Short Communication

Presence of nitrogen fixing *Klebsiella pneumoniae* in the gut of the Formosan subterranean termite (*Coptotermes formosanus*)

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**Abstract**

A gram-negative facultative anaerobic enteric bacterium, *Klebsiella pneumoniae* was isolated from the hindgut of the Formosan subterranean termite (FST). It was characterized using fatty acid methyl ester (FAME) analysis, BIOLOG assay, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and biochemical studies. The role of this isolate seems to be nitrogen fixation because the termite’s diet is nitrogen deficient and the isolate produced significant amounts of ammonia when it was grown on nitrogen deficient medium under anaerobic condition with nitrogen gas in the headspace.

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1. Introduction

The termite gut contains endosymbiotic protozoa and bacteria. The prokaryotic microorganisms found in the termite gut perform a wide range of physiological functions such as cellulose digestion (Hethene et al., 1992), hemicellulose digestion (Taguchi et al., 1993), acetogenesis (Leadbetter et al., 1999), hydrogenogenesis (Taguchi et al., 1993), methanogenesis (Leadbetter et al., 1999), sulfate reduction (Kuhnigk et al., 1996), and nitrogen fixation (Liburn et al., 2001; Ohkuma et al., 1996). The process of cellulose digestion begins with the flagellated protozoans of the gut. The protozoa degrade cellulose into hydrogen, acetate, and carbon dioxide (Nakashima et al., 2002). Acetogenic bacteria of the gut take the hydrogen and carbon dioxide produced by the protozoa and metabolize it into acetate (Leadbetter et al., 1999). The role of methanogenic bacteria is not completely known. Researchers believe that methanogens remove excess hydrogen to enhance cellulose digestion (Shinzato et al., 1999) and to protect hindgut microorganisms from high concentrations of hydrogen, which can be toxic (Leadbetter and Breznak, 1996). The nitrogen fixing bacteria in the hindgut of the termites are important because the termite’s diet is nitrogen poor (Breznak et al., 1973) and may provide the termites' metabolic needs.

Termite hindguts are not totally anoxic. Rather, the hindguts of termites contain complex microhabitats characterized by steep gradients of metabolites and physicochemical conditions (Brune, 1998). Microsensor studies have demonstrated that large amounts of oxygen penetrate the gut epithelium and are rapidly consumed in the gut periphery, which renders the center of the gut anoxic (Kapppler and Brune, 1999). Leadbetter and Breznak (1996) found novel methanogenic bacteria in the hindgut of the lower termite *Reticulitermes flavipes*. Ohkuma et al. (1996) discovered the presence of the dinitrogenase reductase gene (*nifH*) sequence in six termites. The majority of the *nifH* sequences could be assigned to the anaerobic *nif* group (consisting of clostridia and sulfur reducers) or the methanogen group. The objective of this study was to isolate, identify, and characterize facultative anaerobic bac-
teria in the gut of Formosan subterranean termites (FST) and their possible role in the microbial ecology of termite gut ecosystem.

2. Methods

2.1. Materials

The Formosan subterranean termites, Coptotermes formosanus were provided by the United States Department of Agriculture (USDA), Formosan Subterranean Termite Research Unit, Southern Regional Research Center (SRRC), New Orleans, LA.

2.2. Isolation

The FST worker was externally sterilized in 100% ethanol for about 1 min and then was allowed to air dry for 1 min. The hindgut was then opened using flame sterilized fine-tip forceps and placed in an individual anaerobic culture bottle containing 20-mL of tryptic soy broth (TSB) medium. Cultures were grown anaerobically with hydrogen/carbon dioxide (80:20) headspaces at 22°C (Balch and Wolfe, 1976). Cultures were transferred to fresh media every two weeks, and, after three enrichment transfers, pure cultures were isolated using TSA plates (Adams and Boopathy, 2005).

Cultures were streaked for isolation under both aerobic and anaerobic conditions. Isolates that grew aerobically were placed in test tubes containing 10-mL of tryptic soy broth (TSB) in test tubes. Isolates that grew anaerobically were placed in 20-mL TSB in anaerobic culture bottles, under anaerobic conditions described by Balch and Wolfe (1976). Pure cultures were maintained in tryptic soy agar (TSA) slants at 4°C until needed for identification and characterization experiments.

