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Occurrence of rhizobia in the gut of the higher termite *Nasutitermes nigriceps* ☆

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Abstract

Wood-eating termites feed on a diet highly deficient in nitrogen. They must complement their diet with the aid of nitrogen-fixing bacteria. Nitrogen fixation in the gut has been demonstrated, but information about nitrogen-fixing bacteria in pure culture is scarce. From the higher termite *Nasutitermes nigriceps* the symbiotic bacterial strain M3A was isolated, which thrives in the hindgut contents. The Gram-negative strain exhibited similarities to the species of the genus *Ensifer* (including *Sinorhizobium*) on the basis of morphological and physiological/biochemical features. The 16S rRNA gene analysis showed the highest sequence similarity of the isolate M3A to *Ensifer adhaerens* (>99%; ATCC 33499). The DNA–DNA hybridization revealed a similarity of 66% with *E. adhaerens* (NCIMB12342^T). In contrast to the type strain the isolate M3A possesses the capacity to nodulate plant roots. This is the first report on the detailed identification of a rhizobia-related strain from the intestinal tract of animals. Strain M3A has been deposited with two culture collections (DSM10169; ATCC BAA-396).

Keywords: Rhizobia; *Ensifer*; Termites; Intestinal flora; Nitrogen fixation

Introduction

One of the most interesting examples of a complex symbiotic community of prokaryotic and eukaryotic microorganisms with insects is found in the intestinal tract of termites. The gut flora consists of Bacteria, Archaea and Archaezoa [21,22]. Yeasts have also been found to form a significant part of the gut flora [40]. During the last years the systematic relationship of a

Abbreviations: E. = Ensifer.

☆The GenBank accession number for the 16S rRNA gene sequence of strain M3A (DSM 10169; ATCC BBA-396) is AJ298869.

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large portion of culturable and unculturable gut bacteria has been determined with conventional and molecular methods [4,14,24–26,28,29,34–36,43]. Because of their intestinal flora many termites can feed on wood. Wood has a very low content of nitrogen (0.03–0.1%) [6]. It is suggested that wood-feeding termites must supplement their food with nitrogen. N_2-fixing activity has been demonstrated in the termite gut [3,7,13]. It could be accomplished with the aid of nitrogen-fixing bacterial isolates, e.g. *Enterobacter* [7], *Desulfovibrio* [25] and *Treponema* [30] species. With culture-independent methods using oligonucleotide probes specific for nitrogenase [33], the presence of nitrogen-fixing bacteria from different systematic positions has also been shown. We have isolated bacterial strains related to rhizobia [24,48]. Here we describe the more detailed identification of an *Ensifer adhaerens*-related strain from the gut of *Nasutitermes nigriceps*.

Materials and methods

Organisms and culture conditions

Nasutitermes nigriceps (Haldeman) was obtained from the Bundesanstalt für Materialforschung und Materialprüfung (Berlin, Germany). Strain M3A was isolated from the gut in medium I [24]). Medium I contained the following constituents: 10.8 mM K_2HPO_4, 6.9 mM KH_2PO_4, 21.5 mM KCl, 24.1 mM NaCl, 5.3 mM $MgSO_4$, 0.53 mM $CaCl_2$, vitamin and trace element solution (10 ml l^{-1}) [2] and yeast extract (1 g l^{-1}). A mixture of aromatic compounds (4-hydroxycinnamic acid, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid) was added after sterilization to a final concentration of 2 mM each. For inhibiting the growth of Gram-positive bacteria neutral red (0.03 g l^{-1}) and crystal violet (0.001 g l^{-1}) were added. The pH was adjusted to 6.5–7.0. Solid media contained agar (1.5 g l^{-1}). Incubation: 7 d at 28 °C.

Bacteria were routinely grown in tryptic soy broth or tryptic soy agar (TSB, TSA Difco) at 28 °C. *Escherichia coli* strain K12 DHα was grown in LB medium at 37 °C [42]. The type strains of *Ensifer fredii* (DSM 5851^T), *E. meliloti* (DSM 30135^T), *E. terangae* (DSM 11282^T) and *E. xinjiangense* (DSM 5852^T) were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). *E. adhaerens* was obtained from the University of Gent (Casida, LMG 20582), Laboratorium voor Mikrobiologie, Gent, Belgium and from the National Collections of Industrial and Marine Bacteria Ltd. (NCIMB12342^T), Aberdeen, Scotland, United Kingdom. The latter six strains were grown on yeast extract–mannitol medium [47]. Incubation: 7 d at 28 °C.

Determination of phenotypic features

Cell morphology was examined by phase-contrast microscopy with a light-microscope (Leitz, Wetzlar, Germany). Gram staining was performed by using Hucker's modification [15]. Determination of growth at different pH-values, NaCl-concentrations and at 40 °C was performed in yeast extract–mannitol medium [47]. Physiological characterization was performed with the BioLog GN2 MicroPlates (incubation time: 24 h) and the MicroLog computer software (BioLog identification system; Biolog Inc. Hayward, USA). Additional physiological tests in microtiter plates were done as described earlier [18]. The procedure of the "initial numerical identification" is described in detail in Ref. [18]. Here a database on the basis of phenotypic data (physiological tests) is used for comparison. All details on the procedure are given in Ref. [18].

These tests were read after 7 days at 28 °C. Growth in nitrogen-free medium was obtained in semisolid Burk medium [38]. Acetylene reduction was checked after growth in semisolid LNB5 medium [27]. After 3, 6, and 9 days the gas atmosphere was analysed on a Haye Sep T column (2 m, 60 °C; Macherey and Nagel, Düren, Germany) with a gas chromatograph (Fractovap 4100, Carlo Erba, Hofheim, Germany).

Preparation and analysis of fatty acids

The fatty acid methyl esters (FAMEs) were determined according to Kämpfer [17], except that the cells were grown at 28 °C.

Nodulation tests

Nodulation studies were carried out selecting 12 common legumes growing in India (Table 3). Seeds of the legumes were procured from National Bureau of Plant Genetic Resources, IARI, New Delhi. *N. nigriceps* is widespread in Central America. Since we had no legumes from Central America we used legumes from India, because one of the authors (D. K. Maheshwari) regularly performs nodulation tests. Common field soil was used to carry out nodulation studies. The soil was filled in 500 g capacity earthen pots. The pots filled with soil were sterilized (121 °C, 2 h) and surface sterilized seeds of the legumes were then sown in the pots. Four seeds of each legume crop were sown in each pot. A exponentially grown broth culture (10^8 cells ml^{-1}) of the strain M3A was inoculated twice in each of the pots except control [1]. First inoculation was done soon after the seed germination and the second 1-week later. All the experiments were performed in triplicate and a set of uninoculated controls was also maintained. The pots