APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1994, p. 1822–1826
0099-2240/94/$04.00+0

Vol. 60, No. 6

Effect of Host Diet and Hindgut Microbial Composition on Cellulolytic Activity in the Hindgut of the American Cockroach, *Periplaneta americana*

HUUB J. GIJZEN,[1]† CHRIS VAN DER DRIFT,[2] MARTIN BARUGAHARE,[1] AND HUUB J. M. OP DEN CAMP[2]*

Applied Microbiology Unit, Faculty of Science, University of Dar es Salaam, Dar es Salaam, Tanzania,[1] and Department of Microbiology and Evolutionary Biology, Faculty of Science, University of Nijmegen, NL-6525 ED Nijmegen, The Netherlands[2]

Received 18 January 1994/Accepted 31 March 1994

Cellulase activity measured as filter paper digesting activity (FPase) and carboxymethyl cellulase (CMCase) was demonstrated in hindgut extracts of the cockroach *Periplaneta americana*. The highest activities measured amounted to 0.89 and 0.12 U · ml^{-1} for CMCase and FPase, respectively. The cellulolytic capacity of the hindgut population increased dramatically when protozoa were present, and the activities were found to vary depending on the feeding regimen. Cellulose-rich diets induced high protozoal numbers, resulting in a high cellulase activity. A close correlation was found between the number of *Nyctotherus ovalis* organisms, the major protozoans in the hindgut, and both FPase and CMCase activity. Since the numbers of this protozoan also correlated with the methane production of the insect, it appears that *N. ovalis* is responsible for the major part of cellulolytic and methanogenic activity found in the hindgut of *P. americana*.

The ability of many insects to feed on wood, foliage, and detritus has stimulated research on how these species degrade structural carbohydrates. The digestion of cellulose is generally accomplished by the combined action of enzymes with endoglucanase (1,4-β-D-glucan 4-glucanohydrolase; EC 3.2.1.4), exoglucanase (1,4-β-D-glucan cellobiohydrolase; EC 3.2.1.91), and β-glucosidase (1,4-β-D-glucoside 4-glucohydrolase; EC 3.2.1.21) activity. In insects, cellulolytic enzymes may originate from various sources, including the host itself, the gut microbiota, or feed-associated enzymes in the case of fungus-growing termites (21). The endogenous production of endoglucanase and β-glucosidase in the salivary glands of termites and cockroaches has been proven incontrovertibly (21, 23). Data suggesting that insects produce their own exoglucanases are sparse, and this ability is regarded as the exception rather than the rule (5, 21).

Early studies by Hungate (17–19), Cleveland (6, 7), and Trager (24, 25) have demonstrated an intriguing metabolic interaction between several species of cellulolytic flagellated protozoa and phylogenetically lower termites. The presence of these protozoa appeared to be essential to the survival of termites on a diet consisting largely of wood or native cellulose (5). Some of these flagellates have been studied in axenic cultures (27) and have been shown to be the major, and possibly the only, source of cellulolytic enzyme production in termites. A similar situation has been observed in the wood-roach *Cryptocercus punctulatus*, which depends on cellulase generated by protozoa present in the hindgut (8).

Contrary to the situation for wood-roaches and lower termites, cockroaches appeared to be less strictly dependent upon their anaerobic microbiota and their metabolic products (11) since they are omnivorous and able to include both cellulosic and noncellulosic substrates in their diets. Several studies suggest that the presence of a large and diverse hindgut microbial population may contribute to nutritional and developmental processes of the host (1, 2, 9, 12). Other reports, however, indicated that elimination of the anaerobic gut microbiota by applying a high dose of metronidazole had no obviously negative effects on adult cockroaches (3, 4). The extent and importance of nutritional interactions between cockroaches and their intestinal microbiota are not yet fully understood. The effect of host diet on the metabolic interactions between the insect and its intestinal microbiota has not yet been considered in detail. It is likely that the extent of the microbial contribution varies with differences in the diet.

