# Occurrence of poly(hydroxyalkanoate) in the gut homogenate of a phylogenetically higher termite: *Macrotermes carbonarius*[1]

**Kumar Sudesh, Bee-Yong Tay, and Chow-Yang Lee**

**Abstract:** Gas chromatography analysis of a phylogenetically higher termite-gut homogenate detected the presence of trace amounts of poly(3-hydroxybutyrate) (PHB), a prokaryotic storage material. In addition, the gut homogenate smear, stained with Nile blue A, also suggested the presence of PHB-like granules. Chloroform extracts of both soldier and worker classes of this termite were obtained for further spectroscopic analysis. FTIR, $^{1}$H NMR, and 2D $^{1}$H-$^{1}$H NMR analyses confirmed the presence of PHB in both the chloroform extracts. This showed for the first time the occurrence of bacteria capable of accumulating PHB in the termite gut. The results indicated that the physiological environment in the termite gut is suitable for the colonization by PHB-producing bacteria and is probably rich in organic carbon sources, which can be readily assimilated and stored as PHB.

*Key words: Macrotermes carbonarius*, poly(hydroxyalkanoate), PHB, termite

**Résumé :** L'analyse par chromatographie gazeuse d'une préparation homogénéisée d'estomac de termites phylogénétiquement plus élevées a permis de détecter la présence de traces de poly(3-hydroxybutyrate) (PHB), un matériel d'emmagasinage prokaryotique. De plus, un frottis d'une préparation homogénéisée d'estomac, teint avec du bleu du Nile A, suggère aussi la présence de granules ressemblant à du PHB. On a aussi obtenu des extraits au chloroforme des classes tant de soldat que de travailleur de cette termite qui ont pu être analysés par des méthodes spectroscopiques. Des analyses par IR à transformée de Fourier, par RMN du $^{1}$H et par RMN $^{1}$H-$^{1}$H en 2D ont permis de confirmer la présence de PHB dans chacun de ces extraits chloroformiques. Ces résultats démontrent pour la première fois l'existence de bactéries qui sont capables d'accumuler du PHB dans l'estomac des termites. Ils démontrent aussi que l'environnement physiologique dans l'estomac des termites est approprié pour la colonisation par des bactéries pouvant produire du PHB et qu'il est probablement riche en sources de carbone organique qui peut être facilement assimilé et emmagasiné sous la forme de PHB.

*Mots-clés : Macrotermes carbonarius*, polyhydroxyalcanonate, PHB; termite.

[Traduit par la rédaction]

## Introduction

Poly(hydroxyalkanoate) (PHA) is a carbon- and energy-storage material accumulated by a large number of prokaryotes (1). The synthesis of PHA by bacteria usually occurs when carbon source is abundant. The most common type of PHA found naturally in the environment is poly(3-hydroxybutyrate) (PHB). The ability to accumulate PHB has been shown to enhance the survival of bacteria during prolonged period of starvation and (or) under stress conditions (2). PHB also serves as an electron sink and provides energy for the energy-intensive process of sporulation in some bacteria (3, 4). The occurrence of PHB in an environmental sample provides physiological evidence for the presence of prokaryotic microorganisms capable of synthesizing this storage material (5). In addition, it also indicates suitable growth conditions for bacteria and the presence of sufficiently high concentrations of organic carbon sources that can be assimilated and polymerized into PHB.

The intestine of termites is colonized by a variety of microorganisms (6), which contribute to the biomineralization of lignocellulosic matter ingested by termites. These microorganisms have attracted the attention of microbiologists because of their important and interesting symbiotic association with the termite host for the efficient biorecycling of plant litter (7). A major drawback faced by microbiologists in understanding the physiological aspects of termite gut microbiota is the difficulty to cultivate most of the microorganisms in the laboratory (8). To partially solve this problem, culture independent methods have been used to identify and characterize the various types of microorganisms (7, 9).

The primary objective of this study was to determine if termite-gut environment provides conditions favorable for the colonization by PHA producing bacteria. Although molecular methods have revealed the presence of bacteria that

Received 17 October 2007. Accepted 18 December 2007. Published on NRC Research Press Web site at canjchem.nrc.ca on 1 April 2008.

