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Heterotrophic Bacteria of Wood-Eating Termites

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HETEROTROPHIC BACTERIA OF WOOD-EATING TERMITES [*RETICULITERMES FLAVIPES* (KOLLAR)]

By

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ABSTRACT

HETEROTROPHIC BACTERIA OF WOOD-EATING TERMITES

[RETICULITERMES FLAVIPES (KOLLAR)]

By

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To better understand the nature of prokaryotes inhabiting the gut of xylophagous termites, strict anaerobic culture techniques were used to quantitate heterotrophic bacteria present in hindguts of Reticulitermes flavipes. The grand mean number of viable bacteria per hindgut was 0.4×10^5 (1st instar larvae), 1.3×10^5 (3rd instar larvae), 3.5×10^5 (workers), and 1.5×10^5 (soldiers). Of 344 isolates, 66.3% were homolactic streptococci (mostly S. lactis and S. cremoris) and 17.4% were bacteroides which were obtained regardless of the origin of termites, their caste, or length of captivity. Selected strains of bacteroides fermented lactate to propionate and acetate in anaerobic cell suspensions. In vitro cocultures of S. lactis and Bacteroides sp. revealed that lactate formed by S. lactis was fermented as an energy source by Bacteroides sp. to propionate, acetate, and CO_2. Results indicate that not only are streptococci and bacteroides relatively stable components of the hindgut microbiota, but also that cross-feeding of lactate between these microorganisms may constitute one aspect of the overall hindgut fermentation in the termite.

DEDICATION

To my parents

who have given me encouragement and support

in all endeavors, past and present.

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INTRODUCTION

Termites are social insects which ingest cellulosic materials for nutrition. It has long been known that protozoa in the hindgut of evolutionarily "lower" termites are responsible for the digestion of cellulose into useable nutrients for the termite. However, the hindgut also contains an abundance of morphologically diverse bacteria which appear to be an integral part of the gut ecosystem (10). While bacteria in termite hindguts have been implicated in nitrogen fixation (9) and methane production (8), very little is known about their other roles in the hindgut. Uncertainty about the importance of bacteria in termite metabolism arises largely from the paucity of data concerning the numbers and species composition of the bacterial component, and their biochemical capabilities.

It was felt that a knowledge of the major types of heterotrophic bacteria in the termite gut would provide a basis for studying the nutritional aspects of these bacteria in the hindgut environment. Therefore, a study was initiated with the following objectives: 1) to isolate under nonselective conditions, characterize, and quantify the major groups of heterotrophic bacteria found in the wood-eating termite, Reticulitermes flavipes; 2) to compare the bacterial component of different castes and developmental stages of R. flavipes; 3) to determine the influence of laboratory captivity and the origin of R. flavipes on bacterial types; and 4) to investigate, with in vitro

techniques, nutritional aspects of the major groups of bacteria and possible interactions among these bacteria in the termite hindgut.

LITERATURE REVIEW

Biology of Termites

Termites are insects belonging to the order Isoptera which includes five living families: Mastotermitidae, Kalotermitidae, Hodotermitidae, Rhinotermitidae, and Termitidae (90). The first four families are referred to as "lower" termites, since they are phylogenetically more primitive; members of the Termitidae are referred to as "higher" termites. Lower termites all possess intestinal symbiotic protozoa on which they depend for digestion of cellulose. The Termitidae, which contain three-fourths of all known species, harbor protozoa which are neither numerous nor cellulolytic (35).

The termite colony is usually produced by a pair of reproductives and consists of several castes, which are morphologically and functionally different (soldiers, alates, and neotenics), in addition to other individuals in various stages of development (larvae, winged nymphs, and workers) (24). Newly hatched larvae proceed through developmental stages (instars) in which the termite undergoes ecdysis. Depending on the needs of the colony, larvae can differentiate into reproductives or soldiers, or remain undifferentiated (91). Workers are most abundant in the termite colony and consequently, dominate all colony activities except reproduction and defense. However, in many species workers do not constitute a terminal caste and are therefore difficult to define. For example, workers of the genus

Reticulitermes are merely defined as externally undifferentiated larvae past the third instar (24). These forms, like the 1st to 3rd instar larvae, retain the potential to differentiate into soldiers or reproductives.

The diet of termites consists of plant material, either living, dead, or in various stages of decay by fungi. The majority of the lower termites feed on wood, while the Termitidae prefer a more varied diet of grass, humus, dead wood, or plant debris (57). Many termites prefer wood decayed by fungi and benefit from the fungal degradation of lignin and other wood constituents (77). Termite species, however, react differently to fungi species. While some termites might favor a particular fungus, others may be indifferent or repelled by it (3).

Workers obtain and digest crude nutrients to feed themselves and dependent reproductives, soldiers, and larvae. In lower termites, dependent castes solicit stomodeal food (a mixture of saliva and regurgitated raw food) or proctodeal food from the workers (67). Proctodeal food consists of liquid excretions from rectal pouches and is excreted in response to tactile stimuli (57). Higher termites do not practice proctodeal feeding, although coprophagy has been reported (65).

The intestinal tract of a worker termite consists of a foregut, midgut, and hindgut. An esophagus, crop, and gizzard comprise the foregut, while the midgut is a simple tube of uniform diameter. Digestive enzymes, which degrade easily hydrolyzable compounds, are secreted primarily in the midgut (67). In some Termitidae, the midgut is elongated on one of the faces of the intestinal tube, resulting in a "mixed segment" (67). An enteric valve separates the midgut from the hindgut which, in turn, consists of five regions including a

well-developed, bulbous paunch. It is the paunch region which contains the bulk of microorganisms (bacteria and protozoa) responsible for most of the digestion in termites. The paunch may thus be likened to a fermentation chamber superficially similar to the rumen of cattle. The paunch contents displays a pH close to neutrality (31, 49, 67, 75), and products of digestion are absorbed in this region since the enteric valve prevents refluxing of paunch contents to the midgut (67).

Cellulose and hemicellulose, the major polysaccharides of plant material, undergo extensive degradation on passage through the termite gut. Oshima (68) first demonstrated cellulose digestion in termites from analyses of the constituents of wood and feces of Coptotermes formosanus. Hungate (44) compared wood and fecal pellets of Zootermopsis and found that 84% of the cellulose was utilized. Seifert and Becker (78), using five different woods and four species of termites, found that 74 to 99% of the initial cellulose was degraded. Other studies have found hemicelluloses to be substantially attacked (25, 37, 47). Esenther and Kirk (25) fed aspen sapwood to Reticulitermes flavipes and found 84% of the major hemicellulose, xylan, and 77% of the mannan-type hemicellulose was utilized on passage through the termite.

The extent of lignin degradation by termites appears minor. Seifert and Becker (78) obtained values for lignin degradation as high as 77% with Reticulitermes santonensis. However, this estimate appears to be high because of uncontrolled coprophagy. By contrast, studies with R. flavipes (25) and Microcerotermes edentatus (47) revealed no net degradation of lignin after passage through termites, although a slight loss in the methoxyl content of the lignin was

detected. Other studies either indicate that lignin degradation does not occur in termites (20, 37) or provide data of questionable significance (29).

Symbiotic Relationships between Termites and Their Intestinal Microorganisms

As defined by deBary (2), symbiosis is an intimate and relatively stable association of two dissimilar organisms. Embraced by this definition is the relationship between termites and their intestinal microorganisms. The symbiosis between lower termites and their hindgut protozoa can best be described as mutualistic (12). Oxymonad, trichomonad, and hypermastigote flagellates (35) degrade cellulose into useable nutrients for the termite, while the termite provides the protozoa with food and lodging. By contrast, the association between termites and their intestinal bacteria is less well-understood, although several lines of recent evidence suggest that they, too, may be important to the termites' nutrition and vitality. Protozoan and bacterial symbionts will be discussed individually below.

Lower Termites

Protozoan Symbionts

Cleveland (12, 13, 14) recognized the ability of termites to thrive on sound wood or cellulose, and he showed that hindgut protozoa were indispensable for cellulose digestion and survival of the insects. Reticulitermes flavipes could be defaunated (i.e. rid of their protozoa) by incubation at elevated temperatures, or under hyperbaric O_2, or by starvation, without apparent damage to the insect per se (15). However, if such termites were fed a normal diet of

wood, death occurred within 10-20 days. Indefinite survival of defaunated termites could be achieved by refaunation, or by feeding the termites fungus-digested wood or cellulose (12). These data indicated that the cellulose-digesting activity of hindgut protozoa was critical to termite survival. Selective defaunation of Zootermopsis (15, 16), however indicated that not all protozoa were of equal value to termite survival. The presence of Leidyopsis or Trichonympha could keep the termite alive indefinitely, whereas Streblomastix could not. Other workers have used defaunation techniques to determine the importance of protozoa in termite survival (22, 79, 80, 81, 92) or cellulose catabolism and lipid synthesis (62, 64).