Recent studies on the cockroach *Periplaneta americana* have shown that production of organic acids and methane and the number of gut microorganisms varied considerably with the insect's diet (12, 20). The ciliate *Nyctotherus ovalis* was shown to be present in high numbers in the hindgut of this cockroach species (12, 13). The ciliates harbor endosymbiotic methanogenic bacteria which were the major source of methane production in the hindgut. The present study describes the effects of host diet and of induced differences in the hindgut microbial composition on cellulase activity in the hindgut of *P. americana*.

MATERIALS AND METHODS

Insects. Ten different cultures of the American cockroach, *P. americana*, were maintained in separate 5-liter glass containers to evaluate the effects of host diet and hindgut microbial composition. Each of the cultures was kept in duplicate, and each container had a minimum of 20 insects at the start of the experiments. Water and feed were added ad libitum into separate petri dishes which were cleaned and refilled twice every week. The containers were kept in the dark and at room temperature (25 to 28°C). Details on source and culture conditions of cockroaches used in this study were described

* Corresponding author. Mailing address: Department of Microbiology and Evolutionary Biology, Faculty of Science, University of Nijmegen, Toernooiveld 1, NL-6525 ED Nijmegen, The Netherlands. Phone: +31 (0)80 652657. Fax: +31 (0)80 553450. Electronic mail address: huubcamp@sci.kun.nl.

† Present address: International Institute for Infrastructure, Hydraulics and Environment (IHE), 2601 DA Delft, The Netherlands.

TABLE 1. Summary of the culture conditions for *P. americana*

Culture	Diet composition			Addition to water	Description
	Cellulose	Fish flour	Maize flour		
A	+	−	−	NH_4Cl	
B	−	+	−	None	
C	−	−	+	None	Stock culture for G[a]
D	+	+	−	None	
E	+	+	+	None	Stock culture for F, H, I, and J[a]
F	+	+	+	BES	
G	−	−	+	Metronidazole	
H	+	+	+	None	Free of protozoa and bacteria
I	+	+	+	None	Reinfected with complete hindgut suspension
J	+	+	+	None	Reinfected with protozoan-free hindgut suspension

[a] Stock culture means that insects from this culture were used to start the new cultures indicated.

earlier (13). A brief summary of the culture conditions is given in Table 1.

Culture conditions. The effect of host diet was evaluated by feeding five different diets. Culture A received cellulose (shredded white household tissue; cellulose content about 99%), with NH_4Cl (1.5 g · liter^{-1}) added to the drinking water; culture B received fish flour (prepared from *Rastineobola argentea*); culture C received maize flour; culture D received cellulose and fish flour in a ratio of 3:1; and culture E received a mixture of cellulose, fish flour, and maize flour in a ratio of 3:1:0.5. The composition of the feed was chosen to evaluate the effects of different sources of carbon, i.e., starch or cellulose, and of different levels of nitrogen in the diet.

In order to evaluate the effect of hindgut microbial composition, five additional cultures (cultures F to J) were started from cultures E and C, which served as stock cultures. The hindgut microbial composition of insects in these cultures was altered in various ways. Culture F contained cockroaches from culture E which were continuously treated with bromoethanesulfonic acid (BES; 25 mM) in the drinking water to establish the effect of inhibition of methanogenic bacteria. Culture G was started with insects from culture C, which received maize flour as a sole dietary component. In this culture, the antiprotozoal drug metronidazole was added to the drinking water (0.1 mg · ml^{-1}). Culture H contained cockroaches which developed from eggs removed from the stock culture. Before the eggs were transferred to a new container, they were thoroughly washed in a mineral buffer solution (15) to remove possible contamination of gut microorganisms from the stock culture. The cockroaches that developed in this way remained free of protozoa and methanogenic bacteria. Culture I was started with insects taken from culture H, which were subsequently infected with normal hindgut microbiota. Infection was induced by adding hindgut homogenates from insects from stock culture E to the feed. Culture J was similar to culture I, but in this case, the hindgut suspension was made free of protozoa before addition to the feed. This was achieved by applying osmotic shock, followed by filtration through a 7-μm-pore-size nylon gauze. Cultures F, H, I, and J were all kept on the cellulose-rich diet as for stock culture E, while culture G was kept on a maize flour diet. A detailed description of the conditions of cultures A to J was presented earlier (12, 13). All cultures were kept for a minimum period of 3 months before cellulase activity and other parameters were analyzed. At frequent intervals, three insects from each culture were dissected for analysis of cellulolytic enzymes and ciliate numbers in their hindguts. Methane production was analyzed by using intact cockroaches. Only adult insects were used for observations and data collection.