**K. Sudesh,[2] B.Y.Tay, and C.Y. Lee.** School of Biological Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia.

[1]This article is part of a Special Issue dedicated to Professor R. Marchessault.

[2]Corresponding author (e-mail: ksudesh@usm.my).

 doi:10.1139/V08-007 

are phylogenetically related to PHA producers (10), there is still no evidence for the occurrence of PHA-producing bacteria in termite gut. Furthermore, it is not known if the nutritive conditions in the termite gut favor the growth of PHA producing bacteria and that substantial amounts of suitable carbon sources are available to the bacteria for the accumulation of PHA. Here, we report for the first time the occurrence of PHA in a phylogenetically higher termite, *Macrotermes carbonarius*. Quantitative monitoring of PHA in the termite gut might be useful in defining the recent nutritional history of the gut microbiota. In addition, knowledge of the microorganisms in the termite gut will provide new insights into the possibility to convert lignocellulosic matter to PHA.

## Materials and methods

Live worker and soldier termites of *M. carbonarius* were collected from mound material dug from various locations in Universiti Sains Malaysia. The termites were transported to the laboratory together with their fungus comb. The termites were then separated into workers and soldiers based on their obvious morphological characteristics (11, 12). One hundred workers were cleaned using sterile distilled water and 70 % ethanol before extracting their whole guts for gas chromatography (GC) analysis. The freeze-dried gut homogenate was methanolyzed in a screw-capped test tube at 100 °C for 140 min in a mixture of 2 mL chloroform, 1.7 mL methanol, and 0.3 mL concentrated sulfuric acid. Upon completion, 1 mL of distilled water was added to the cooled mixture and vortexed for 1 min. After phase separation, the organic phase (bottom layer) was subjected to GC analysis. A Shimadzu GC-14B capillary gas chromatograph system equipped with flame-ionization detector (FID) was used along with a fused silica capillary column (SPB$^{TM}$-1, Supelco) for the analysis.

The remaining workers (1368) and soldiers (706) were homogenized separately prior to lyophilization. A total freeze-dried mass of 14.5 g whole soldier homogenate and 4.5 g whole worker homogenate were obtained and subjected to extraction with hot chloroform for 4 h. The brownish chloroform extracts were then concentrated to approximately 10 mL and added to 100 mL of rapidly stirred cold methanol. This resulted in the formation of a fluffy precipitate, which was recovered by filtration and dried in vacuo before subjecting to spectroscopic analyses. For $^{1}$H, $^{13}$C NMR, and 2D $^{1}$H-$^{1}$H NMR spectroscopy analyses, the dried precipitate was dissolved in $CDCl_3$, and the spectra were recorded using a Bruker 400 MHz NMR spectrometer. The same solution was used for FTIR analysis. For the latter, a thin layer of film was made on a ZnSe window by evaporating the solvent. After thorough drying, the IR spectrum of the resulting film was recorded using a PerkinElmer System 2000 FTIR. For comparison purposes, pure PHB was synthesized and purified from *Delftia acidovorans* according to a method described recently (13).

## Results and discussion

GC analysis of the lyophilized whole-gut extract of *M. carbonarius* workers revealed the presence of trace amounts (<0.5% of the gut dry mass) of PHB. Nile blue A staining of the gut homogenate smear also suggested the presence of PHA-like granules, which showed the typical bright orange fluorescence when viewed under a UV-light microscope (results not shown). These results provided early indication for the presence of PHA in the termite gut. To obtain conclusive evidence, large quantities of lyophilized whole-termite homogenate were subjected to chloroform extraction to dissolve any PHA that may be present. During the precipitation step in methanol, the formation of white fluffy material was a further indication for the presence of relatively high molecular mass PHA. Upon filtration, the white precipitate was co-purified with a brownish pigment. To remove the brownish pigment, the above process of dissolving in chloroform and precipitating in methanol was repeated. This resulted in lesser amount of the brownish pigment, as indicated by its lighter color intensity. However, it also reduced the amount of the white fluffy precipitate. No further attempts were made to remove the brownish pigment, as it may decrease the concentration of any PHA that was extracted.

