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Ultrastructural and Single-Cell-Level Characterization Reveals Metabolic Versatility in a Microbial Eukaryote Community from an Ice-Covered Antarctic Lake

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ABSTRACT

The McMurdo Dry Valleys (MCM) of southern Victoria Land, Antarctica, harbor numerous ice-covered bodies of water that provide year-round liquid water oases for isolated food webs dominated by the microbial loop. Single-cell microbial eukaryotes (protists) occupy major trophic positions within this truncated food web, ranging from primary producers (e.g., chlorophytes, haptophytes, and cryptophytes) to tertiary predators (e.g., ciliates, dinoflagellates, and choanoflagellates). To advance the understanding of MCM protist ecology and the roles of MCM protists in nutrient and energy cycling, we investigated potential metabolic strategies and microbial interactions of key MCM protists isolated from a well-described lake (Lake Bonney). Fluorescence-activated cell sorting (FACS) of enrichment cultures, combined with single amplified genome/amplicon sequencing and fluorescence microscopy, revealed that MCM protists possess diverse potential metabolic capabilities and interactions. Two metabolically distinct bacterial clades (*Flavobacteria* and *Methylobacteriaceae*) were independently associated with two key MCM lake microalgae (*Isochrysis* and *Chlamydomonas*, respectively). We also report on the discovery of two heterotrophic nanoflagellates belonging to the Stramenopila supergroup, one of which lives as a parasite of *Chlamydomonas*, a dominate primary producer in the shallow, nutrient-poor layers of the lake.

IMPORTANCE

Single-cell eukaryotes called protists play critical roles in the cycling of organic matter in aquatic environments. In the ice-covered lakes of Antarctica, protists play key roles in the aquatic food web, providing the majority of organic carbon to the rest of the food web (photosynthetic protists) and acting as the major consumers at the top of the food web (predatory protists). In this study, we utilized a combination of techniques (microscopy, cell sorting, and genomic analysis) to describe the trophic abilities of Antarctic lake protists and their potential interactions with other microbes. Our work reveals that Antarctic lake protists rely on metabolic versatility for their energy and nutrient requirements in this unique and isolated environment.

The microbial loop links the transfer of nutrients and carbon between the microorganisms of the food web and includes complex ecological interactions between microbial eukaryotes, bacteria, archaea, and viruses. Microbial activity is influenced by bottom-up (e.g., availability of nutrient and energy sources) and top-down (e.g., predation, parasitism, and viral lysis) controls, as well as microbe-microbe interactions (e.g., bacterial consortia). Interaction partnerships between microorganisms lead to combinations of win, loss, or neutral outcomes for each microbial partner. Parasitism and predation are examples of win-loss outcomes (1), while mutualistic interactions within biofilms and syntrophic interactions (cross-feeding of metabolic products between two species) between an alga and a heterotrophic bacterium are examples of win-win partnerships (2). Understanding microbial interactions and the influences of environmental factors on microbial distribution patterns is critical for deciphering global nutrient fluxes and biogeochemical cycles.

Single-cell eukaryotic microorganisms (i.e., protists) are ubiquitous in every ecosystem on earth and play critical ecological roles in food web dynamics and global carbon and nutrient cycles (3). Diverse protist lineages possess multiple nutritional modes, playing key roles as producers, decomposers, parasites, and predators. Phototrophic protists are important producers that contribute to global primary production and incorporate a significant proportion of inorganic carbon into the food web (4). Predatory protists are major consumers of planktonic phytoplankton and bacteria, providing control over the abundance of these organisms as well as linking primary producers/consumers with higher trophic levels (i.e., metazoans) (5). Mixotrophic protists (with combined abilities for photosynthesis and phagotrophic ingestion of food particles) are widespread in aquatic ecosystems (6, 7). Recent molecular surveys from marine and freshwater environments have revealed a great diversity of 18S rRNA sequences that are unrelated to existing cultured protists (8, 9). The application of high-throughput sequencing has begun to reveal protist biogeography (10–12), as well as seasonal variability (13, 14).

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Antarctica, represent a polar desert, with average air temperatures of −20°C and precipitation rates of <10 cm per year (15, 16). Numerous marine-derived, perennially ice-covered lakes are the only source of year-round liquid water for life on the Antarctic continent. Permanent ice caps prevent wind mixing and significant nutrient inputs; MCM lake chemistry is vertically stratified in the water column, and lakes exhibit oxygen-rich ultraoligotrophic surface waters that are separated by permanent chemoclines from ancient anoxic saline waters (17). Thus, the MCM lakes represent aquatic environments with strong selective pressures on microbial evolution, including low temperatures, low annual levels of photosynthetically active radiation (PAR), and limited nutrient (nitrogen and phosphorus) availability (18). Salinity, oxygen, and light appear to play important roles in the biogeography of Antarctic lake microorganisms (19). Each lake supports simple food webs that harbor few to no metazoans, with the exception of small numbers of copepods and rotifers in a few lakes (20). Protists play dominating roles in carbon and nutrient cycling in the MCM microbial food web. The majority of dissolved organic carbon is autochthonous (i.e., derived from new photosynthetic activity within the water column). Protists represent the major producers of organic matter in the MCM lake food web (21, 22), while heterotrophic nanoflagellates (HNFs) and ciliates are the top predators of bacteria and smaller protists (23, 24). Despite the energetic costs of maintaining and regulating both photosynthetic and heterotrophic cellular apparatuses, mixotrophy appears to be very prevalent in MCM aquatic food webs (23, 25). Mixotrophic metabolism is a survival strategy for MCM protists to exploit alternative sources of either nutrients (i.e., under oligotrophic conditions) or energy (i.e., in the absence of light) (26).

As part of the NSF Long Term Ecological Research (LTER) Program, several lakes within the MCM have been the focus of more than 2 decades of intensive study. Lake Bonney is one of the most well-studied MCM lakes, and the physical and chemical characteristics of the lake have been thoroughly described (27). Lake Bonney is divided into two basins (the west and east lobes), which are separated by a narrow passage that allows mixing of the upper photic zones for a few weeks in the summer. The shallow waters of both lobes are generally freshwater; however, salinity levels increase steeply at the permanent chemocline (18- to 20-m depth for the east lobe). Below the chemocline, the deep anoxic zone is hypersaline (maximum salinity, 125 to 150 PSU). Photosynthetically active radiation (PAR) is relatively low under the ice (<50 μmol m^{-2} s^{-1}), due to the attenuation of the ice cover, and declines below detectable levels at depths of >25 m (28). Recently, the diversity and spatial distribution of the planktonic microbial eukaryotic communities residing in Lake Bonney have been reported (28–31). Protist populations residing in the MCM lakes are vertically stratified and exhibit lake-specific differences in distribution patterns. The water column of Lake Bonney is dominated by large chlorophytes (genus *Chlamydomonas*) in the shallow, nutrient-poor depths, while the dominant photosynthetic protists are nanoplankton (haptophytes and stramenopiles), which exhibit peak abundances within the permanent chemocline, where PAR is extremely limited (29). While these studies have begun to describe the biogeographic distribution of the protist communities residing in these ice-covered polar lakes, a full understanding of the trophic abilities of MCM protists and potential interactions with other MCM microorganisms is currently lacking.