Cellulase extraction. For each cellulase determination, the hindguts of three insects were removed, cut into pieces, and pooled in a test tube. After addition of 1 ml of 0.4 M acetate-phosphate buffer (pH 6.0) to the semisolid gut content, samples were homogenized with a glass rod homogenizer. The homogenized gut suspension was incubated with 0.2% Triton X-100 at room temperature (25 to 28°C) for 30 min, with vortexing at 5-min intervals. Preliminary trials had demonstrated an improved liberation of cellulase activity, following Triton X-100 treatment (results not shown). After incubation, the suspension was used to prepare enzyme extracts by applying ultrasonic treatment and centrifugation as described previously for rumen samples (14). The supernatant obtained was used for enzyme assays. The extraction procedure was repeated on five different days for each cockroach culture.

Enzyme assays. Cellulase activity was measured as filter paper digesting activity (FPase) and as carboxymethyl cellulase activity (CMCase; 1,4-β-D-glucan 4-glucanohydrolase; EC 3.2.1.4; endoglucanase). FPase was assayed by measuring the release of reducing sugars from filter paper (Whatman no. 1). The standard assay mixture contained 30 mg of filter paper strips, 0.55 ml of distilled water, 0.25 ml of 0.4 M acetate-phosphate buffer (pH 6.0), and 0.2 ml of enzyme extract. Samples and controls were always prepared in duplicate and were incubated for 1 h at 50°C. The reaction in the test samples was stopped by addition of 1.0 ml of dinitrosalicylic acid reagent (22) and by subsequently placing the reagent tubes in a water bath at 100°C for 15 min. After centrifugation (15,000 × *g*, 10 min), the supernatants were used for analysis of reducing sugars according to the method of Miller (22), with glucose as a standard. The controls were prepared as for the test samples, except that 1.0 ml of dinitrosalicylic acid reagent was added shortly before addition of the enzyme extract.

CMCase was assayed by measuring the release of reducing sugars from carboxymethyl cellulose (sodium salt, low viscosity, degree of substitution of 0.7, Sigma C 8758 [Sigma, St. Louis, Mo.]). The assay mixture was prepared by combining 0.25 ml of carboxymethyl cellulose solution (2% [wt/vol]), 0.3 ml of distilled water, 0.25 ml of 0.4 M acetate-phosphate buffer (pH 6.0), and 0.2 ml of enzyme extract in a reagent tube. Controls were prepared in the same way, but 1.0 ml of dinitrosalicylic acid was added before addition of the enzyme extracts. Samples and controls were incubated in duplicate for 30 min at 50°C. Termination of the reaction, centrifugation, and determination of reducing sugars were carried out as described for the FPase activity.

CMCase and FPase activities were expressed in units per milliliter of original hindgut suspension, 1 U being defined as 1 μmol of glucose equivalent released per min.

TABLE 2. Effect of diet and hindgut microbial composition on cellulolytic activity in the hindgut of *P. americana*[a]