Cellulase activity was first demonstrated by Trager (88) in guts of Reticulitermes flavipes, Zootermopsis angusticollis and in the xylophagous cockroach, Cryptocercus punctulatus. When extracts of the guts were incubated with purified cellulose, an increase in reducing sugars was observed. Analysis of extracts of foregut, midgut, and hindgut showed that cellulase activity was confined to the hindgut region (19, 37, 76, 88). That the protozoa were the source of the enzyme was shown by the lack of cellulase activity in both defaunated termites (37) and cockroaches (19, 88). In addition, cellulase activity has been demonstrated in cultures of Trichomonas termopsidis, a flagellate of Zootermopsis (88, 92). While most data indicate that the protozoa are the sole source of cellulase, Yokoe (93) found that about 20% of the original cellulase activity was maintained in defaunated Leucotermes speratus. He suggested that the termite produced the cellulase, however no mention was made as to whether the bacterial component was also affected by the defaunation techniques.

Cleveland (13) postulated that the protozoa benefitted the termite by degrading cellulose to cellobiose and then to glucose. By using an extract of the protozoan T. termopsidis, Trager (88) showed that glucose was a product of cellulose breakdown. It was thus supposed that the protozoa excreted glucose which was absorbed by the termite for carbon and energy. However, it was not until Hungate's in vitro experiments with protozoa from Zootermopsis, that the products of cellulose digestion were quantified (38, 40). He found that 70-75% of the cellulose carbon could be accounted for in CO_2 and acetic acid. H_2 was also produced, but did not appear to be utilized by the termite. Only small amounts of lactic acid were detected. Hungate (38, 40, 42) suggested that the protozoa obtained energy through anaerobic fermentation of cellulose, liberating CO_2, H_2, and organic acids (primarily acetic). Furthermore, his respirometric data with live termites suggested that acetate was subsequently absorbed by the termite and oxidized for energy. Examination of gut contents revealed the presence of acetate in hindguts of Zootermopsis (40) and propionate (in addition to acetate) in hindguts of unidentified lower termites (11). These data suggest that defaunated termites should be able to survive if fed acetate. However, such attempts have been unsuccessful (21, 42), despite the permeability of the termite gut to this compound (40).

More extensive knowledge of the nutrition and metabolism of termite flagellates is limited, primarily because axenic culture conditions have not been developed. Trager (89) succeeded in culturing Trichomonas termopsidis from Zootermopsis anaerobically on a cellulose-based medium for over 3 years, although his cultures also contained

a noncellulolytic bacillus. Other attempts to cultivate cellulolytic protozoa have been less successful (34, 44, 89). Trichonympha sphaerica from Zootermopsis survived as long as 6 weeks on an anaerobic cellulose medium, but growth was not maintained in subcultures (89).

Bacterial Symbionts

Besides protozoa, bacteria are also abundant in the intestinal tract of lower termites. Direct microscopic counts by Breznak et al. (9) revealed an average of 3×10^6 bacteria per gut in workers of Coptotermes formosanus. Most of the bacteria have been found to reside in the hindgut (9, 52, 53). Electron microscopy studies by Breznak and Pankratz (10) revealed that the paunch epithelium of R. flavipes and C. formosanus is densely colonized by morphologically diverse bacteria, many of which possess holdfast elements that secure them to the epithelium and other bacterial cells. Aggregation of bacteria near indentations on the epithelial surface, possibly involved in ion absorption, suggest that bacteria could be nutritionally or biochemically important to the termite. In addition, Breznak and Pankratz (10) found that the midgut of R. flavipes and C. formosanus contained endospore-forming bacteria of a single morphological type.

Despite the abundance of bacteria in termite guts, few attempts have been made to isolate them with nonselective media and to identify them. Krasil'nikov and Satdykov (53) isolated gut bacteria from two species of Anacanthotermes by plating diluted intestinal contents on eleven different media. They found that Enterobacter aerogenes, Enterobacter cloacae, and Streptococcus faecalis were always isolated, ranging from about 4×10^5 to 1×10^9 viable bacteria per gut depending on the caste of termite or its stage of development. In addition,

Escherichia coli, Pseudomonas liquifaciens, and Bacillus subtilis were also isolated from intestines, but less frequently (53). Mauldin et al. (63) estimated that approximately 3×10^5 viable bacteria were present per hindgut of C. formosanus by using a tryptone-glucose-yeast extract medium, but he did not identify the isolates. Eutick et al. (26) recently isolated gut bacteria from 9 different species of Australian termites, by using plates of reinforced clostridial medium. Quantitation and identification of the bacteria revealed that each termite species possessed one major bacterial type in concentrations of approximately 10^7 cells per ml of termite gut. Streptococcus was isolated as the major bacterium from families Mastotermitidae and Kalotermitidae, while Enterobacter was found in 4 species of Rhinotermitidae. Streptococcus was also isolated from 3 species of Rhinotermitidae, but in quantities 10- to 100-fold less than that of Enterobacter. Although isolation plates were incubated under both aerobic and anaerobic conditions, no strict anaerobes were detected (26).

Most previous studies of termite gut bacteria have focussed on their possible involvement in cellulose digestion. Accordingly, many investigators have employed enrichment cultures as a means of detecting such forms. Cleveland (12, 13) was unsuccessful in more than 50 attempts to detect cellulolytic bacteria in guts of R. flavipes. Likewise, Dickman (23) and Hungate (36) used anaerobic and aerobic media and failed to detect cellulolytic bacteria in gut contents of Zootermopsis. Eutick et al. (26) found no evidence of cellulolytic bacteria in 9 different species of termites, although cellulolytic bacteria were readily isolated from soil.

Several investigators have obtained limited success with

enrichments for cellulolytic bacteria. Beckwith and Rose (4) inoculated gut contents of 7 species of lower termites into cellulose media. While no cellulose decomposition was evident under anaerobic conditions, 9 of 64 aerobic cultures indicated degradation of the polysaccharide. The time necessary for degradation varied from 10 days to 3 months, and microscopic examination revealed gram negative rods and some micrococci. Mannesmann (58) also used enrichment cultures to detect cellulolytic bacteria in gut contents of Reticulitermes virginicus and C. formosanus. Although some destruction of filter paper was evident after 10 weeks, bacteria grew in all cultures including many control tubes containing no cellulose. Other workers (28, 54, 55, 82, 83, 84) also claimed the isolation of cellulolytic bacteria from guts of lower termites. Unfortunately, a lack of quantitative methodology has made it difficult to ascertain the importance of such isolates in cellulose digestion in situ. To date, there is no convincing evidence that cellulolytic bacteria are important to the nutrition of lower termites which possess cellulolytic protozoa.

Attention has also focussed on the importance of bacteria in the nitrogen economy of termites. This is not surprising inasmuch as wood, the food of many termites, is low in combined N (i.e. 0.03-0.05% N w/w; 39). Cleveland (14) postulated that atmospheric nitrogen (N_2) was fixed by termites. He analyzed air samples from tubes containing live Zootermopsis, but could not detect any change in the amount of N_2 present. Likewise, nitrogen balances for colonies of Zootermopsis showed no net gain in combined nitrogen indicating that N_2 was not fixed (39, 41). By contrast, a specific assay for N_2 fixation, acetylene reduction, has been used to confirm N_2 fixation in termites

(5, 9, 30). Breznak (8) estimated that the amount of N_2 fixed by young larvae could allow such forms to double their protoplasmic N in the period of a year if their fixation rate remained constant. In addition, Breznak et al. (9) found that gut bacteria were responsible, since elimination of bacteria corresponded to a loss in the termite's ability to fix N_2. Whereas bacteria have long been implicated in N_2 fixation (33, 69, 70, 85, 86), the isolation and characterization of such bacteria had not been done. Recently, however, Potrikus and Breznak (72) isolated N_2-fixing Enterobacter agglomerans from hindguts of C. formosanus and implicated this species in N_2 fixation in situ. The recoveries of E. agglomerans from guts was 100-fold lower than expected, based on N_2 fixation rates of E. agglomerans in vitro and that of the intact termites. However, E. agglomerans was the only N_2-fixer isolated under a variety of conditions. French et al. (30) isolated N_2-fixing Citrobacter freundii from guts of C. lacteus and Mastotermes darwiniensis, however the bacteria were not quantitated. Likewise, no quantitative data are available for N_2-fixing Enterobacter from several species of lower termites (26).

Bacteria have also been implicated in the recycling of nitrogenous excretory compounds in the termite colony. Leach and Granovsky (56) first postulated that microorganisms in the hindgut of termites could utilize urates and other nitrogenous waste products of termites. Isolation of uricolytic bacteria from R. flavipes has revealed numbers as high as 6×10^4 cells per hindgut (73). Isolates were identified as strains of Streptococcus, Bacteroides, and Citrobacter and were found to degrade uric acid under anaerobic conditions.

Termites have been shown to emit methane, which appears to be produced by gut bacteria (8). When R. flavipes larvae were fed a diet of nest wood, 27-73 nmoles of methane were emitted per hour per gram of fresh weight. Bacteria seem to be responsible for the production of methane since feeding larvae antibacterial drugs abolished methane emission and detectable bacteria, but not protozoa (8).

