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ScienceDirect
European Journal of Protistology 46 (2010) 86–95
European Journal of PROTISTOLOGY
www.elsevier.de/ejop

Saccamoeba lacustris, sp. nov. (Amoebozoa: Lobosea: Hartmannellidae), a new lobose amoeba, parasitized by the novel chlamydia '*Candidatus* Metachlamydia lacustris' (Chlamydiae: *Parachlamydiaceae*)

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Received 17 June 2009; received in revised form 22 September 2009; accepted 22 October 2009

Abstract

An amoeba isolated from an aquatic biotope, identified morphologically as *Saccamoeba limax*, was found harbouring mutualistic rod-shaped gram-negative bacteria. During their cultivation on agar plates, a coinfection also by lysis-inducing chlamydia-like organisms was found in some subpopulations of that amoeba. .Here we provide a molecular-based identification of both the amoeba host and the two bacterial endosymbionts. Analysis of the 18S rRNA gene revealed that this strain is the sister-group to *Glaeseria*, for which we proposed the name *Saccamoeba lacustris*. The rod-shaped endosymbiont was identified as a member of *Variovorax paradoxus* group (*Comamonadaceae*, Beta-*Proteobacteria*). No growth on bacteriological agars was recorded, hence this symbiont might be strictly intracellular. The chlamydia-like parasite was unable to infect *Acanthamoeba* and other amoebae in coculture, showing high host specificity. Phylogenetic analysis based on the 16S rDNA indicated that it is a new member of the family *Parachlamydiaceae* (order *Chlamydiales*), for which we proposed the name '*Candidatus* Metachlamydia lacustris'.

Keywords: Amoebozoa; *Saccamoeba*; *Metachlamydia*; *Variovorax*; endosymbiont

Introduction

Amoebae and other protists very frequently harbour endosymbionts, either prokaryotic or eukaryotic (e.g., Horn and Wagner 2004; Dyková et al. 2003, 2008). The nature of such association is largely described as mutualistic or parasitic, depending on the interaction of the bionts. It is mostly monospecific., Amoebae hosting more than one endosymbiont at the same time have only occasionally been described. For example, Heinz et al. (2007) isolated a strain of

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doi:10.1016/j.ejop.2009.11.002

Acanthamoeba (Amoebozoa) from a lake, which was found harbouring two bacterial endosymbionts in different vacuoles of the same cells. They were identified as *Procabacter* sp. (Beta-*Proteobacteria*) and *Parachlamydia acanthamoebae* (*Chlamydiae*, *Parachlamydiaceae*, strain OEW1). The two endosymbionts resided within the amoebae for several months, suggesting a stable three-partner symbiosis (Heinz et al. 2007). Michel and collaborators described various amoebae harbouring multiple endosymbionts. They found an environmental strain of *Naegleria clarki* (Excavata) harbouring a cytoplasmic chlamydia-like organism and an intranuclear endosymbiont. The chlamydia-like organism seemed to interfere with the encystment, but not with the formation of flagella. Cysts could be obtained only after the elimination of the chlamydial symbiont (Michel et al. 1999; Walochnik et al. 2005). Another chlamydia-like symbiont was found coinfecting a subpopulation of an environmental strain of '*Hartmannella*' *vermiformis* (Amoebozoa) along with rod-shaped gram-negative bacteria, while other subpopulations of the same strain were found infected by only one of the two symbionts (Hoffmann and Michel 2001). An additional chlamydia-like organism was discovered infecting subpopulations of an amoeba. It was identified morphologically as *Saccamoeba limax*, and it was already harbouring rod-shaped gram-negative symbionts (Michel et al. 2006). This chlamydia-like organism was described as a parasite since it was responsible for the lysis of infected amoebae (Michel et al. 2006). The aim of this study is the identification of this amoeba strain and its bacterial symbionts on a molecular level,

Materials and Methods

Amoebae

The strain SL-2 originated from an aquatic biotope containing mud and plant detritus in Hardert, Germany. Isolation and first description of this amoeba as *Saccamoeba limax*, and its symbionts, were performed by Michel et al. (2006). Amoebae were maintained as clonal subpopulations, at room temperature on 1.5% non-nutritive agar (NNA) covered with *Enterobacter cloacae* or *Escherichia coli*. All clones naturally harboured rod-shaped endosymbionts, in apparently healthy status. Moreover, some subpopulations were found to be infected also by a lysis-inducing chlamydia-like organism, named CHSL. Amoebae infected by CHSL needed to be periodically transferred to fresh NNA plates containing uninfected SL-2 amoebae, allowing the chlamydia-like parasite to find new hosts (Michel et al. 2006).

Amoeba coculture

In order to isolate and propagate the chlamydia-like symbiont CHSL, amoeba coculture was mainly performed as previously described (Corsaro et al 2009). *Acanthamoeba* sp. genotype T4 (ATCC 30010), '*Hartmannella*' *vermiformis*, and *Naegleria clarki* were prepared as host cells in 24-well microplates (Costar, Corning, New York) at 5×10^5 amoebae ml^{-1}, in Page's Amoeba Saline (PAS) buffer. Extracts of infected SL-2 amoebae (100 µl), harbouring the chlamydia-like parasite, were inoculated into the wells at different dilutions. The microplates were centrifuged at $1{,}500 \times g$ for 30 min, incubated either at room temperature or at 32 °C, in a humidified atmosphere in the dark. 6 days post-infection, a fresh coculture (coculture II) was inoculated by a 100-µl aliquot from the first coculture, and after another 6 days a coculture III was inoculated from the coculture II. At the time of each coculture, a 100-µl aliquot of each well was screened by chlamydia-specific 16S rRNA gene PCR (see below).

Infection of amoeba SL-2 with chlamydiae

Parachlamydia acanthamoebae (strain Bn9) and *Protochlamydia amoebophila* (strain UWE25) were grown into *Acanthamoeba* ATCC 30010. Amoebal lysates (10 µl) were added separately to bacterized NNA containing amoeba SL-2, and incubated at room temperature.

DNA amplification, sequencing and phylogenetic analysis

Amoebae were harvested from the agar plates, suspended in PAS, and centrifuged three times at $200 \times g$ to eliminate *E. coli*. Additional centrifugations were performed on the pelleted amoebae after freezing-thawing in order to separate the cytoplasmic content (i.e. the bacterial symbionts).

The almost complete amoebal 18S rRNA gene was amplified by using the primers eukF (5′-GACTGGTTGATCCTGCCAG-3′) and eukR (5′-TGATCCTTTCGCAGGTTCAC-3′), in reaction conditions of 5 min at 94 °C, followed by 40 cycles of 94 °C (1 min), 58 °C (30 sec), 72 °C (2 min), and one last elongation at 72 °C for 10 min. Bacterial 16S rRNA genes were amplified by using the pan-chlamydia primers 16SIGF (5′-CGGCGTGGATGAGGCAT-3′) and rP2chlam (5′-CTACCTTGTTACGACTTCAT-3′), as well as the eubacterial primers fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rP2 (5′-ACGGCTACCTTGTTACGACTT-3′), for the coccoid and the