Culture	No. of *N. ovalis* organisms/hindgut	Cellulolytic activity ($U \cdot ml^{-1}$)[b]		Hindgut vol (μl)	Insect wt (g)
		CMCase	FPase		
A	2,806 ± 1,012	0.79 ± 0.25	0.106 ± 0.03	52 ± 5	1.38 ± 0.24
B	1,262 ± 787	0.59 ± 0.12	0.053 ± 0.01	61 ± 4	1.41 ± 0.19
C	3,102 ± 1,023	0.72 ± 0.20	0.089 ± 0.004	66 ± 3	1.53 ± 0.28
D	3,970 ± 1,839	0.84 ± 0.26	0.108 ± 0.03	65 ± 4	1.49 ± 0.30
E	3,500 ± 1,150	0.85 ± 0.17	0.120 ± 0.02	65 ± 6	1.47 ± 0.25
F	3,605 ± 1,088	0.89 ± 0.24	0.119 ± 0.01	67 ± 9	1.51 ± 0.23
G	0	0.27 ± 0.07	0.004 ± 0.0004	64 ± 3	1.20 ± 0.17
H	0	0.15 ± 0.03	0.003 ± 0.0004	43 ± 4	0.68 ± 0.11
I	3,210 ± 228	0.82 ± 0.15	0.096 ± 0.01	58 ± 8	0.91 ± 0.13
J	0	0.22 ± 0.06	0.004 ± 0.0004	61 ± 7	0.73 ± 0.13

[a] Protozoal numbers, hindgut volumes, and insect weights were partially adapted from references 12 and 13.
[b] Values represent means of separate cellulase assays at different days ($n = 5$) ± standard deviation.

Other parameters. Microscopic observations and gas chromatographic analysis of methane were as described previously (12, 13).

RESULTS

Hindgut extracts from cockroaches collected near the University Campus (Dar es Salaam, Tanzania) were shown to possess both CMCase (0.51 ± 0.11 $U \cdot ml^{-1}$) and FPase (0.15 ± 0.04 $U \cdot ml^{-1}$) activity. To gain more insight into the origin of this activity, insects were subcultured under a variety of conditions (cultures A to J).

Effect of diet on hindgut cellulolytic activities. The effect of diet composition was studied in cultures A to E (Table 2). Hindgut cellulolytic activity was strongly affected by the composition of the diet. Both CMCase and FPase activities were highest in cultures in which cellulose was a major or the sole dietary component. The activities in insect hindguts from culture A, which received only cellulose, were not significantly different ($P > 0.05$) from those of cultures D and E, which received, besides cellulose, maize flour and/or fish flour. The average activity levels of CMCase and FPase in these cultures amounted to 0.83 and 0.10 $U \cdot ml^{-1}$, respectively. The lowest activity was observed in culture B, which was kept on a fish flour diet. CMCase and FPase activities in the hindgut of these cockroaches amounted to 71 and 48%, respectively, of the average activity in cellulose-fed cultures (A, D, and E). Enzyme activities in culture C, kept on maize flour, were 93 and 78% of the activity found in cellulose-fed cultures for CMCase and FPase activities, respectively.

Effect of hindgut microbial composition on cellulolytic activities. Culture E served as a stock culture for studying the effect of alterations in hindgut microbial composition. Microbial composition was altered in three ways: (i) BES treatment resulted in the specific inhibition of methanogens (culture F); (ii) metronidazole treatment at low dosage was applied to remove protozoa from the gut system (culture G); and (iii) cockroaches lacking protozoa, methanogens, and possibly other relevant gut microorganisms were developed from surface-washed eggs (culture H).

The BES treatment resulted in an almost immediate reduction of methane production to about 6% of the control value. As demonstrated previously (13), methanogenesis was almost completely inhibited by BES, but methanogens remained viable. Long-term exposure to BES did not reveal any effect on CMCase or on FPase activity in hindgut extracts (Table 2). The numbers of the symbiotic protozoan *N. ovalis* were not affected by BES treatment (Table 2). A major decrease in CMCase and FPase activity to 31.8 and 3.3%, respectively, of the activity in the original stock culture (culture E) was observed upon treatment with the antiprotozoal drug metronidazole. Frequent microscopical observations revealed that application of metronidazole resulted in complete removal of *N. ovalis* organisms together with their endosymbiotic methanogenic bacteria from the hindgut.

Neither *N. ovalis* nor methanogens were observed in the hindgut of cockroaches raised from washed eggs (culture H), and no methane production could be detected. The activities against carboxymethyl cellulose and filter paper cellulose in the hindgut of these insects were only 17.6 and 2.5% of values found in stock culture E, respectively.

