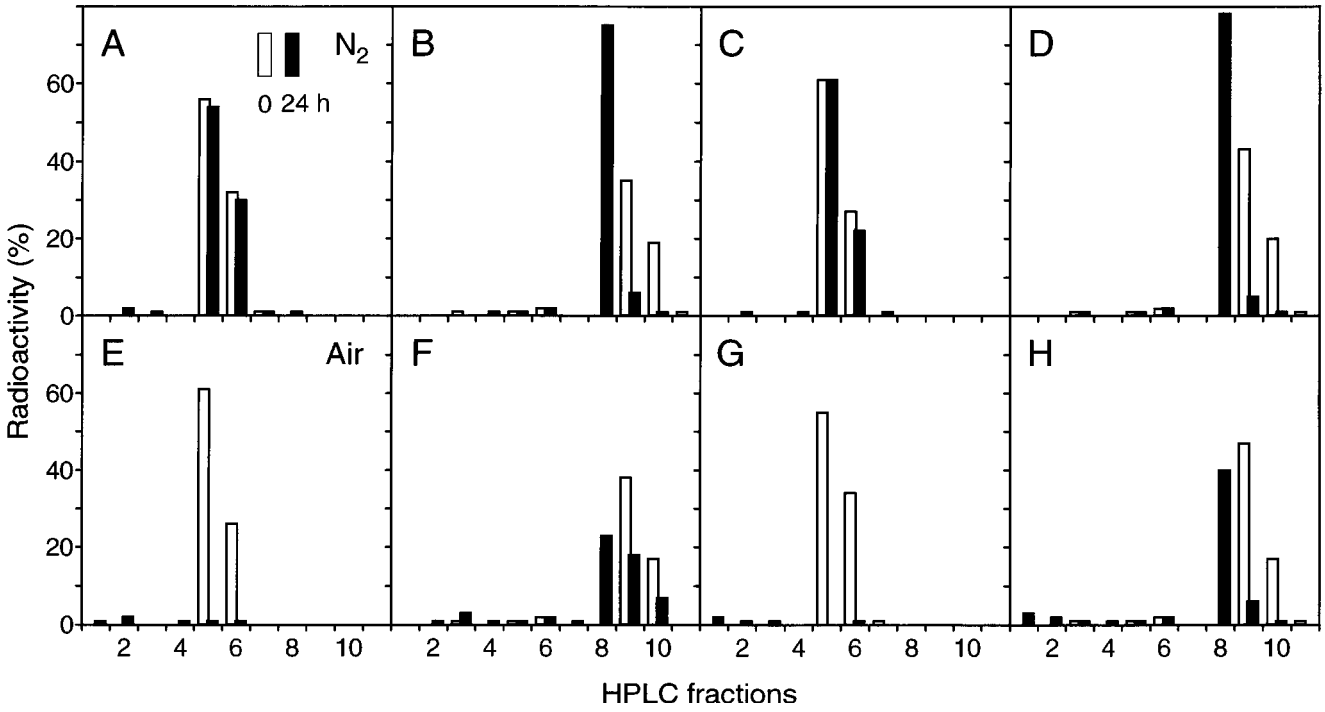

FIG. 3. Distribution of radioactivity in reaction mixtures containing gut homogenates of *N. lujae* (A, B, E, and F) or *R. flavipes* (C, D, G, and H) and the substrate [*ring*-U-^{14}C]benzoate (A, E, C, and G) or [*ring*-U-^{14}C]cinnamate (B, F, D, and H) under an N_2 atmosphere (A to D) or air (E to H). The soluble portions of reaction mixtures were analyzed by HPLC immediately after the substrate was added (open bars) and again after 24 h of incubation (solid bars). HPLC fractions (0.5 ml) were collected to determine levels of radioactivity. An HPLC analysis of standards revealed that they eluted in the following fractions: benzoate, fractions 5 and 6; cinnamate, fractions 9 and 10; and phenylpropanoate, fraction 8.

TABLE 4. Three-tube MPN estimates of the numbers of bacteria in the gut of *N. lujae* capable of degrading monoaromatic compounds under oxic and anoxic conditions

Substrate	MPN (no. of cells gut^{-1})[a]	
	Oxic conditions	Anoxic conditions
Benzoic acid	8.6×10^4	0
Cinnamic acid	8.6×10^4	1–3[b]
Phenylpropanoic acid	8.6×10^4	0
4-Hydroxyphenylpropanoic acid	4.8×10^4	0
3,4-Dihydroxyphenylpropanoic acid	4.8×10^4	0
Ferulic acid	1.5×10^5	9–43[b]

[a] Cultures were incubated for a minimum of 1 month. In the positive cultures, the aromatic substrate (2 mM) completely disappeared and there was no accumulation of any other aromatic product.