Many bacteria in the hindguts of termites are associated with protozoa, either as residents within the cytoplasm or attached to the protozoan surface. A variety of bacteria have been reported to be endosymbiotic (6, 45, 46, 69). Bloodgood (6) found rod-shaped or fusiform-shaped bacteria, which showed no signs of degeneration, enclosed in membrane vesicles within the cytoplasm of Pyrsonympha and Trichonympha of R. flavipes. He suggested that essential nutrients were transferred from the protozoan cytoplasm to the prokaryotes. The prokaryotes, on the other hand, may confer on protozoa the ability to degrade cellulose or fix atmospheric nitrogen (6, 69, 70). Hungate (37) attempted to culture such intracellular bacteria, but without success.

Many bacteria, including spirochetes, are associated with protozoa by attachment to the protozoan surface. Bloodgood (6) found that spirochetes, associated with the surface membrane of Pyrsonympha of R. flavipes, provided the specialized structure for attachment to the protozoa. On the other hand, Urinympha and Barbulanympha of C. punctulatus provided attachment structures promoting the association of rod-shaped bacteria. Little is known of the roles of the attached bacteria, although the associations are believed to be advantagous to one or both partners (6). The attachment of

spirochetes to the flagellated Mixotrichia paradoxa is the only case where a role for the prokaryote has been established. Cleveland and Grimstone (18) found that the host protozoan derives its motility from synchronous movement of its adherent spirochetes. Margulis et al. (60) hypothesized that adherent spirochetes, originally selected to confer motility on the protozoan, evolved into eukaryotic flagella. Consistent with this notion is the recent evidence for microtubules in certain spirochetes (and a gliding bacterium) from hindguts of subterranean and drywood termites (60). No comparable tubules were seen in any of the other bacteria from the termite hindgut.

The biochemical capabilities of spirochetes in the termite hindgut are not known, primarily because the spirochetes have never been isolated in pure culture (7). Cleveland (17) eliminated spirochetes from the guts of lower termites by feeding them filter paper moistened with a solution of 5% acid fuchsin. He discovered that the removal of spirochetes did not affect the vitality of the termite. Eutick et al. (27) attempted to eliminate spirochetes from C. lacteus and the higher termite, Nasutitermes exitiosus, but found that none of the flora, including the spirochetes, were affected by acid fuchsin. All attempts that eliminated the spirochetes in C. lacteus also removed the protozoa. However, selective removal of spirochetes in N. exitiosus significantly reduced the termite's life span.

Higher Termites

Higher termites do not possess the cellulolytic protozoa found in lower termites, although they do have a substantial bacterial population in their guts. The majority of bacteria are located in the

paunch region, although the mixed segment, when present, usually contains bacteria of a single morphological type (32, 50). Potts and Hewitt (74) described a variety of paunch bacteria, including spirilla, small rods, and cocci in Trinervitermes trinervoides. They also found, in agreement with other workers (32, 50), no bacteria in the foregut or midgut and "pure cultures" of rods in mixed segments. Microscopic examination of guts of Macrotermes subhyalinus revealed an abundance of cocci and small gram positive rods in the paunch (1). Pochon et al. (71) found the paunch of Sphaerotermes sphaerothorax to contain cocci, rods, fusiforms, and spiral-shaped bacteria, many of which were similar to forms seen in the rumen of cattle.

Several genera of bacteria have been isolated from guts of higher termites. Strains of Pseudomonas (59) and cellulolytic Achromobacter (28) were isolated from guts of Nasutitermes exitiosus, but were not quantitated. Eutick et al. (26) isolated Staphylococcus from three species of Nasutitermes in concentrations of about 10^7 cells per ml of termite gut. Hungate (43) isolated a cellulolytic actinomycete, Micromonospora propionici, from Amitermes minimus in quantities of about 500 colony-forming-units per gut. However, he concluded the bacterium to be of limited importance in the digestion of cellulose in situ.

It is still speculative whether bacteria play a role in the digestion of cellulose. Misra and Rangathan (66) suggested that the cellulase and cellobiase found in the hindgut of Termes obesus were derived from bacteria, but they presented no conclusive evidence for this. Enrichment cultures for cellulolytic bacteria from guts of S. sphaerothorax indicated growth of cocci, primarily diplococci, rods,

and some vibrio-shaped bacteria (71). Analysis of the mixed culture fluid revealed the presence of acetate and butyrate, but no lactate or succinate. Tracey and Youatt (87) confirmed the presence of cellulase in guts of N. exitiosus, but did not determine its source. Kovoor (51) found cellulase activity in all intestinal regions of Microcerotermes edentatus and attributed it to the bacteria as well as synthesis by the termite. Other workers found cellulase activity localized in the midgut, a region essentially devoid of bacteria (1, 61, 74). Potts and Hewitt (74) found approximately 70% of the cellulase activity in the midgut of T. trinervoides, with 40% of this activity associated with the midgut wall. They concluded that cellulase was synthesized by the termite, although about 50% of the cellobiase was found in the paunch and could have been of bacterial origin.

Cellulolytic activities have recently been studied in fungus-growing termites, Macrotermes. Abo-Khatwa (1) found C_x cellulase (active against soluble derivatives, such as carboxymethylcellulose) and cellobiase equally distributed between the midgut and paunch and concluded that bacteria in the hindgut may contribute to digestion by providing C_x enzymes to the termite. Martin and Martin (61) implicated the midgut as the major site for these enzymes and concluded from analyses of midgut tissue and salivary glands that one source of C_x cellulase and cellobiase may be the termite itself. Abo-Khatwa (1) and Martin and Martin (61) found the vast majority of C_1 cellulase (active against crystalline cellulose) located in midgut contents. Fungi grown by Macrotermes sp. in nest material on "fungus combs" were analyzed for the presence of cellulolytic enzymes. Conidiophores

of the fungus Termitomyces, which are ingested by the termites, were found to contain high levels of C_1 cellulase and C_x cellulase (1, 61). If termites were starved or fed combs without fungus nodules, C_1 cellulase activity significantly decreased. It was suggested that the ingested condidiophores contributed C_1 cellulase to aid the termite in the initial stages of cellulose digestion.

Regardless of the origin of the cellulase, an active fermentation appears to take place in the paunch region of higher termites. Kovoor (48) detected acetic, propionic, and butyric acids in hindguts of Microcerotermes endentatus. Mannesmann (59) quantitated acids in the hindgut fluid of Nasutitermes nigriceps and found 4.7 mg of acetate per gram of gut fluid. Lactate levels approached only 0.5 mg per gram of hindgut fluid. The presence of acetate, propionate, butyrate, and only small amounts of lactate in guts of higher termites indicate an overall fermentation similar to that occurring in hindguts of lower termites.

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ARTICLE 1

HETEROTROPHIC BACTERIA PRESENT IN HINDGUTS OF WOOD-EATING TERMITES [*RETICULITERMES FLAVIPES* (KOLLAR)]

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Heterotrophic Bacteria Present in Hindguts of Wood-Eating Termites [*Reticulitermes flavipes* (Kollar)]†

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Strict anaerobic culture techniques were used to quantitate heterotrophic bacteria present in hindguts of *Reticulitermes flavipes*. The grand mean number of viable cells per hindgut was 0.4×10^5 (first-instar larvae), 1.3×10^5 (third-instar larvae), 3.5×10^5 (workers), and 1.5×10^5 (soldiers). Of a total of 344 isolates, 66.3% were streptococci that were always obtained regardless of the origin of termites, their developmental stage or caste, or their length of captivity. Most of the remaining isolates were strains of *Bacteroides* and *Enterobacteriaceae*. A small percentage were strains of *Lactobacillus*, *Fusobacterium*, and unidentified anaerobic gram-positive rods. Recovery of bacteria from worker hindguts was 13.0% of the direct microscopic count. Isolations performed aerobically failed to reveal strict aerobes. Attempts to isolate cellulolytic bacteria were uniformly unsuccessful. Of 145 streptococcal strains isolated from freshly collected termites, almost all were *Streptococcus lactis* and *S. cremoris*. *Enterobacteriaceae* isolates from the same termite specimens were indole-positive *Citrobacter*, citrate-negative *Citrobacter*, and *Enterobacter cloacae*. The possibility of in situ interspecies lactate transfer, between lactate producers (e.g., streptococci) and lactate fermenters (*Bacteroides*), is discussed.

The hindgut of xylophagous lower termites harbors a microbiota consisting of both protozoa and bacteria. Although much attention has been focused on the protozoa (because many are cellulolytic and exist in a mutualistic relationship with the termites [3, 29]), our understanding of the bacterial component is meager. However, available evidence suggests that procaryotes are also important elements of the gut biota and important to the vitality of termites. First, they are abundant (3), and their presence appears necessary for persistence of the protozoa and for normal termite nutrition (4, 36). Second, N_2 fixation by some of the bacteria has implicated them in the N economy of termites (4, 18, 33). Third, many exist in intimate physical association with the gut epithelium, a situation presumably reflecting biochemical interaction as well (5).