The vast diversity of microbial eukaryotes has been largely inaccessible with conventional cultivation methods. Over the past decade, the technology of single-cell genomics has been exploited to recover genomic information from single uncultivated cells collected from their natural habitats and has revealed cell-specific interactions such as symbiosis, predation, and parasitism (32–34). In this study, single cells of protists originating from an enrichment culture from the zone of maximum primary production in the permanent chemocline of Lake Bonney (east lobe) were isolated on the basis of fluorescence properties, which were related to their potential nutritional mode (photosynthetic, heterotrophic, or mixotrophic). We chose to utilize an enrichment cultivation approach to allow us to work with live organisms and to avoid the logistical issues associated with preserving and shipping natural protist communities from Antarctica to our U.S. laboratory. Our hypothesis was that MCM lake protists possess broad metabolic versatility, which drives nutritional mode-dependent microbial interactions. Specifically, we addressed the following open questions regarding the ecology of the MCM lake microbial eukaryote communities. What is the range of trophic versatility in the MCM planktonic protist communities? Do MCM phototrophic and heterotrophic protists interact with specific microbial partners? In this study, whole-genome amplification (WGA) was performed to produce single amplified genomes (SAGs) from a variety of the sorted eukaryotic cells (with their microbial partners). Combining Illumina sequencing with various microscopic methods, we were able (i) to identify the nutritional mode and metabolic potential of several key MCM protists, (ii) to reveal a diversity of potential microbial interactions, including predation, parasitism, and endosymbiosis, and (iii) to provide new information for understanding microbial metabolic strategies for survival under permanent light-limiting and nutrient-poor conditions.

MATERIALS AND METHODS

Site description, sample collection, and enrichment cultures. Samples were collected from the water column of the east lobe of Lake Bonney, an ice-covered meromictic lake located in the Taylor Valley, McMurdo Dry Valleys. Samples were collected through the ice hole located in the middle of the lake (77°42.825′S, 162°26.832′E) on 21 December 2012, during the austral summer. A sampling depth (piezometric depth of 13 m) was selected to reflect the depth of maximum phytoplankton biomass (29), which was verified by *in situ* chlorophyll fluorescence with a submersible FluoroProbe (BBE Moldaenke GmbH) (28). Lake water samples were collected with a 5-liter Niskin bottle (General Oceanics), transferred to 1-liter amber bottles, and stored at 4°C in the dark until processing.

An enrichment cultivation approach was employed to allow for the transport of live cultures with greater biomass to our U.S. laboratory (Miami University, Oxford, OH, USA). To ensure maximum recovery of protist diversity, a series of enrichment cultures was started in the presence of a number of autotrophic medium types (35–37) and growth medium concentrations (10 to 100%). Lake water was inoculated into 25-ml sterile culture flasks and incubated in a photoincubator, at 5°C and 25 μmol photons m^{-2} s^{-1}, at McMurdo Station, Antarctica, for 2 weeks prior to shipment. Cultures were regularly inspected under the microscope for growth and the presence of microbial eukaryotes. Cultures were then shipped to our U.S. laboratory (Miami University) at 4°C in the dark. Upon arrival, enrichment cultures were transferred to a photoincubator set at the same temperature and light conditions until cell sorting. While cryopreservation in the presence of glycerol buffer works well for preserving bacterial communities (38, 39), preliminary experiments showed that this preservation approach failed to preserve the internal structures of eukaryotic cells and caused lysis of some wall-less protists.

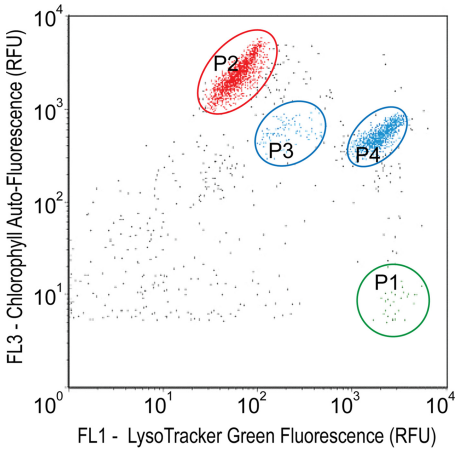

FIG 1 Sample cytogram showing flow cytometric sorting regions for microbial eukaryotes from an enrichment culture (MCM87) generated from the Antarctic Dry Valley of Lake Bonney. The sample was stained with LysoTracker prior to sorting, and samples were gated on green fluorescence (LysoTracker-stained food vacuoles) versus red fluorescence (chlorophyll autofluorescence). Ovals, groups selected for single-cell sorting. Red, high levels of chlorophyll autofluorescence; green, high levels of LysoTracker fluorescence; blue, intermediate levels of both types of fluorescence. P1 to P4, sorted 96-well plates 1 to 4.

Single-cell sorting. Prior to cell sorting, all enrichment cultures were visually inspected by light microscopy for growth and morphological diversity of the protist communities. Based on these microscopic observations, one enrichment culture (grown in 10% F medium) that exhibited relatively great protist morphological diversity was chosen to be shipped to Oak Ridge National Laboratory for single-cell sorting. Protist food vacuoles were stained using a pH-sensitive probe (LysoTracker Green DND-26; Invitrogen, Carlsbad, CA, USA) (40). A 2-ml aliquot of the culture was gently homogenized with several inversions and incubated for 15 min in the dark on ice in the presence of 75 nmol liter^{-1} of LysoTracker. Microbial eukaryotic cells of various sizes, morphological types, and trophic abilities (phototrophic, heterotrophic, or mixotrophic) were identified and sorted with a Cytopeia Influx sorter (BD, Franklin Lakes, NJ, USA) in a class 1000 clean room, using a 488-nm argon laser for excitation. Green (528 to 538 nm) and red (670 to 730 nm) fluorescent emission indicated LysoTracker green fluorescence and chlorophyll autofluorescence, respectively. Targeted single eukaryotic cells were sorted into four 96-well plates, containing 3 μl UV-sterilized TE buffer in each well (32). Individual cells were sorted from discrete sectors using three criteria; plate 1 contained cells showing high levels of LysoTracker fluorescence only (i.e., heterotrophic or phagotrophic), plate 2 contained cells exhibiting high levels of red fluorescence (i.e., photosynthetic) and the absence of green fluorescence, and plates 3 and 4 contained smaller cells exhibiting intermediate levels of red and green fluorescence (i.e., mixotrophic) (Fig. 1). Plates were stored at −80°C until whole-genome amplification.

DNA template preparation for PCR. For DNA preparation, single sorted eukaryotic cells were lysed using alkaline treatment with the addition of 3 μl of buffer consisting of 0.13 M KOH, 3.3 mM EDTA (pH 8.0), and 27.7 mM dithiothreitol (DTT), heated to 95°C for 30 s, and immediately placed on ice for 10 min, followed by the addition of 3 μl of neutralization buffer (0.13 M HCl, 0.42 M Tris [pH 7.0], 0.18 M Tris [pH 8.0]). Genomic DNA from the cell lysates was amplified using multiple displacement amplification (MDA) with the addition of 11 μl of MDA master mix containing 90.9 μM random hexamers with two protective phosphorothioate bonds on the 3′ end (Integrated DNA Technologies, Coralville, IA, USA), 1.09 mM deoxynucleoside triphosphates (dNTPs) (Roche, Indianapolis, IN, USA), 1.8× phi29 DNA polymerase buffer (New England BioLabs, Ipswich, MA, USA), 4 mM DTT, and ~100 U phi29 DNA polymerase (purified in-house) (41). Whole-genome amplification was performed at 30°C for 10 h, followed by inactivation at 80°C for 20 min. MDA products were diluted 100-fold in sterile 1× TE buffer and then screened by PCR and sequencing.