[b] Under anoxic conditions, cinnamic acid was reduced to phenylpropanoic acid, and ferulic acid was converted to 3,4-dihydroxyphenylpropanoic acid and several other unidentified aromatic products. In neither case were the aromatic nuclei attacked.

converted exclusively to phenylpropanoate (Fig. 3B and D, fraction 8), indicating that cinnamate metabolism in the absence of oxygen was limited to reduction of the propenoic side chain and did not involve cleavage of the aromatic ring. In the presence of air, a significant amount of the cinnamate ring carbon was oxidized to CO_2 (Table 3); however, a substantial level of reduction of the propenoic side chain occurred as well (Fig. 3F and H). Whether side chain reduction is part of an overall pathway leading to O_2-dependent oxidation of the cinnamate ring or was the result of O_2 limitation in the reaction vial is not yet known.

In separate attempts to demonstrate mineralization of nonlabeled ferulic, cinnamic, and 4-hydroxycinnamic acids by dense gut homogenates, we generally observed that, under anoxic conditions, phenylpropanoids (i.e., phenylpropanoic, 4-hydroxyphenylpropanoic, and 3,4-dihydroxyphenylpropanoic acids and several unidentified peaks with similar retention times and UV spectra) were formed in excess of the added substrate. These compounds presumably arose from endogenous material present in the gut contents. However, in parallel incubations under an air atmosphere, the added substrates disappeared completely, and no accumulation of endogenous phenylpropanoids was observed when we performed an HPLC analysis (data not shown).

Enumeration of aromatic compound-degrading gut microbes. The numbers of termite gut microbes capable of attacking the aromatic nucleus were determined by using the MPN method in the presence and in the absence of oxygen. When gut homogenates of *N. lujae* were used as inocula, approximately 10^5 bacteria that were capable of mineralizing various phenylpropanoids under oxic conditions were present in each gut (Table 4). In contrast, if MPN analyses were performed by using strictly anoxic incubation conditions, no mineralization of the aromatic nucleus occurred. Under such conditions, only modifications of aromatic ring substituents were observed. These involved mainly reduction of the phenylpropenoid side chain of cinnamic acid and demethylation of the methoxy group of ferulic acid, leading to the formation of saturated phenylpropanoids. Neither benzoic acid, phenylpropanoic acid, nor hydroxylated derivatives of phenylpropanoic acid were utilized under anoxic conditions. The inclusion of pyruvate, lactate, or H_2 in anoxic cultures did not promote degradation of the aromatic nuclei, nor did the inclusion of viable cells of *Clostridium mayombei* (15) as hydrogen scavengers promote such degradation.

Preliminary experiments performed with gut homogenates of *R. flavipes* revealed similar high numbers of bacteria capable of mineralizing benzoic or phenylpropanoic acid under oxic conditions. However, it is noteworthy that MPN determinations with ferulic and trimethoxybenzoic acids under anoxic conditions revealed substantially higher numbers of demethylating and side chain-reducing bacteria ($>4.8 \times 10^3$ bacteria per gut) than the numbers observed with *N. lujae* (Table 4).

DISCUSSION

Our results show that the ability to mineralize ingested monoaromatic compounds is common in termites belonging to the major feeding guilds (i.e., wood feeders, soil feeders, and fungus cultivators) (Tables 1 and 2) and that some or possibly all of this activity is associated with the termite gut microbiota. The evidence for this included (i) the retardation of monoaromatic compound mineralization when termites were prefed antibacterial drugs (Fig. 2A); (ii) the association of aromatic compound-degrading activity with crude homogenates of microbe-packed guts (Table 3; Fig. 3); and (iii) the presence of relatively high numbers of aromatic compound-degrading bacteria in guts of both higher and lower termites, as determined by quantitative culture methods (Table 4) (see above). Considering the size of the termite guts examined (the gut volumes were on the order of 1 to 2 μl) and considering that because of the presence of food particles only a fraction of the gut volume represents liquid volume, the observed populations of microbes that degrade aromatic compounds (approximately 10^5 cells per gut) translate to densities of at least 10^8 cells per ml of gut fluid, densities which must be considered significant. However, our studies also revealed that the presence of oxygen appears to be necessary for mineralization of the aromatic ring; this was certainly the case for crude gut homogenates and also for aromatic compound-degrading gut microbes in culture. Under anoxic incubation conditions, only modification of some of the aromatic substrates (e.g., demethylation and/or side chain reduction [Tables 3 and 4; Fig. 3]) was observed, and ring cleavage was not observed.

