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Ultrastructural studies of the termite (*Odontotermes obesus*) gut microflora and its cellulolytic properties

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The major gut microflora colonizing the hind gut of a higher termite, *Odontotermes obesus*, included morphologically diverse bacteria, both coccoid and rod-shaped, along with spirochaetes, pseudomonads and actinomycetes. Flagellated protozoa were totally absent. When the gut extract was inoculated on plates containing carboxymethyl cellulose or cellobiose, higher numbers of bacteria grew than on plates without cellulosic sources. The gut homogenate exhibited strong hydrolytic activity when carboxymethyl cellulose, *p*-nitrophenyl-β-D-glucoside or xylan were used as substrate, indicating the role of gut microbiota in the process of cellulose and hemicellulose digestion. Activities were highest in the hind gut, and the paunch was probably the major site of polysaccharide digestion in this higher termite. *In vitro* cultivation of some of the isolates revealed both cellulase and xylanase activities. To our knowledge, this is the first report on ultrastructural studies of the higher termite *Odontotermes obesus*.

Key words: Electron microscopy, celluloytic microorganisms, termite gut.

There is limited information regarding the bacterial population inhabiting the higher termite *Odontotermes obesus*, which occurs in the semi-arid zone of northern India. Absence of a xylophagous, protozoan assemblage from the gut of higher termites makes the study more significant, considering the extent to which wood polysaccharides are dissimilated in termites. Attempts to enumerate and identify bacteria from the termite gut have been reviewed by Breznak (1984). Bacterial isolates from lower, as well as higher, termites have been described as aerobes and facultative or strict anaerobes and were predominantly strains of *Streptococcus*, *Staphylococcus*, *Bacteroides*, Enterobacteriaceae and *Bacillus*. The biochemical characteristics of these bacteria, as they relate to symbiotic interaction with the termites, have been discussed in the context of cellulose digestion. Cellulolytic actinomycetes belonging to the genera *Streptomyces* and *Micromonospora* have been isolated from the hind gut of four different termites: *Macrotermes*, *Amitermes*, *Odontotermes* and *Microcerotermes* (Pasti & Belli 1985). Among other species, spirochaetes are abundant in the hind gut of all termites that have been examined and exhibit considerable diversity in terms of their size range (Breznak 1984). Lower survival of higher termites (*Nasutitermes exitiosus*) was observed by Eutick *et al.* (1978) when the bacterial population was eliminated using an antibiotic. Recently, the H_2/CO_2 acetogen, *Sporomusa termitida*, was isolated from the wood-feeding higher termite *Nasutitermes nigriceps* by Breznak *et al.* (1988). Clearly, more research on this group of termites is needed for a clear understanding of the role of gut microflora in the process of cellulose digestion.

In this study we have examined the hind gut microflora of *Odontotermes obesus* using electron microscopy and have quantified the cellulose-digesting bacterial population and activity present in different parts of the gut.

Materials and Methods

Removal of Termite Gut and Preparation of Extract

Worker termites of *Odontotermes obesus*, belonging to the family Termitidae and subfamily Macrotermitinae, were collected from live termite mounds and infested tree barks. The termites were surface-sterilized and then decapitated using sterile tweezers and needles. The gut was removed without rupturing (Figure 1) and

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washed in sterile phosphate buffer (pH 7.0). Individual guts were dissected, using a dissecting microscope, into their component parts: fore gut (FG), including the crop and muscular gizzard; mid gut (MG), which was tubular; and the voluminous hind gut (HG), comprising the paunch, colon and rectum. These segments were homogenized separately in 5 ml of phosphate buffer (50 mM). The extract was then centrifuged at 20,000 ***g*** for 10 min and the supernatant was dialysed against the same buffer (500 ml) for 16 h. Gut extraction was carried out at 4°C.

Enzyme Assay

Diluted gut extract, 0.5 ml, was incubated either with 1% (w/v) carboxymethyl cellulose for measuring endoglucanase activity or xylan for measuring xylanase activity. β-Glucosidase activity was measured using *p*-nitrophenyl-β-D-glucoside (PNPG) as substrate. Production of enzymes was estimated following the method of Wood & Bhat (1988). One unit of activity was defined as the amount of enzyme which produced 1 mg of reducing sugar per 20 min per ml of extract.