Numerous attempts have been made to isolate cellulolytic bacteria from termites. Hungate (22), for example, isolated an anaerobic, cellulolytic actinomycete (*Micromonospora propionici*) from guts of *Amitermes minimus*, but concluded that the bacterium was of limited importance in situ. Other such attempts have been unsuccessful (7, 11, 20) or have yielded positive results of uncertain significance, either because the methods were not fully described or because no quantitation of bacteria was made (2, 17, 27, 30, 32, 35, 37, 38). By contrast, Krasil'nikov and Satdykov (25) used relatively nonselective media to quantitate bacteria present in guts of *Anacanthotermes ahngerianus* and *A. turkestancius*. They found 1.3×10^6 to 4.3×10^9 viable cells per hindgut, depending on the developmental stage or caste of termite examined. However, these workers apparently used only aerobic conditions for bacterial isolation and could have overlooked a numerically significant population of gut anaerobes.

It was felt that a better understanding of the nature and possible roles of bacteria in the termite gut required not only a comprehensive and quantitative approach, but attention to strict anaerobic methodology as well. Accordingly, the present study was initiated. In this paper we report on heterotrophic bacteria present in hindguts of *Reticulitermes flavipes*, the common eastern subterranean termite.

MATERIALS AND METHODS

Termites. *R. flavipes* was collected in Janesville, Wis., and Spring Arbor, Mich., and maintained in the laboratory as previously described (5), except that commercial lumber or paper towels were not supplied. The contents of termite containers were periodically moistened by spraying the surface of infested wood with distilled water.

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Terminology used to indicate the developmental stage or caste of termites was that of Esenther (14).

Media. Strict anaerobic techniques (19, 23) were used for preparation of all media unless indicated otherwise. Media included: supplemented brain heart infusion (BHI); chopped meat with meat particles (CMMP); Sweet E; and peptone-yeast-glucose (PYG). All were compounded according to Holdeman and Moore (19) except for PYG, which contained 0.5% each of peptone, yeast extract, and glucose, 0.05% cysteine·HCl, 10^{-4}% resazurin, and inorganic salts (12). In addition, a medium (MB medium) designed for isolating saccharolytic rumen bacteria was also used. The composition of MB medium was described by Bryant (6) and was used herein with 0.5% glucose, maltose, cellobiose, or soluble starch or with 1.0% α-cellulose (Sigma Chemical Co., St. Louis, Mo.) as sole fermentable carbohydrate. Variations of MB medium also tried were: (i) omission of the volatile fatty acid solution; (ii) substitution of volatile fatty acids with bovine rumen fluid (10% [vol/vol], final concentration); and, for cellulose-containing media, (iii) incorporation of 0.1% Trypticase into variations (i) and (ii).

Solid media contained 1.5 or 2.0% agar. MB media were prepared as roll tubes (23) with 100% CO_2 as gas phase and were contained in anaerobic screw-cap culture tubes (Hungate type; Bellco Glass Inc., Vineland, N.J.). Just before use, sterile reducing agent (cysteine-sulfide solution) was added by syringe (23). All other media were prereduced and anaerobically sterilized (19) under 95% N_2-5% CO_2 and were contained in rubber-stoppered 18- by 142-mm culture tubes (broths) or in plastic petri plates (solid media) that were filled while held in an anaerobic glove box (1; Coy Manufacturing Co., Ann Arbor, Mich.). Media used under aerobic conditions contained no added reducing agents or resazurin. The initial pH of media ranged from 6.7 to 7.2.

Isolation of bacteria. Guts were removed anaerobically from groups of 10 termites and were homogenized as previously described (33). Serial 10-fold dilutions of gut homogenate were made, and 0.1 ml of each dilution was spread in duplicate on plates of isolation medium. Broth solutions, used for homogenizing guts and diluting the homogenate, were homologous to the plating medium but lacked agar. Plates were then either left directly in the glove box atmosphere (90% N_2-10% H_2) or placed in a GasPak jar (BBL, Cockeysville, Md.) containing an activated GasPak H_2 + CO_2 generator envelope. Jars were also retained in the glove box. Incubation was at 23 to 25°C for at least 7 days. Isolated colonies were then picked at random and were considered pure cultures after three successive passages on streak plates.

For isolation of bacteria by the roll tube method (23) a similar approach was used, except that dilution of gut homogenates and inoculation of molten agar media were performed by syringe.

Control experiments used the following source materials, which were plated on BHI agar: (i) surface washes of intact termites; (ii) homogenates prepared from degutted termite bodies; and (iii) homogenates prepared from termite midguts, portions of which frequently remained attached to extracted hindguts (5). Midguts were obtained by severing extracted guts just anterior to the enteric valve.

Enumeration of bacteria. The number of cultivable bacteria per gut was calculated from the average number of colonies present on primary isolation plates or in roll tubes. The acridine orange-epifluorescence method (16) was used for determining the total number of bacteria present in gut homogenates.

Characterization of isolates. Isolates were characterized by standard microbiological methods (8). Since all were found to grow in BHI, plates of this medium were used to test for aerobic growth.

Anaerobes were identified to the genus level by using criteria outlined in the VPI *Anaerobe Laboratory Manual* (19). Acidic fermentation products were determined after cells were grown anaerobically for 96 h in BHI broth. Organic acids were extracted from the spent medium (19) and analyzed by gas chromatography (33).

Streptococci were identified to species according to the criteria of Deibel and Seeley (10). Ammonia formation from arginine was assessed as described by Deibel (9). Lancefield precipitin tests were performed by using autoclaved cells according to the bulletin accompanying the commercial antiserum (Difco Laboratories, Detroit, Mich.). Sugar fermentation reactions were evaluated by determining the pH of cultures 72 h after inoculation into a basal medium containing 1.0% (xylose or cellobiose) or 0.5% (xylan) of the test sugar. The basal medium contained 1.0% tryptone, 0.1% yeast extract, and 4.0% (vol/vol) salts solution (19) lacking $NaHCO_3$. The ability of cells to develop a final pH at least 1.0 unit lower than that in control medium (lacking sugar) was interpreted as a positive result. Xylan (Sigma Chemical Co.) was twice extracted with boiling distilled water before use in fermentation tests.

Enterobacteriaceae were identified according to established criteria and tests (13, 26). In addition, presumptive tests for the ability of strains to fix N_2 were made by using GSV medium and methods previously described (33).

Lactate fermentation by *Bacteroides* strains. Formation of propionate and acetate from lactate by growing cells was determined by using PY and PYL media (19).

Lactate fermentation by cell suspensions was assessed by using strains grown anaerobically in BHI broth. Strict anaerobic techniques were used throughout. Cells were harvested by centrifugation, resuspended in 0.1 M potassium phosphate buffer (pH 7.2) containing 1 mM dithiothreitol, and added to reaction mixtures (3.0 ml, final volume) containing (millimolar): potassium phosphate buffer (pH 7.2), 93.3; dithiothreitol, 2.6; and sodium-DL-lactate, 17.0. Reaction mixtures were held under O_2-free N_2 in 5-ml-capacity serum vials equipped with rubber stoppers. Incubation was for 2 h at 37°C, after which reactions were terminated by addition of 0.2 ml of 5 N H_2SO_4. Acidified mixtures were centrifuged to obtain cell-free supernatant fluids, which were then used for assay of organic acids. Results were corrected for propionate and acetate formation by endogenous cell metabolism. Heat-treated cell suspensions (100°C for 15 min) served as controls. Organic acids and cell protein were assayed as previously described (33).

Other bacterial strains. A variety of known bacteria were used as positive and negative controls, either for the efficacy of the anaerobic methodology or for physiological/biochemical tests. Such organisms, and their origin, were: *Peptostreptococcus anaerobius, Bacteroides fragilis, Bacillus licheniformis, Escherichia coli, Proteus mirabilis, Enterobacter cloacae,* and *Pseudomonas aeruginosa* (culture collection, Department of Microbiology and Public Health, Michigan State University); *Streptococcus cremoris* ATCC 19257, *S. lactis* ATCC 19435, and *S. faecalis* ATCC 19433 (American Type Culture Collection, Rockville, Md.).

RESULTS

Bacteria isolated from guts of *R. flavipes* workers. A summary of isolates obtained with BHI medium is given in Table 1. No marked differences were observed for termites collected from sites 237 miles (ca. 381 km) apart, whether isolations were performed immediately after collection or 17 months thereafter. The grand mean number of cultivable bacteria per gut was $3.5 \pm 2.8 \times 10^5$ ($n = 13$). The mean number obtained by using N_2/H_2 incubation atmosphere was not significantly different at the $\alpha_{0.05}$ level from that observed with GasPak incubation (Student's *t* test; Table 1, footnote *c*). Moreover, the distribution of physiological types retrieved was virtually identical under either incubation atmosphere (data not shown). Streptococci were always obtained and were clearly the predominant cultivable organisms, accounting for 66.3% (138/208) of all isolates. Most of the remainder were represented by strains of *Bacteroides* and *Enterobacteriaceae,* which together accounted for 27.9% of the total and which were isolated almost routinely. Strains of *Lactobacillus, Fusobacterium,* and unidentified anaerobic rods constituted a minority of isolates.

