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Antibiotic resistance genes and taxa analysis from mat and planktonic microbiomes of Antarctic perennial ice-covered Lake Fryxell and Lake Bonney

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Abstract: The perennial ice-covered lakes of the Antarctic McMurdo Dry Valleys harbour oligotrophic microbial communities that are separated geographically from other aquatic systems. Their microbiomes include planktonic microbes as well as lift-off mat communities that emerge from the ice. We used the ShortBRED protein family profiler to quantify the antibiotic resistance genes (ARGs) from metagenomes of lift-off mats emerging from ice and from filtered water samples of Lake Fryxell and Lake Bonney. The overall proportion of ARG hits was similar to that found in temperate-zone rural ponds with moderate human inputs. Specific ARGs showed distinct distributions for the two lakes and for mat *vs* planktonic sources. Metagenomic taxa distributions showed that mat phototrophs consisted mainly of cyanobacteria or Betaproteobacteria, whereas the water column phototrophs were mainly protists. An enrichment culture of the Betaproteobacterium *Rhodoferax antarcticus* from a Lake Fryxell mat sample showed an unusual mat-forming phenotype not previously reported for this species. Its genome showed no ARGs associated with Betaproteobacteria but had ARGs consistent with a minor *Pseudomonas* component. The Antarctic lake mats and water showed specific ARGs distinctive to the mat and water sources, but overall ARG levels were similar to those of temperate water bodies with moderate human inputs.

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Key words: microbial mat, *Rhodoferax*, Taylor Valley

Introduction

The McMurdo Dry Valleys in Antarctica, one of Earth's coldest dry deserts, contain numerous ice-covered lakes with unique geochemistry and microbial communities (Spigel & Priscu 1998, Roberts *et al.* 2004, Cavicchioli 2015, Sohm *et al.* 2020). The microbial genomic analysis of these lakes remains limited (Koo *et al.* 2018, Dillon *et al.* 2020, W. Li *et al.* 2020). There are a number of reports on antibiotic resistance genes (ARGs) in Antarctic soil or marine bacteria (Tam *et al.* 2015, Wang *et al.* 2016, van Goethem *et al.* 2018, Na *et al.* 2019, Antelo *et al.* 2021) but few studies of ARGs in Antarctic lake water or their mat communities (Jara *et al.* 2020). Antibiotic biosynthesis and resistance are natural phenomena that predate the human discovery of antibiotics. Today, the human use of antibiotics has amplified the environmental prevalence of resistance. The prevalence of ARGs in relatively pristine habitats is of importance for human health, as it provides a baseline for assessment of human-associated ARG contamination (Allen *et al.* 2010, D'Costa *et al.* 2011). Antimicrobial resistance is a leading cause of death worldwide (Murray *et al.* 2022).

We report on the taxonomic composition and ARG prevalence in the water of Lake Fryxell and Lake Bonney and in the phototrophic mat communities of Lake Fryxell (Fig. 1a). Lake Fryxell extends along the Taylor Valley, 10 km downstream of Lake Bonney and 80 km across the Ross Sea from McMurdo Station. No permanent stream connects the two lakes; their main hydrology involves glacial meltwater, and each lake has an underground brine aquifer (Mikucki *et al.* 2015). Their food webs are entirely microscopic, with no fauna larger than nematodes. The lakes and their ecosystems are studied as models for ancient Mars (Head & Marchant 2014) and as indicators of climate change (Hall *et al.* 2017).

Lakes Fryxell and Bonney have permanent stratified layers in the water column that give rise to oligotrophic microbial communities (Roberts *et al.* 2004, Li *et al.* 2016, Kwon *et al.* 2017). The lack of turnover allows for

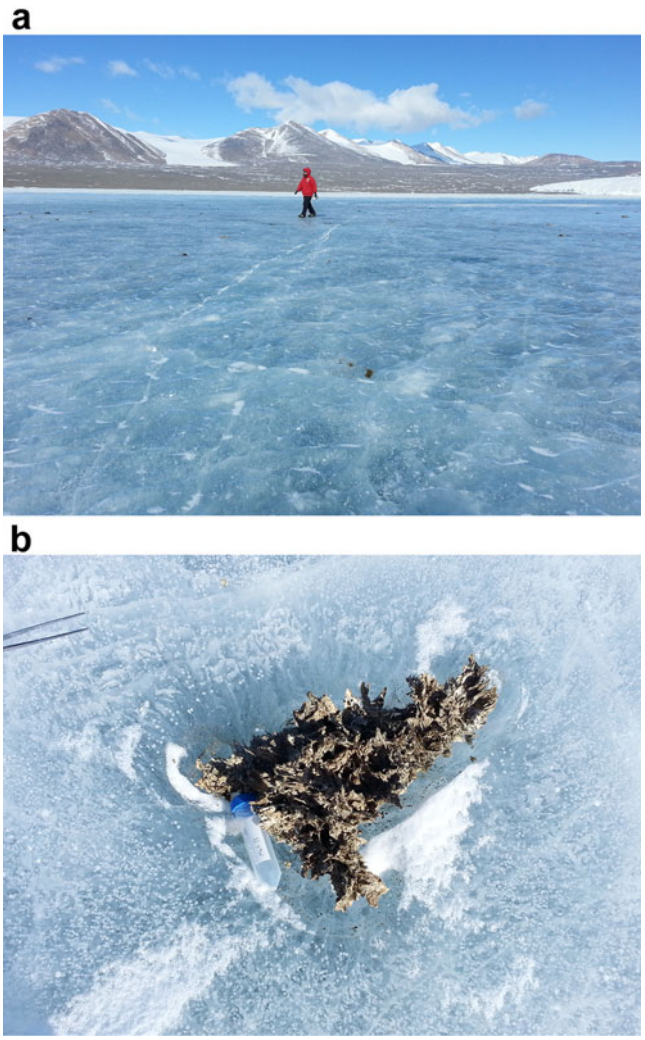

Fig. 1. Lake Fryxell uplift mats. **a.** Lake Fryxell with permanent ice cover, weathered by katabatic winds (10 December 2014). **b.** Source of Mat-4 DNA. Uplift mat emerges from the ice (~40 cm across).

the growth of benthic microbial mats that form flame-shaped towers several centimetres tall (Hawes *et al.* 2013, Jungblut *et al.* 2016). The microbial mats have been studied with primary emphasis on the role of cyanobacteria in mat morphology, such as *Microcoleus* and *Oscillatoria* (Taton *et al.* 2003, Jungblut *et al.* 2016). Metagenomes of lake water, however, reveal a high abundance of proteobacteria (Dillon *et al.* 2020) and protists (Bielewicz *et al.* 2011, Li *et al.* 2016). The Fryxell sedimentary biofilm includes a layer of phototrophic purple bacteria beneath the cyanobacterial layer (Buffan-Dubau *et al.* 2001).