Sanger sequencing. A fragment of the 18S rRNA gene was amplified from each of the SAGs using the universal eukaryote primers EK-82F (5′-GAAACTGCGAATGGCTC) and EK-1520R (5′-CYGCAGGTTCAC CTAC) (42), to generate PCR products for sequencing. PCR was performed in triplicate using 25 cycles of 95°C for 1 min, 52°C for 1 min, and 72°C for 2 min. Sequencing reactions were performed using the BigDye Terminator v3.1 cycle sequencing kit (ABI) with the M13R primer, and the fragments were sequenced on an Applied Biosystems 3730xl DNA analyzer (ABI) located in the Center for Bioinformatics and Functional Genomics (CBFG) at Miami University.

Illumina sequencing. Following successful identification of the individual eukaryote SAGs, 79 samples were selected for sequencing of the associated bacterial communities on an Illumina MiSeq platform at the CBFG. The 16S rRNA genes from the SAGs were amplified using a primer set (F515/R806) that encoded sequences against the highly variable V4 region of 16S rRNA, barcodes, and linkers. PCRs and MiSeq sequencing reactions strictly followed the protocol provided by the Earth Microbiome Project (http://www.earthmicrobiome.org) (42–45). Samples were sequenced using a 300-cycle MiSeq v2 reagent kit (Illumina), according to the manufacturer's recommendations, in a paired-end run (2 by 150 bp) in the presence of 25% PhiX DNA.

Analysis of sequences. For 18S rRNA gene sequences derived from Sanger sequencing, representative and closest sequences were selected from GenBank and aligned using MUSCLE. A maximum likelihood tree was generated using MEGA6 software. Bootstrapping was used to estimate the node support of 1,000 replicate trees.

The 16S rRNA gene sequences generated with the MiSeq system were analyzed with QIIME (v1.8.0). Operational taxonomic units (OTUs) were identified at 97% cutoff using the Greengenes database (v13.8) (46). All OTUs with one sequence per sample were discarded. Samples were rarefied to 120 sequences/sample based on the number of sequences in the library with the lowest sequence number. Alpha diversity (number of OTUs, Chao1 index, and Shannon index) was assessed (data not shown). Beta diversity was integrated using the weighted UniFrac distance metric (47, 48). Principal-coordinate analysis (PCoA) and analysis of similarities (ANOSIM) were performed to identify the coexistence of eukaryotic and prokaryotic organisms in the single-cell samples (49–52).

Confocal laser scanning microscopy. *Isochrysis* sp. MDV and *Chlamydomonas* sp. ICE-MDV cultures were treated with the LysoTracker Green DND-26 probe using the same procedure as described above for fluorescence-activated cell sorting (FACS). A Zeiss LSM-710 confocal laser scanning microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) was used for the images in Fig. 3. A 488-nm argon ion laser was used as the elimination/excitation source. LysoTracker fluorescence and chlorophyll fluorescence were detected at 520 to 540 nm and 650 to 750 nm, respectively. Differential interference contrast (DIC) images were also generated, using the transmission light mode. Images were pseudocolored with Zeiss ZEN 2011 software (Carl Zeiss Microscopy GmbH).

Scanning electron microscopy. Samples from the original enrichment culture were fixed with 1% paraformaldehyde and 1.25% glutaraldehyde, and a secondary fixation was performed with 1% osmium tetroxide. Specimens were dehydrated through an ethanol series, critical point dried with CO_2, and sputter coated with gold. Micrographs were generated with a Zeiss Supra 35 FEG scanning electron microscope (Carl Zeiss Microscopy GmbH).

Nucleotide sequence accession numbers. The 79 high-quality partial 18S rRNA sequences (~600 bp) recovered were submitted to GenBank under accession numbers KU196097 to KU196166 (NCBI BioProject accession no. PRJNA304193).

RESULTS AND DISCUSSION

Identities and trophic modes of sorted eukaryotes. To overcome the logistical constraints of transporting fresh samples from Antarctica to our U.S. laboratory, as well as low biomass issues associated with MCM planktonic communities, we used an enrichment cultivation-based approach to test FACS/single-cell sorting with McMurdo Dry Valley lake protist communities. A series of enrichment cultures was started in Antarctica using a range of growth media, as described in Materials and Methods, and cultures were incubated for 2 weeks prior to shipment to our U.S. laboratory. When the cultures arrived in our U.S. laboratory, we monitored algal diversity using a BBE FluoroProbe (see Table S2 in the supplemental material) and visually inspected each culture using bright-field and fluorescence microscopy, to assess protist diversity qualitatively. Inspection of one of the cultures (enrichment MCM87) revealed large (>15-μm) biflagellate chlorophytes, several nanophytoplankton identified as haptophytes and cryptophytes (~5 μm), dinoflagellate cells exhibiting variable pigmentation (owing to the presence or absence of phytoplankton prey), and several nonpigmented heterotrophic nanoflagellates (2 to 5 μm). Based on our recent work on natural protist diversity in Lake Bonney (29), we concluded that MCM87 harbored representatives of several key members of the Lake Bonney protist community, including chlorophytes, haptophytes, choanoflagellates, dinoflagellates, and nanoflagellates, all of which have been detected in environmental sequence libraries generated from the photic zone of the east lobe of Lake Bonney.

Based on LysoTracker and chlorophyll fluorescence signals combined with cell size information, we gated four eukaryote populations for single-cell sorting, i.e., a group with high levels of LysoTracker fluorescence (i.e., the presence of protist food vacuoles; plate 1) (Fig. 1, green group), a group with high levels of red fluorescence (i.e., the presence of chlorophyll; plate 2) (Fig. 1, red group), and two groups with smaller cells that exhibited intermediate levels of red and green fluorescence (plates 3 and 4) (Fig. 1, blue groups). Targeted single cells were deposited into four 96-well plates according to distinct gates that separated various types of organisms on the basis of the size and fluorescence characteristics mentioned above. Following cell lysis and whole-genome amplification using phi29 DNA polymerase, 65% (250 of 384 samples) of the SAGs were successfully amplified using 18S rRNA primers, based on electrophoretic separation of the PCR products on a 1% agarose gel. All SAGs that exhibited a band of the correct size (~1,400 bp) were subjected to Sanger sequencing. We recovered 79 high-quality partial 18S rRNA sequences (~600 bp) for phylogenetic analysis. Representative sequences were aligned with the SILVA and GenBank databases using SINA and BLAST. Phylogenetic analysis revealed that the SAG library harbored a total of 16 OTUs (based on a cutoff value of 97% similarity) related to the Stramenopila supergroup and several other major phyla, including Chlorophyta, Choanoflagellida, Dinoflagellata, Cryptophyta, and Haptophyta. The majority of SAG 18S rRNA sequences were identical to uncultivated eukaryote clones recovered in an earlier study on natural protist populations in Lake Bonney (29).