Bacterial Isolation

Selective media were used to isolate bacteria capable of producing any of the three cellulases. Agar plates contained Skinner cellulose medium B (Skinner 1971) with Hoagland trace element solution and 1% (w/v) Whatman powdered cellulose, 0.5% (w/v) carboxymethyl cellulose (CMC) or 0.5% (w/v) cellobiose (CB), with 1% (w/v) agar and cycloheximide (0.1 mg per ml) to inhibit fungi. Media containing the above reagents but no cellulose served as controls. Serial dilutions of the gut isolates were spread on three replicate plates. The plates containing CMC or CB were incubated at 35°C for 4 days, whereas those containing Whatman cellulose were grown for 10 days, before observations were made. As a presumptive test, CMC-grown plates were flooded with 1% Congo Red followed by washing with 2 M NaCl to observe the zone of clearing by CMC-degrading bacteria.

The basal medium used to study the enzyme activities in the isolates contained (g/l): NaCl, 3.0; KH_2PO_4, 1.0; K_2HPO_4, 1.0; $(NH_4)_2SO_4$, 2.0; $MgSO_4$, 0.05; $CaCl_2$, 0.05; yeast extract, 0.5 and powdered bagasse, 10; pH was adjusted to 6.5.

Gut Preparation for Electron Microscopy

Hind guts were withdrawn and immersed immediately in a drop of 3% (w/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) kept on a wax sheet. The paunch region was punctured with a needle to allow rapid penetration of the fixative into the lumen. Guts were then transferred to a glass vial containing the fixative and kept overnight at 4°C, followed by washing several times with phosphate buffer. The samples were then post-fixed for 2 h in 1% (w/v) OsO_4 in 0.2 M collidine buffer (pH 7.4), dehydrated in an ethanol series and embedded in Spurr's low viscosity resin. Sections cut on an ultramicrotome were stained with 2% (w/v) aqueous uranyl acetate for 45 min followed by 0.5% (w/v) lead citrate for 15 min. Sections were then viewed with a Phillips EM 300 electron microscope.

Results

Ultrastructural Studies

Electron microscopic observations of hind guts of *Odontotermes obesus* revealed a high incidence of bacteria and spirochaetes but a total absence of flagellated protozoa, confirming the fact that *Odontotermes obesus* belongs to the higher termite group.

Transmission electron microscopy (Figure 2), revealed the epithelial layers of the paunch and small rod-shaped bacteria (R_1) with both vegetative cells and endospores. The cuticle of the epithelium and the circular muscle were found to be contiguous. Aggregation of the bacteria near the epithelial surface was not a very common feature. The associated microbiota also included actinomycetes, presumably *Micromonospora* (M_1), as well as slender, flexous, helically-coiled, unicellular bacteria—the spirochaetes (S_1). Pleomorphic coccoid-shaped bacteria were quite common (C_1) besides the long rods (R_2) and (R_3) (Figures 3, 4 and 5). Rods with subterminal endospores, presumed to be *Bacilli*, were also quite common. R_3 cells showed wavy margins and abundant electron-translucent granules. Some of the rods exhibited a fuzzy appearance (Figure 4) due to fibrous materials around the cells which may help the cells to aggregate (R_4). The paunch was also found to be colonized by rather few cells (R_{Cy}) which contained numerous cytoplasmic inclusions, similar to polyhydroxybutyrate, which occupied major cytoplasmic spaces (Figure 3). Spherical cells, occurring in pairs (C_2) with the typical endospores, were also observed (Figure 6) and resembled *Sporosarcina*; the broken cell wall between each of the pairs was also visible.

Bacterial Isolates

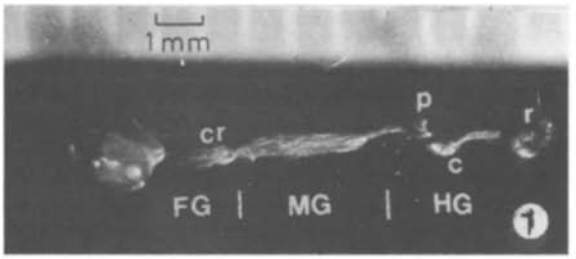
In vitro cultivation of the organisms from the hind gut extract revealed the morphological and biochemical characteristics of some of the isolates (Table 1). Following standard descriptions, they were assigned generic names. Strict anaerobes were not screened for in the present study. Repeated trials failed to grow spirochaetes in pure culture.