The benthic cyanobacterial biofilms form 'lift-off' mats that reach upward, buoyed by oxygen bubbles derived from photosynthesis. Pieces break off and float upward to the under-surface of the ice cover (Parker *et al.* 1982, Moorehead *et al.* 1999). During winter, water freezes beneath the mat fragments, adding to the ice layer, but above the lake the valley's dry winds ablate the ice. Ice sections reveal six to eight annual layers of freezing below followed by ablation above (Parker *et al.* 1982), leading to exposure of the desiccated mats (Fig. 1b). Surviving several years trapped in ice, the mat material remains viable, and psychrophiles may continue growing (Boetius *et al.* 2015). When mat organisms reach the surface of ablated ice, they can blow off and enter perimeter 'moats' of melted ice that occur in summer, surrounding the persisting ice in the main part of the lake. The mat material carries communities that include microscopic arthropods and nematodes; thus, the mats can act as vectors for the transport of entire ecosystems (Parker *et al.* 1982, Brambilla *et al.* 2001, Dillon *et al.* 2020).

Molecular genetic data on McMurdo Dry Valley microbes remained limited until recently (Taton *et al.* 2003, Vick-Majors *et al.* 2014, Kwon *et al.* 2017). In December 2014 (i.e. during the summer), we obtained water samples from Fryxell and Bonney as well as desiccated mat samples emerging from the ice cover of Fryxell. For the present study, we applied tools of metagenome analysis to compare the taxonomic diversity and ARG abundance of mat and lake samples. We also sequenced the genome of a novel ecotype of *Rhodoferax antarcticus* from a mat-forming enrichment culture, whose phenotype differs markedly from planktonic isolates of this species (Madigan *et al.* 2000, Jung *et al.* 2004, Baker *et al.* 2017).

Our study addressed the important question of ARG prevalence in relatively pristine Antarctic lakes. A modest level of antibiotic resistance is an ancient, widespread phenomenon naturally and historically occurring in all environments (Allen *et al.* 2010, D'Costa *et al.* 2011). Non-anthropogenic processes can select for ARGs in pristine habitats; for example, cyanobacterial blooms drive increases in bacterial ARG prevalence and diversity (Zhang *et al.* 2020). But inputs of human origin can add substantially to the native ARG pool as well as add additional types of ARGs (Tam *et al.* 2015, Jara *et al.* 2020, Antelo *et al.* 2021). We surveyed our mat and water metagenomes for the presence of ARGs, referenced to the Comprehensive Antibiotic Resistance Database (CARD; Alcock *et al.* 2020).

Methods

Sample collection and culture

Microbial communities were sampled in December 2014 from two meromictic lakes of the Taylor Valley, Victoria Land, Antarctica. Lake Fryxell has a maximum depth of 20 m (Lawrence & Hendy 1985), while Lake Bonney has a depth of 40 m (Priscu & Spigel 1996). Both lakes are

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Table I. Physical, chemical and biological parameters from lakes Fryxell and Bonney (east lobe).

Site	Conductivity	Temperature	PAR	NH_4^+	SRP	Chlorophyll *a*
	(mS cm^{-1})	(°C)	(µmol m^{-2} s^{-1})	(µM)	(µM)	(µg/l)
Lake Fryxell, 9 m depth	3.23	2.67	1.67	1.04	0.08	6.85
Lake Bonney, 15 m depth	17.21	4.58	8.46	6.07	0.04	0.72

Data were retrieved from the McMurdo Dry Valleys Long-Term Ecological Research (LTER) programme (Priscu 2021a, 2021b, 2022a, 2022b).
PAR = photosynthetically active radiation; SRP = signal recognition protein.

covered by a perpetual ice layer that is ~4 m thick (Priscu 2018), although summer melting occurs near the shoreline.

Microbial lift-off mat samples (Mat-01–06) were collected from independent mat tufts emerging separately from the Lake Fryxell ice surface, within the GPS area of: -77.60491, 163.16315; -77.60473, 163.16290; -77.60463, 163.16405; -77.60495, 163.16495. Each sample consisted of a separate tuft of desiccated microbial mat, collected with alcohol-sterilized forceps and stored at -20°C (4 weeks) then at -80°C (indefinitely).

Lake water was sampled from the permanent chemoclines of Lake Fryxell (-77.605, 163.163, 9 m depth; samples FRY-01, -02, -03) and Lake Bonney, east lobe (-77.719, 162.283, 15 m depth; samples BON-01, -02, -03). Samples were obtained using ice holes established by the McMurdo Dry Valleys Long-Term Ecological Research (LTER) programme (Priscu 2021a, 2021b, 2022a, 2022b). The LTER geochemical data are presented in Table I. Water samples were collected in 5 l cubitainers pre-washed in 10% HCl. Each water sample was concentrated by filtration onto 47 mm Pall Supor® 450 polyethersulfone membranes (0.45 µm pore size; Pall Corporation, NY, USA).

Enrichment culture for anaerobic phototrophs was performed using Harwood photosynthetic medium (PM) supplemented with 10 mM succinate (Kim & Harwood 1991, Rey *et al.* 2006, Fixen *et al.* 2019). Screwcap Pyrex tubes were filled with medium and inoculated with ~0.05 g desiccated material from sample Mat-04. Sealed tubes were incubated under ~10% photosynthetically active radiation at 10°C for 5 weeks. Portions of biofilm were serially sub-cultured for 2 week periods, then frozen at -80°C. Gram staining was performed using standard methods (Remel Gram Stain Kit; ThermoFisher Scientific, MA, USA).

Testing for the growth range of pH and NaCl amendment was performed using a *Rhodoferax* medium modified from references (Tayeh & Madigan 1987, Madigan *et al.* 2000). The medium contained yeast extract (0.5 g/l), ethylenediaminetetraacetic acid (EDTA; 20 mg/l), malic acid (4 g/l), $(NH_4)_2SO_4$ (1 g/l), $MgSO_4{\cdot}7H_2O$ (200 mg/l), $FeSO_4{\cdot}7H_2O$ (12 mg/l), K_2HPO_4 (0.9 g/l), KH_2PO_4 (0.6 g/l), 10 mM sodium succinate and 100 mM 3-(*N*-morpholino)propanesulphonic acid (MOPS) adjusted to pH 7.0 with KOH. In addition, 1 ml/l of a trace element solution was added (H_3BO_3 (2.8 g/l), $MnSO_4{\cdot}H_2O$ (1.6 g/l), $Na_2MoO_4{\cdot}2H_2O$ (0.76 g/l), $ZnSO_4{\cdot}7H_2O$ (240 mg/l), $Cu(NO_3)_2{\cdot}3H_2O$ (40 mg/l), $CoCl_2{\cdot}6H_2O$ (200 mg/l)). For pH 6.0, the MOPS buffer was replaced with 100 mM 2-(*N*-morpholino)ethanesulphonic acid (MES) and for pH 8.0, the buffer was 100 mM (tris(hydroxymethyl) methylamino)propanesulphonic acid (TAPS). All buffered media were adjusted for pH with KOH. Growth of the enriched culture of *R. antarcticus* JLS was compared with culture of the type strain *R. antarcticus* ANT.BR obtained from the American Type Culture Collection (ATCC 700587).