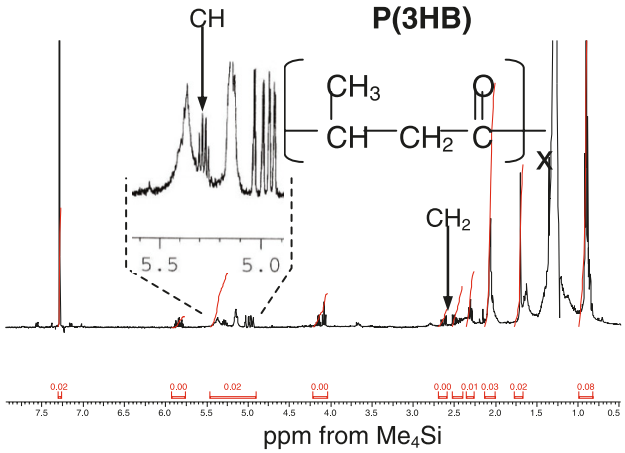
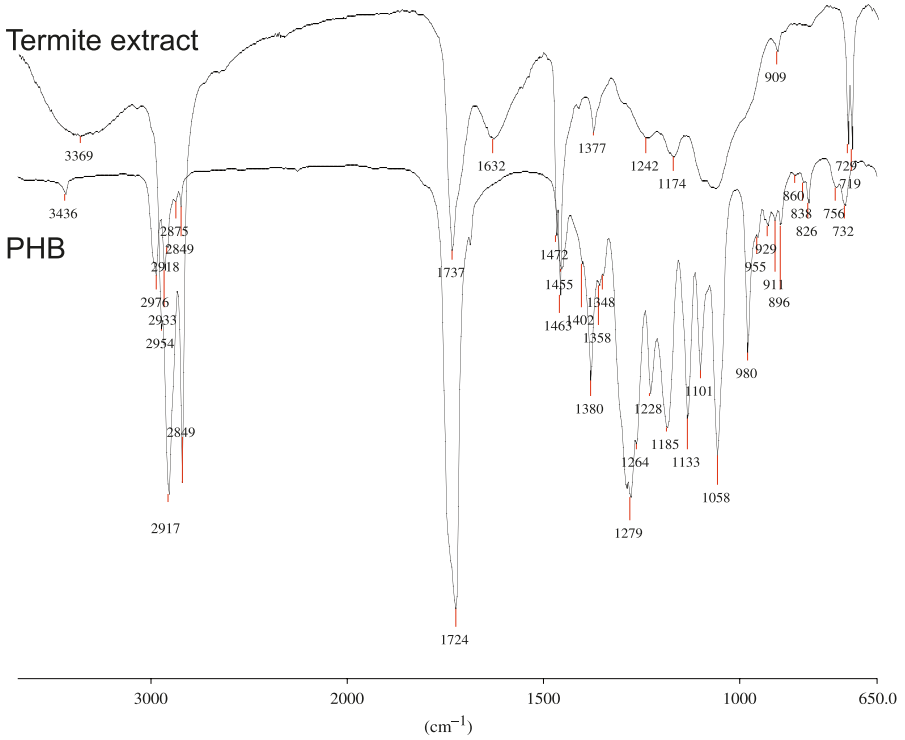



**Fig. 1.** $^{1}$H NMR spectrum of chloroform extract of whole worker homogenate recorded at 25 °C in $CDCl_3$. Inset shows an expanded spectrum in the vicinity of methine proton resonance of PHB.

Figure 1 shows the $^{1}$H NMR spectrum of the extract recorded in $CDCl_3$ at 25 °C. The chemical shifts are in ppm from TMS. The predominant peaks at around 0.9 ppm and 1.4 ppm can be attributed to methyl and methylene protons of long-chain alkanes. This is further supported by the symmetric–asymmetric stretching bands of methyl–methylene at around 2900 cm$^{-1}$ in the FTIR spectrum (Fig. 2) of the chloroform extract. The carbonyl (C=O) stretching band at 1724 cm$^{-1}$ (appears as a faint shoulder near the band at 1737 cm$^{-1}$) is a characteristic band of PHB, as shown in the spectrum obtained from pure PHB. However, the bands at 2900 cm$^{-1}$ are more intense than that at 1724 cm$^{-1}$, indicating that most of the extracted products may be long-chain alkane compounds. Therefore, the peak from methyl protons of PHB (1.27 ppm) in the NMR spectrum may be overlapping with the peak at 1.4 ppm due to the methylene protons of long-chain alkanes. Because of the significant amount of co-purified long-chain alkane compounds, the presence of PHB could not be confirmed from the FTIR spectrum.