When surface washes of live termites or degutted termite bodies were used as inocula, colony counts with BHI medium were 2×10^1-fold less, indicating that the isolates described in Table 1 were not extraintestinal contaminants. Moreover, 100-fold fewer colonies were obtained from midgut homogenates, indicating that isolates were virtually all derived from hindguts.

The ability to isolate strict anaerobes from guts served as an internal control for the methodology used. However, an additional control consisted of known strains of *B. fragilis* and *P. anaerobius* that were manipulated in a manner simulating the isolation procedure with guts. Both strains formed isolated colonies after 4 days of incubation in either the N_2/H_2 or GasPak atmosphere.

During the initial phase of this study, a variety of isolation media were screened for their suitability. It was found that recovery of bacteria on CMMP, Sweet E, or PYG medium was not significantly different from that obtained with BHI, either in terms of the number of viable cells per gut or the nature of the isolates. By contrast, recovery of bacteria with the various MB media (containing soluble sugar) was only 1/10 to 1/3 that obtained with BHI; however, the isolates were not characterized. Ultimately BHI medium was chosen for routine use because it permitted rapid and luxurious colony development, and it was readily prepared.

Aerobic isolation attempts revealed an average of 1.4×10^5 viable cells per gut (one experi-

TABLE 1. *Summary of bacteria isolated from guts of R. flavipes workers*[a]

Location and date of collection	Length of captivity (mo)[b]	Viable bacteria/gut ($\times 10^{-5}$)[c]	No. of isolates characterized	Generic affiliation of isolates						
				Streptococcus	*Bacteroides* Group 1	*Bacteroides* Group 2	"Enteric type"[d]	*Lactobacillus*	*Fusobacterium*	Unidentified anaerobic rods
Janesville, Wis.										
June 1977	0	2.1	44	28	14	1	1	0	0	0
June 1976	2	2.0	33	19	8	1	4	0	0	1
June 1976	9	3.0	16	13	1	0	2	0	0	0
June 1975	9	8.6	38	17	4	0	9	6	0	2
June 1973	17	5.8	23	15	0	3	2	1	2	0
Spring Arbor, Mich.										
May 1977	0	1.4	30	27	2	0	1	0	0	0
July 1976	8	1.6	24	19	0	0	5	0	0	0

[a] All isolates were obtained by using BHI medium.

[b] Zero months refers to termites used within 24 h of collection.

[c] Average of two experiments (one using N_2/H_2 isolation atmosphere, the other using GasPak) for each period of captivity, except for 9-months captivity of June, 1976, Janesville collection (one experiment; N_2/H_2 atmosphere). Grand mean, $3.5 \pm 2.8 \times 10^5$ ($n = 13$). Mean value with N_2/H_2 atmosphere, $3.1 \pm 2.0 \times 10^5$ ($n = 7$); with GasPak atmosphere, $4.0 \pm 3.7 \times 10^5$ ($n = 6$).

[d] Assigned to the family *Enterobacteriaceae.*

ment). Of 41 random isolates examined, all were facultative anaerobes, but they were not characterized further. All attempts to isolate cellulolytic bacteria were unsuccessful, although control experiments (using sheep rumen contents as inoculum) yielded such organisms at high dilution.

Direct microscopic counts revealed an average of 2.7×10^{11} bacteria per gut ($n = 4$). Comparison of this datum with the grand mean number of viable cells per gut indicated a recovery of about 13%. Gram-stained preparations of gut homogenates revealed numerous gram-negative rods and spiral-shaped cells along with gram-positive cocci. Gram-positive rods were rarely observed, and most protozoa were disrupted by the homogenization procedure.

General properties of bacteria isolated from *R. flavipes* workers. Results are presented in Table 2. All isolates were acidogenic and developed a final pH of about 4.5 in BHI broth as a result of organic acid production.

Streptococci occurred as short chains of coccoid (i.e., football-shaped) cells. All were homolactic fermenters, forming no detectable gas in BHI broth. All but one strain were facultative anaerobes. Cells were consistently variable with respect to the Gram reaction, even when 8-h broth cultures were examined. In addition, all strains fermented xylose and cellobiose; none fermented xylan.

Bacteroides isolates were separable into two groups based on acidic fermentation products. Most group 1 strains were aeroduric anaerobes inasmuch as they could initiate growth in the primary streak area of aerobic plates. However, such growth was slow and sparse, and isolated colonies were never formed. Group 1 isolates also formed small amounts of propionate and acetate from lactate during anaerobic growth in PYL broth. However, growth in PYL was not significantly greater than in medium lacking lactate (i.e., PY broth). These observations suggested that lactate fermentation by group 1 strains provides little or no energy for growth. Nonetheless, to confirm the ability of such strains to ferment lactate, experiments were performed with cell suspensions. In fixed-time assays, two strains tested (strains S34C and SS10C) formed 0.52 to 0.70 μmol of propionate and 0.29 to 0.31 μmol of acetate per μmol of lactate fermented per 2 mg of cell protein. No lactate was fermented by heat-treated cell suspensions.

Bacteria present in guts of larvae and soldiers. To determine whether bacteria present in guts of workers were also present in other colony members, isolations were performed with larvae and soldiers freshly collected from the two sites. Results are presented in Table 3, which includes data from workers for ease of comparison. The number of viable bacteria per gut was generally less than that for workers (see also Table 1), particularly in the case of first-instar larvae, which were, in turn, considerably smaller in size than workers. However, the major groups of bacteria isolated from larvae and soldiers were strikingly similar to those obtained from workers (see also Table 1). Streptococci again represented the most abundant and consistently isolatable group, and *Bacteroides* strains were obtained in almost every instance. All groups of isolates displayed characteristics analogous to those described in Table 2.

Identification to species of streptococci and *Enterobacteriaceae*. Because of the preponderance of streptococci among termite gut isolates, an assessment of their phenotypic diversity seemed warranted. Accordingly, an attempt was made to identify to species 145 strains isolated from freshly collected termites (Table 3). Most streptococci (124/145) were identified

TABLE 2. *General properties of bacteria isolated from guts of R. flavipes workers*[a]

Generic affiliation	Morphology	Gram reaction	Relation to oxygen	Catalase	Motility	Acidic fermentation products
Streptococcus	C	var.	F(A)	−	−	L(af)
Bacteroides						
Group 1	R	−	AA(A)	+	−	APsiv
Group 2	R	−	A	+	−	apibbiv
"Enteric type"[b]	R	−	F	+	+ or −	LAS
Lactobacillus	R	+	F(A)	−	−	L(afs)
Fusobacterium	R	−	A	ND	−	SABl(p)
Unidentified	R	+	A	ND	−	lapibbiv

[a] Symbols: (+) Positive reaction; (-) negative reaction; (C) coccoid; (R) rod; (var.) variable; (F) facultative; (A) strict anaerobe; (AA) aeroduric anaerobe; (ND) not determined. Acidic fermentation products: (A, a) acetic; (L, l) lactic; (P, p) propionic; (S, s) succinic; (B, b) butyric; (f) formic; (ib) isobutyric; (iv) isovaleric. Upper-case and lower-case letters refer to major and minor amounts, respectively (19). Parentheses indicate results with some strains.

[b] See Table 1, footnote *d*.

as either *S. lactis* or *S. cremoris*. The remainder were represented by unidentified group N streptococci and by red-pigmented strains whose group affiliation was not determined. Curiously, Janesville termites had a larger proportion of *S. lactis* in their hindguts than *S. cremoris*, whereas Spring Arbor termites had large numbers of *S. cremoris* but no demonstrable *S. lactis*. Detailed physiological characteristics of the streptococcal isolates are given in Table 4.

Attempts were also made to identify *Enterobacteriaceae* isolated from both Janesville and Spring Arbor termites. Of 21 randomly selected strains, 12 were indole-positive *Citrobacter*, 4 were citrate-negative *Citrobacter*, and 5 were identified as *Enterobacter cloacae*. In addition, none showed evidence of N_2 fixation ability.

DISCUSSION

Results herein clearly show that an abundance of viable, heterotrophic bacteria is present in the hindgut of *R. flavipes*. Approximating the hindgut of workers to a right circular cone, with a base radius of 0.4 mm and height of 4 mm (5), its volume is about 0.7 mm^3. Thus, the grand mean number of viable cells per hindgut (3.5 ×

TABLE 3. *Bacteria isolated from guts of freshly collected larvae, soldiers, and workers of R. flavipes*[a]

Bacteria collected from:	Viable bacteria/gut (× 10^{-5})[b]	No. of isolates characterized	Generic affiliation of isolates								
			Streptococcus				*Bacteroides*		"Enteric type"[c]	*Lactobacillus*	Unidentified anaerobic rods
			S. lactis	*S. cremoris*	Unidentified group N	Red-pigmented strains	Group 1	Group 2			
Janesville, Wis.											
Larvae											
1st instar	0.4	25	14	1	3	1	2	0	0	4	0
3rd instar	1.6	23	11	3	0	2	0	2	2	2	1
Soldiers	2.0	30	9	4	3	0	4	4	2	2	2
Workers	2.1	44	18	6	4	0	14	1	1	0	0
Spring Arbor, Mich.											
Larvae											
1st instar	0.4	17	0	9	1	0	3	0	4	0	0
3rd instar	1.1	22	0	13	4	0	4	0	1	0	0
Soldiers	1.0	19	0	12	0	0	7	0	0	0	0
Workers	1.4	30	0	24	3	0	2	0	1	0	0

[a] Termites collected from Janesville and Spring Arbor in June and May, 1977, respectively. Isolation medium was BHI in all cases.