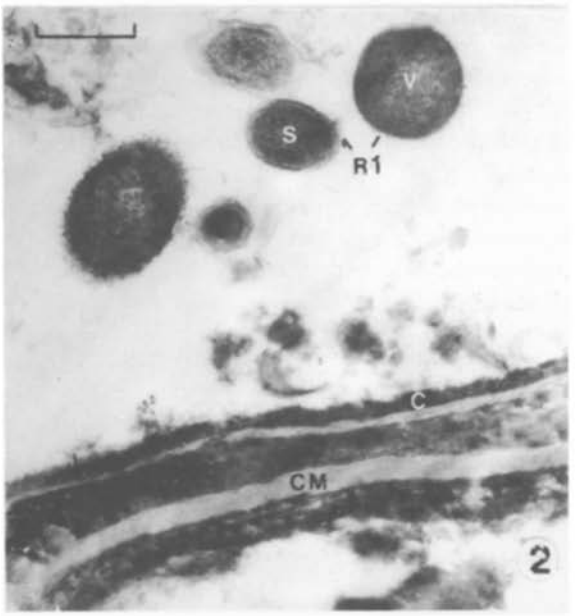
Cellulolytic Bacteria

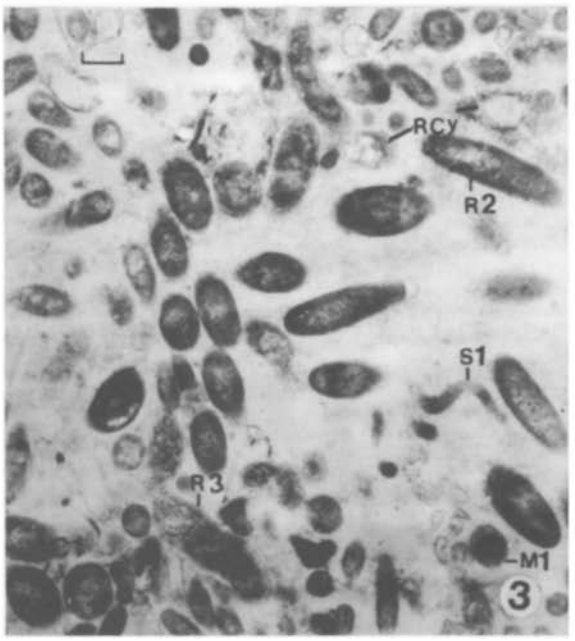
The method employed for *in vitro* cultivation of cellulolytic bacteria from different parts of the gut was standardized so that 1 ml of gut homogenate was isolated from 10 termites at a time. Control plates without added cellulose supported only a few organisms when compared with the plates with added cellulose. Both mid-gut and hind-gut extracts supported high number of colonies when cellobiose was used as a substrate (Table 2). Colonies degrading microcrystalline cellulose were always few in number but 80% of bacteria growing on carboxymethyl cellulose established zones of clearing on addition of Congo Red (data not shown).

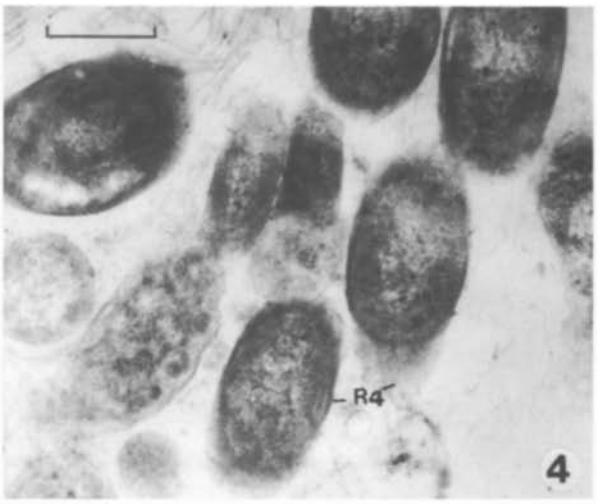
Enzyme Assay

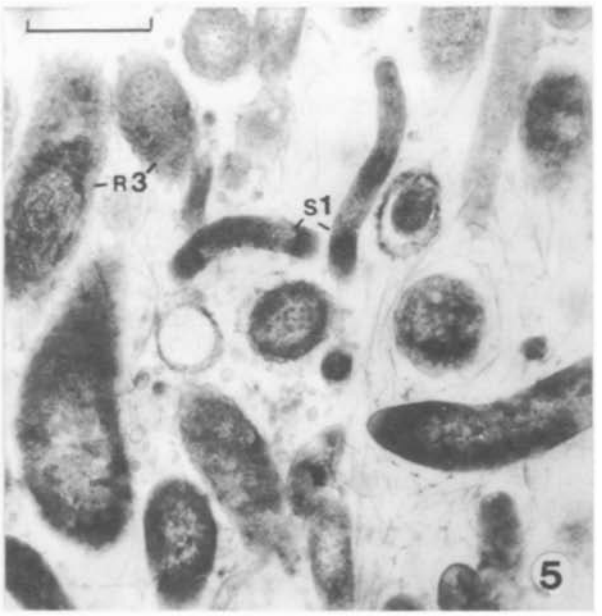
Degradation of crystalline cellulose was very low in all gut extracts (Table 3). However, endoglucanase (CMC-degrading) and β-glucosidase (PNPG-degrading) activities were quite high in hind-gut extracts. Surprisingly, very low