DNA isolation and sequencing

Mat DNA was extracted using the PowerBiofilm kit (MO BIO, Carlsbad, CA, USA). From each sample, ~20 mg of material was extracted, and 0.75–1.50 µg DNA (measured using a Qubit fluorometer; ThermoFisher Scientific) was sent for sequencing. From lake water filters, 300–400 ng of DNA was obtained by extraction using an MP FastDNA SPIN DNA kit (MP Biomedicals, CA, USA) (Bielewicz *et al.* 2011). All metagenomic DNA was sequenced at the Department of Energy (DOE) JGI (Joint Genome Institute Community Science Program award 1936). Shotgun metagenomic library construction and sequencing were carried out at JGI using standard protocols on the Illumina (San Diego, CA, USA) HiSeq 2500 platform. The raw reads were quality filtered using JGI standard protocols, and 1.2 Tb of total sequences were obtained. Illumina reads were further processed using *Trimmomatic* (Bolger *et al.* 2014) to remove adapters and low-quality sequences. *Trimmomatic* counts the number of reads per *FASTQ* file, and it was used to determine the total reads per sample metagenome.

Cultured *Rhodoferax* biofilm DNA was isolated using the PowerBiofilm kit (MO BIO). Sequencing was performed by the Michigan State University Genomics Core. Libraries were prepared using the Illumina TruSeq Nano DNA Library Preparation Kit. Sequencing was performed on an Illumina MiSeq done in a 2 × 250 bp format using an Illumina 500 cycle v2 reagent cartridge. The raw reads were quality filtered and 1 Tb of total sequences was obtained.

Identification of ARGs by ShortBRED

ShortBRED (Kaminski *et al.* 2015) is a pipeline that identifies protein family sequences by generating specific

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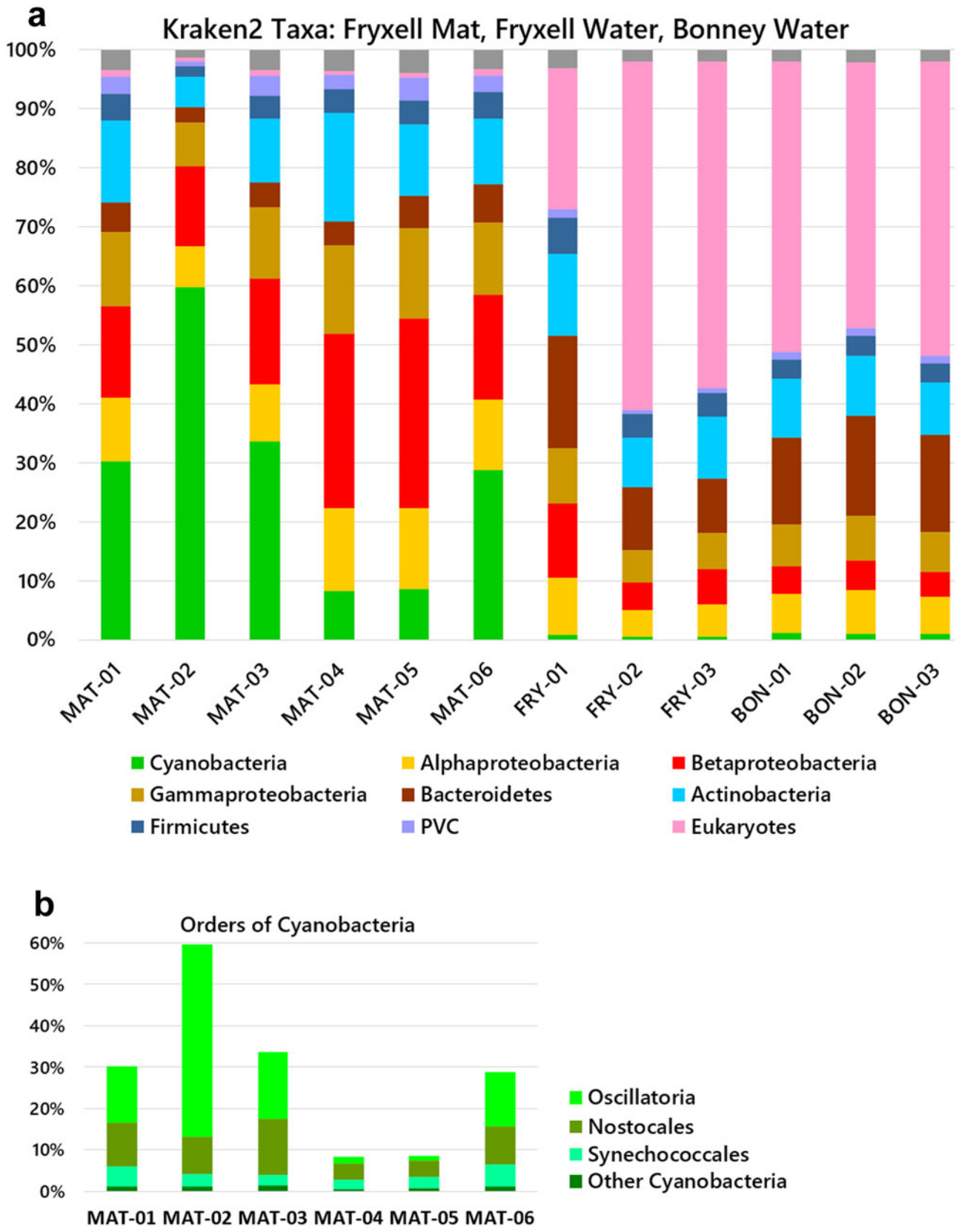

Fig. 2. Microbial community compositions of the mat and water samples. Relative abundances of phyla were determined by read alignment to marker genes using the Kraken 2-Bracken pipeline. **a.** Phyla and classes. **b.** Orders of cyanobacteria in mat samples. MAT (Lake Fryxell, mat samples); FRY (Lake Fryxell, water samples); BON (Lake Bonney, water samples). MAT samples were from the ice surface and unfiltered. The water samples were collected on a 0.45 μm filter. PVC = Planctomycetes-Verrucomicrobia-Chlamydiae.

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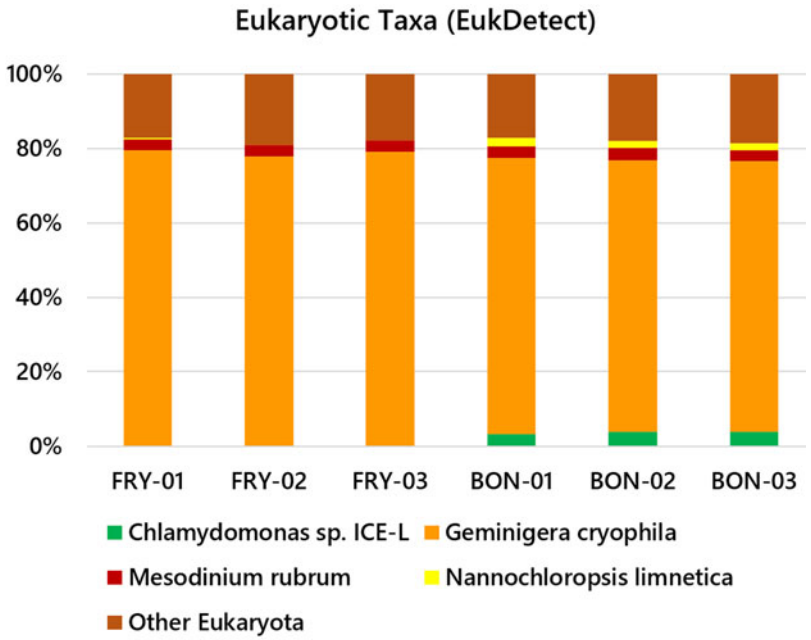

