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Isolation of methanotrophic bacteria from termite gut

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ABSTRACT

The guts of termites feature suitable conditions for methane oxidizing bacteria (MOB) with their permanent production of CH_4 and constant supply of O_2 *via* tracheae. In this study, we have isolated MOB from the gut contents of the termites *Incisitermes marginipennis*, *Mastotermes darwiniensis*, and *Neotermes castaneus* for the first time. The existence of MOB was indicated by detecting *pmoA*, the gene for the particulate methane monooxygenase, in the DNA of gut contents. Fluorescence *in situ* hybridization and quantitative real-time polymerase chain reaction supported those findings. The MOB cell titer was determined to be 10^2–10^3 per gut. Analyses of the 16S rDNA from isolates indicated close similarity to the genus *Methylocystis*. After various physiological tests and fingerprinting methods, no exact match to a known species was obtained, indicating the isolation of new MOB species. However, MALDI-TOF MS analyses revealed a close relationship to *Methylocystis bryophila* and *Methylocystis parvus*.

Introduction

Aerobic methane-oxidizing bacteria (MOB) are widely distributed and appear in habitats where methane and oxygen occurs, such as soils, oceans, deserts, rice paddies, or landfills (Heyer et al. 2002). Termites also present acceptable conditions for MOB, as they produce methane (2–4% of the worldwide methane emissions) while digesting polysaccharides from wood (Sanderson 1996; König and Varma 2005; Brune 2014). Furthermore, the presence of oxygen, which is obligatory for the methane monooxygenase (MMO), is ensured by diffusion to the inner fringe of the gut epithelium *via* trachea (Brune et al. 1995; Berchtold et al. 1999). It is well-known that termites accommodate a numerousness and great variety of microorganisms with up to 10^8 bacteria per gut (Noda et al. 2005). They participate, *inter alia*, in the digestion of polysaccharides and are often crucial for the survival of their host (Radek 1999). The exceptional complex and tangled relation between termites as host and their symbionts is an actual field of research in which new discoveries are reported annually (Paul et al. 2012; Reuß et al. 2013, 2014; Rosenthal et al. 2013). As the termites produce such a great amount of methane in their gut, the question arose whether the intestines host organisms that are capable of exploiting the methane for their growth.

MOB, in general, possess the unique ability to use methane as the sole source of carbon and energy (Dunfield et al. 2003). They play an important role in the regulation of global warming as they act as the major sink of atmospheric, terrestrial, and marine methane, a greenhouse gas 26 times more potent than the carbon dioxide often discussed (Lelieveld et al. 1993; Hanson and Hanson 1996). MOB are divided into type I and type II methanotrophs based on several physiological and morphological characteristics, mainly the way of carbon fixation and the structure of intracytoplasmic membranes (Wartiainen et al. 2006). Type I MOB belong to the γ-proteobacteria and apply the ribulose monophosphate pathway to assimilate carbon. Their intracytoplasmic membranes (ICM) are arranged in bundles of vesicular disks (Dunfield et al. 2003). By contrast, type II MOB are part of the α-proteobacteria and use the serine pathway for carbon assimilation (Hanson and Hanson 1996). Their ICM are organized in paired membranes at the periphery of the cell (Wartiainen et al. 2006). The key enzyme for methane oxidation and the first step of the conversion of methane to CO_2 by both types of MOB is MMO, which occurs in a membrane-bound particulate form, the pMMO (Chen et al. 2007). A soluble type, the sMMO, is only produced by some species as a reaction to copper limitation (Cheng et al. 1999; Kolb et al. 2003). The MMO converts methane to methanol, which is further oxidized to formaldehyde by the

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periplasmic methanol dehydrogenase. A huge part of the reducing energy, which is needed for the oxidation of methane, is generated by the oxidation of formaldehyde *via* formate to CO_2 which is catalyzed by a aldehyde dehydrogenases and formate dehydrogenase, respectively. The formaldehyde can also be used to form intermediates of which cell material can be assimilated (Hanson and Hanson 1996).

Despite the widespread distribution of MOB, this is the first study known so far that describes the isolation of methanotrophic bacteria from the termite gut and, in general, from the intestines of animals.

Methods

Reference bacterial strains and termites

Methylocystis echinoides (IMET 10491), *Methylocystis bryophila* (DSM 21852), *Methylocystis heyeri* (DSM 16984), *Methylocystis hirsuta* (DSM 18500), *Methylocystis rosea* (DSM 17261), and *Methylocystis parvus* (NCIMB 11129) were used as reference strains. *Methylobacter luteus* (NCIMB 11914) was also used as a reference for type I MOB. All strains were purchased from the corresponding culture collection. *Escherichia coli* str. K-12 substr. MG 1655 (CGSC 6300) was kindly provided by C. Monzel (IMW) and used as a negative control. The gut contents of *Incistitermes marginipennis*, *Mastotermes darwiniensis*, and *Neotermes castaneus* (kindly provided by the Bundesanstalt für Materialforschung und -prüfung BAM, Berlin, Germany) were tested for the presence of MOB. The gut contents differed between about 3 μl per gut (*Incisitermes marginipennis* and *Neotermes castaneus*) and about 14 μl per gut (*Mastotermes darwiniensis*).

Growth conditions and isolation of MOB

Methylocystis echinoides, *Methylocystis parvus*, *Methylocystis rosea*, *Methylocystis hirsuta*, *Methylocystis heyeri*, and *Methylobacter luteus* were isolated and cultivated in nitrate mineral salts (NMS) medium, as described by Whittenbury et al. (1970), and *Methylocystis bryophila* in M2 medium, developed by Belova et al. (2013). All MOB were cultivated in a gas atmosphere of 25% CH_4 in artificial air (20% O_2, 80% N_2) at 200 rpm and 30 °C, except for *Methylocystis heyeri*, which was grown at 20 °C. *E. coli* was grown in LB medium at 37 °C, according to the instructions of Bertani (1951).