Infection of cockroaches from culture H at adulthood with complete hindgut homogenate obtained from stock culture E almost completely restored the number of *N. ovalis* organisms (Table 2) and the level of methane production to normal values. CMCase activity in the hindgut could also be almost completely restored, whereas FPase activity increased to 80% of the activity level in the stock culture. In contrast to this, CMCase and FPase activities in hindgut extracts were not affected upon infection with a protozoan-free hindgut homogenate (culture J). This indicates that the bacterial hindgut population probably contributes only to a limited extent to overall hindgut cellulase activity.

Correlation between protozoal numbers and cellulolytic activities. The above-mentioned results suggest that a major portion of both CMCase and FPase activity is closely linked with the presence of protozoa in the hindgut. The CMCase and FPase activities from hindgut extracts from cultures A to J were plotted against the number of *N. ovalis* organisms present in the hindgut (Fig. 1). A close correlation between the two parameters ($r = 0.943$ and 0.960, respectively) was found. The y-axis intercepts were 0.25 and 0.007 $U \cdot ml^{-1}$ for CMCase and FPase, respectively.

DISCUSSION

Cockroaches feed and survive on whatever feed source is available. What reaches the microbiota of the hindgut is fiber, undigested cellulose, and excretory products of the Malpighian tubules of the insect. These remains support a complex and active anaerobic population of microorganisms (10, 11). The hindgut of the *P. americana* strain we used was previously shown to contain high numbers of the protozoan *N. ovalis* (up to 6.1×10^4 cells per ml), which in turn possessed endosymbiotic methanogens (13). It was demonstrated that hindgut anaerobic microbiota play an essential role in the early devel-

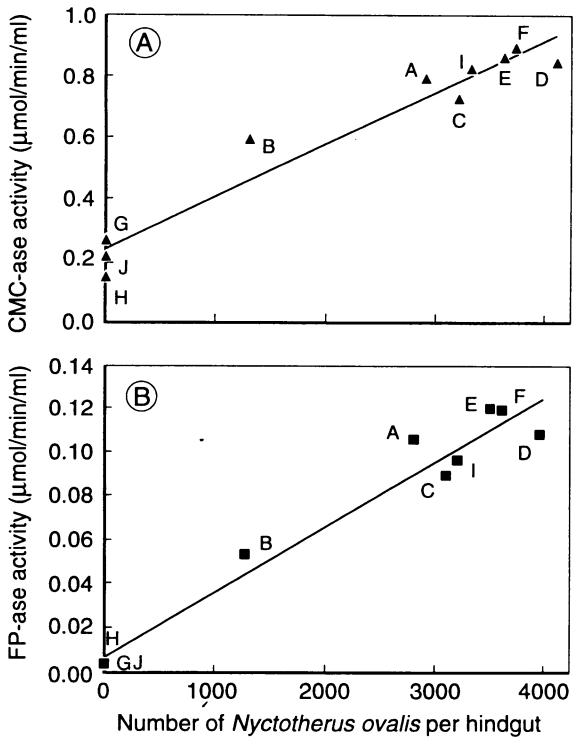

FIG. 1. Relation between number of *N. ovalis* organisms and CMCase (A) and FPase (B) activity in the hindgut of *P. americana*. Points marked A to J represent the different cultures; the solid line is the calculated linear regression line.

opmental stages of the American cockroach fed on a cellulose-rich diet (12). In growing nymphs reared from buffer-washed eggs, which as a consequence lacked a normal hindgut microbial population, retarded insect development and low methanogenic activity were observed. The results of these experiments further suggested that this effect could be mainly ascribed to the protozoal rather than to the bacterial hindgut population.