Fig. 3. Eukaryotic taxa classified by EukDetect. Relative abundances of protist taxa were determined by read alignment to marker genes using EukDetect (Lind & Pollard 2021).

peptide markers from a database of interest, then screening the markers for specificity against the UniProt universal database of protein sequences (Bateman *et al.* 2021). The *shortbred_identify* command scans a reference database for protein family-specific peptide sequences. It was used to scan UniRef (the UniProt protein reference database) for sequences of the ARGs in version 3.0.7 of the CARD (Alcock *et al.* 2020). The minimum marker length accepted was 8 amino-acid residues, and the maximum length for combined markers for a given protein was 200. Markers that matched non-specific genes in UniProt were discarded from the marker dataset.

The ShortBRED marker set was then used by the *shortbred_quantify* command to assign ARGs in the 150 kb reads from lake water and mat samples. From each target read, six reading frames were translated using *tblastn*. A marker hit required a 95% match to the target. Sample class differences for ARG abundance were tested for significance ($P \leq 0.05$) using the Kruskal-Wallis test.

Taxa classification of metagenomic reads

The 150 kb sequence reads from mat and water metagenomes were assigned to taxonomic classes using the Kraken 2 classifier pipeline (Wood & Salzberg 2014, Wood *et al.* 2019). Kraken associates genomic *k*-mers (short sequence strings) in the reads with the lowest common ancestor taxa. The read classifications were then used to calculate taxa percentages via the Bracken abundance estimator (Lu *et al.* 2017). The *k*-mer length was set at 150. For the Kraken 2-Bracken pipeline, the reference database used was the 5/17/2021 Standard Collection accessed at https://benlangmead.github.io/aws-indexes/k2.

Eukaryotic taxa were further characterized using the pipeline EukDetect (Lind & Pollard 2021). This pipeline assigns reads to a database of 521,824 marker genes from 241 gene families out of 3713 genomes and transcriptomes of fungi, protists and invertebrates. EukDetect identified protist species such as *Geminigera cryophila* (van den Hoff *et al.* 2020), *Mesodinium rubrum* (Yih *et al.* 2004), *Nannochloropsis limnetica* (Kong *et al.* 2012) and *Chlamydomonas* sp. ICE-L (Lizotte *et al.* 1996).

For an alternative classifier, mat and water metagenomes were mapped to core taxonomic markers using the MetaPhlAn2 pipeline (Segata *et al.* 2012, Truong *et al.* 2015). MetaPhlAn2 assigns short reads to taxa using a set of marker genes identified from ~17,000 microbial reference genomes, primarily bacteria and archaea.

Genome assembly by breseq

The pipeline breseq 0.35.6 (Deatherage & Barrick 2014) was used to assemble reads from two samples of the enrichment culture obtained from Mat-04. The reads were mapped to the *R. antarcticus* ANT.BRT (DSMZ24876) reference genome (Baker *et al.* 2017).

Results

Taxa abundance of mat and water samples

Metagenomes were sequenced from three microbial sources: the water columns of Lake Fryxell (FRY-01, -02, -03) and Lake Bonney (BON-01, -02, -03) and the lift-off mat from the ice surface of Lake Fryxell (Mat-01–06). A total of 4 billion reads were sequenced, with a range of

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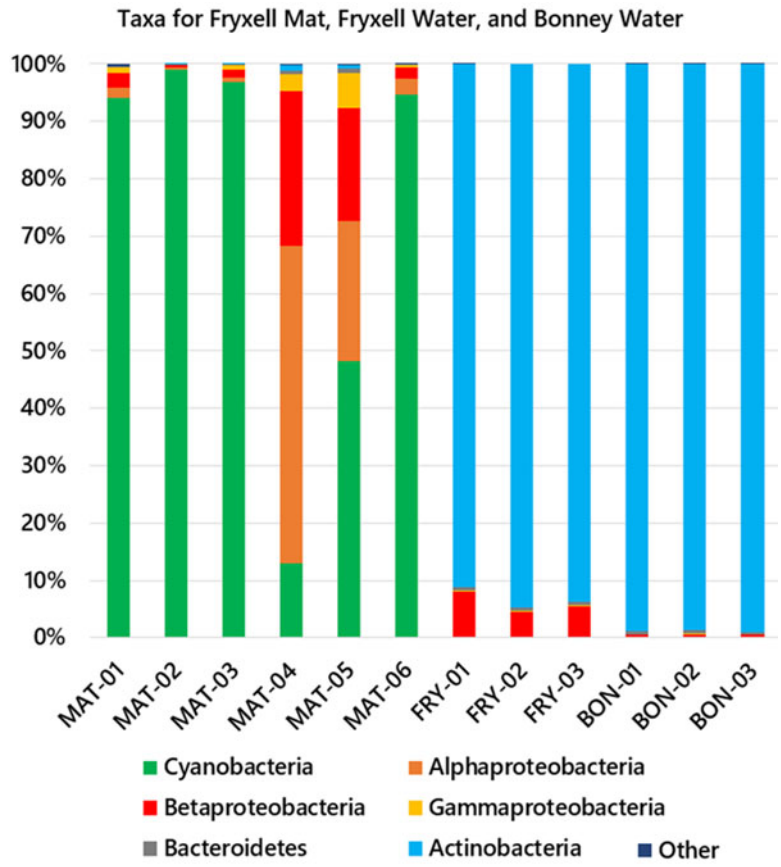

Fig. 4. Microbial community compositions via MetaPhlAn2. Relative abundances of class-level taxa were determined by read alignment to marker genes using MetaPhlAn2 (Segata *et al.* 2012, Truong *et al.* 2015). Samples were as indicated for Fig. 2.

between 210 and 420 million reads per metagenome. The average read length was 147 bp (SD = 26).

From the three groups of samples, we characterized the taxa abundance using the Kraken 2-Bracken pipelines. The ice-surface mat samples (Mat-01–06) showed substantial amounts of cyanobacteria, ranging from 9% to 60% of the total (Fig. 2a). The predominant orders of cyanobacteria were Oscillatoriales and Nostocales (Fig. 2b). A high abundance of Oscillatoriales and Nostocales is consistent with previous reports for Fryxell mats obtained from benthic samples (cited in the 'Introduction' section). For Mat-01, Mat-02, Mat-03 and Mat-06, the cyanobacterial assignments were primarily Oscillatoriales and Nostocales. Mat-04 and Mat-05, however, showed depletion of Oscillatoriales, with a predominant abundance of Betaproteobacteria. Throughout the six mat samples, other taxa with significant abundance included Actinobacteria, Bacteroidetes and Alphaproteobacteria, with smaller abundances of Planctomycetes, Bacteroidetes and Firmicutes. Thus, the ice-trapped, air-exposed community showed a composition remarkably similar to that of the benthic mat samples from which lift-off mats originate.

