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Short communication

A clinical *Acanthamoeba* isolate harboring two distinct bacterial endosymbionts

Anneliese Müller[a], Julia Walochnik[b], Martin Wagner[a], Stephan Schmitz-Esser[a,*]

[a] *Institute for Milk Hygiene, University of Veterinary Medicine Vienna, Veterinaerplatz 1, 1210 Vienna, Austria*
[b] *Institute of Specific Prophylaxis and Tropical Medicine, Medical University of Vienna, Kinderspitalgasse 15, 1090 Vienna, Austria*

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Abstract

Acanthamoebae feed on bacteria but are also frequent hosts of bacterial symbionts. Here, we describe the stable co-occurrence of two symbionts, one affiliated to the genus *Parachlamydia* and the other to the candidate genus *Paracaedibacter* (Alphaproteobacteria), within a clinical isolate of *Acanthamoeba hatchetti* genotype T4. We performed fluorescence *in situ* hybridization (FISH) and transmission electron microscopy (TEM) to describe this symbiosis. Our study adds to other reports of simultaneous co-occurrence of two symbionts within one *Acanthamoeba* cell.

Keywords: *Acanthamoeba*; Intracellular bacteria; *Paracaedibacter*; *Parachlamydia*; Protozoa; Symbiosis

Introduction

Since it has been discovered that acanthamoebae can harbor endosymbionts, bacteria occurring within *Acanthamoeba* spp. seem to be the rule rather than the exception: Between 24% and 59% of environmental and also clinical *Acanthamoeba* isolates harbor intracellular bacteria (Fritsche et al. 1993; Iovieno et al. 2010). Acanthamoebae are ubiquitous free-living amoebae and opportunistic pathogens (Schuster and Visvesvara 2004). Considering their cosmopolitan distribution human infections caused by *Acanthamoeba* spp. are rare, but can lead to severe diseases such as granulomatous amoebic encephalitis (Schuster and Visvesvara 2004). More common is *Acanthamoeba* keratitis (AK) (Iovieno et al. 2010; Schuster and Visvesvara 2004). *Acanthamoeba* spp. feed on bacteria and play an important role in nutrient cycling and in controlling microbial community composition (Rodriguez-Zaragoza 1994). Yet some bacteria are able to prevent digestion and survive within their predators (Barker and Brown 1994; Weekers et al. 1993): They either live a limited time within their hosts (facultative intracellular bacteria), a state addressed as transient symbiosis, or establish a permanent symbiosis and lose their ability to live outside their hosts (obligate intracellular bacteria). Transient symbioses within amoebae have been described for a number of human pathogens, for instance: *Legionella pneumophila* or *Mycobacterium avium* (Greub and Raoult 2004; Thomas et al. 2010). Additionally, *L. pneumophila* and *M. avium* have been shown to be more virulent after intra-amoebal growth (Cirillo et al. 1997, 1999). Intracellular survival provides protection as well as distribution of the pathogens (Barker and Brown 1994). Furthermore, pathogenic bacteria might have developed their infectious potential in

*Corresponding author. Current address: Department of Animal Science, Iowa State University, 3222 NSRIC, 1029 North University Boulevard, Ames, IA 50011, USA. Tel.: +1 515 294 2739.
E-mail address: sse@iastate.edu (S. Schmitz-Esser).

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unicellular organisms before being able to infect multicellular organisms (Barker and Brown 1994; Molmeret et al. 2005).

Obligate intracellular symbionts of amoebae have until now been affiliated to the Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Bacteroidetes and Chlamydiae (Horn and Wagner 2004; La Scola et al. 2004; Schmitz-Esser et al. 2008). Occurrence of more than one endosymbiont within the same protozoan host cell has been reported for isolates of *Naegleria clarki*, *Saccamoeba limax*, various ciliates and several *Acanthamoeba* isolates (Fokin 2004, 2012; Heinz et al. 2007; Iovieno et al. 2010; Lagkouvardos et al. 2014; Matsuo et al. 2010; Michel et al. 2006; Walochnik et al. 2005).

Here, we describe the stable co-occurrence of two obligate intracellular symbionts within the clinical *Acanthamoeba* isolate *A. hatchetti* 3ST (Walochnik et al. 2000).