All SAGs recovered from the photosynthetic group (i.e., high autofluorescence levels and low LysoTracker fluorescence levels; $n = 13$) (Fig. 1, P2) were related to a large biflagellate chlorophyte, *Chlamydomonas*, which was closely related (99% identity) to an Antarctic marine species, *Chlamydomonas* sp. Antarctic 2E9, and two Antarctic ice *Chlamydomonas* spp., ICE-W and ICE-L (53). The *Chlamydomonas* SAGs were also closely related to sequences from clone libraries generated from the depth of maximum productivity (13 m depth) in the east lobe of Lake Bonney (29) (Fig. 2). Recent studies on phylogenetic and functional genes indicated that *Chlamydomonas* sp. ICE is the dominant chlorophyte in the east and west lobes of Lake Bonney (29, 30). Lastly, our FACS results, which suggested that this organism is photoautotrophic, were supported by earlier physiological studies on *Chlamydomonas* sp. ICE (53), as well as a highly studied Lake Bonney chlorophyte, *Chlamydomonas* sp. UWO241 (22, 54).

SAG sequences related to a nanoflagellate haptophyte (*Isochrysis galbana*; 97% identity) represented a significant proportion (80%) of the samples recovered from SAG plate 4 ($n = 17$) (Fig. 2), which represented one of two plates with intermediate levels of green and red fluorescence (Fig. 1, P4). The 18S rRNA sequences from these SAGs were very similar to sequences recovered from clone libraries generated from 13-m-deep waters from the east and west lobes of Lake Bonney (29), and recent reports suggested that haptophyte communities related to this organism are key primary producers in this lake (28, 30, 55). Communities of *Isochrysis* dominate the chemoclines of the east and west lobes of Lake Bonney (28). Several recent studies using quantitative PCR analyses to monitor the abundance of photosynthetic functional genes (*rbcL* and *psbA*) indicated that, in contrast to chlorophyte populations, the abundance (DNA) and activity (RNA) of haptophyte populations are not correlated with light availability (28, 55). These data suggest that the Dry Valley *Isochrysis* may rely on alternative metabolic strategies, such as mixotrophy.

Stramenopiles represent a poorly understood supergroup of microbial eukaryotes that exhibit broad trophic abilities (e.g., phototrophy, mixotrophy, predation, and parasitism). The marine stramenopile (MAST) lineage is a diverse group of microbial eukaryotes and represents a large proportion of heterotrophic nanoflagellates (HNFs) in marine environments (56). HNFs are small microbial eukaryotes (2 to 20 μm) that graze on bacteria and picophytoplankton and are recognized as the dominant consumers of picoplanktonic biomass in marine environments (7, 57). Our 18S rRNA gene sequence library contained several SAGs that were related to stramenopiles (Fig. 2). The most abundant stramenopile SAGs were closely related to *Pteridomonas danica* (99% identification, $n = 22$; SAG plate 4) and *Pirsonia verrucosa* (94 to 100% identification, $n = 22$; SAG plate 3). Sequences related to both stramenopiles were recovered in clone libraries from the west and east lobes of Lake Bonney (29) (Fig. 2). Recent work reported that stramenopiles make up significant proportions of the protist population (up to 60%) in both lobes of Lake Bonney (28, 31). *Pirsonia* is a phycovorous nanoflagellate that feeds on marine centric diatoms (58). *Pteridomonas* is a ubiquitous marine heterotrophic nanoflagellate (5). In addition, two OTUs within the Stramenopila supergroup (SAG clones P3E2 and P4C7) exhibited very low levels of similarity (<81%) to the closest related identified sequence in the database (*Thraustochytrium* sp.). Both OTUs, however, were closely related to the sequences generated for Lake Bonney clone libraries in a previous study (29).

Several other protist groups were represented in the SAG libraries at low abundance. A single SAG was recovered for two choanoflagellates and one dinoflagellate. One SAG related to the psychrophilic marine cryptophyte *Geminigera cryophila* was also recovered. *G. cryophila* is abundant in the shallow layers of Lake