The filtered water from both lakes Fryxell and Bonney contained abundant bacterial taxa of Actinobacteria, Bacteroides, Betaproteobacteria and Alphaproteobacteria, similarly to previous reports of Fryxell water and other glacier-associated water bodies (see the 'Introduction' section). The water column showed almost no cyanobacteria but a high proportion of eukaryotes, in some cases > 50% of the reads classified by Kraken 2-Bracken. By contrast, the mat samples showed virtually no detectable eukaryotic DNA. The one-sided Mann-Whitney *U* test confirms that water from each lake contains more eukaryotic DNA than the mat samples ($P = 0.01$) and that the mat samples contain more cyanobacteria than the water microbiomes of either lake ($P = 0.01$).

To classify the eukaryotes, we used EukDetect, a pipeline that matches short read data to marker genes from fungal, protist and invertebrate genomes (Fig. 3; full output provided in Table S1). In the lake water, EukDetect identified the protist species *G. cryophila*, *M. rubrum*, *N. limnetica* and *Chlamydomonas* sp. ICE-L. The two lakes showed similar taxa profiles, except that *Chlamydomonas* was found only in Lake Bonney. The mat samples had too few eukaryotic read counts to classify them.

The validity of taxa classifier pipelines is highly dependent on their algorithm and taxa database. For comparison with the Kraken 2-Braken output, our mat and water reads were mapped to core taxonomic markers using an alternative pipeline, MetaPhlAn2 (Fig. 4). Four of the six mat samples showed mainly cyanobacteria, consistent with longstanding reports of

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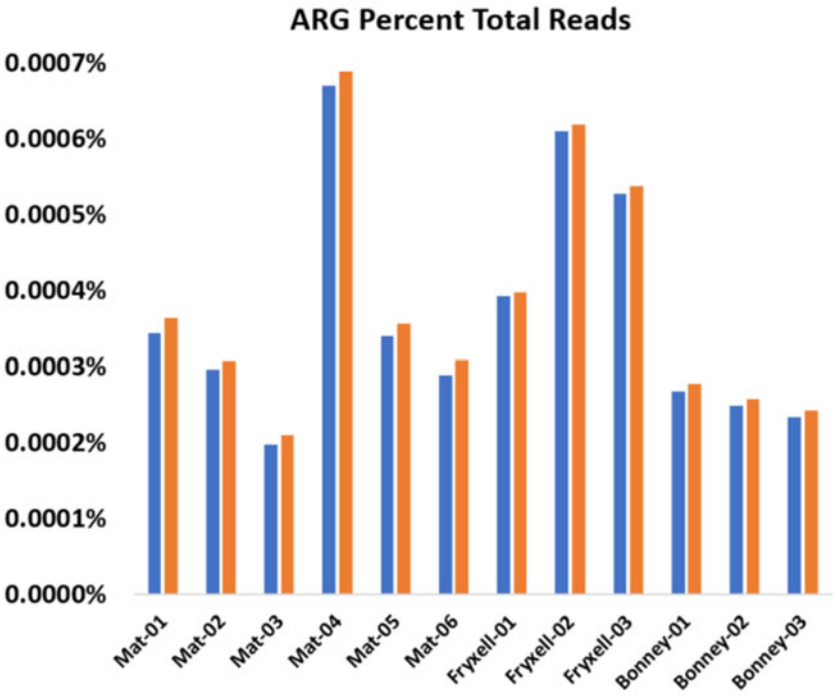

Fig. 5. Antibiotic resistance gene (ARG) abundances in lake samples. Percentages of reads matched to ShortBRED markers. Total reads were counted using *Trimmomatic*. Bars indicate total ARG hits per sample (orange) and top 60 ranked ARG hits (blue).

cyanobacterial mats (see the 'Introduction' section). Consistent with the Kraken 2 analysis, the Mat-04 and Mat-05 samples showed major amounts of Betaproteobacteria and Alphaproteobacteria, with depletions of cyanobacteria. For the filtered water samples, the MetaPhlAn2 markers include a limited content of Eukarya, and the pipeline did not find the eukaryotic taxa predicted by Kraken 2 (Fig. 2). The filtered water from both lakes showed mainly Actinobacteria, with some Betaproteobacteria. Overall, MetaPhlAn2 appeared less effective than Kraken 2-Bracken at classifying our samples.

ARG composition characterized using ShortBRED

Gene sequences encoding various forms of antibiotic resistance, including genomic loci as well as mobile elements, are collected in the CARD database. We used the ShortBRED pipeline to identify ARGs in our samples based on a set of marker peptides matching CARD sequences. The percentage of reads from each sample that matched ARGs ranged from 0.0001% to 0.0006% (Fig. 5). The overall ARG abundances were similar amongst the three sample classes (Fryxell mat, Fryxell water, Bonney water).

The specific ARGs identified by ShortBRED were sorted by abundance across samples (Tables II & S2). Eight of the top 20 sorted ARGs showed a difference among the three sample classes (Kruskal-Wallis test, $P \leq 0.05$); of these results, seven of eight are significant. Of the top-ranked ARGs, matches to CARD families BUT-1 and vancomycin resistance genes *vanYA*, *vanTG* and *vanI* appeared mainly in the water column. BUT-1 is a class-C beta-lactamase reported in *Buttiauxella agrestis*, an environmental Gammaproteobacterium found in environmental water sources. The vancomycin resistance genes occur in *Enterococcus* and other Gram-positives. By contrast, matches to the beta-lactamase AAC(3)-Ia occurred only in the mat samples. AAC(3)-Ia is encoded on *Pseudomonas aeruginosa* integrons as well as in other Proteobacteria. Other ARGs showed possible differences in prevalence between Lake Bonney and Lake Fryxell water; for example, matches to the *Streptomyces*-associated beta-lactamase AAC(3)-VIIa were more abundant in Bonney water, whereas *vanYA* matches were more abundant in Fryxell water.

Overall, the three sample classes each had distinctive ARG-abundance signatures, as indicated by Kruskal-Wallis tests. Ten of the top 20 ranked ARGs were associated with Proteobacteria, which corresponds approximately with the proportion of bacterial taxa abundances predicted by Kraken 2-Bracken (Fig. 2).

Enrichment of mat-forming R. antarcticus

The relatively high proportions of Betaproteobacteria and Alphaproteobacteria in Mat-04 differed from those of other samples, and the colour of the sample material was more red than green. We cultured the various samples anaerobically (in closed tubes) at 10°C with illumination. From Mat-04, red spots of biofilm were obtained. There was no evidence of planktonic growth

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Table II. Mat and water antibiotic resistance genes (ARGs) identified using ShortBRED marker peptides.