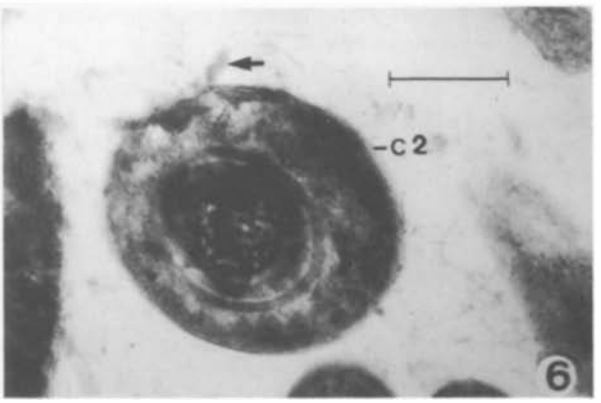


Figure 1. Anatomical regions of the gut extracted from the termite *Odontotermes obesus*: FG—fore gut; cr—crop; MG—mid gut; HG—hind gut; p—paunch; c—caecum; r-rectum.

Figures 2–6. Transmission electron micrographs of the paunch epithelium and associated bacteria. Bar—0.3 μm in each.

Figure 2. Rod-shaped bacteria (R_1) with vegetative cell (V) and spore (S), near hind gut surface showing circular muscle fibres (CM) and cuticle (C).

Figure 3. Rod (R_2) with subterminal endospore, dividing rods with wavy margins (R_3) cell with cytoplasmic inclusions (R_{Cy}) and coccoid bacteria (C_1).

Figure 4. Small rods (R_4) with fibrous material around the cells.

Figure 5. Spirochaetes (S_1) and rods (R_3).

Figure 6. Coccoid cell (C_2) with endospore; the arrow indicates the broken cell wall of the pair.

Table 1. Morphological and biochemical characteristics of the gut isolates.

Isolate no.	Morphological characteristics	Biochemical characteristics	Assigned genus
M_1	Colonies brown in colour, dark pigmented spores occurring singly, well defined	Aerobic, growth on Czapeks sucrose agar, α-melibiose utilized, hydrolyse arabinose and xylose	*Micromonospora*
C_2	Cells spherical, non-motile, endospores formed, tetrads	Chemoorganotrophs, aerobic, Gram-positive	*Sporosarcina*
S_1	Slender, helically coiled, unicellular (3 to 500 μm in length)	Pure culture techniques not available	*Spirochetes*
R_{Cy}	Orange colonies producing pigment, rods, motile by polar flagella	Gram-negative, accumulated polyhydroxybutyrate, utilize arginine	*Pseudomonas*
R_2	Cells rod-shaped, endospore forming, 0.3 to 2.2 by 2 to 7.0 μm, motile, flagellated	Gram-positive, catalase positive	*Bacillus*
S_2	Cells spherical, 0.5 to 1.5 μm in diameter, occurring singly, non-motile	Gram-positive, catalase positive, wide range of carbohydrates utilized, facultative anaerobe	*Staphylococcus*

levels of activity were detected when xylan was used as a substrate.

Moderate amounts of endoglucanase and xylanase activities were produced by the isolates S_1, M_1 and S_2, whereas R_2 produced high activities when all were grown in a medium containing bagasse as a cellulosic source (Table 4).

Table 2. Bacterial population from different parts of the gut growing on cellulosic sources.

Cellulosic source (concentration w/v)	Bacterial population per ml of gut extract in buffer (±S.E.)		
	Fore gut ($\times 10^{-3}$)	Mid gut ($\times 10^{-5}$)	Hind gut ($\times 10^{-6}$)
Whatman powdered cellulose (1%)	0.1 ± 0.05	0.15 ± 0.09	0.75 ± 0.25
CMC (0.5%)	0.2 ± 0.04	0.65 ± 0.3	3.0 ± 0.5
Cellobiose (0.5%)	1.25 ± 0.25	1.75 ± 0.63	4.5 ± 2.0

Table 3. Distribution of enzyme activity in the gut of *Odontotermes obesus*.