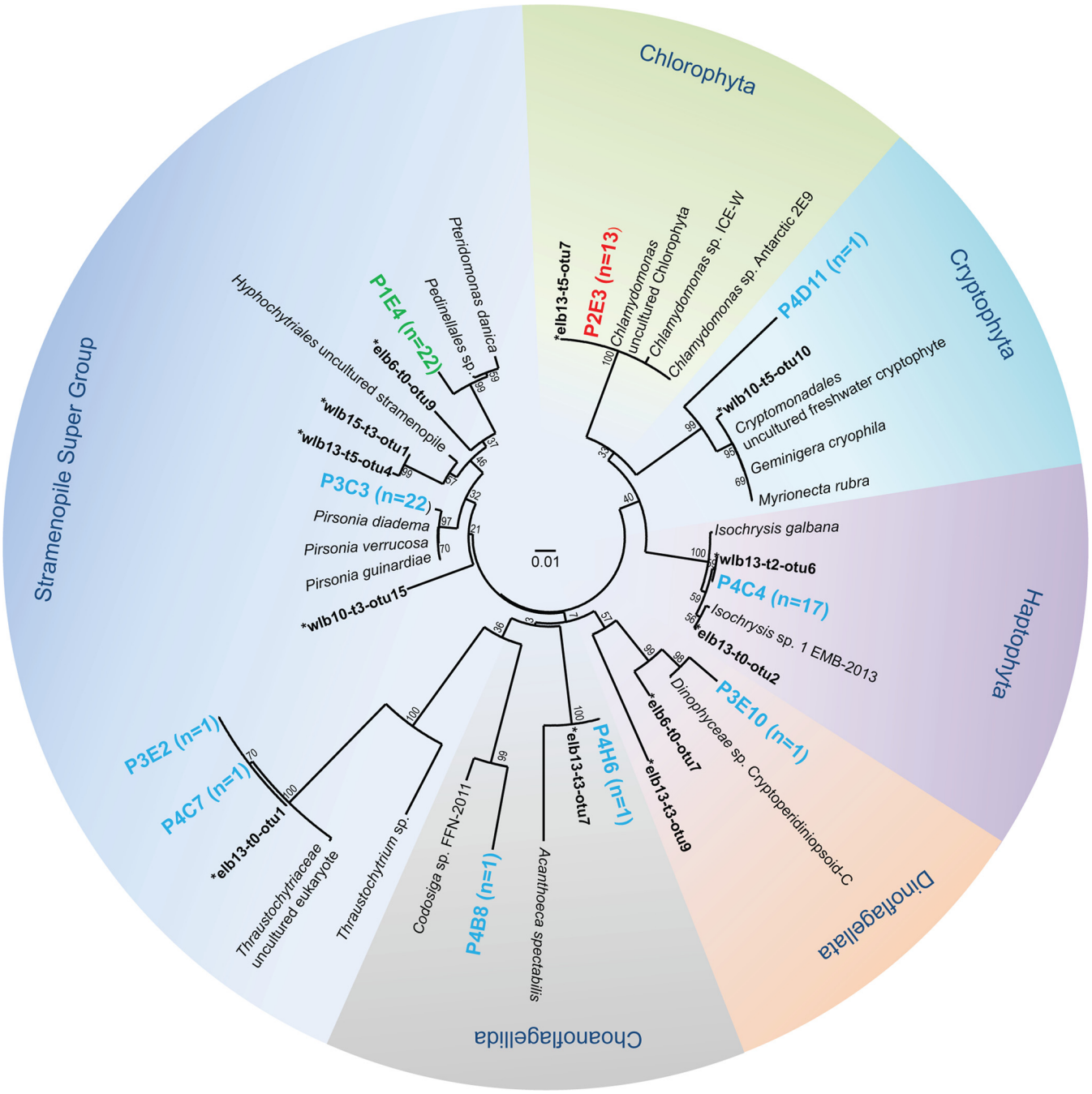

FIG 2 Maximum likelihood tree (1,000 bootstrap replicates) showing the identity of Lake Bonney microbial eukaryote single amplified genomes (SAGs) recovered from enrichment culture MCM87. The SILVA and GenBank databases were used to assign the phylogenetic positions of eukaryote SAGs. The colors of the SAG sequences are correlated with the sorting parameters in Fig. 1. *, sequences from the natural eukaryote populations in Lake Bonney generated in a previous study (29).

Bonney (28, 29). Dinoflagellates have been detected in relatively high abundance in the west lobe of Lake Bonney, while sequences related to choanoflagellates were less abundant (29, 31). All low-abundance SAGs were recovered from the two mixotrophic groups (Fig. 1, P3 and P4) and were closely related to sequences recovered from clone libraries generated from the east or west lobe of Lake Bonney, with the exception of one choanoflagellate sequence (SAG P4B8) (Fig. 2).

Isolation and description of two key photosynthetic protists. Recent work on the natural communities of Lake Bonney suggested that a chlorophyte related to *Chlamydomonas* sp. ICE-L and a haptophyte related to *Isochrysis galbana* occupy important but distinct roles in the Antarctic lake food web (28–31). Our FACS analyses indicated that the chlorophyte is a pure photoautotrophic species, while the haptophyte may possess the ability to combine photosynthetic metabolism with heterotrophic activity,

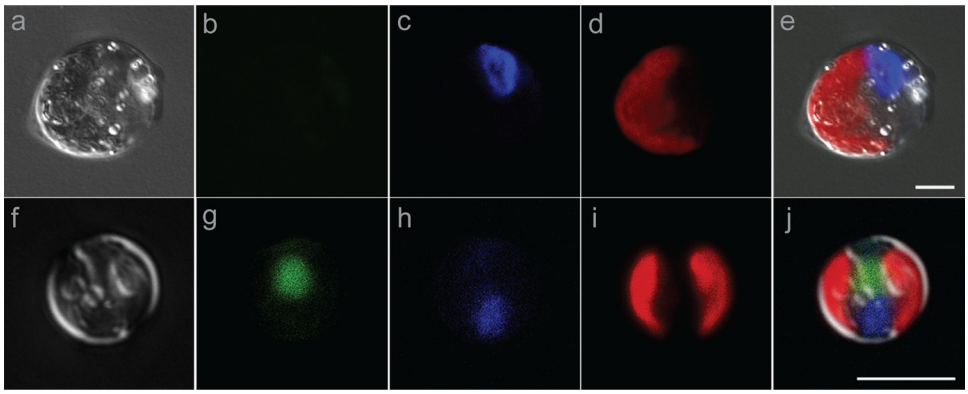

FIG 3 Confocal microscopic images of *Chlamydomonas* sp. ICE-MDV (a to e) and *Isochrysis* sp. MDV (f to j) isolates stained with LysoTracker Green and 4′,6-diamidino-2-phenylindole (DAPI). (a and f) DIC images. (b and g) LysoTracker fluorescence. (c and h) DAPI fluorescence. (d and i) Chlorophyll autofluorescence. (e and j) Overlays of a to d and f to i, respectively. Bars, 5 μm.

such as digestion of captured bacterial prey or particulate carbon. To confirm our hypotheses regarding the trophic capabilities of these key MCM protists, we purified isolates of both organisms from enrichment cultures. A culture of the *Chlamydomonas* sp. (named *Chlamydomonas* sp. ICE-MDV) was recovered by plating of an enrichment culture on Bold's basal medium (59) with 1.5% agar. The *Isochrysis* strain (named *Isochrysis* sp. MDV) was isolated by dilution to extinction of an enrichment culture in 0.5× seawater supplemented with F2 medium. The identity of both strains was confirmed by 18S rRNA gene sequencing (data not shown). Confocal microscopy showed that *Chlamydomonas* sp. ICE-MDV had large (15- to 20-μm) biflagellate cells exhibiting high levels of autofluorescence (Fig. 3a, d, and e). The LysoTracker probe is a fluorescent acidotrophic probe; therefore, we wondered whether the acidic compartment (lumen) of chloroplasts (60) might cause false-positive fluorescence signals in flow cytometry studies. We did not detect green fluorescence in the presence of LysoTracker in *Chlamydomonas* sp. ICE-MDV cells, despite the presence of a large chloroplast (Fig. 3d and e). This finding also confirms that it is unlikely that any of the SAGs sorted in the mixed-SAG plates (Fig. 1, P2 and P3) were products of spurious chloroplast staining. *Isochrysis* sp. MDV is a small (2- to 5-μm) brown biflagellate alga. It possesses bilobed chloroplasts, which exhibited high levels of autofluorescence (Fig. 3i and j). In contrast to the chlorophyte strain, green fluorescence was localized to a single vacuole when cells of *Isochrysis* sp. MDV were treated with the LysoTracker probe (Fig. 3g). The presence of green fluorescence was associated only with LysoTracker-stained cells, inasmuch as neither strain exhibited green fluorescence in the absence of LysoTracker (see Fig. S1 and S2 in the supplemental material). We also confirmed in growth experiments that *Isochrysis* sp. MDV can grow either in the dark (heterotrophic) or in the light (phototrophic) but it grows optimally in the presence of a variety of organic carbon sources and low irradiance (mixotrophic). In contrast, *Chlamydomonas* ICE-MDV cannot grow in the dark in the presence of organic carbon but exhibits the ability to grow under a broader range of light intensities (for a description of the basic growth physiology, see Table S1 in the supplemental material). Therefore, the chlorophyll and LysoTracker fluorescence information from confocal microscopy, combined with growth physiology, confirmed results from the FACS analyses indicating that *Chlamydomonas* sp. ICE-MDV is a pure photosynthetic protist, while *Isochrysis* sp. MDV is likely capable of mixotrophic metabolism. These results fit well with data from functional gene analyses indicating that Lake Bonney chlorophyte populations that occupy the under-ice layers of the water column are strongly correlated with light availability on spatial and seasonal scales (55). In contrast, haptophyte populations dominating planktonic communities in the permanent chemocline are not influenced by light availability but can supplement light-dependent photosynthesis with ingestion of bacterial prey or particulate carbon.