**Fig. 2.** FTIR spectra of chloroform extract of whole worker homogenate and pure poly(3-hydroxybutyrate) (PHB).




Further clues for the presence of PHB was obtained from a faint peak at around 5.27 ppm in the $^{1}$H NMR spectrum (Fig. 1). By comparison with previously reported $^{1}$H NMR spectrum of PHB (14), the peak near 5.27 ppm may be a resonance from the methine proton of PHB. In addition, weak resonance peaks (2.45–2.65 ppm) corresponding to the methylene protons of PHB can also be detected from the $^{1}$H NMR spectrum (14). The identities of the peaks also were confirmed by comparing with the $^{1}$H NMR spectrum of pure PHB sample (see Supplementary Data).[3] To obtain further confirmation, 2D $^{1}$H-$^{1}$H NMR analysis was performed, and the result is displayed in Fig. 3.

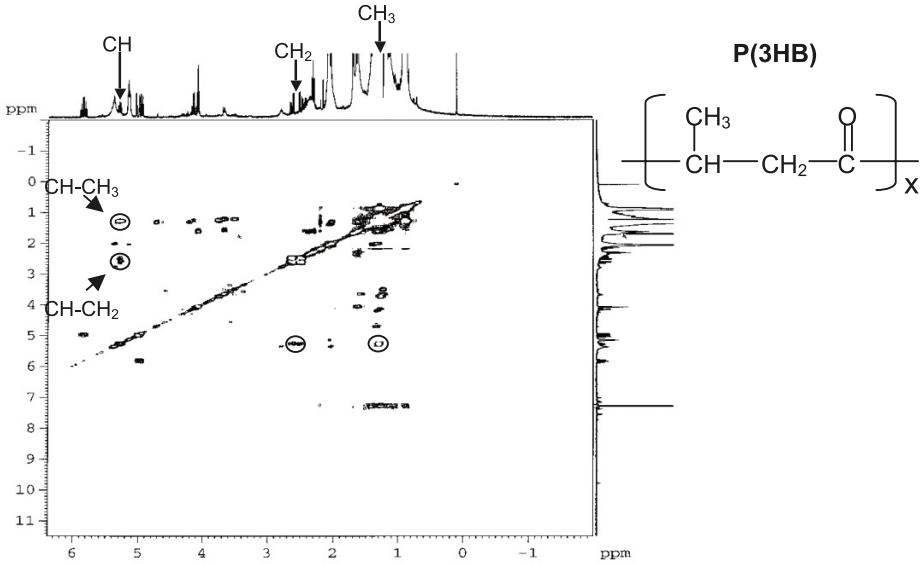
Clear correlation peaks were seen between the neighboring protons of methine and methylene. In addition, correlation peaks were also observed between methine and methyl protons showing that they are adjacently located as is in the case of PHB. These results provided strong evidence for the presence of PHB in the chloroform extract of the worker-class termite homogenate.

Another interesting type of PHA has also been identified in many organisms, including eukaryotes. This PHA is characterized by its low molecular mass, because it is consisted of less then 150 monomeric units of either 3-hydroxybutyrate or 3-hydroxyvalerate. Unlike the high molecular mass storage PHA synthesized by most bacteria, the low molecular mass PHA is a non-storage PHA and is usually complexed with other cellular macromolecules; hence they are referred to as complexed PHA (15). It is possible that some of the non-storage PHAs may have been co-extracted from our samples. However, we have also carried out ultrastructural studies on the termite gut, which revealed the presence of many bacterial cells that contained discrete granules resembling PHAs (see Supplementary Data).[3] Based on all these evidences, the PHB detected in this study is most likely the storage PHB accumulated by bacteria in the termite gut.

