

Novel lineages of free-living anaerobic eukaryotes identified through cultivation of predators and extremophiles

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Anaerobic protists are of particular interest within evolutionary cell biology, and controversy still rages over the history of major adaptations for anaerobiosis among eukaryotes. Anaerobic groups have evolved multiple times independently, and range from very diverse clades like Metamonads to recently-discovered taxa with few described species, like *Breviatea*, *Creneis*, and *Brevimastigomonas*. This pattern of ongoing discovery suggests that there may be many more major anaerobic groups awaiting identification, both new major lineages within known groups and entirely independent clades. We aimed to cultivate and characterise potentially novel free-living anaerobic protists, focusing on predatory (eukaryvorous) anaerobes, which are little studied, and cells from environments with extreme salinity and/or pH. At least four significant lineages were identified based on microscopy and molecular phylogenies: 1) Strains RC and RCL are bacterivorous biflagellates isolated from alkaline sediments. They form a novel clade that branches deeply within Metamonads, outside the three primary divisions currently recognised. 2) Strain BLO is a bacterivore with a groove and two flagella. The long posterior flagellum points backwards when swimming, and generates a current when the cell settles with its anterior flagellum pointing towards the substrate. Unexpectedly, BLO proves to be related to *Breviatea*, despite bearing little morphological similarity to *Breviatea* etc. 3) Strains of SSF are predatory biflagellate cells. They represent an independently-derived anaerobic lineage within endomyxean rhizarians. 4) “Protist X” is a tetraflagellate obligate predator with no obvious morphological affinities. Phylogenetic analyses currently place it outside all recognised major lineages (i.e. supergroups) of eukaryotes. The number of major new lineages cultivated in this study suggests there remains a considerable undiscovered diversity of anaerobic eukaryotes. Future study of our isolates may reveal independent innovations in metabolism, cell biology, and genome structure, and contribute to our understanding of the evolution of anaerobiosis in eukaryotes.