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# Identification of *Pelomyxa palustris* Endosymbionts

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***Pelomyxa palustris* is a giant anaerobic/microaerobic amoeba, characterized by a number of exceptional cytological and physiological features, among them the presumed absence of energy producing organelles and the presence of endosymbiotic bacteria. These endosymbionts have been previously distinguished as: a large rectangular-shaped Gram-variable rod with a central cleft; a slender Gram-negative rod; and a slender Gram-positive rod. Using DNA extracted from *P. palustris* cysts, we have obtained three SSU rRNA gene sequences. We have determined that these sequences are affiliated to three different prokaryotic genera: *Methanosaeta* (a methanogenic archaea), *Syntrophorhabdus* (a syntrophic Gram-negative bacteria) and *Rhodococcus* (an aerobic chemoorganotrophic Gram-positive bacteria). To our knowledge, it is the first time that *Syntrophorhabdus* has been described as an endosymbiont in association with a methanogen. Strikingly, no traces of *Methanobacterium formicicum* could be detected, despite this methanogen had allegedly been isolated from trophozoites of *P. palustris*. It seems that the host and the endosymbionts have established a multipartite syntrophic consortium resembling to some extent those found in sewage treatment plants.**



**Key words:** Methane; endosymbiosis; SSU rRNA; FISH; autofluorescence; amoeba.

## Introduction

*Pelomyxa palustris* (Greef 1874) is a free-living protist. Specifically, it is a giant methane-producing amoeba that can reach up to 5 mm in length. *P. palustris* lives as an anaerobe/microaerobe in freshwater sapropel (from greek *sapros* and *pélos*, meaning rotten silt).

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**Abbreviations:** FISH, fluorescence in situ hybridization; SSU rRNA, small subunit ribosomal RNA; RDP, Ribosomal Database Project.

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*P. palustris* has attracted the attention of protistologists due to its peculiar cytological features (Chapman-Andresen 1971; Daniels and Breyer 1967; Griffin 1988), among them: i) multiple nuclei, ranging in number from two to several thousand; ii) highly vesicular and vacuolate cytoplasm; iii) it lacks, apparently, Golgi bodies, mitochondria, hydrogenosomes, and contractile vacuole; iv) endoplasmic reticulum restricted to the perinuclear region; v) glycogen bodies well distributed throughout the cytoplasm; vi) shape and size change throughout the life cycle (Fig. 1A); vii) flagella with unusual set of microtubules in axoneme; and viii) prokaryotic endosymbionts in the cytoplasm (Fig. 1B). It is important to note that, although no mitochondrion-related organelles have been revealed in *Pelomyxa* by electron microscopy, such structures as mitosomes are quite difficult to be found by this technique (Whatley and Chapman-Andresen 1990).

The taxonomic position of *Pelomyxa* was clarified when the sequence of the small subunit ribosomal RNA (SSU rRNA) gene was reported (Milyutina et al. 2001). These authors made phylogenetic analyses, using only well conserved SSU rRNA gene regions, and concluded that *Pelomyxa* is closely related to genera *Entamoeba* and *Mastigamoeba*, members of the Archamoebae. The phylogenetic position of *Pelomyxa* has been subsequently confirmed by several studies (Fahrni et al. 2003; Lahr et al. 2011; Pánek et al. 2016; Ptáčková et al. 2013; Zadrobílková et al. 2015).

Despite the potential attractiveness of *P. palustris*, knowledge about this microorganism is scarce, since it is refractory to laboratory cultivation. In addition, until recently all pelomyxae were included in a single species, while, nowadays, more than ten species can be recognized. Consequently, it is very difficult to determine which *Pelomyxa* species was used in former investigations (Chistyakova et al. 2016). Thus, despite the fact that the literature about *P. palustris* endosymbionts is profuse, contradictions among papers hamper the interpretation of the actual diversity and nature of prokaryotes in this giant amoeba. The presence of endosymbionts in the cytoplasm of *P. palustris* was first observed by Greeff (1874) and subsequently by Penard (1902). Leiner and Bohwmick (1967) further demonstrated that *P. palustris* contained three types of endosymbionts. At that time, the three endosymbionts could be distinguished by their morphology and Gram staining (see Fig. 1B, also reviewed in Chapman-Andresen 1971): a very large rectangular-shaped Gram-variable rod, a slender Gram-negative rod with round ends and a slender Gram-positive rod with conically pointed ends. It was also observed that the distribution pattern of the three endosymbionts varied with the phase of the *P. palustris* life cycle (Fig. 1A, B) and that this pattern was related to oxygen tolerance (Whatley and Chapman-Andresen 1990). The slender Gram-positive endosymbiont was more abundant during the oxygen-tolerant (microaerobic) phase, while the other two types prevailed during the oxygen-intolerant (anaerobic) phase of the life cycle of *P. palustris*.

The exact nature of the three microbial endosymbionts has also been widely debated and disputed. van Bruggen et al. (1983) observed that the slender endosymbionts in *P. palustris* specimens showed autofluorescence, i.e. emitted a blue-green light when irradiated with UV (Fig. 1B). Regarding this feature as the hallmark of methanogens, the authors concluded that one or both of the slender microbes were methanogenic. The rectangular-shaped microbe was considered non-methanogenic, since it did not emit autofluorescence (van Bruggen et al. 1983). Afterwards, the same group of researchers reported the successful isolation of one of the endosymbionts upon squashing a *P. palustris* specimen and culturing the fluid in a mineral anaerobic medium aerated with $H_2/CO_2$ and supplemented with formate. The isolated microbe was identified as *Methanobacterium formicicum* DSM3637 (Methanobacteria), which was proposed to be one of the slender rods. In contrast, other authors (Whatley 1976; Whatley and Chapman-Andresen 1990) suggested that the rectangular-shaped endosymbiont was likely a methanogen (Fig. 1B). The contradiction has not been solved, since identification of microbes at the time, when all these papers were published, was cumbersome. Although the first reports using pioneer DNA techniques for the microbial identification date back to the mid-80s (reviewed in Amann et al. 1995), the new approach was only incipient and not yet widely used.

Here we report identification of all three species of prokaryotic endosymbionts living in *P. palustris*. For that we amplified, cloned and sequenced 16S rRNA genes of these microbes from DNA isolated from *Pelomyxa*'s cysts. In order to correlate the obtained sequences with the three morphological types of symbionts we observed microbial autofluorescence as well as applied FISH (fluorescence in situ hybridization).