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Colonization of termite hindgut walls by oxymonad flagellates and prokaryotes in *Incisitermes tabogae*, *I. marginipennis* and *Reticulitermes flavipes*

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Abstract

We studied the colonization of the paunch wall of three lower termites, *Reticulitermes flavipes*, *Incisitermes tabogae*, and *Incisitermes marginipennis*, by light and electron microscopy. In addition to various prokaryotes, oxymonad flagellates were attached to the wall of the paunch in all three species. The prokaryotic layer found in *R. flavipes* is relatively thin, since most organisms are attached laterally. Large members of the flagellate genus *Pyrsonympha* protrude into the gut lumen. The prokaryotes are very abundant on the gut wall in *I. tabogae* and *I. marginipennis*, forming a thick carpet of mostly vertically attached rods and wavy spirochetes. The adhering oxymonads are relatively small and almost hidden in the thick bacterial biofilm. Three small morphotypes were seen in *I. tabogae*; two possessing a short rostellum and one amoeboid. The only oxymonad found in *I. tabogae* so far, *Oxymonas clevelandi*, is not identical to any of the present oxymonads. *I. marginipennis* contains a mid-sized oxymonad with ectobiotic spirochetes, probably identical to *Oxymonas hubbardi*, and a tiny unknown morphotype. The spatial organization of the pro- and eukaryotic microorganisms on the gut wall of the three termites is described and discussed concerning oxygen stress.

Keywords: Bacteria; Flagellate; *Incisitermes*; *Oxymonas*; *Reticulitermes flavipes*; Termite

Introduction

The dilated hindgut of lower termites is inhabited by large numbers of symbiotic flagellates and prokaryotes, with densities reaching up to 10^{11} cells ml^{-1} (Brugerolle and Radek, 2006; Ohkuma and Brune, 2011). Whereas the flagellates are essential for cellulose digestion, the diverse prokaryotes fulfill many physiological functions and create suitable conditions for the flagellates (Brune and Ohkuma, 2011; Tokuda et al., 2000). The termites absorb the released nutrients (mainly acetate) via the wall of the paunch. The different gut segments are generally termed according to the nomenclature of Noirot (1995). In addition to crop (C) and midgut (M), there are five more or less well-developed hindgut segments (P1–P5).

A considerable number of the gut prokaryotes are associated with the flagellates as endo- or ectobionts or are attached to the cuticle of the gut wall (Breznak and Pankratz, 1977; Brune, 2006; Radek et al., 1992). For example, in the thin-walled anterior region of the paunch (=P3a) of *Mastotermes darwiniensis*, 90% of the prokaryotes were associated with flagellates and 2% with the gut wall, whereas in the thick-walled posterior region of the paunch (=P3b) and the colon (=P4), only 6–8% were associated with flagellates and more than 85% of the prokaryotes were attached to

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the wall (Berchtold et al., 1999). Many termite species are known to each have several hundred prokaryotic phylotypes in their guts, which are generally specific for this habitat (Hongoh et al., 2003a,b; Schultz and Breznak, 1978; Yang et al., 2005). In addition, at least 20–30 different bacterial morphotypes have been found in the gut of *Reticulitermes flavipes*, *Coptotermes formosanus* (Breznak and Pankratz, 1977) and *Pterotermes occidentis* (To et al., 1980). For example, the spore-forming filaments described by Leidy (1881) as *Arthromitus* or the large spirochetes can easily be morphologically differentiated (Bermudes et al., 1988; Breznak, 1984; Margulis et al., 1990).

We are especially interested in the microorganisms associated with the wall of the paunch. On the one hand this habitat offers valuable attachment sites for gut microorganisms, but on the other hand the physicochemical conditions are a challenge. The fact that oxygen can diffuse into the gut is problematic for the generally anaerobic organisms living in the paunch. Microelectrode investigations have revealed that oxygen may penetrate 50–200 μm into the lumen of the termite hindgut, leading to a micro-oxic peripheral zone (Brune, 1998; Ebert and Brune, 1997; Kappler and Brune, 1999; Schmitt-Wagner and Brune, 1999). It is assumed that the gut prokaryotes maintain anoxic conditions in the center of the paunch by removing the oxygen (Veivers et al., 1982). The result is a steep gradient of oxygen partial pressure toward the gut periphery. Microorganisms encounter higher partial pressure with increasing proximity to the gut wall. Prokaryotes colonize the cuticle of the gut epithelium of all lower and higher termites which have been investigated to date (Breznak and Pankratz, 1977; Czolij et al., 1985; To et al., 1980; Yara et al., 1989).