Community composition of organisms cosorted with Lake Bonney eukaryotes. To gain further insight into the trophic modes and potential microbial partners of the MCM microbial eukaryotes, we selected a number of eukaryote SAGs of different trophic potentials for 16S rRNA amplicon community sequencing. From the population of successfully amplified and sequenced SAGs, we sequenced a fragment of the 16S rRNA gene from 79 eukaryote SAGs using the Illumina MiSeq platform. We recovered both bacterial 16S and plastid small-subunit (SSU) rRNA genes from these sequencing libraries. The plastid sequences were filtered from the 16S rRNA gene libraries of all SAG OTUs known to be photosynthetic, including *Chlamydomonas* sp. ICE-MDV, *Isochrysis* sp. MDV, and *Geminigera*. To assess both the diversity and cooccurrence patterns of organisms that cosorted with the individual eukaryote SAGs, we generated a heat map of 16S rRNA genes associated with each individual SAG sample (see Fig. S3 in the supplemental material). The distribution of recovered 16S rRNA gene sequences was highly variable and was dependent on both the trophic mode and the identity of the microbial eukaryote partner. Bacterial sequences associated with the photosynthetic *Chlamydomonas* sp. ICE-MDV and the mixotrophic *Isochrysis* sp. MDV were generally diverse, with a predominance of the phyla *Actinobacteria* and *Bacteroidetes*. In contrast, 16S rRNA gene sequences recovered from the two stramenopile SAGs related to *Pteridomonas* and *Pirsonia*, which dominated the heterotrophic population (plate 1) and one of the mixotrophic populations (plate 4), respectively, were enriched with plastid sequences (see Fig. S3).

Principal-coordinate analysis (PCoA) based on UniFrac distance metrics of 16S rRNA OTUs from the four most abundant SAGs (*Isochrysis* sp. MDV, *Chlamydomonas* sp. ICE-MDV, *Pter-*

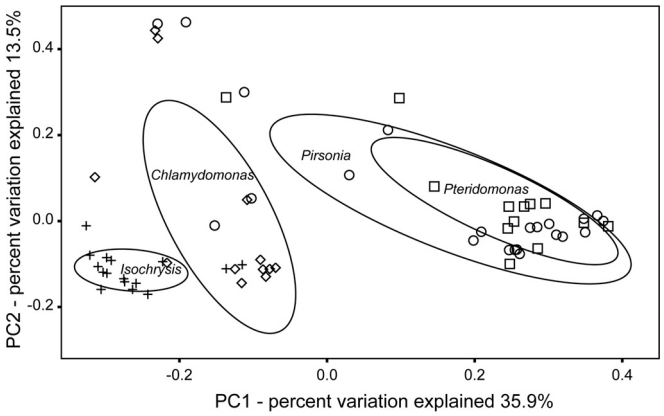

FIG 4 Principle-coordinate analysis (PCoA) of weighted UniFrac distances of 16S rRNA genes associated with eukaryote SAGs of *Isochrysis* sp. MDV (+), *Chlamydomonas* sp. ICE-MDV (◇), *Pteridomonas* (□), and *Pirsonia* (○). Each data point represents one SAG sample. Ovals, 50% similarities within each sample group.

idomonas, and *Pirsonia*) demonstrated clustering by protist trophic mode (Fig. 4). Similarity analysis using the ANOSIM method indicated that bacterial OTUs associated with individual eukaryote SAGs were significantly different from each other ($P < 0.001$, $R^2 = 0.40$). Bacterial OTUs associated with *Isochrysis* sp. MDV and *Chlamydomonas* sp. ICE-MDV were clustered separately from each other but were more closely related than either of the stramenopile SAGs. The 16S rRNA gene libraries generated from SAGs of the heterotrophic nanoflagellates (*Pteridomonas* and *Pirsonia*) were related more to each other than to either of the photosynthetic protists or the environmental samples (Fig. 4).

Potential interactions between Dry Valley protists and bacteria. Complex ecological interactions between protists and other microorganisms exist, including predation of bacteria by heterotrophic protists and syntrophic interactions between photosynthetic algae and heterotrophic bacteria (2, 61). Traditionally, heterotrophic protists were identified via culture-dependent and microscopic methods (62–65). Recent studies have shown that a single-cell genomic approach can provide direct evidence of specific predator-prey interactions (66, 67). We recovered 22 samples that had high levels of LysoTracker probe-associated fluorescence and were closely related to the stramenopile *Pteridomonas danica* (Fig. 1 and 2). *Pteridomonas* is a ubiquitous heterotrophic nanoflagellate in marine systems and is an important link between bacteria and higher trophic levels in the food web (68–70). Interestingly, we observed that more than 90% of 16S rRNA gene sequences from *Pteridomonas* SAGs were closely related to a stramenopile chloroplast sequence that was neither reported in environmental sequence libraries (29, 31) nor detected in other SAG 16S rRNA libraries in this study. A previous study reported that *Pteridomonas danica* harbors nonpigmented nonphotosynthetic plastids or vestigial chloroplasts (71); therefore, it is likely that the chloroplast sequences we recovered in 16S rRNA gene sequence libraries generated from *Pteridomonas* SAGs represent plastid sequences from vestigial chloroplasts. This interpretation was supported by a lack of pigmentation in *Pteridomonas* cells under light microscopy (data not shown). We removed the stramenopile plastid sequences from the 16S rRNA gene libraries generated from *Pteridomonas* SAGs and investigated the remaining 16S rRNA gene sequences in the *Pteridomonas* libraries (Fig. 5a). The most abundant sequences were related to a haptophyte plastid rRNA gene (32% ± 23% of total OTUs) and a firmicute, *Streptococcus* (13% ± 8% of total OTUs) (Fig. 6a). HNFs represent a diverse ecological group capable of various trophic preferences (e.g., bacteriovorous, phycovorous, or omnivorous). *P. danica* feeds on both bacteria and picophytoplankton and therefore is an omnivore (72); however, we did not find any reports of this genus preying on haptophytes. Picocyanobacteria are largely absent from the water column of the MCM lakes (28, 30); therefore, we suggest that, in addition to heterotrophic bacteria, the haptophyte population that dominates the photic zone of Lake Bonney could be the natural prey for the MCM *Pteridomonas*.

A number of feeding strategies for heterotrophic flagellates, such as filtration of food particles with an array of radial pseudopodia or tentacles or direct interception of prey by flagella, have been reported. In addition, flagellates are often attached to particles such as marine snow when feeding (73, 74). Examination of the Lake Bonney *Pteridomonas* by scanning electron microscopy (SEM) revealed the presence of a single long flagellum surrounded by a radial array of tentacles (Fig. 5b to d), which are characteristic morphological features of *Pteridomonas* spp. (68). In addition, we observed particles attached to the tentacles, and cells were often attached to the surface of a particle by a stalk (Fig. 5b). To our knowledge, our images are some of the first published SEM images of this genus. Thus, the Lake Bonney *Pteridomonas* appears to rely on a filter feeding mechanism and may be particle attached within the water column. This would be an advantageous strategy within the permanent chemocline, where potential prey would be abundant and particulate organic carbon from the upper photic zone may accumulate. In support of this suggestion, Roberts et al. (24) reported that HNFs dominated heterotrophic protozoa in Lake Bonney and peaked within the permanent chemocline of the west lobe of Lake Bonney.