Material and Methods

Culturing of acanthamoebae

Acanthamoeba hatchetti 3ST, genotype T4, was isolated from a corneal scraping of a patient suffering from keratitis (ATCC: PRA-114) as described in (Walochnik et al. 2000). *A. hatchetti* 3ST was grown axenically in tissue culture flasks (Nunc) containing peptone-yeast extract-glucose medium (PYG: 20 g proteose peptone, 18 g glucose, 2 g yeast extract per liter H_2O_{dd} and 3.4 mM sodium citrate-dihydrate, 4 mM $MgSO_4$ $7H_2O$, 2 mM Na_2HPO_4 $2H_2O$, 1.7 mM KH_2PO_4, 0.05 mM $Fe(NH_4)_2(SO_4)_2$ $6H_2O$) at room temperature.

DNA extraction and PCR assays

DNA was isolated from 4 ml culture with the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. 16S rRNA genes were amplified with the universal primers 616F (5′-AGAGTTTGATYMTGGCTC-3′) and 1492R (5′-GGYTACCTTGTTACGACTT-3′) (Loy et al. 2005). PCR was performed with the TaKaRa Ex Taq Hot Start (TaKaRa Bio Europe). Cycling conditions were: initial denaturation for 5 min at 95 °C; 25 cycles of: 40 s at 94 °C, 40 s at 52 °C and 1 min at 72 °C; final elongation for 7 min at 72 °C. Final concentrations in 50 μl reaction volume were: TaKaRa Ex Taq Hot Start 0.625U, dNTP Takara-Mix 0.8 mM, 0.2 pmol/μl of each primer. Furthermore, a PCR with primers 16SigF (5′-CGGCGTGGATGAGGCAT-3′) (Everett et al. 1999) and rP2chlam (5′-CTACCTTGTTACGACTTCAT-3′) (Thomas et al. 2006) targeting the 16S rRNA genes of *Chlamydiales* was performed. Final concentrations and PCR conditions were the same as described above except for an annealing temperature of 54 °C.

Cloning

16S rDNA PCR products were cloned using the StrataClone PCR Cloning Kit following the manufacturer's instructions. Plasmids of 7 and 12 clones of the *Candidatus* (*Ca.*) Paracaedibacter-like and the chlamydial symbiont, respectively, were sequenced (LGC Genomics).

Calculation of phylogenetic trees

Phylogenetic relationships were calculated with ARB (Ludwig et al. 2004) using the SILVA 111 SSU Ref NR release (Quast et al. 2013) applying 50% conservation filters for the respective phyla and the maximum parsimony and RaxML treeing algorithms included in ARB. All trees were calculated with 1000× bootstrapping.

Nucleotide sequence accession numbers

The 16S rRNA gene sequences of both bacterial endosymbionts were deposited in the European Nucleotide Archive (ENA) and are available *via* the following link: http://www.ebi.ac.uk/ena/data/view/HG813251-HG813252 or accession numbers HG813251 and HG813252.

Fluorescence *in situ* hybridization

Four ml of a two and a four days old culture were mixed, pelleted and resuspended in 100 μl 1× Page's amoebic saline (PAS: 20 mM NaCl, 0.16 mM $MgSO_4 \cdot 7H_2O$, 0.27 mM $CaCl_2 \cdot 2H_2O$, 10 mM Na_2HPO_4, 10 mM KH_2PO_4). FISH was performed according to (Daims et al. 2005), using the following probes: Chls-0523, targeting the order *Chlamydiales* (Poppert et al. 2002), and its unlabelled competitor probe: CC23a, targeting *Caedibacter caryophilus* and other *Caedibacter*-related endosymbionts (Springer et al. 1993) and EUK 516 targeting the 18S rRNA of *Eukarya* (Amann et al. 1990). Information on probes and hybridization conditions can be found in probeBase (Greuter et al. 2016). Images were taken using a Confocal Laser Scanning Microscope (CLSM) (LSM 710 ConfoCor3, Carl Zeiss).

Transmission electron microscopy

Four ml of a two and a four days old culture were mixed, centrifuged and washed with 1× PAS. For TEM analysis samples were fixed in cold Karnovsky solution, post-fixed with cold phosphate buffered osmium tetroxide and embedded in Epon followed by polymerization (2 days at 60 °C). Ultrathin sections were stained with methanolic uranyl acetate and lead citrate (Wodak et al. 2011). Images were taken with a transmission electron microscope (Carl Zeiss). Preparation of the samples and TEM analysis was carried out at the Institute of Pathology at the University of Veterinary Medicine Vienna.