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ScienceDirect
European Journal of Protistology 44 (2008) 308–316
European Journal of PROTISTOLOGY
www.elsevier.de/ejop

Living together: The marine amoeba *Thecamoeba hilla* Schaeffer, 1926 and its endosymbiont *Labyrinthula* sp.

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Received 30 January 2008; received in revised form 12 April 2008; accepted 12 April 2008

Abstract

Two protists isolated simultaneously from the same sample of gill tissue of *Psetta maxima* (L.) were identified as *Thecamoeba hilla* Schaeffer, 1926 and *Labyrinthula* sp. A *Labyrinthula* strain (LTH) derived from a mixed culture of both organisms was well established in a short time, while subcultures of *T. hilla* continued to be associated with *Labyrinthula* cells despite all efforts to eliminate them. Ultrastructural examination, repeated several times in the course of long-lasting subculturing of amoebae, revealed that trophozoites of *T. hilla* host in their cytoplasm multiplying labyrinthulid cells. Comparison of SSU rDNA sequences of the *Labyrinthula* strain LTH and those from labyrinthulid endosymbionts from *T. hilla* verified the assumption that the extra- and intra-cellularly multiplying *Labyrinthula* cells are identical organisms. The association of the marine amoeba *T. hilla* and *Labyrinthula* sp. displayed signs of mutualistic symbiosis.

Keywords: Amoebozoa; *Thecamoeba hilla*; *Labyrinthulomycota*; *Labyrinthula sp.*; Symbiotic association

Introduction

In quite a long history of studies of interactions of free-living amoebae with other microorganisms that share their common environment, special attention was paid to symbiotic associations of amoebae with bacteria. *Acanthamoeba* and *Hartmannella* strains of environmental and clinical origins were of primary interest; among endosymbionts, bacteria pathogenic for humans were those most frequently studied. Current knowledge of bacterial endosymbionts of free-living amoebae has been reviewed in detail by Horn and Wagner (2004). In contrast to the great diversity of bacterial endosymbionts of amoebae, scarce data are available on symbiotic associations of free-living amoebae with algae (Karpov et al. 1991; Lee et al. 1985) and other protists (Ossipov et al. 1997).

In a short overview on free-living amoebae isolated from fish as hosts of other organisms, Dyková and Lom (2004) mentioned several findings of facultative and obligate bacterial endosymbionts in clonal cultures of *Acanthamoeba* species. Except for *Nuclearia pattersoni* isolated from gills of *Rutilus rutilus*, which was found to host *Rickettsia* sp. related to rickettsial endosymbionts of leeches (Dyková et al. 2003b), no other prokaryotic endosymbionts have been identified to date

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0932-4739/$ - see front matter © 2008 Elsevier GmbH. All rights reserved.
doi:10.1016/j.ejop.2008.04.001

in fish-isolated strains. More attention was paid to eukaryotic endosymbionts resembling *Perkinsela amoebae* Hollande, 1980. They were found in trophozoites of all *Neoparamoeba* strains isolated from gills of fish (*Psetta maxima*, *Dicentrarchus labrax*, *Salmo salar*) and from invertebrates, sea urchins and crabs (Dyková et al. 2003a, 2007). The association of these two eukaryotic organisms was classified in accordance with definitions by Reisser et al. (1985) as stable and hereditary. Another example of a symbiotic association of a free-living amoeba with a eukaryotic heterotrophic organism that we have found was mentioned briefly in a review article on amphizoic amoebae infecting fish (Dyková and Lom 2004). This study is aimed at describing in detail both partners of this association, i.e., a marine *Thecamoeba* sp. isolated from fish and *Labyrinthula* sp. living together.

