Nitrogen Fixation by Bacteria from the Hindgut of Termites

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SUMMARY

Anaerobically grown bacteria isolated from the hindgut contents of the termites *Coptotermes lacteus* (Froggatt), *Mastotermes darwiniensis* Froggatt and *Nasutitermes exitiosus* (Hill) were nitrogenase-positive as assayed by acetylene reduction. Nitrogen fixation, confirmed with $^{15}\text{N}_2$, was highest in the isolate from *M. darwiniensis*. All isolates were identified as *Citrobacter freundii* (Braak) Werkman & Gillen.

INTRODUCTION

Peklo (1946) suggested that insects have endosymbiotic nitrogen-fixing bacteria, but unequivocal experimental evidence was lacking. Recently, using the acetylene reduction assay, nitrogen fixation was demonstrated in termites (Breznak *et al.*, 1973). The data suggested that bacteria in the gut of *Coptotermes formosanus* Shiraki reduced atmospheric nitrogen to a form of nitrogen available to the termite (Breznak *et al.*, 1973). Preliminary experiments using the acetylene reduction assay confirmed that three Australian termite species fixed atmospheric nitrogen. Of these, two species (*Coptotermes lacteus* and *Mastotermes darwiniensis*) possessed hindgut bacteria and protozoa, whereas the third (*Nasutitermes exitiosus*) possessed only bacteria (Noirot & Noirot-Timothee, 1969). We now report the isolation and identification of these hindgut bacteria and confirm that they are capable of nitrogen fixation when cultured anaerobically.

METHODS

The species of termites used were *Coptotermes lacteus* (Froggatt) (Isoptera: Rhinotermitidae), *Mastotermes darwiniensis* Froggatt (Isoptera: Mastotermitidae) and *Nasutitermes exitiosus* (Hill) (Isoptera: Termitidae). *Coptotermes lacteus* was collected from mounds near Canberra in the Australian Capital Territory, *M. darwiniensis* from Townsville in Queensland, and *N. exitiosus* from near Seymour in Victoria.

Before examination, *C. lacteus* and *N. exitiosus* were maintained separately in 1 l jars containing wood and their own mound materials (Gay *et al.*, 1955). *Mastotermes darwiniensis* was maintained in perspex boxes on heartwood sawdust and wood of *Eucalyptus regnans* F. Muell (Howick & Creffield, 1975).

Live workers of the termite species were transferred to stoppered 13.5 ml bottles and the air was replaced by a mixture of 20 % O_2 , 16 % C_2H_2 and 64 % argon. After incubation

Table 1. Ethylene production by live workers of *C. lacteus*, *M. darwiniensis* and *N. exitiosus* assayed by acetylene reduction

Termite species	No. of live workers/assay	Treatment prior to assay	Ethylene produced per termite (nmol h ⁻¹)	
			After 1 h	After 4 h
<i>C. lacteus</i> *	50	Fed on filter paper for 24 h	0.05	0.03
	50	Fed on mound material	0.01	0.01
<i>M. darwiniensis</i> †	10	Starved for 48 h	0	0.60
	10	Fed on wood	1.00	5.50
<i>N. exitiosus</i> *	50	Fed on filter paper for 24 h	0.15	0.11
	50	Fed on wood	0	0
Blank control	—	—	0	0

* Incubation temperature, 25 °C. † Incubation temperature, 30 °C.

(25 °C) for 1 and 4 h, 200 µl gas samples were assayed for acetylene and ethylene using a Philips PV 4000 gas chromatograph fitted with a Porapak R column operating at 50 °C and a flame ionization detector.

The intestinal tracts were dissected from 10 termites of each species and the contents of the hindguts streaked on N-free agar (Hino & Wilson, 1958). Single colonies were sub-cultured on N-free agar slants and in broth cultures under an atmosphere of nitrogen. All bacteria were examined and tested according to the Society of American Bacteriologists' *Manual of Microbiological Methods* (1957) and *Bergey's Manual of Determinative Bacteriology* (1974). All isolates were incubated at 28 °C for between 6 and 10 days before being tested for nitrogen fixation. This was measured by the increase in total culture nitrogen, by ¹⁵N₂ incorporation (Bergersen & Hipsley, 1970), and by the acetylene reduction assay (Hardy *et al.*, 1968). The last test was carried out in 13.5 ml reaction bottles using cultures in N-free broth and on N-free agar slants (Bergersen & Hipsley, 1970), and ethylene was measured by gas chromatography.

RESULTS AND DISCUSSION

Nitrogen fixation by live termite workers

The acetylene reduction assay using live workers indicated nitrogen fixation in all three species of termite (Table 1). Estimated nitrogenase activity per termite was highest (5.5 nmol C₂H₄/h) in the *M. darwiniensis* workers fed on wood for 48 h prior to assay. In contrast, *N. exitiosus* workers showed no nitrogenase activity when fed on wood. *Mastotermes darwiniensis* workers starved for 48 h did not reduce acetylene in the first hour of test, but traces of ethylene were detected after 4 h incubation. Only low nitrogenase activity per termite was observed in the *C. lacteus* workers (0.03 nmol C₂H₄/h) which agrees with data on the estimated activity in the related termite *C. formosanus* (Breznak *et al.*, 1973). Of the smaller termites (4 to 8 mg fresh wt/worker), *N. exitiosus* was more efficient in fixing atmospheric nitrogen than the species of *Coptotermes*. In the large *M. darwiniensis* workers (50 to 55 mg fresh wt/worker), nitrogenase activity was higher than in the smaller termites (Table 1).