From the above-mentioned results, it follows that cellulolytic activity may be of major importance in the hindgut, especially on fiber-rich diets. Cellulases in the digestive tract of cockroaches were demonstrated to be of both insect and microbial origin (11, 26). Thus far, only CMCase (endoglucanase) and β-glucosidase activity have been demonstrated. The insect cellulase activity was located in the salivary gland, whereas the microbial cellulase activity was found mainly in the midgut and hindgut. Thus far, there is no firm evidence for the presence of exoglucanase activity in insects (23). Carboxymethyl cellulose-degrading bacterial strains isolated from the intestinal tract of cockroaches were unable to digest filter paper (9). In this study, it was clearly demonstrated that hindgut extracts contained both endoglucanase (CMCase) and FPase and that these activities were affected by the presence or absence of cellulose in the diet. The strongest effects were observed on FPase activity. On the basis of FPase activity measured in hindgut extracts, a degradation of about 2 mg of cellulose per insect per day can be calculated.

When the hindgut microbial composition was manipulated by BES or metronidazole treatment, only the specific removal of protozoa by the latter resulted in a dramatic decrease in CMCase and FPase activity. The important role of protozoa in cellulolytic activity was further substantiated by the reinfection experiments (cultures H to J, Table 2). Defaunation before reinfection, achieved by a gentle treatment not affecting the bacterial population, did not restore the cellulolytic activity. A clear correlation between protozoal numbers and both CMCase and FPase was obtained by combining results from diet and hindgut microflora studies (Fig. 1). FPase activity appeared to be exclusively associated with the presence of protozoa, while CMCase activity dropped to a basal level of about 0.20 $U \cdot ml^{-1}$. This value equals the activity reported earlier for hindgut CMCase in *P. americana* (9), and it is likely that the insect strain used by these authors did not contain protozoa. Remarkably, the average weight of their adult insects was 0.9 g while our insects had an average weight of 1.45 g (cultures A to D). We observed decreases in body weight only when protozoa were absent.

The significance of enteric protozoa for insect physiology is not well understood yet (21, 23). The presence of intestinal protozoa is not obligatory, but acquisition of protozoa with endosymbiotic methanogens was shown to improve methane production in cockroaches (12, 13, 16). When the methane production values from references 12 and 13 were plotted against the protozoal numbers, a positive correlation was found ($r = 0.889$, $n = 7$). Only a few free-living methanogens were observed in the hindgut. In accordance with this finding, Kane and Breznak (20) found low methane production in their strain of *P. americana*, which appeared to be free from protozoa. From our study, it is clear that the cellulolytic capacity of the hindgut population increases dramatically when protozoa are present. Cellulose-rich diets induced high protozoal numbers, resulting in a high cellulase activity. In general, it can be stated that *N. ovalis* is responsible for the major part of cellulolytic and methanogenic activity found in the hindgut of *P. americana*. Thus far, no endosymbionts other than methanogenic bacteria have been observed (15), and since these bacteria are not cellulolytic, the protozoan is most likely responsible for the cellulose breakdown.

REFERENCES

1. **Bignell, D. E.** 1977. An experimental study of cellulose and hemicellulose degradation in the alimentary canal of the American cockroach. Can. J. Zool. **55**:579–589.
2. **Bignell, D. E.** 1977. Some observations on the distribution of gut flora in the American cockroach, *Periplaneta americana*. J. Invertebr. Pathol. **29**:338–343.
3. **Bracke, J. W., D. L. Cruden, and A. J. Markovetz.** 1978. Effects of metronidazole on the intestinal microflora of the American cockroach, *Periplaneta americana* L. Antimicrob. Agents Chemother. **13**:115–120.
4. **Bracke, J. W., D. L. Cruden, and A. J. Markovetz.** 1979. Intestinal microbial flora of the American cockroach, *Periplaneta americana*. Appl. Environ. Microbiol. **38**:945–955.
5. **Breznak, J. A.** 1982. Intestinal microbiota of termites and other xylophagous insects. Annu. Rev. Microbiol. **36**:323–343.
6. **Cleveland, L. R.** 1923. Symbiosis between termites and their intestinal protozoa. Proc. Natl. Acad. Sci. USA **9**:424–428.
7. **Cleveland, L. R.** 1924. The physiological and symbiotic relationships between the intestinal protozoa of termites and their host, with special reference to *Reticulitermes flavipes* Kollar. Biol. Bull. **46**:178–227.
8. **Cleveland, L. R., S. R. Hall, E. P. Sanders, and J. Collier.** 1934. The wood feeding roach *Cryptocercus*, its protozoa, and the symbiosis between protozoa and roach. Mem. Am. Acad. Arts Sci. **17**:185–342.
9. **Cruden, D. L., and A. J. Markovetz.** 1979. Carboxymethyl cellulose decomposition by intestinal bacteria of cockroaches. Appl. Environ. Microbiol. **38**:369–372.
10. **Cruden, D. L., and A. J. Markovetz.** 1984. Microbial aspects of the cockroach hindgut. Arch. Microbiol. **138**:131–139.