The guts of *Incistitermes marginipennis*, *Mastotermes darwiniensis*, and *Neotermes castaneus* were separated from the termite, according to the instructions of Trinkerl et al. (1990). Ten guts were transferred to 10 ml nitrate mineral salt (NMS) medium and incubated at 30 °C and 200 rpm in an atmosphere of 25% CH_4 and 75% artificial air for up to three weeks (Whittenbury et al. 1970; Lindner et al. 2007). After five subsequent serial dilutions, bacteria were plated on solid NMS medium containing 1% Phytagel (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) as the gelling agent and 0.001% cycloheximide. The plates were again incubated at 30 °C in an atmosphere containing 25% CH_4 and 75% artificial air in steel cylinders. Single colonies were restreaked repeatedly and strain purity was confirmed as described below.

Verification of strain purity

A first check of the strain's purity was made by monitoring the isolates with phase-contrast microscopic techniques. Furthermore, contamination of the isolates was excluded by plating them on complex LB medium (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl) and NMS medium supplemented with 0.1% glucose and incubation at 30 °C for four weeks (Dunfield et al. 2003). Additionally, DNA from the isolates was extracted and analyzed by denaturing gradient gel electrophoresis (DGGE) after amplifying partial 16S rRNA fragments *via* polymerase chain reaction (PCR) by using the universal bacterial primers Eubak1070rev (5′-AGCTGACGACAGCCAT-3′) and EubakGC519for (5′-[CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCGGCCGCC]AGCAGCCGCGGTAATA-3′), as described by Knopf (2010). DGGE was performed according to Stantscheff et al. (2014) with a denaturing gradient of 30–70% in the polyacrylamide gels.

Morphological observations and electron microscopy

Cells of cultures were inspected under a phase-contrast light microscope (Axioplan, Carl Zeiss Microscopy GmbH, Jena, Germany) after two weeks of incubation. Electron microscopy was performed using the CM 12 transmission microscope (FEI Co., Eindhoven, The Netherlands) with an acceleration voltage of 120 keV. Cell preparation and freeze-etching were performed following the protocol described in detail in Rachel et al. (2010).

Fluorescence in situ *hybridization*

Fluorescence *in situ* hybridization (FISH) was applied to verify the culture-independent presence of MOB within the guts of *Incistitermes marginipennis*, *Mastotermes darwiniensis*, and *Neotermes castaneus*. Therefore, the protocols of Eller et al. (2000) and Hirschhäuser et al. (2005) were used. The content of one single termite gut was transferred to 10 ml nitrate mineral salt (NMS) medium (Whittenbury et al. 1970) and pelleted at 10,000 rpm (centrifuge 2–16 K, Sigma, Osterode, Germany). The cells were washed three times with 0.9% (w/v) NaCl, transferred to diagnostic microscope slides (5 μl per well, Erie Scientific Company, Portsmouth, USA) and allowed to dry at room temperature for 1 h. The slides were then incubated at 90 °C for 10 min and washed in 50, 70, and twice in 96% aqueous ethanol for 5 min each. Two μl of the type II MOB solution targeting Mα450 probe (5′-ATCCAGGTACCGTCA-3′) were mixed with 58 μl hybridization buffer (20 mM Tris, 900 mM NaCl, 0.01% SDS, 10% dextran sulfate). For type I specific hybridization, 2 μl of probe Mγ84 (5′-CCACTCGTCAGCGCC-3′) and Mγ705 (5′-CTGGTGTTCCTTCAGATC-3′) each were mixed with 56 μl hybridization buffer. Each probe was labeled with Cy3 at the 5′ site. The mixtures were applied to the wells and incubated at 72 °C for 10 min. The hybridization was accomplished in a water-saturated atmosphere chamber at 46 °C overnight. The slides were incubated in washing buffer (20 mM Tris, 900 mM NaCl, 0.01% SDS) at 48 °C for 20 min to remove unbound probes. Hereafter, the slides were stained with 0.2% (w/v) 4′,6-diamidino-2-phenylindole (DAPI) in the dark for 20 min and afterwards rinsed with distilled water. The slides were covered in antifading solution (80% (v/v) glycerol, 2.34% (w/v) 1,4-diazabicyclo[2.2.2]octan) and the samples were analyzed by fluorescence microscopy (Biozero BZ8000, Keyence Neu-Isenburg, Deutschland) with a Texas Red chromatic filter ($\lambda_{absorption,max}$ = 550 nm, $\lambda_{emission,max}$ = 570 nm).

DNA extraction and phylogenetic analyses

The template DNA of the reference MOB and termite isolates were isolated using the DNeasy Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany). The evidence of MOB in termite guts was provided by verifying the gene *pmoA* in the extracted DNA samples by PCR with the primer pair described by Cheng et al. (1999): pmof1 (5′-GGGGGAACTTCTGGGGITGGAC-3′) and pmor (5′-GGGGGRCIACGTCITTACCGAA-3′). The 50 μl PCR mixtures contained 10 pmol of each primer, 800 μmol dNTP mix, 1 U *Taq* DNA polymerase, 5 μl PCR buffer, and 2 μl template DNA. The PCR was performed in a Mastercycler gradient module (Eppendorf AG, Hamburg, Germany) under the following conditions: The initial