Besides prokaryotes, oxymonad flagellates are attached to the gut wall in many lower termites as well. Members of six genera, *Barroella*, *Microrhopalodina*, *Oxymonas*, *Sauromonas*, *Streblomastix*, and *Pyrsonympha*, are able to attach to the cuticle of the paunch by anterior attachment organelles (Brugerolle and Lee, 2000). Most oxymonads have one karyomastigont (nucleus plus four flagella and their root structures). The four flagella are arranged in two pairs which are connected by a paracrystalline preaxostyle. An axostyle composed of parallel rows of microtubules extends through the cell body and is associated with an anterior microtubular plate, the pelta. Further microtubular bundles may extend into an elongated anterior attachment organelle called a rostellum. While the parabasalid flagellates that are also usual inhabitants of lower termite guts possess further cell organelles such as Golgi bodies (parabasal bodies) and hydrogenosomes, these structures are not found in oxymonads. There are indications that hydrogenosomal/mitochondrial derivatives were once present in oxymonads (Carpenter et al., 2008; Vacek et al., 2011). The metabolic capabilities of oxymonads are largely unexplored. Many species have ecto- or endobiotic prokaryotes (Dolan, 2001; Noda et al., 2009; Stingl et al., 2005).

The hindgut epithelium including the paunch is covered by a thin cuticle, the intima. This cuticle is lost during the molt, together with many/most of its attached microorganisms (Honigberg, 1970). The gut microbiota is regained by proctodeal trophallaxis or through feeding on the exuvia.

We investigated the hindgut wall of three termites of different systematic affiliations and lifestyles in respect to their colonization with oxymonads and prokaryotes. *Reticulitermes flavipes* (Rhinotermitidae) is a widespread and destructive subterranean termite (and therefore has been well investigated), whereas *Incisitermes tabogae* and *I. marginipennis* (Kalotermitidae) are dry wood termites (Constantino, 1998). *R. flavipes* has spread from America to Europe, where it was erroneously given a new species name: *R. santonensis* (Austin et al., 2005). Our main aim was to characterize the spatial organization of the gut biofilms and to describe their key components in the three investigated termite species. Furthermore, the structure of the paunch wall serving as substrate for the biofilm should be elucidated.

Material and Methods

Termites

False workers (pseudergates) of *Incisitermes tabogae*, *I. marginipennis* and *Reticulitermes flavipes* were obtained from the Federal Institute for Materials Research and Testing (BAM) in Berlin, where they are in culture.

Light microscopy

The hindguts were removed using a pair of tweezers and the paunch was then opened in 0.6% NaCl for live observations.

Scanning electron microscopy (SEM)

Extracted hindguts were put into fixative (2.5% glutaraldehyde in 50 mM cacodylate buffer, pH 7.1) and their paunches cut into anterior and posterior portions using razor blades. After fixation in the same fixative for 30–60 min, the gut pieces were washed three times in 50 mM cacodylate buffer, pH 7.1, and fixed in 1% OsO_4 for 30 min. The samples were washed again three times and transferred into small cups covered with planktonic gauze. After dehydration in a graded series of ethanol, the samples were dried with a BAL-TEC Critical Point Dryer (CPD) 030 and coated with gold in a Balzer SCD 040. The gut pieces were examined using a FEI Quanta 200 ESEM.

For enabling a more direct view of the cuticle of the paunch, several termites of each species were treated with antibiotics to remove the attached prokaryotes. The termites were kept in Petri dishes with some sand. Filter paper soaked in a solution containing 6 mg/ml penicillin – K^+-salt and 10 mg/ml streptomycin-sulfate was added as the sole food.