NMR and FTIR analyses of chloroform extract of the soldier-class termite homogenate also produced similar results (results not shown). Thus, the occurrence of PHB was confirmed in both the soldier and worker homogenates. The results indicated the presence of PHB producing microorganisms in the gut of both soldier and worker classes of this termite. It is known that the soldiers are not capable of feeding themselves because of their huge mandible. The soldiers have to be fed by the workers by a process called trophallaxis. Therefore, the intestinal microbiota of *M. carbonarius* soldiers must have originated from the workers and therefore may be quite similar. The occurrence of PHB in the guts of both these classes provides good evidence for the similarities of gut physiological conditions in workers and soldiers. In addition, this study has also shown that the physiological environment of the guts of both soldiers and workers allow for the growth and proliferation of PHB producing bacteria and that suitable types and concentrations of carbon sources are readily available for PHB biosynthesis. We have since successfully isolated three morphologically different bacteria capable of producing PHB from the gut of *M. carbonarius* (manuscript in preparation). By understand-

[3] Supplementary data for this article are available on the journal Web site (canjchem.nrc.ca) or may be purchased from the Depository of Unpublished Data, Document Delivery, CISTI, National Research Council Canada, Ottawa, ON K1A 0R6, Canada. DUD 3729. For more information on obtaining material, refer to cisti-icist.nrc-cnrc.gc.ca/irm/unpub_e.shtml.

**Fig. 3.** 2D $^{1}H$-$^{1}H$ NMR spectrum of chloroform extract of whole worker homogenate. Signals confirming the presence of poly(3-hydroxybutyrate) (PHB) are circled.




ing the PHB biosynthesis process in these bacteria, it would provide additional insight into the physiological environment of the termite gut. In addition, this knowledge might enable the development of novel processes for the conversion of lignocellulosic wastes into PHB.

## Acknowledgements

The authors thank Goh Yik Pheng and Chee Jiun Yee for assisting with the collection and dissection of termites. Dr. Hideki Abe's help with the interpretation of the NMR spectra is gratefully acknowledged. Universiti Sains Malaysia supported this study through the Research University grant.

## References

1. A.J. Anderson and E.A. Dawes. Microbiol. Rev. **54**, 450 (1990).
2. J.A. Ruiz, N.I. Lopez, R.O. Fernandez, and B.S. Mendez. Appl. Environ. Microbiol. **67**, 225 (2001).
3. M.A. Cevallos, S. Encarnacion, A. Leija, Y. Mora, and J. Mora. J. Bacteriol. **178**, 1646 (1996).
4. W.J. Page, A. Tindale, M. Chandra, and E. Kwon. Microbiology, **147**, 483 (2001).
5. R.H. Findlay and D.C. White. Appl. Environ. Microbiol. **45**, 71 (1983).
6. A. Brune and M. Friedrich. Curr. Opin. Microbiol. **3**, 263 (2000).
7. M. Ohkuma. Appl. Microbiol. Biotechnol. **61**, 1 (2003).
8. B.S. Stevenson, S.A. Eichorst, J.T. Wertz, T.M. Schmidt, and J.A. Breznak. Appl. Environ. Microbiol. **70**, 4748 (2004).
9. A. Brauman, J. Dore, P. Eggleton, D. Bignell, J.A. Breznak, and M.D. Kane. FEMS Microbiol. Ecol. **35**, 27 (2001).
10. T. Thongaram, Y. Hongoh, S. Kosono, M. Ohkuma, S. Trakulnaleamsai, N. Noparatnaraporn, and T. Kudo. Extremophiles, **9**, 229 (2005).
11. Y.P. Tho. Malayan Forest Records, **36**, 1 (1992).
12. C.Y. Lee, B.T. Forschler, and T.M. Jenkins. Proceedings of the 5th International Conference on Urban Pests, July 9-14, Singapore, 2005. pp. 205-211. ISBN 983 42607 0 9.
13. W.H. Lee, M.N.M. Azizan, and K. Sudesh. Polym. Degrad. Stab. **84**, 129 (2004).
14. Y. Doi, M. Kunioka, Y. Nakamura, and K. Soga. Macromolecules. **19**, 1274-1276 (1986).
15. R.N. Reusch. Non-storage poly-(*R*)-3-hydroxyalkanoates (complexed PHAs) in prokaryotes and eukaryotes. In Y. Doi and A. Steinbüchel (*Editors*). Biopolymers, vol. 3a. Wiley-VCH Verlag GmbH, Weinheim, Germany. p123 (2002).