ARG family	MAT-01	MAT-02	MAT-03	MAT-04	MAT-05	MAT-06	FRY-01	FRY-02	FRY-03	BON-01	BON-02	BON-03	Total	*P*
ARO_3004294 BUT-1 *Buttiauxella agrestis*	0	1	2	0	0	0	554	1720	967	266	262	323	4095	0.027
ARO_3002541 AAC(3)-VIIa *Streptomyces rimosus*	89	235	114	173	152	54	44	31	31	323	313	231	1790	0.038
ARO_3002528 AAC(3)-Ia *Pseudomonas aeruginosa*	302	189	131	492	238	196	0	0	0	0	0	0	1548	0.022
ARO_3003167 CTX-M-159 *Proteus mirabilis*	36	9	25	1032	111	18	1	0	0	0	0	3	1235	0.052
ARO_3001461 OXA-156 *Pandoraea pulmonicola*	43	18	75	146	70	36	14	15	19	70	60	53	619	0.099
ARO_3002894 otrC *Streptomyces rimosus*	40	122	16	27	13	23	162	73	81	11	20	10	598	0.079
ARO_3003599 OXA-443 *Ralstonia mannitolilytica*	41	260	13	42	33	16	64	23	12	2	3	4	513	0.061
ARO_3001338 SHV-100 *Klebsiella pneumoniae*	58	1	49	144	56	44	4	1	5	10	6	11	389	0.241
ARO_3002955 vanYA *Enterococcus faecium*	0	3	2	1	1	0	101	59	47	19	29	31	293	0.027
ARO_3001855 ACT-35 *Enterobacter cloacae*	4	7	26	32	37	5	30	22	22	40	22	30	277	0.214
ARO_3000410 sul1 *Vibrio fluvialis*	72	0	32	19	34	62	1	0	0	0	0	0	220	0.199
ARO_3003022 dfrB3 *Klebsiella oxytoca*	13	4	9	19	23	8	54	21	18	9	12	18	208	0.069
ARO_3002972 vanTG *Enterococcus faecalis*	1	0	1	0	2	0	10	9	13	50	53	55	194	0.027
ARO_3001517 OXA-329 *Acinetobacter calcoaceticus*	2	2	1	2	0	1	76	52	24	1	15	5	181	0.054
ARO_3002701 Rfas_cmr *Rhodococcus fascians*	6	5	17	22	19	2	39	47	16	3	2	2	180	0.038
ARO_3003723 vanI *Desulfitobacterium hafniense*	0	0	0	0	0	0	53	50	40	9	16	10	178	0.024
ARO_3003942 abcA *Aspergillus fumigatus*	31	75	2	9	17	20	0	0	0	7	4	5	170	0.055
ARO_3003583 basS *Pseudomonas aeruginosa*	12	1	30	8	7	12	1	0	0	47	32	19	169	0.046
ARO_3004652 Erm(O)-lrm *Streptomyces lividans*	28	8	16	37	35	10	8	7	4	5	2	5	165	0.061
ARO_3001299 tlrB *Streptomyces fradiae*	18	8	10	30	26	8	2	2	0	18	18	22	162	0.034

Sample class differences for ARG abundance were tested for significance ($P \leq 0.05$) using the Kruskal-Wallis test. Colour-shaded rows represent sample classes for which the given ARG shows significant difference in abundance amongst the Mat, Lake Fryxell and/or Lake Bonney samples. Under the *P* column, yellow highlighting represents significant difference.

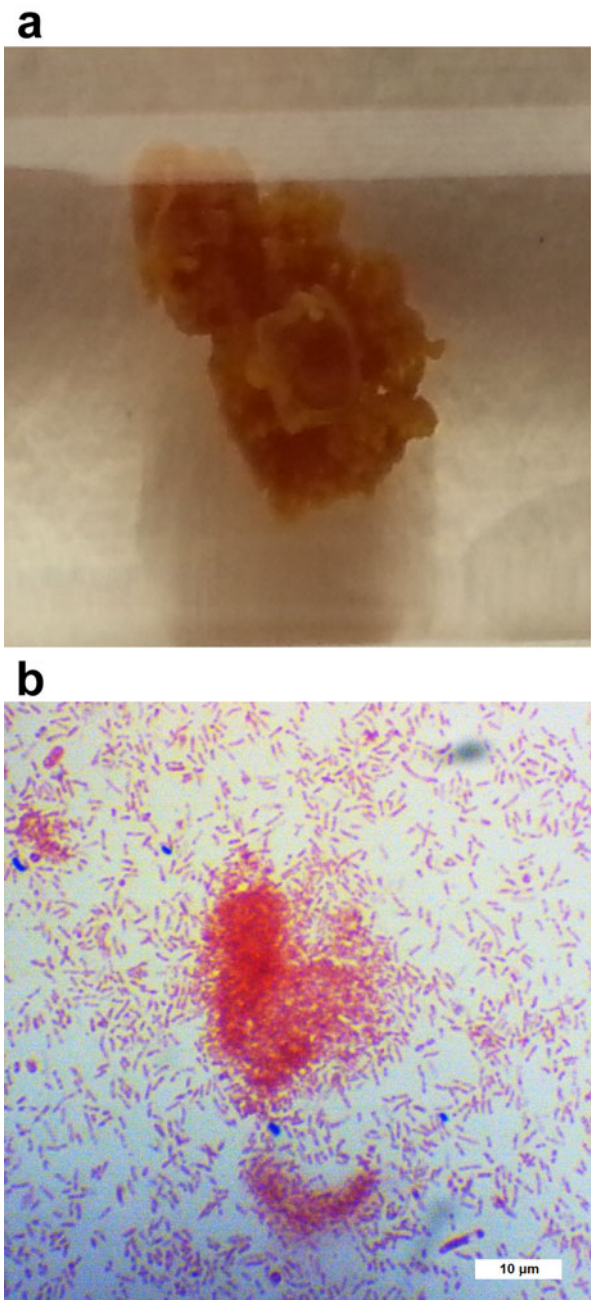

Fig. 6. Mat-forming enrichment of Mat-04 *Rhodoferax antarcticus* JLS. **a.** Biofilm of cultured *R. antarcticus* within a closed tube of medium, showing a globular form of growth. The initial Mat-04 sample was sub-cultured serially three times in Harwood photosynthetic medium in horizontal closed tubes at 10°C with 30 µmol photons m^{-2} s^{-1} illumination. **b.** Gram stain of culture, 1000× with oil immersion. Courtesy of Emma Stuart-Bates.

(cloudiness) or cell motility within the culture medium, but the organism grew as a mat, forming red blobs with a 'red nose' appearance. In addition, the culture extended as a red film-like mat growing along the inside of the culture tube (Fig. 6a). It was not possible to obtain isolated colonies, but serial culture of the 'red noses' showed consistent biofilm formation and Gram-stain morphology (Fig. 6b). This finding is consistent with Buffan-Dubau *et al.*'s (2001) report of a layer of phototrophic purple bacteria beneath the cyanobacterial layer. Ours is the first report of such a biofilm cultured from material at the ice surface rather than from its inferred origin in the sediment at the bottom of the lake.