A second highly abundant SAG ($n = 22$) within the Stramenopila supergroup exhibited a relatively large proportion of chloroplast sequences within the 16S rRNA gene sequence libraries (Fig. 6; also see Fig. S3 in the supplemental material). These SAGs were recovered from plate 4, which exhibited relatively high levels of green fluorescence and intermediate levels of autofluorescence, and were closely related to a parasitoid nanoflagellate, *Pirsonia* sp. (Fig. 1 and 2). Parasitoid protists represent a diverse taxonomic group and are thought play major roles in controlling the abundance of their prey; however, their roles in microbial food webs are poorly understood (75). Plastid sequences recovered from the *Pirsonia* SAGs were all related to *Chlamydomonas* chloroplast sequences (Fig. 6a); however, unlike *Pteridomonas*, *Pirsonia* is not known to harbor a vestigial plastid. Therefore, the presence of plastid sequences within the 16S rRNA gene sequencing libraries suggested that *Pirsonia* potentially interacts with *Chlamydomonas* in a predator-prey relationship. However, microscopic examination indicated that the average size of *Pirsonia* was ~5 μm, which was noticeable smaller than *Chlamydomonas* (~15 μm) (Fig. 6b and c). Previous studies observed that *Pirsonia* species are host specific for marine centric diatoms. The feeding mechanism involves attachment to a host diatom cell, formation of a trophosome inside the host cell, and transfer of digested material into the parasite cell (58, 76). We observed an extracellular organelle in the Dry Valley *Pirsonia* (Fig. 6b, arrowhead), which likely functions as part of this feeding mechanism. While diatoms are abundant in

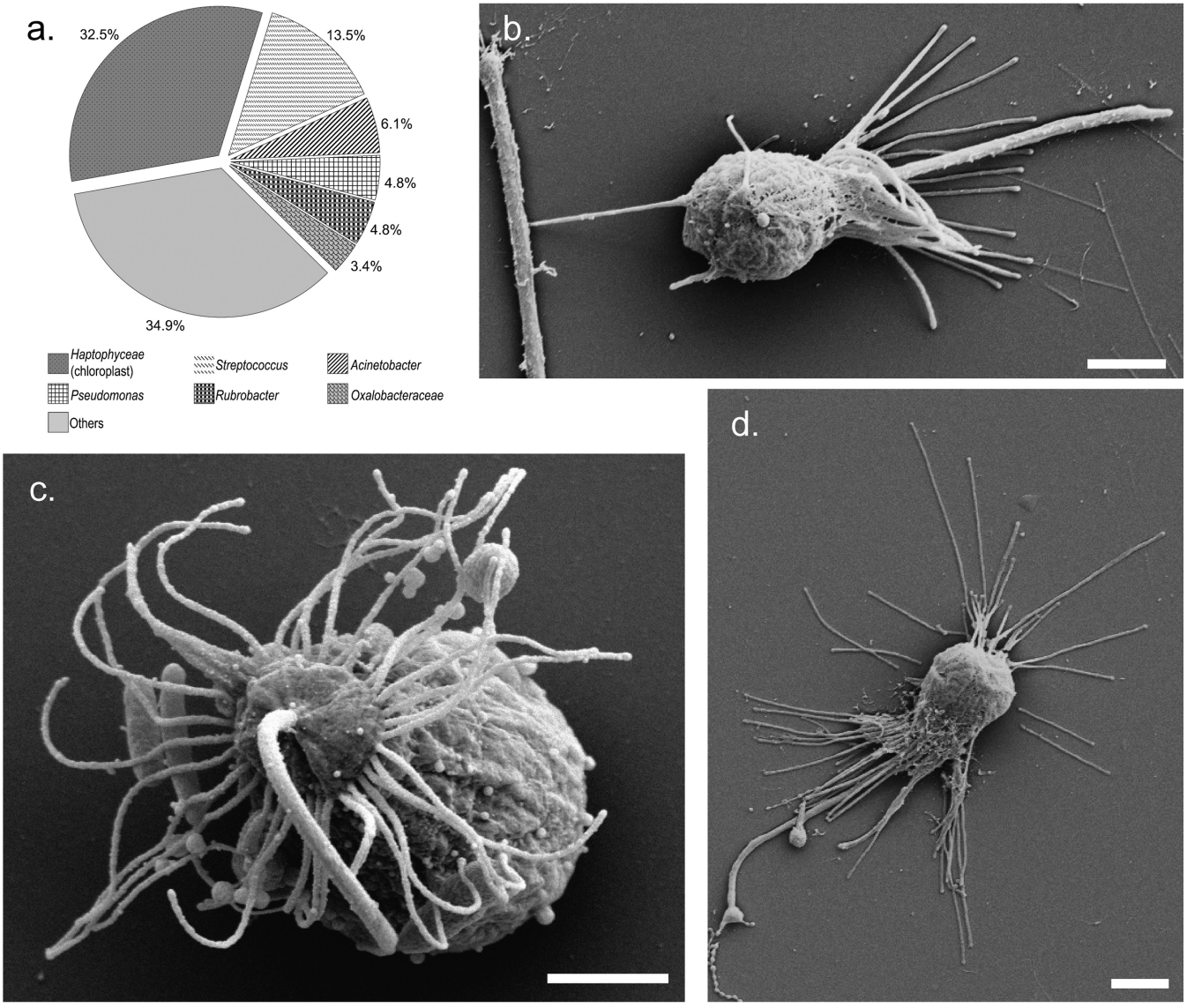

FIG 5 SEM images and the diversity of microbial partners associated with the heterotrophic nanoflagellate *Pteridomonas* ($n = 22$). (a) Chart showing the diversity of 16S rRNA gene OTUs recovered from *Pteridomonas* eukaryote SAGs. (b to d) SEM images of *Pteridomonas* sp. cells from the enrichment culture. Bars, 1 μm.

the ephemeral streams and microbial mats around the Dry Valleys (77–79), they are rare in MCM lake water columns (28, 29, 31). To our knowledge, there are no reports of *Pirsonia* interacting with chlorophytes; however, we observed numerous *Pirsonia* cells attached to *Chlamydomonas* cells (Fig. 6d and e), which is a critical step for infection of host cells.

Phytoplankton-bacteria interactions in aquatic environments are well known and are largely driven by the dependence of heterotrophic bacteria on the production of alga-derived organic substrates in the form of extracellular phytoplankton products or decaying algal biomass. There is clear evidence that phytoplankton community distribution and dynamics influence specific bacterial assemblages (80–82). The interactions of algae and bacteria involve either free-living, non-particle-attached communities of heterotrophic bacteria or cell-to-cell contact between specific bacteria and algal hosts; bacterial groups associated with the two types of interactions appear to be significantly different from each other (83, 84). Bacterioplankton can exhibit different abilities for incorporation of specific alga-derived substrates (85). The Dry Valley lakes are essentially closed systems for most of the year, and thus heterotrophic bacteria are heavily reliant on newly fixed carbon from phytoplankton production. Recent studies based on environmental sequencing of phytoplanktonic and bacterial communities have shown that spatial distribution of planktonic communities varies greatly within and between lakes (28, 29, 31); however, interactions between MCM phytoplankton and heterotrophic bacteria have not been resolved. In the present study, SAGs of either the chlorophyte *Chlamydomonas* sp. ICE-MDV or the haptophyte *Isochrysis* sp. MDV were associated with a number of bacterial OTUs (Fig. 5; also see Fig. S3 in the supplemental material). In general, the evidence for strong interactions between algae and bacteria was lacking. However, we did note two poten-

