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Characterization of *Pharyngomonas kirbyi* (= “*Macropharyngomonas halophila*” nomen nudum), a Very Deep-branching, Obligately Halophilic Heterolobosean Flagellate

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The tetraflagellate *Pharyngomonas* is among the most commonly reported morphotypes of halophilic protozoa. We have established two cultures of *Pharyngomonas kirbyi*, SD1A and AS12B, from 300‰ and 210‰ salinity waters from the USA and Australia, respectively. 18S rRNA gene phylogenies confirm that *Pharyngomonas* is the same entity as ‘*Macropharyngomonas*’ (nomen nudum), and represents the deepest branch in the heterolobosean lineage. *Pharyngomonas kirbyi* (Strain SD1A) has flattened/discoidal cristae, and lacks conspicuous Golgi dictyosomes. It also has a heterolobosean ‘double bikont’ flagellar apparatus, with two right roots, each associated with an ‘I’ fibre and part of a rhizoplast-like complex. One right root splits shortly after its origin, and supplies most of the microtubules that support both the ventral groove, and the sub-anterior cytopharynx. Interestingly, *Pharyngomonas* has some potentially ancestral features not found in typical Heterolobosea, including elongated left roots associated with multilayered ‘C’ fibres, orthogonal basal bodies, and a spur structure that might represent a ‘B’ fibre homolog. Both isolates are obligate halophiles that grow best at 100–200‰ salinity and do not grow below 75‰ salinity. *Pharyngomonas* is therefore of considerable evolutionary importance, both as a deep-branching, plesiomorphic heterolobosean, and a borderline extreme halophile. © 2011 Elsevier GmbH. All rights reserved.

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Introduction

A wide range of microorganisms live in hypersaline environments. Although there is a steep decrease in diversity in habitats with >200‰ salinity (Benlloch et al. 2002; Javor 1989), some halophiles/extreme halophiles grow well at or above 200‰ salinity. The best known of these are

halophilic prokaryotes, principally *Salinibacter* and various Haloarchaea, and certain species of the chlorophyte alga *Dunaliella* (Antón et al. 2000; Javor 1989). Much less recognized is the presence in these environments of predatory microbes, that is, protozoa. There is some evidence that protozoa can be key grazers of prokaryotes in brine (Park et al. 2003), and several heterotrophic protozoa that grow in culture at >200‰ salinity have been recently isolated and characterized. These are 1) the bicoseocid *Halocafeteria seosinensis*

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(Park et al. 2006), 2) a species of the anaerobic ciliate genus *Trimyema* – *Trimyema koreanum* (Cho et al. 2008), 3) the heterolobosean flagellate *Pleurostomum flabellatum* (Park et al. 2007), 4) the heterolobosean amoeba *Tulamoeba peronophora* (Park et al. 2009b), and 5) *Euplaesiobystra hypersalinica* (Park et al. 2009b), which is also an heterolobosean and has both amoeba and flagellate phases. None of these isolates could grow at <75‰ salinity in laboratory culture, and none of these species have been reported in natural environments at marine salinity. With the exception of *T. koreanum* they are also assigned to distinct genera on the basis of sub-cellular morphology and molecular phylogeny.

Nonetheless, these forms represent only some of the protozoan diversity that can inhabit highly saline habitats. Park et al. (2009b) recorded at least 25 additional morphologically distinguishable protozoa that had been reported in saturated or near-saturated brines in previous light microscopy-based studies, and speculated that many or most of these were capable of growth at very high salinities. Additional protozoan species have been reported from various hypersaline environments in the 100‰ to 250‰ salinity range (Hauer and Rogerson 2005). Therefore, it is likely that most of the protozoan biota in hypersaline habitats has not yet been examined using modern methods.

One of the more frequently reported morphotypes in hypersaline habitats is the collection of organisms now assigned to the genus *Pharyngomonas* Cavalier-Smith (see Cavalier-Smith and Nikolaev 2008). These protozoa have four flagella that insert at the head of a ventral groove, and were first described by Entz (1904) as *Trichomastix salina*, and later observed by Kirby (1932) under the name *Tetramitus salinus*. The organisms have usually been found at salinities ranging from 110‰ to 150‰ (Kirby 1932; Post et al. 1983), and sometimes occur at 220‰ salinity, or possibly higher (Post et al. 1983). The morphology of this organism was studied in some detail by Gunderson (1981), but no molecular data was obtained and the culture employed is no longer available. The organism known by the nomen nudum '*Macropharyngomonas halophila*' apparently also corresponds to this morphotype, and was isolated from a ~140‰ salinity crude culture (Tom Nerad, personal observations, as reported by Cavalier-Smith and Nikolaev 2008). There are no published morphological data on '*Macropharyngomonas*', but an 18S rRNA gene sequence is available (Genbank accession number AF011465). Phylogenies of 18S rRNA genes,

and the absence of the heterolobosean-specific helix 17_1 place '*Macropharyngomonas*' as the deepest branch within Heterolobosea sensu lato (Cavalier-Smith and Nikolaev 2008; Nikolaev et al. 2004; Park et al. 2007, 2009b), making this an organism of evolutionary interest, in addition to its intriguing status as a probable obligate halophile (note that we here use the taxon name Heterolobosea in a broad sense that includes the *Pharyngomonas*/ '*Macropharyngomonas*' lineage; this is identical in composition to the taxon Percolozoa sensu Cavalier-Smith and Nikolaev 2008). Unfortunately our knowledge of *Pharyngomonas*/ '*Macropharyngomonas*' has been restricted, with no direct linkage of published microscopy data and gene sequence data.

We have established two monoeukaryotic cultures of *Pharyngomonas*, Strain SD1A from 300‰ salinity water from a solar saltern in California, USA, and Strain AS12B from a 210‰ salinity hypersaline puddle in Western Australia. The 18S rRNA gene sequences from these isolates are very similar to one another, and also very similar to the sequence from '*Macropharyngomonas halophila*' (AF011465), confirming that *Pharyngomonas* and '*Macropharyngomonas*' are one. The ultrastructural characteristics of Strain SD1A, and basic growth physiology of both isolates are described.

Results

Light Microscopy

Pharyngomonas kirbyi strains SD1A and AS12B were both usually spindle-shaped (Fig. 1A-H), but with the posterior end of the cell more pointed than the anterior end and with the dorsal side more convex than the ventral side (Fig. 1A, D, E, F, H). Strain SD1A (Fig. 1A-D) ranged from 9 to 14 µm long (mean ± SD of 11.6 ± 1.4 µm, n=20), and was between 3 and 6 µm wide (mean ± SD of 4.6 ± 1.0 µm, n=20). The average ratio of length to width was 2.6 (range 1.9-4.1). Strain AS12B (Fig. 1E-H) was larger, with length varying from 11 to 18 µm (mean ± SD of 15.0 ± 1.8 µm, n=20), and width between 3 and 8 µm (mean ± SD of 5.3 ± 1.1 µm, n=20). The average ratio of length to width was 2.9 (range 1.7-3.8). Two anterior and two posterior flagella inserted within a broad bulge at the anterior end of the cell (Fig. 1A, C, E-H). The two anterior flagella inserted more or less apically, and were directed anteriorly during swimming, and actively beat. The two posterior flagella were directed laterally/ posteriorly during swimming, and

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