The Mat-04 biofilm culture was subjected to genome analysis. DNA was sequenced and short reads were used to BLAST the National Center for Biotechnology Information (NCBI) database. The predominant hits were to the genome of *R. antarcticus* ANT.BRT, a Betaproteobacterium originally isolated by Madigan *et al.* (2000) from a saltwater pond at Cape Royds, Ross Island, Antarctica. Kraken 2-Bracken analysis of sequenced reads agreed with this classification, assigning the samples as 95% *R. antarcticus* with ~1% *Pseudomonas.*

We used the breseq pipeline to assemble reads from two non-axenic samples (7-RN1 and 8-RN2) whose DNA sequence reads were assembled to the *R. antarcticus* ANT.BRT reference genome (Tables S3 & S4). For both samples, 7-RN1 and 8-RN2, 78% of reads matched the ANT.BRT reference genome. Tables S3 & S4 present 'mutations'; that is, all of the differences from the ANT.BR reference sequence that show 100% read coverage. The two samples, 7-RN1 and 8-RN2, showed 276 and 271 sequence differences, respectively, in their main chromosomes compared with that of Baker *et al.*'s (2017) reference genome (length 3,809,266 bp). Their 16S rRNA sequences showed 100% identity in breseq output. The sample genomes showed average nucleotide identity values of > 99.99% identity with the main chromosome and > 99% identity with one plasmid in the reference genome. A large part of the observed sequence differences from the reference genome were silent mutations. These observations confirm the close relatedness of our culture to the published strain.

The *R. antarcticus* enrichment culture (designated *R. antarcticus* JLS) was tested for ARG abundance using ShortBRED (Table III). More than 30 ARGs showed hits, but nearly all were associated with *P. aeruginosa*. By contrast, no ARGs matched those from CARD database that were associated with *Rhodoferax*.

Characterization of the mat-forming R. antarcticus *JLS*

The *R. antarcticus* JLS biofilm was further tested for growth range in terms of pH and salinity. The biofilm was sub-cultured anaerobically with illumination in a malate-succinate *Rhodoferax* medium modified from that of references as described in the 'Methods' section (Fig. 7a–c). After 45 days, at pH 7, the bacteria formed globules as well as a red coating along the glass. At pH 6, spots of biofilm grew slowly, and at pH 8 little growth

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Table III. *Rhodoferax* enrichment culture antibiotic resistance genes identified using ShortBRED.

Culture A: *Rhodoferax* (95.0%), *Pseudomonas* (1.5%)	Hits	Marker length	Culture B: *Rhodoferax* (95.0%), *Pseudomonas* (0.7%)	Hits	Marker length
ARO_3003679 TriA *Pseudomonas aeruginosa*	8	242	ARO_3003680 TriB *Pseudomonas aeruginosa*	3	144
ARO_3001796 OXA-50 *Pseudomonas aeruginosa*	4	116	ARO_3003031 mexW *Pseudomonas aeruginosa*	1	56
ARO_3003681 TriC *Pseudomonas aeruginosa*	3	113	ARO_3002320 KPC-10 *Acinetobacter baumannii*	1	66
ARO_3003710 mexL *Pseudomonas aeruginosa*	3	134	ARO_3003583 basS *Pseudomonas aeruginosa*	1	290
ARO_3003030 mexV *Pseudomonas aeruginosa*	3	176	ARO_3004777 CMH-1 *Enterobacter cloacae*	1	25
ARO_3002507 PDC-8 *Pseudomonas aeruginosa*	3	155	ARO_3003705 mexN *Pseudomonas aeruginosa*	1	63
ARO_3000802 OprJ *Pseudomonas aeruginosa*	2	36	ARO_3000808 mexI *Pseudomonas aeruginosa*	1	118
ARO_3003680 TriB *Pseudomonas aeruginosa*	2	144	ARO_3004054 CpxR *Pseudomonas aeruginosa*	1	26
ARO_3000379 OprM *Pseudomonas aeruginosa*	2	35			
ARO_3000805 OprN *Pseudomonas aeruginosa*	2	102			
ARO_3002985 arnA *Pseudomonas aeruginosa*	2	102			
ARO_3003692 mexJ *Pseudomonas aeruginosa*	2	120			
ARO_3003031 mexW *Pseudomonas aeruginosa*	2	56			
ARO_3004038 emrE *Pseudomonas aeruginosa*	2	51			
ARO_3003705 mexN *Pseudomonas aeruginosa*	2	63			
ARO_3003698 mexP *Pseudomonas aeruginosa*	2	85			
ARO_3002645 APH(3)-IIb *Pseudomonas aeruginosa*	2	153			
ARO_3000377 MexA *Pseudomonas aeruginosa*	2	106			
ARO_3001214 mdtM *Escherichia coli*	1	71			
ARO_3004077 PmpM *Pseudomonas aeruginosa*	1	109			
ARO_3000804 MexF *Pseudomonas aeruginosa*	1	102			
ARO_3004072 OpmB *Pseudomonas aeruginosa*	1	111			
ARO_3004075 MuxC *Pseudomonas aeruginosa*	1	177			
ARO_3003693 mexK *Pseudomonas aeruginosa*	1	44			
ARO_3000809 opmD *Pseudomonas aeruginosa*	1	116			
ARO_3003682 OpmH *Pseudomonas aeruginosa*	1	79			
ARO_3002695 cmlA5 uncultured bacterium	1	99			
ARO_3003583 basS *Pseudomonas aeruginosa*	1	290			
ARO_3000795 mdtE *Escherichia coli*	1	54			
ARO_3004612 ampH *Escherichia coli*	1	68			

For enrichment cultures A and B, taxa proportions were estimated using the Kraken 2-Bracken pipeline.

was seen. Growth was also tested for cultures buffered at pH 7 with NaCl amendment (Fig. 7d–f). The fullest growth was seen in the absence of NaCl amendment (the core medium contains ~10 mM Na^+ ions). Less growth was seen with added NaCl (23 or 46 mM).

Despite the high sequence identity, the *R. antarcticus* JLS enrichment culture differed from Madigan *et al.*'s (2000) *R. antarcticus* ANT.BR strain in its growth phenotype, under all conditions of pH and NaCl concentration that were tested for both strains. Unlike the motile, planktonic *R. antarcticus* ANT.BR, our culture showed no motility and little sign of planktonic single-celled growth. Instead, the sub-cultured material grew entirely as a biofilm, in globular spots and as a film-like growth along the interior surface of the glass tube.

The type strain ANT.BR was cultured at the same time, under all conditions. The original strain never formed a biofilm; it appeared planktonic and motile under all conditions of pH and NaCl concentration.

Discussion

Identification of ARGs from relatively pristine environments is of interest for several reasons. Long before the human introduction of high-dose antibiotics, environmental bacteria evolved multidrug pumps to efflux toxic products of their own metabolism, as well as antimicrobial substances produced by their competitors (Allen *et al.* 2010, Wright 2019). Many antibiotics possess signalling capabilities and other unknown functions. Phylogeny dates the origin of beta-lactamases to hundreds of millions of years ago. Vancomycin resistance genes are found in 30,000 year-old permafrost (D'Costa *et al.* 2011). But the specific kinds of ARGs found in environmental sources may differ from those prevalent in human microbiomes - those conferring resistance to the drugs we depend on for therapy (Zeng *et al.* 2019).