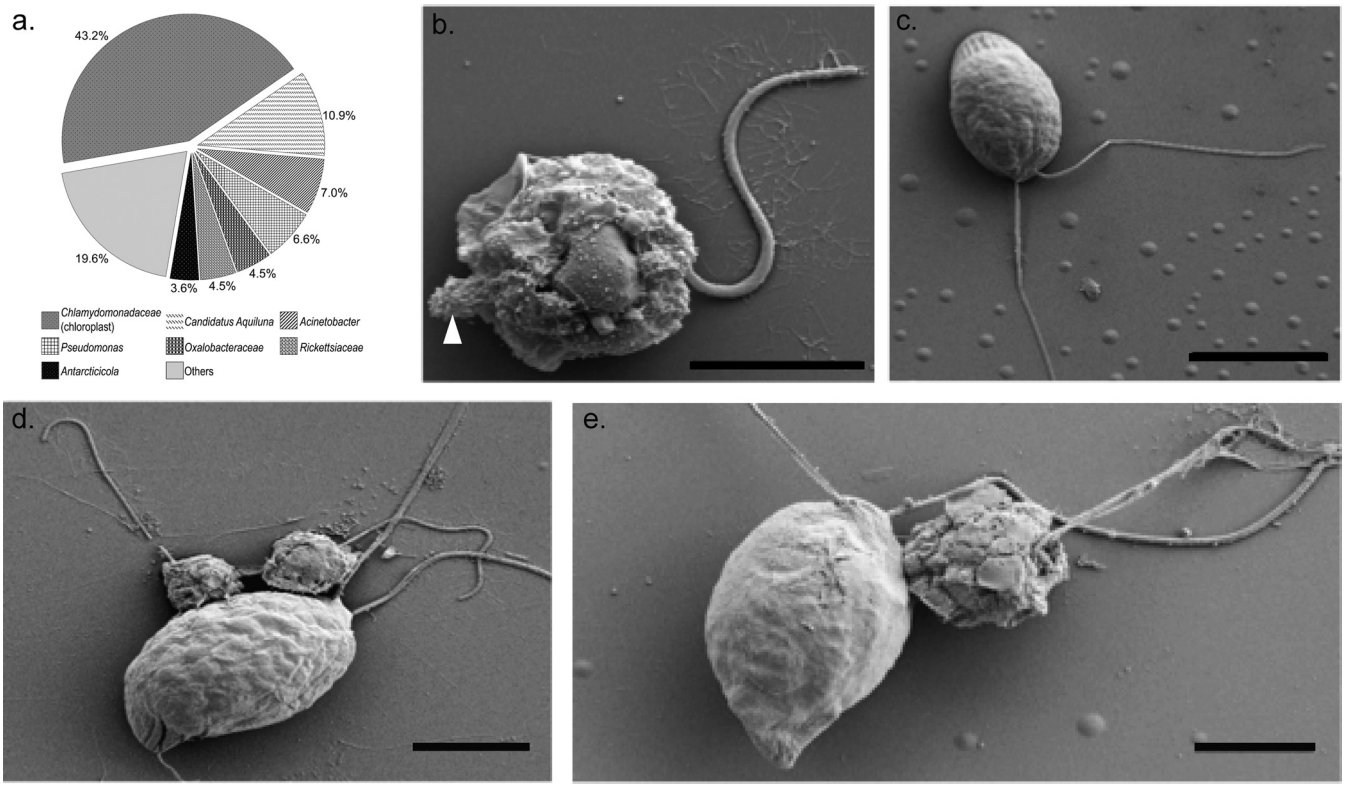

FIG 6 Evidence of parasite-host interactions among Lake Bonney microbial eukaryotes. (a) Chart showing the diversity of 16S rRNA gene OTUs recovered from *Pirsonia* eukaryote SAGs ($n = 22$). (b and c) SEM images showing the sizes of *Pirsonia* and *Chlamydomonas* sp. ICE-MDV, respectively. Arrowhead, extrusive organelle of *Pirsonia*. (d and e) SEM images showing *Pirsonia* cells attached to *Chlamydomonas* sp. ICE-MDV cells. Bars, 5 μm.

tial bacterial associations with the Lake Bonney phototrophic SAGs. First, a significant proportion of *Chlamydomonas* ICE-MDV single cells were associated with OTUs related to the Methylobacteriaceae. Members of this group are obligate methylotrophs that can oxidize a number of methylated compounds, including methanol and methylamines. These organisms are relatively abundant in marine environments, particularly during phytoplankton blooms (86, 87). Many marine microalgae produce a number of single-carbon compounds as osmolytes (88); however, less is known about alga-methylotroph interactions in freshwater environments (89). While the presence of methylotrophy in the MCM lakes has not been reported, algal production of osmolytes would be a likely adaptation for survival under harsh conditions such as low temperatures combined with high salinity.

In contrast to the *Chlamydomonas* sp. ICE-MDV SAGs, *Isochrysis* sp. MDV SAGs were not generally associated with methylotrophic bacterial sequences. Instead, the majority of haptophyte single cells were associated with a number of bacterial sequences from Flavobacteriaceae (see Fig. S3 in the supplemental material). In the marine environment, *Flavobacteria* represent dominant *Bacteroidetes* organisms, which break down complex organic matter and biopolymers by direct attachment to algal cells or alga-derived detritus (90, 91). Haptophyte blooms in the Southern Ocean are associated with peaks in abundance of *Flavobacteria* (92), and sequences related to *Flavobacteria* are highly abundant in the bacterioplankton communities in the chemocline of Lake Bonney (31). Our work supports a putative interaction between Lake Bonney haptophytes and *Flavobacteria*.

The relatively large number of bacterial OTUs associated with the phototrophic SAGs could also represent evidence of low levels of alga-bacterium coupling in the MCM lakes. Phytoplankton exudate quantity and composition are influenced by environmental factors, including nutrient limitations, light availability, and temperature (91, 93). The conditions of the MCM lakes (e.g., limited light and nutrients and low temperatures) might have selected for algal species that excrete a small fraction of alga-derived compounds, and thus bacterioplankton rely more heavily on exudate release during algal lysis or death. In support of the latter hypothesis, we have observed heavy recruitment and colonization of bacteria on dead and dying algal cells in our enrichment cultures, an interaction that would have been excluded in the FACS analysis. There is also evidence that natural bacterioplankton communities obtain a significant fraction of organic carbon from ancient relict pools of organic matter (94).

Conclusions. Microbial eukaryotes represent the majority of diversity across the eukaryotic tree of life and are capable of complex metabolic modes and interactions. While important in marine ecosystems, their influence reaches profound levels in aquatic systems in which the microbial loop dominates. In the current study, we discovered that the MCM protists possess diverse metabolic capabilities (from pure photosynthesis to mixotrophy, heterotrophy, and parasitism), which likely contributes to the strong vertical layering of key protist communities in the water column of Lake Bonney. The metabolic versatility of MCM protists underpins specific microbe-microbe interactions in some cases (e.g., *Pteridomonas*-chlorophytes), while interactions with hetero-

trophic bacteria do not appear to be an important survival strategy for other protists (e.g., haptophytes).

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