2.3. Identification

BIOLOG® (Hayward, CA) and fatty acid methyl ester (FAME) analyses were used to identify the isolates according to manufacturer’s specifications (Biolog, 2002). FAME analysis (Miller and Berger, 1985) was conducted using fresh isolate growth plated on TSA plates. The FAME profiles were then compared to a database for the closest match that could be made for species identification. For SDS-PAGE analysis, the Laemmli method (Laemmli, 1970) was followed. A low molecular weight marker (Amersham Bioscience Co., Piscataway, NJ) was run concurrently as a size marker. Basic biochemical tests such as oxidase, urease, gelatinase, and fermentation reactions were conducted as per the method described by Benson (2002).

2.4. Nitrogen fixation assay

An experiment was performed to see whether the isolate was capable of nitrogen fixation. A basic mineral salt medium (Adams and Boopathy, 2005) was prepared in anaerobic culture bottles in duplicates. The basic mineral salt medium was supplemented with 0.05 g/100 mL NaMo and 0.001 g/100 mL Fe₂(SO₄)₃. These two co-factors are needed for nitrogen fixing function by bacteria (Shah and Brill, 1977). However, the media did not contain yeast extract or ammonium sulfate since both compounds contain nitrogen. The bacteria were grown anaerobically with nitrogen or helium in the headspace in duplicate. Nitrogen (100%) or helium (100%) was used to fill the headspace (Balch and Wolfe, 1976) prior to sterilization. To remove any nitrogen present in the stock culture, 1.5 mL of pure culture was centrifuged at 10,000 rpm for 5 min. The supernatant was removed and the isolate was then resuspended in 1.0 mL of sterile basic salt media and 1 mL of re-suspended isolate was used as an inoculum. The tubes were incubated at 37°C in a shaker set at 100 rpm. Bacterial growth was monitored at 600 nm using a spectrophotometer. Ammonia in the culture media was analyzed according to Hach method using Hach Instructional manual (Hach Co, Loveland, CO).

3. Results and discussion

3.1. Identification of isolate

No obligate anaerobes were cultured from the gut of the Formosan termite under the isolation conditions used in this project. However, several facultative anaerobes were isolated and one isolate was positively identified and studied further. A gram stain indicated that the organism was a gram-negative rod. BIOLOG® identification indicated that the organism was Klebsiella pneumoniae sub. pneumoniae. The FAME analysis confirmed the identification by BIOLOG® (Fig. 1). FAME analysis results showed 98% match of our isolate to K. pneumoniae sub. pneumoniae. The SDS-PAGE showed protein banding, which matched the banding pattern of two environmental strains of K. pneumoniae obtained from the American type culture collection (ATCC # 13883) and Food and Drug Administration (FDA) laboratory in Jefferson, AR (FDA Strain KP 12) (Fig. 2).

3.2. Biochemical tests

The basic biochemical tests confirmed the isolate as K. pneumoniae. The isolate was urease positive, gelatinase negative, oxidase negative, and non-motile. The isolate fermented glucose, lactose, and sucrose. The isolate produced acid slant and acid butt, produced gas and negative for hydrogen sulfide in triple sugar iron agar (TSIA).

3.3. Nitrogen fixing activity

The isolate was able to grow in nitrogen free medium with nitrogen gas in the headspace under anaerobic condition. However, the isolate did not grow when the headspace contained helium instead of nitrogen (Fig. 1). The
ammonia analysis showed the production of a significant amount of ammonia in the culture media (Fig. 1). This experiment demonstrated that this isolate has the ability to fix nitrogen under anaerobic condition and may play a key role by providing a nitrogen source for the FST.

Under anaerobic or microaerobic conditions *K. pneumoniae* is considered an associative nitrogen fixer (Ladha et al., 1983). The presence of the facultative anaerobe *K. pneumoniae* in non-FST has been observed by Kuhnigk et al. (1994). Isolates of *Klebsiella* have been found in living or decaying wood, bark, and composted wood (Descamps et al., 1983). Therefore, it is reasonable to assume that the termite ingests *K. pneumoniae*. One possible role that *K. pneumoniae* may play in the hindgut microbial ecology is that of a nitrogen fixer. The presence of a nitrogen fixing spirochete has also been identified in the termite gut (Lilburn et al., 2001). The role of the *K. pneumoniae* in the FST gut has not been reported before and, to our knowledge, this is the first report on the presence of *K. pneumoniae* in the FST gut. Further research is needed to better understand the ecology of this microbe. It remains to be determined whether this bacterium is consistently found in the FST from various locations or whether it is loosely associated with the termite based upon its environment.

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**References**