11. **Cruden, D. L., and A. J. Markovetz.** 1987. Microbial ecology of the cockroach gut. Annu. Rev. Microbiol. **41:**617–643.
12. **Gijzen, H. J., and M. Barugahare.** 1992. Contribution of anaerobic protozoa and methanogens to hindgut metabolic activities of the American cockroach, *Periplaneta americana*. Appl. Environ. Microbiol. **58:**2565–2570.
13. **Gijzen, H. J., C. A. M. Broers, M. Barugahare, and C. K. Stumm.** 1991. Methanogenic bacteria as endosymbionts of the ciliate *Nyctotherus ovalis* in the cockroach hindgut. Appl. Environ. Microbiol. **57:**1630–1634.
14. **Gijzen, H. J., H. J. Lubberding, M. J. T. Gerhardus, and G. D. Vogels.** 1988. Contribution of rumen protozoa to fibre degradation and cellulase activity in vitro. FEMS Microbiol. Ecol. **53:**35–44.
15. **Gijzen, H. J., K. B. Zwart, P. T. van Gelder, and G. D. Vogels.** 1986. Continuous cultivation of rumen microorganisms, a system with possible applications to the anaerobic degradation of lignocellulosic waste materials. Appl. Microbiol. Biotechnol. **25:**155–162.
16. **Hackstein, J. H. P., and C. K. Stumm.** Methane production in terrestrial arthropods. Proc. Natl. Acad. Sci. USA, in press.
17. **Hungate, R. E.** 1938. Studies on the nutrition of *Zootermopsis*. II. The relative importance of the termite and the protozoa in wood digestion. Ecology **19:**1–25.
18. **Hungate, R. E.** 1939. Experiments on the nutrition of *Zootermopsis*. III. The anaerobic carbohydrate dissimilation by the intestinal protozoa. Ecology **20:**230–245.
19. **Hungate, R. E.** 1943. Quantitative analysis on the cellulose fermentation by termite protozoa. Ann. Entomol. Soc. Am. **36:**730–739.
20. **Kane, M. D., and J. A. Breznak.** 1991. Effect of host diet on production of organic acids and methane by cockroach gut bacteria. Appl. Environ. Microbiol. **57:**2628–2634.
21. **Martin, M. M.** 1983. Cellulose digestion in insects. Comp. Biochem. Physiol. **75A:**313–324.
22. **Miller, G. L.** 1959. Use of dinitrosalicylic acid as reagent for the determination of reducing sugars. Anal. Chem. **31:**426–428.
23. **Slaytor, M.** 1992. Cellulose digestion in termites and cockroaches: what role do symbionts play? Comp. Biochem. Physiol. **103B:**775–784.
24. **Trager, W.** 1932. A cellulase from the symbiotic intestinal flagellates of termites and of the roach, *Cryptocercus punctulatus*. Biochem. J. **26:**1762–1771.
25. **Trager, W.** 1934. The cultivation of a cellulase digesting flagellate *Trichomonas termopsidis* and of certain other termite protozoa. Biol. Bull. **66:**182–190.
26. **Wharton, D. R. A., M. L. Wharton, and J. E. Lola.** 1965. Cellulase in the cockroach, with special reference to *Periplaneta americana* (L.). J. Insect Physiol. **11:**947–959.
27. **Yamin, M. A., and W. Trager.** 1979. Cellulolytic activity of an axenically-cultivated termite flagellate, *Trichomitopsis termopsidis*. J. Gen. Microbiol. **13:**417–420.