Nitrogen fixation by termite hindgut bacteria

Bacterial colonies on N-free medium inoculated from termite hindgut contents were usually uniform. Only from *C. lacteus* were two colony types isolated. Three replicates of

each pure bacterial colony were isolated on N-free agar slants (Table 2). The acetylene reduction of isolates assayed anaerobically is shown in Table 2. Aerobic growth was slight to good in all cultures (Table 2), but no acetylene reduction occurred. Anaerobically, several bacterial isolates, particularly M-A3 (*M. darwiniensis*) and N-A1 (*N. exitiosus*), showed relatively high nitrogenase activity.

Three replicates of each nitrogenase-positive isolate (C-A2, C-B3, M-A3 and N-A1) were inoculated on to N-free agar slants and tested for $^{15}\text{N}_2$ incorporation (Bergersen & Hipsley, 1970) under anaerobic conditions. After incubation under $^{15}\text{N}_2$ for 1 week at 28 °C, the bacterial growth in the replicate bottles was pooled and analysed (Table 2). All isolates showed $^{15}\text{N}_2$ incorporation: the highest value was recorded in M-A3, isolated from *M. darwiniensis*.

Species characterization of the nitrogen-fixing cultures

All the cultures were Gram-negative, facultative anaerobic rods. The N-A1 isolate from *N. exitiosus* failed to grow on culture media after its ability to fix nitrogen had been determined, thus we report only the characteristics of isolates from *C. lacteus* and *M. darwiniensis*.

On nutrient agar, the isolates formed abundant, irregular, creamy-grey colonies; all were motile, reduced nitrate, were catalase and methyl red positive, and fermented sucrose, xylose, arabinose and galactose with gas production. None of the cultures produced gas from arabinose during the first two days. All three cultures were Kovacs' oxidase-negative, phenylalanine deaminase-negative, gluconate-negative, Thornley's arginine-positive, Simon's citrate-positive, H_2S -positive, KCN-positive, and all produced acid and gas from salicin. All isolates were identified as *Citrobacter freundii* (Braak) Werkman & Gillen, synonym *Escherichia freundii* (Braak) Yale. Isolates C-B3 (*C. lacteus*) and M-A3 (*M. darwiniensis*) were identical in all tests. C-A2 (*C. lacteus*) was slightly different in appearance and was probably a different strain.

Citrobacter freundii is frequently found on vegetable material, where it is presumably a saprophyte. This report appears to be the first record of nitrogen fixation by this species, although there are well substantiated reports of nitrogen fixation by closely related members of the Enterobacteriaceae.

There was high variability between replicate cultures (Table 2). Good growth of bacterial colonies under anaerobic conditions did not signify correspondingly high nitrogenase activity, and vice versa. The variability between replicates could well have been caused by the use of colonies grown on a solid medium. The lack of correlation of growth with acetylene-reducing capacity might be because the isolates were grown on N-free medium for 6 to 10 days before acetylene reduction tests were carried out. In a test of this kind one would expect to find that fast-growing nitrogenase-positive isolates would show good growth but low acetylene reduction (e.g. culture C-A2), while isolates which grow more slowly would show only moderate growth but high acetylene-reducing capacity (e.g. culture C-B3). However, cultures M-A3 and N-A1 appeared to be exceptions. Statistical analysis of the ethylene assayed was deemed unnecessary considering the methods used and the values obtained. Limitations of time prevented a repeat of the assays. In any further studies, quantification would be preferable. Known quantities of bacteria would need to be isolated on to N-free agar slants introducing $^{15}\text{N}_2$ at the same time, prior to incubation. The data presented however clearly demonstrate that anaerobically grown cultures of these bacteria isolated from the termite hindgut do fix atmospheric nitrogen.

Our estimates of nitrogenase activity by live workers of *C. lacteus* and *N. exitiosus* (Table 1) are similar to the values reported by Breznak *et al.* (1973) and Benemann (1973)

for a species of *Coptotermes*. It seems that nitrogen-fixing ability is similar in worker termites of similar size, in northern and southern hemispheres. It is noteworthy that the same species of nitrogen-fixing bacteria was isolated from three different termite species, collected from different locations in Australia. Termites live in a meagre nitrogen environment (Lee & Wood, 1971) in which metabolizable carbon compounds are continuously generated from cellulose by the anaerobic activity of gut organisms. It is in these conditions that nitrogen-fixing bacteria would be expected to thrive, although it is somewhat surprising that those found belong to a species in which, to the best of our knowledge, nitrogen fixation has not previously been reported.

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