Materials and methods

An amoeba strain AF12B was isolated together with other microorganisms from gill tissue of *Psetta maxima* (Linnaeus, 1758) collected in a farm in Northwest Spain. The primary isolate was obtained in December 2000, and the clonal culture of amoebae was derived in May 2001 from the passage no. 12. The methods of isolation, agar plate culturing and cloning followed those described in previous papers (Dyková et al. 1997, 1998, 2000; Dyková and Lom 2004). Malt & Yeast Extract–75% Seawater Agar (1.5% MY75S) was used in the beginning of subculturing; later on, when subculturing was combined with the effort to purify the culture, 2.5–3% MY75S was also used. Live amoebae were examined and documented in hanging drop preparations with an Olympus BH2 microscope equipped with Nomarski differential interference contrast. For the study of ultrastructure, cell pellets prepared after fixation of trophozoites *in situ* on agar plates were embedded in Spurr resin. This procedure described by Dyková et al. (2000) was repeated several times in the course of subculturing. A JEOL JEM 1010 electron microscope operating at 80 kV was used for ultrastructural studies.

Simultaneously with primary isolation of amoebae from fish gills, small spindle-shaped cells appeared on the surface of the agar. The amoebae were our primary interest; nevertheless, the bushy appearance of colonies of cells resembling labyrinthulids stimulated us to separate and culture them on MY75S. The resulting, well-established strain, denominated LTH, was the source of material for light microscopy, ultrastructural studies and molecular characterisation of this organism.

Both organisms, the amoeba clone AF12B/I and the labyrinthulid strain LTH characterised in this study, are cryopreserved and stored in the collection of the Institute of Parasitology, Biology Centre, Academy of Sciences of the Czech Republic, České Budějovice. The SSU rRNA gene sequences of *Labyrinthula* sp. have been deposited in the GenBank database under the accession numbers EU431329 (LTH) and EU431330 (LTH/AF12B).

DNA isolation, amplification and sequencing

In the course of long-running subculturing of amoebae aimed also at clearing them from free labyrinthulid cells, six samples of DNA were gradually extracted from harvested trophozoites. Owing to substantial difference in size, amoeba trophozoites were easily separated from remaining free labyrinthulid cells by repeated centrifugation. Samples of DNA were isolated using the DNeasy^{TM} Tissue Kit (Qiagen, Germany). The standard phenol/chloroform extraction technique with ethanol precipitation (Sambrook et al. 1989) was used to extract DNA from labyrinthulid strain LTH. The methods for DNA amplification and sequencing were essentially the same as those in Dyková et al. (2002) except for the PCR conditions, which for *Labyrinthula* strains were as follows: initial denaturation at 94 °C for 5 min followed by 30 cycles of 94 °C for 1 min, 48 °C for 1 min and 72 °C for 1.5 min with final extension at 72 °C for 10 min. To amplify SSU rDNA of amoeba strain (AF12B/I) morphologically assigned to *Thecamoeba* spp., the following primers were designed: Thec1F: 5′-ATATGGCTCATTAAATCGGTTG-3′, Thec1R: 5′-ACAATAATTGCAATGATCTAT-3′, Thec2F: 5′-ATGGTKGTARWCYCTCACAA-3′, Thec2R: 5′-TCCGAGGACTTTGCCYGGMCG-3′. These primers were used also in the nested PCR together with the universal ERIB1 and ERIB10 primers (Barta et al. 1997). Conditions of PCRs were repeatedly modified: the temperature for annealing primers was tested within the range of 40–60 °C using a gradient XP cycler (Bioer); various extension times were used (up to 2.5 min) to amplify longer PCR products; the PCR mixtures were prepared with the different concentrations of Mg^{2+} (from 0.8 to 2.5 mM of $MgCl_2$).

Phylogenetic analyses

Seventeen SSU rDNA sequences of *Labyrinthula* species, three aplanochytrids and two thraustochytrids retrieved from GenBank were analysed together with two newly obtained sequences of *Labyrinthula* sp. The alignment was performed using the Clustal_X program (Thompson et al. 1997) with arbitrarily chosen parameters (8.0 for gap opening penalty and 2.0 for gap extension penalty). Ambiguous positions were checked by eye and then removed from analyses.