[b] Average of two experiments (one using N_2/H_2 isolation atmosphere, the other using GasPak) for each caste or developmental stage.

[c] See Table 1, footnote *d*.

TABLE 4. *Physiological reactions of Streptococcus isolates*[a]

Isolate	Growth at:		Heat tolerance[b]	Growth in milk containing:		Growth in BHI medium containing:		Hemolysis	Hydrolysis of:		Ammonia from arginine	Precipitin reaction with:	
	10°C	45°C		0.1% methylene blue	0.3% methylene blue	4.0% NaCl	6.5% NaCl		Esculin	Hippurate		Group N antiserum	Group D antiserum
S. cremoris	+	−	−	d	−	−	−	γ	−	−	−	+	−
S. lactis	+	−	−	+	+	+	−	γ	d	−	+	+	−
Unidentified group N strains	+	−	−	+	−	+	−	α	−	−	−	+	−
Red-pigmented strains	−	−	−	+	−	−	−	α	−	−	+[w]	ND	ND

[a] Symbols: +, Positive; −, negative; d, different biochemical types (+, −, +[w]); +[w], weak positive; ND, not determined.

[b] 60°C, 30 min.

10^5) translates to 5.0 to 10^8 viable cells per ml of hindgut fluid. This estimate is quite conservative, however, and probably low by 10- to 100-fold, because the calculation ignores the appreciable volume of the hindgut taken up by protozoa (5). Our estimate of 13% recovery of the direct microscopic count is also conservative in view of the method used to enumerate cells. Homogenization of termite guts generates considerable debris, derived not only from gut tissue but also from disrupted protozoa. Yet during microscopic counts, questionable particles were scored as bacteria. Notwithstanding, there are numerous bacteria with unique morphologies (5) that were not isolated, e.g., spirochetes. Hopefully such organisms will be obtained in future attempts, as our understanding of the hindgut ecosystem increases and leads to medium formulations that are more habitat-simulating.

The absence of cellulolytic bacteria in *R. flavipes* guts is consistent with Hungate's data (21), which attribute cellulolysis in xylophagous termites to the protozoa. In addition, our inability to demonstrate strict aerobes is consistent with a concept of the hindgut being an anaerobic niche (3). Although most of our isolates were facultative anaerobes, they presumably use a fermentative mode of energy generation in situ. A challenge to this presumption is afforded by Eutick et al. (15), who concluded that the gut of certain Australian termites was "aerobic." While their termite specimens were different from *R. flavipes*, it should also be noted that these investigators did not measure the in situ pO_2, but inferred the E_0' of guts excised from termites fed redox dyes. Consequently, extension of their data to intact animals should be made with caution.

Present findings parallel those of Krasil'nikov and Satdykov (25), who isolated numerous streptococci from *Anacanthotermes* species. However, predominant streptococci in *Anacanthotermes* were strains of *S. faecalis*, whereas in *R. flavipes* they were *S. lactis* and *S. cremoris*. Our results present only an apparent contradiction to those of Martin and Mundt (28), who failed to demonstrate streptococci in *R. flavipes*. The latter investigators used azide-containing media to select for enterococci; azide inhibits the growth of *S. lactis* and *S. cremoris* (24).

The preponderance of streptococci in *R. flavipes* collected from widely separate sites, and their persistence in the gut even after long captivity of the insects, suggests a stable relationship between these bacteria and their host. However, any claim for autochthony (34) of streptococci in *R. flavipes* would be premature, since some castes (winged forms and neotenics) remain to be examined. Presence of streptococci in guts of first-instar larvae, however, suggests colonization early in the animals' life. Acquisition of streptococci by larvae and soldiers is probably via proctodeal food (31) solicited from workers.

The abundance of streptococci in *R. flavipes* hindguts, along with other bacteria that formed lactic acid in pure culture (Table 2), suggests that lactate could be a significant fermentation product in situ and constitute an oxidizable energy source for the insect. Substrates for lactate formation might be xylose and/or cellobiose, which served as fermentable substrates for 145 streptococcal strains tested, and which could arise during hemicellulose or cellulose hydrolysis by other members of the biota (e.g., protozoa). However, preliminary experiments (C. F. Hirsch and J. A. Breznak, unpublished data) revealed that no lactate was formed when suspensions of hindgut microbiota were incubated anaerobically with [U-^{14}C]cellulose. Relative amounts of organic acids that were produced were: acetate > formate > propionate > butyrate. Thus, either lactate is not formed by streptococci in situ, or it is formed but rapidly dissimilated by lactate fermenters. The presence in guts of *Bacteroides* strains (Tables 1 and 3), capable of fermenting lactate to propionate and acetate, is consistent with the latter suggestion and renders tenable the hypothesis that interspecies lactate transfer occurs between streptococci and *Bacteroides*. This hypothesis is currently being examined in our laboratory.

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ARTICLE 2

CROSS-FEEDING OF LACTATE BETWEEN *STREPTOCOCCUS LACTIS* AND *BACTEROIDES* SP. ISOLATED FROM TERMITE HINDGUTS

By. J. E. Schultz and John A. Breznak

ABSTRACT

Streptococcus lactis and *Bacteroides* sp., isolated from hindguts of *Reticulitermes flavipes* termites were grown anaerobically in monoculture and coculture. When grown in a glucose medium, *S. lactis* monoculture produced lactate as the major fermentation product with small amounts of formate, acetate, ethanol, and CO_2. In coculture, glucose was completely consumed during growth of *S. lactis*. Lactate, produced by *S. lactis*, then supported growth of *Bacteroides* and was fermented to propionate, acetate, and CO_2. Small amounts of succinate were formed during growth of *Bacteroides* in the coculture, but little change in the formate or ethanol concentration was observed. Monoculture growth of *Bacteroides* in a tryptone-yeast extract medium revealed that small amounts of lactate (20 to 40 μmoles/ml) increased cell yields and production of ogranic acids. However, initial lactate concentrations greater than 40 μmoles/ml suppressed not only growth of *Bacteroides* but acidic product formation as well. Results suggest that cross-feeding of lactate between streptococci and bacteroides constitutes one aspect of the overall hindgut fermentation in the termite.

INTRODUCTION

The hindgut of wood-eating termites contains an abundant, heterogeneous population of bacteria whose roles in carbon, nitrogen, and energy metabolism are not well-understood. Isolation and characterization of heterotrophic bacteria from hindguts of Reticulitermes flavipes (family Rhinotermitidae) has revealed a predominance of streptococci and Bacteroides species which appear to be relatively stable components of the hindgut microbiota (14). Almost all of the streptococci isolated from R. flavipes were S. lactis and S. cremoris. They were saccharolytic and displayed a homolactic fermentation of glucose. Bacteroides isolates were also saccharolytic, but selected strains additionally possessed the ability to ferment lactate to propionate and acetate as major soluble products (14).

These findings suggested that cross-feeding of lactate from streptococci to Bacteroides might be one feature of the overall hindgut fermentation occurring in the insect. To test the feasibility of this hypothesis, strains of S. lactis and Bacteroides sp. were examined during in vitro culture. In this paper we report on the growth, substrate utilization, and product formation by S. lactis in monoculture and in coculture with Bacteroides sp. In addition, the effect of lactate on growth and product formation by pure cultures of Bacteroides was examined.

MATERIALS AND METHODS

Organisms. Streptococcus lactis strain JW1 and Bacteroides sp. strain JW20 were isolated from hindguts of Reticulitermes flavipes workers collected from Janesville, Wi. (14). Strain JW20 was assigned to group 1 on the basis of acidic fermentation products (14).

Media and growth conditions. Strict anaerobic techniques (5) were used for preparation of media, growth of cells, and sampling of culture contents. Growth media were TY, TYC, and TYG. TY medium contained (g/100 ml): tryptone, 1.0; yeast extract, 0.1; $(NH_4)_2SO_4$, 0.2; KH_2PO_4, 0.34; Na_2HPO_4, 0.66; hemin, 5×10^{-4}; and resazurin, 10^{-4}. The initial pH was 7.0. TYC was identical to TY, but also contained cysteine-HCl (0.05 g/100 ml). TYG was identical to TY, but also contained glucose (0.1 g/100 ml). When glucose-UL-^{14}C was used to TYG medium, it was sterilized separately by filtration and incorporated at an activity of 0.5 μCi/ml.