Gut section	Enzyme activity (mean + S.E.) (units/ml of extract)				
	Crystalline cellulose	CMC	PNPG		Xylan
Fore gut	0.005 ± 0.001	2.31 ± 0.24	0.62	0.12	0.31 ± 0.05
Mid gut	0.003 ± 0.002	2.81 ± 0.31	0.85	0.22	0.71 ± 0.10
Hind gut	0.121 ± 0.015	6.52 ± 1.41	3.12	1.21	2.31 ± 0.56

CMC—Carboxymethyl cellulose; PNPG—*p*-nitrophenyl-β-D-glucoside.

Discussion

With the aid of ultrastructural studies, we propose that morphologically-distinct bacteria belonging to the genera *Bacillus, Staphylococcus* and *Sporosarcina*, spirochaetes, and actinomycetes belonging to the genera *Micromonospora* colonize the paunch region of the hind gut of *Odontotermes obesus* isolated from semi-arid regions of India. Since these detrivores feed on soil, plant litter and other items found on the soil surface, their feeding pattern enables them to acquire varied microbial flora. The study of specific associations of prokaryotes with the symbiotic flagellate protozoa in the hind gut of the termite *Reticulitermes* revealed the presence of rods as well as spirochaetes (Bloodgood & Fitzharris 1976). The present ultrastructural studies of the gut, conducted on the higher termite *Odontotermes obesus*, confirm the clear distinction in the gut microflora of lower and higher termites, the higher termites having no flagellated protozoa. However, typical spirochaetes, bacterial rods with 'fuzzy' surfaces and rod-shaped to coccoidal forms with endospores seem to be common to all termites, when our data are compared with the bacterial morphotypes found in the paunch region of the lower

Table 4. Cellulase and xylanase activities of isolates grown *in vitro*, measured in the culture broth after 48 h of growth.

Isolate	Endoglucanase (units/ml)	Xylanase (units/ml)
S_2	3.56 ± 0.86	0.42 ± 0.21
R_2	8.22 ± 2.31	14.31 ± 3.42
M_1	3.24 ± 1.24	0.42 ± 0.21
C_2	0.8 ± 0.25	0.31 ± 0.12

termite (Breznak & Pankratz 1977). This perhaps establishes the fact that this bacterial population can be considered as an autochthonous flora.

The observed increase in the bacterial populations on media containing microcrystalline cellulose, CM-cellulose or cellobiose as the sources of carbon and energy, compared with growth on control media; indicates that the termites harbour cellulose-digesting bacteria. This seems to be in contrast to an earlier report, by Eutick *et al.* (1978), of failed attempts to isolate cellulose-degrading bacteria from the gut of a higher termite, *Nasutitermes exitiosus*. However, cellulose-degrading bacteria were claimed to have been isolated from *Odontotermes* sp. as early as 1954, by Mishra & Ranganathan (1954). The present study, as well as our earlier reports on *in vitro* cultivation of *Staphylococcus saprophyticus* (Paul *et al.* 1986) and *Micrococcus* sp. (Saxena *et al.* 1991), both isolated from *Odontotermes obesus* guts, revealed cellulose-digesting properties. Therefore, it is concluded that the bacteria present in the gut probably play a crucial role in the process of cellulose digestion by the termites.

Inconclusive evidence is available regarding the synthesis of cellulases in higher termites. Whether these enzymes are synthesized by termite cells, and/or by their endosymbionts, remains unknown as yet (Rouland *et al.* 1988). It is now well established that, in at least the genera *Macrotermes* (Martin 1984; Veivers *et al.* 1991), the fungus-growing termites can be distinguished from other genera by the presence of *Termitomyces* nodules as a part of their dietary component. These nodules serve as a source of cellulase (C_1) that is active against crystalline cellulose. We, however, have been able to detect only very low C_1 activity in gut homogenates, for reasons that are not yet clear. Though *Odontotermes* do not harbour *Termitomyces*, an earlier study revealed the presence of the fungus *Cunninghamella echinulata* (Rajgopal & Varma 1980) in their gut and this showed cellulase activity. Therefore, although it seems that the bacterial population in the gut of *Odontotermes obesus* contribute to plant material degradation by producing endoglucanase, β-glucosidase and xylanase enzymes, but perhaps acquire C_1 enzyme from the fungal flora of the gut.

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