How oxygen-mediated ring cleavage by termite gut microbes might occur in a habitat that is generally thought to be anoxic puzzled us, so we examined, with the aid of microelectrodes, the concentrations of oxygen (and pH values) in various regions of termite guts. The results of this study, which are reported in the accompanying paper (6), revealed that oxygen can penetrate up to 200 μm inward from the hindgut epithelium and that microbes present in that region constitute an oxygen sink. Therefore, the notion that oxygen-mediated degradation of aromatic compounds by some members of the termite gut flora occurs is not inconsistent with typical, anaerobic metabolic processes (e.g., fermentation, homoacetogenesis, methanogenesis) carried out by other members of the microbiota. In fact, the ring modification reactions observed under anoxic incubation conditions (see above) are typical of homoacetogenic anaerobes (26) and in part also of a wider range of fermenting bacteria (reviewed in reference 27); strains of both groups have been commonly isolated from termite guts (3, 4). It may well be that under anoxic conditions such microbes contribute to the degradation of aromatic compounds whose complete mineralization ultimately requires oxygen.

The rates of mineralization of aromatic compounds fed to termites enabled us to make a crude estimate of the extent to which such processes might contribute to the respiratory activity of the insects, as follows. The respiration rate of *R. flavipes* (0.36 to 0.44 μl of O_2 h^{-1} mg^{-1} [23]), which is similar to the

respiration rates of other wood-feeding termites (24), is equivalent to 1.2 to 1.5 μmol of O_2 termite^{-1} day^{-1} for a typical 4-mg termite. Since the respiratory quotient of *R. flavipes* (like that of most wood-feeders [24]) is roughly 1.0, individuals of *R. flavipes* evolve 1.2 to 1.5 μmol of CO_2 day^{-1}, which reflects, for the most part, mineralization of 0.2 to 0.3 μmol of the major monosaccharide component of wood, glucose, per day (23). The value presented above for the mineralization of [U-^{14}C] cellulose by *R. flavipes* in feeding experiments (0.29 μmol of glucosyl units termite^{-1} day^{-1}) (Table 2) is consistent with such previously determined values. Therefore, the rates of mineralization of aromatic compounds reported in Table 2 for *R. flavipes* and other wood feeders of similar size suggest that oxidation of those compounds might account for 2 to 3% of the respiratory activity of xylophagous termites. This may seem small, but it must be remembered that the bulk of the lignocellulosic plant material digested by wood-feeding termites is cellulose, which is itself a relatively refractory food resource. Thus, even a small contribution by aromatic compounds to the accessible nutrient pool of the system may be important.

Our findings are consistent with previous results reported for *Nasutitermes exitiosus* termites fed ring-labeled aromatic compounds, which also implicated gut microbes as agents of degradation (7, 9), and with results reported for a variety of other soil invertebrates (isopods, millipedes, gastropods, and earthworms) (21, 22). Kaplan and Hartenstein showed that the mineralization of monoaromatic compounds in isopods is not attributable to the host tissue (16), and they isolated several aerobic gut bacteria that possess ring-cleaving dioxygenases. Inasmuch as insect tissues may possess high levels of cytochrome *P*-450-dependent monooxygenase activities (13) but appear to lack ring-cleaving dioxygenases, the body of evidence suggests that members of the gut microflora were responsible for the aromatic ring mineralization observed in our feeding trials. However, our overall understanding of invertebrate gut microbes that catalyze degradation of aromatic compounds is still meager. Kuhnigk et al. (18) recently isolated 52 strains of strictly or facultatively aerobic, ring-degrading bacteria from termite guts, but they did not describe the quantitative significance of the isolates in situ. On the other hand, our results affirmed the quantitative significance of aromatic compound-degrading bacteria in the guts of wood-feeding termites, but complete characterization of the isolates is still in progress.

It must be emphasized that in this study we focused on the degradation of monoaromatic compounds by termites and their gut microbes. Hence, free monoaromatic compounds present in plant residues and monoaromatic acids and phenolic compounds that may be linked to plant cell wall polymers by easily hydrolyzable ester linkages are probably prime candidates for such dissimilatory activity. In contrast, more work is still needed to clarify the involvement of gut microbes in dissimilation of the major polyaromatic complex in lignocellulose, lignin. The ambiguous issue of lignin degradation by termites has been reviewed recently by Breznak and Brune (4). These authors concluded that although lignin is not inert as it passes through the gut, there is no convincing evidence yet that insoluble, high-molecular-weight core lignin is degraded by termites or their gut microbes. Depolymerization of such material is the rate-limiting step in its degradation (30). However, the only known biological mechanism for depolymerization of lignin, which consists largely of ether-linked and direct C—C-linked aromatic monomers, is an oxidative attack initiated by lignin and manganese peroxidases. These enzymes, which so far have been found only in fungi and a few actinomycetes, are indirectly, but strictly, dependent on molecular oxygen to produce their hydrogen peroxide cosubstrate. Whether the recently demonstrated oxygen consumption in termite guts is related to some kind of peroxidative depolymerization of lignin is not known (6). However, it seems clear that the gut microbiota is equipped to deal with aromatic monomers released by such depolymerizing activities, whether they are formed internally or externally prior to ingestion.

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