Cells were usually grown in 300 ml Nephelo flasks (Bellco Glass Inc., Vineland, N.J.) equipped with a serum-stoppered sampling port and containing 100 to 200 ml of medium. However, for some experiments cells were grown in rubber-stoppered 18 x 142 mm anaerobe tubes (Bellco) containing 10 ml of medium. The initial gas phase in all cultures was O_2-free N_2. Inocula consisted of exponential phase cells growing in homologous media, and the incubation temperature was 20°C.

Analysis of growth and fermentation products. Growth of cells was monitored turbidimetrically by measuring the absorbance of cultures

at 660 nm with a Spectronic 20 colorimeter (Bausch & Lomb). In addition, viable cell counts were determined by plating appropriate dilutions of culture onto plates of supplemented brain heart infusion (BHI) agar and incubating them in GasPak jars as previously described (14). Under these conditions, colonies of S. lactis JW1 were 5 mm in diameter and round with a slightly erose margin, whereas those of Bacteroides JW20 were only 1.5 mm in diameter and were round with an entire margin. Thus, viable cells of each strain could be easily quantified, even when growing together in the same vessel (i.e. in coculture; Fig. 1).

For time-course analyses of fermentations, culture samples were centrifuged at 16,300 x g for 10 min at 10°C, and the resulting supernatant fluids were frozen until ready for assay. Glucose and lactic acid were assayed by the methods of Spiro (15) and Barker (2), respectively. The molecular configuration of lactic acid was determined enzymatically (5). Other organic acids were quantified by using gas chromatography (13). Ethanol was estimated enzymatically (Ethyl Alcohol Reagent Set; Worthington Biochemical Corp., Freehold, N.J.). Results with TYG medium were corrected for small amounts of products produced during growth of cells in the absence of glucose (i.e. in TY medium).

Products formed from glucose-UL-^{14}C were determined after cells had grown for 81 h. At that time a small volume of culture was removed for determination of viable cell number, and the remainder of the culture was acidified with H_2SO_4 to a final concentration of 0.12 N. The headspace gas was then swept into a phenethylamine/methanol (1:1, vol/vol) trap by using N_2 as carrier. Radioactivity

Figure 1. Surface colonies of Streptococcus lactis strain JW1 (large colonies) and Bacteroides sp. strain JW20 (smaller colonies) on a 100 mm diameter plate of supplemented BHI medium. Incubation was at 25°C for 5 days.

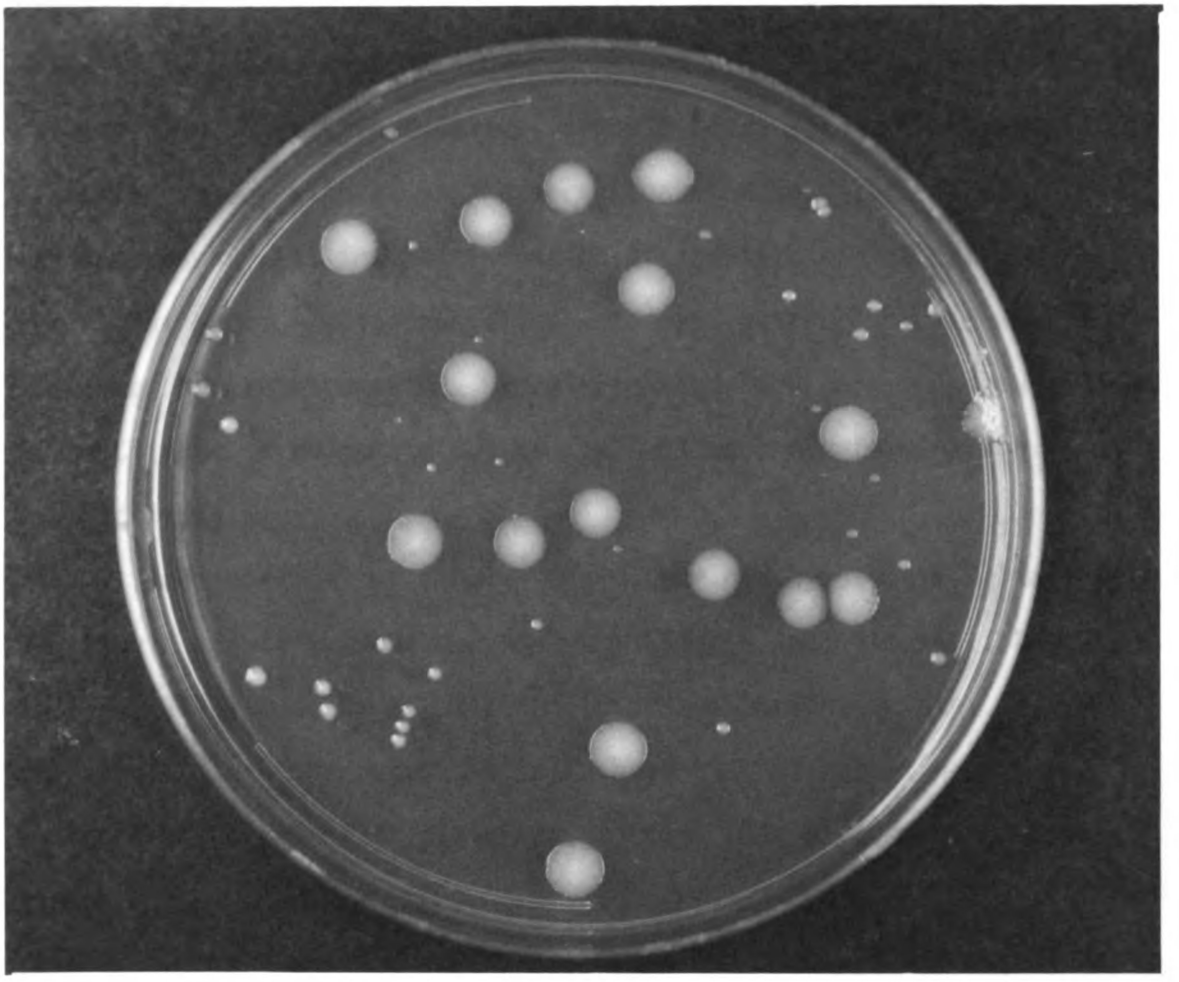

Figure 1.

trapped in this way was assumed to be due solely to $^{14}CO_2$, whose quantity was calculated from the specific activity of glucose-UL-^{14}C. The acidified fermentation liquor was then clarified (11) and assayed as described above. In addition, organic acids were extracted from the clarified liquor with ether and were separated by silicic acid column chromatography (11) for determination of specific radioactivity. For determination of ^{14}C incorporation into cell material, cells were harvested by centrifugation, hydrolyzed with NaOH (4), then neutralized with HCl and 1 M phosphate buffer (pH 7.0) before determination of radioactivity.

Radioactivity of samples was determined by using a Delta model 300 liquid scintillation spectrometer (Searle Analytic Inc., Des Plaines, Ill.) and ACS scintillant (Amersham, Arlington Hts., Ill.). All samples were corrected for self absorption of radiation by the channels ratio method (17); toluene-^{14}C was used as a radioactivity standard.

Chemicals. Tryptone and yeast extract were obtained from Difco Laboratories, Detroit, Mi.; glucose-UL-^{14}C and toluene-^{14}C from New England Nuclear, Boston, Ma.; and sodium-DL-lactate (syrup) from Sigma Chemical Co., St. Louis, Mo. All other chemicals were of reagent grade and were obtained from commercial sources.

RESULTS

Growth of S. lactis JW1 and Bacteroides sp. JW20 in monoculture and coculture. S. lactis grew readily as a monoculture in TYG medium, exhibiting specific growth rates of about 2.0 x hr^{-1} and yields ranging from 2.0 x 10^8 to 2.5 x 10^8 viable cells per ml. During growth glucose was consumed and lactic acid production ranged from 7.6 to 8.0 μmole/ml, thereby accounting for 69 to 72% of the glucose carbon fermented. The molecular configuration of lactic acid was L(+). These events were completed during the first 10 to 15 h of incubation; thereafter the number of viable cells and lactic acid concentration remained constant for up to 80 h. Small amounts of ethanol, acetate, formate, and CO_2 were also produced, and about 6% of the glucose carbon was incorporated into cell material (Table 1; column 1). When glucose-UL-^{14}C was used as substrate, the specific activity of lactate produced indicated that virtually all of the acid was derived from glucose (Table 1; footnote a).

When S. lactis JW1 was cocultured with Bacteroides JW20, the first 10 h of incubation was dominated by growth of JW1 and by glucose consumption and lactate production (Fig. 2). Thereafter, strain JW20 commenced growth at a specific rate of about 0.5 x hr^{-1} and achieved populations ranging from 6.0 x 10^8 to 8.6 x 10^8 viable cells per ml. During the latter stages of growth in particular, lactate previously produced from strain JW1 decreased markedly while propionate and acetate concentration increased (Fig. 2; acetate not depicted). CO_2 and small amounts of succinate were also formed during growth of Bacteroides in the coculture, whereas little change in formate or

Table 1. Fermentation of glucose-UL-^{14}C by S. lactis JW1 in monoculture and in coculture with Bacteroides sp. JW20.

Products[a]	µmol/100 µmol glucose-UL-^{14}C fermented			µmol/100 µmol lactic acid fermented
	S. lactis monoculture	S. lactis + Bacteroides coculture	Coculture minus monoculture	Coculture minus monoculture
Lactate	138.5	36.0	-102.5	--
Propionate	0.0	80.9	80.9	78.9
Acetate	7.2	26.9	19.7	19.2
CO_2	3.6	30.6	27.0	26.3
Ethanol	12.6	9.8	--	--
Formate	5.3	5.4	--	--
Succinate	0.0	2.7	--	--
Carbon recovery (%)				
in products	77.3	78.4	100.5	100.5
in cells[b]	5.8	6.1	--	--
Total carbon recovery (%)	83.1	84.5	--	--

[a]Specific activity of glucose-UL-^{14}C substrate was 31,500 dpm/µmole carbon. Specific activities of lactate in the monoculture and coculture were 30,550 and 28,000 dpm/µmole carbon, respectively. Specific activities of propionate and acetate in coculture were 22,540 and 20,260 dpm/µmole carbon, respectively.

[b]Determined in separate experiments under identical conditions.

Figure 2. Growth, substrate utilization, and product formation by a coculture of Streptococcus lactis strain JW1 and Bacteroides sp. strain JW20. The growth medium was TYG.

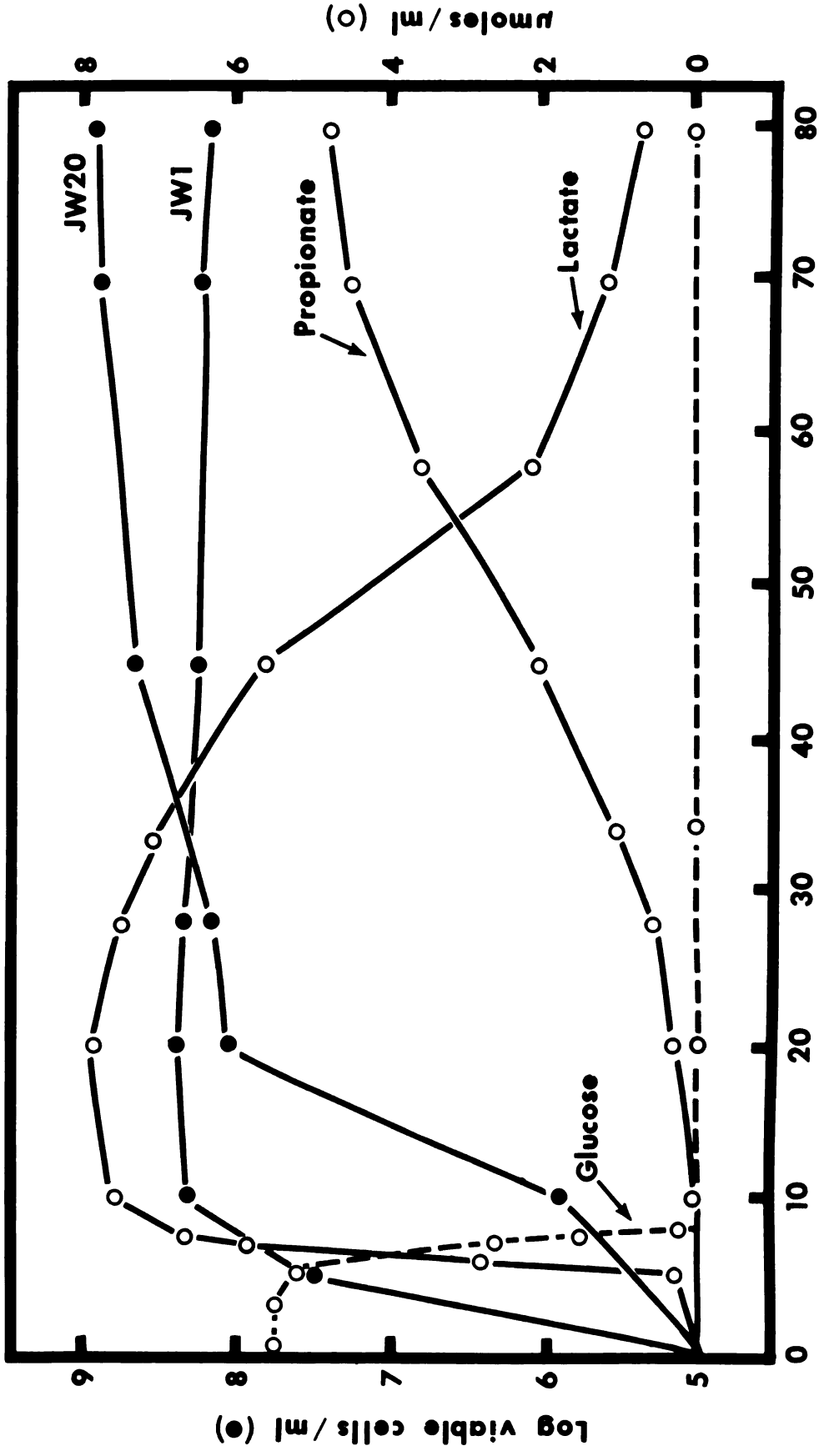
µmoles / ml (○)
0
2
4
6
8
JW20
JW1
Propionate
Lactate
Glucose
Log viable cells / ml (●)
5
6
7
8
9
0
10
20
30
40
50
60
70
80
Hours

Figure 2.

ethanol concentration was observed (Table 1; column 2). When the quantity of products formed by S. lactis monoculture was subtracted from that produced in coculture, then normalized to lactate consumption, propionate, acetate, and CO_2 formation by strain JW20 accounted for 100.5% of the lactate carbon dissimilated (Table 1; columns 3 and 4). The specific activities of propionate and acetate in the coculture were 81 and 72%, respectively, that of lactate carbon (Table 1; footnote a). These data indicated that lactate was the major source of propionate and acetate during growth of JW20, although a slight dilution of label occurred. Nevertheless, the apparent stoichiometry of lactate dissimilation by strain JW20 (100 lactate--→ 79 propionate + 19 acetate + 26 CO_2) agreed reasonably well with that anticipated (100 lactate--→ 66 propionate + 33 acetate + 33CO_2) for the dissimilation of lactate by either the acrylate (16) or succinate pathway (9).

Bacteroides JW20, grown as a monoculture in TY medium (i.e. lacking added glucose or lactate), exhibited a growth rate of 0.3 x hr^{-1} and yielded only 2.0 x 10^8 viable cells per ml. Likewise, only small amounts of acetate (0.20-0.23 μmoles/ml) and propionate (0.50-0.60 μmoles/ml) were formed. The addition of cysteine to TY medium (i.e. TYC medium) had no effect on the cell yield, but resulted in a faster growth rate comparable to that of Bacteroides in the coculture. Comparison of cell yields indicated that growth of JW20 was 4-fold greater in cocultures that in TY or TYC monocultures. From these data we conclude that lactate, produced by S. lactis JW1, was used as an energy source for growth of Bacteroides JW20.

Effect of lactate on growth of Bacteroides JW20 in monoculture. The importance of lactate as an energy source for strain JW20 in co-culture prompted an examination of its effect on JW20 in monoculture. Results are presented in Table 2. Incorporation of small amounts of lactate (20 to 40 μmoles/ml) in TYC medium increased cell yields 3-fold and markedly increased production of propionate, acetate, and succinate. However, higher initial levels of lactate supressed not only final cells yields, but organic acid production as well. At an initial concentration of 120 μmoles/ml, the cell yield was scarcely above that obtained with TYC medium alone. By contrast, incorporation of 11 mM glucose into TYC medium resulted in cell yields greater than those achieved with lactate and also shifted the fermentation pattern in favor of succinate production. The apparent growth inhibition by lactate at concentrations $\geq$ 60 mM was not due to a loss of the buffering capacity of the medium; final pH values of all cultures ranged from 6.5 to 6.9 (Table 2; footnote a).

Table 2. Effect of lactate on growth and product formation by Bacteroides JW20 in monoculture

Addition to TYC medium (μmoles/ml)	Maximum Yield[a]		Products (μmoles/ml)		
	$O.D._{660}$	Viable cells per ml ($\times 10^8$)	Propionate	Acetate	Succinate
No addition	0.12	2.0	0.60	0.20	0.10
Sodium-DL-lactate[b]					
20	0.31	5.7	11.43	2.89	0.49
40	0.33	6.1	15.85	3.91	0.50
60	0.26	4.8	12.00	3.14	0.38
80	0.21	3.8	9.14	2.41	0.33
100	0.18	3.2	7.51	1.97	0.25
120	0.14	2.4	5.81	1.60	0.13
Glucose, 11	0.82	15.5	1.51	1.18	4.10

[a]All values for optical density (O.D.) were maximum at termination (120 h), except for TYC with glucose and TYC alone which attained maximum $O.D._{660}$ at 24 and 58 h, respectively. Final pH values ranged from 6.5 to 6.9.

[b]The molarity of commercial sodium-DL-lactate used to prepare the media was determined by assay (2) against standard zinc lactate. The amount of lactate remaining at termination was not determined.