In our Taylor Valley lake metagenomes, the top-scoring ARG was BUT-1, a cephalosporinase related to sequences previously found in a clinical isolate of *Buttiauxella* (Fihman *et al.* 2002). Two other ARGs related to those of clinical origin (*vanYA*, *vanTG*) encode vancomycin resistance components in *Enterococcus* (Boyd *et al.* 2006, Courvalin 2006). The possible finding of clinical ARGs in Antarctic water bodies is concerning. The rest of the top 20 ARGs we found appear common in environmental organisms. Eight were beta-lactamases,

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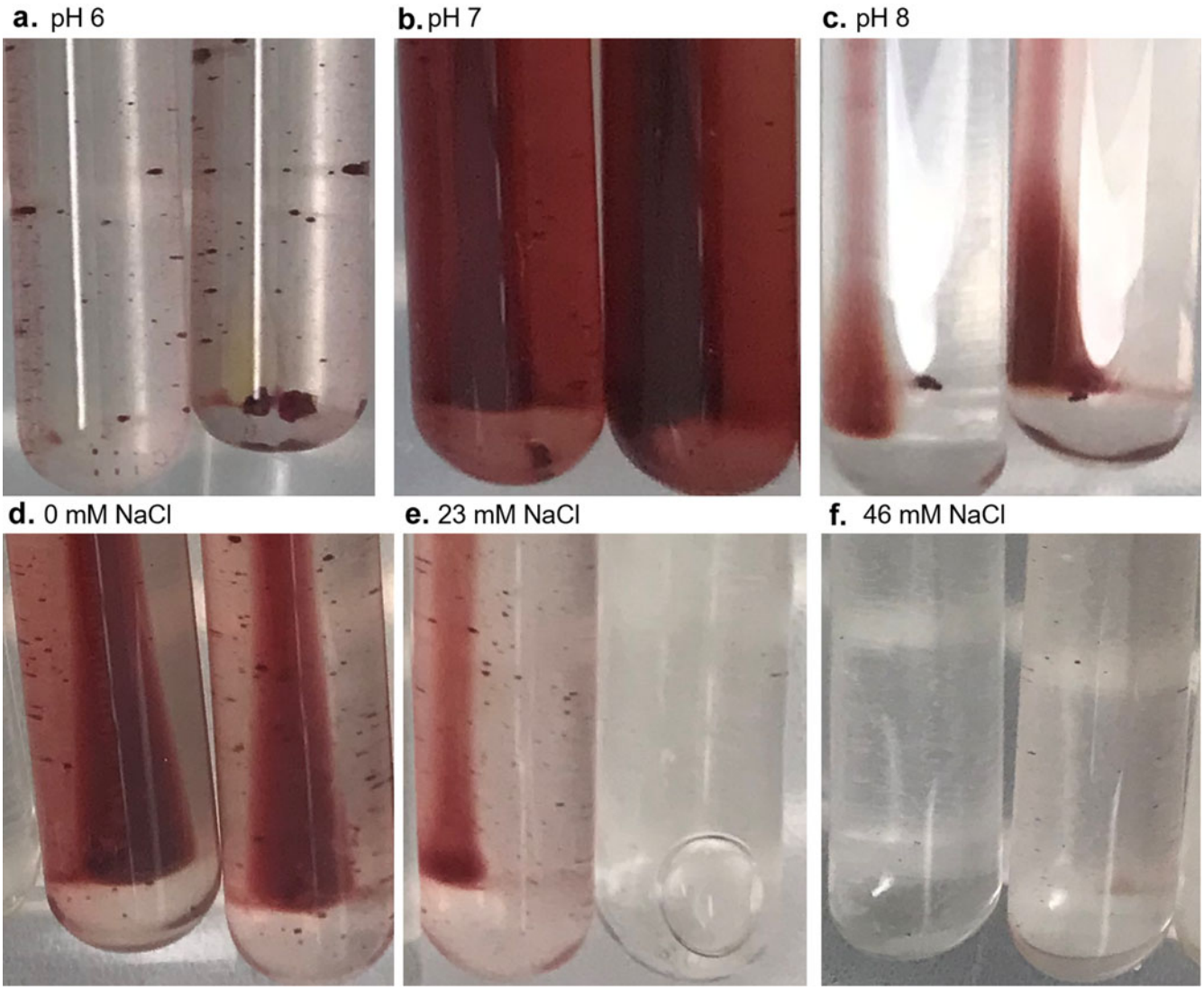

Fig. 7. pH and NaCl dependence of *Rhodoferax antarcticus* JLS. **a.–c.** Harwood photosynthetic medium containing **a.** 100 mM 2-(*N*-morpholino)ethanesulphonic acid (MES) pH 6, **b.** 100 mM 3-(*N*-morpholino)propanesulphonic acid (MOPS) pH 7 and **c.** 100 mM (tris(hydroxymethyl)methylamino)propanesulphonic acid (TAPS) pH 8. Cultures were incubated for 45 days. **d.–f.** PM medium amended with **d.** no NaCl added, **e.** 23 mM NaCl and **f.** 46 mM NaCl. Cultures were incubated for 45 days.

which are commonly found in environmental organisms but can also be readily transferred between environmental and pathogenic strains (Hooban *et al.* 2020).

In polar regions, previous metagenomic studies reveal a range of naturally occurring ARGs. Surface soils of the Antarctic Mackay Glacier region show ARGs encoding multidrug pumps, beta-lactamases and aminoglycoside inactivators, largely associated with Gram-negative bacteria (van Goethem *et al.* 2018). A study of Tibetan soils showed a number of abundant ARGs, most notably encoding vancomycin resistance (B. Li *et al.* 2020). Our study of ARGs from Antarctic lakes adds to this picture, showing that both water and microbial mat sources contain familiar ARGs, and probably contain others not yet discovered in Antarctic strains.

It was interesting to compare the total ARG abundance of our Antarctic lake samples with those of temperate-zone Ohio rural water bodies with moderate human inputs from a study in which samples were prepared using the same method and analysed using the same ShortBRED marker set (Murphy *et al.* 2021). The overall ARG abundance is approximately the same for the Antarctic *vs* Ohio environmental sources, except for river samples obtained just downstream of a wastewater effluent pipe, where the total ARG prevalence is increased approximately five-fold. This result is consistent with a model that natural microbial communities generally

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harbour a small, balanced prevalence of ARGs, which gets amplified by concentrated human input. Note, however, that a database such as CARD can only represent a fraction of the actual ARGs out there; new drug resistance families and related mobility agents are continually being discovered.

The mat lift-off samples we obtained from the ice surface showed a range of DNA taxa consistent with those reported for samples obtained from benthic mats (Dillon *et al.* 2020). Given that our collected mat organisms had survived several years within ice, followed by air desiccation and prolonged ultraviolet radiation exposure, it is impressive how many of the Antarctic mat microbiomes retain viability with intact DNA. Even ciliated protists and invertebrate worms were obtained alive from samples cultured after months of storage at -80°C (not shown). It is probable that some of these organisms are psychrophiles that continue to grow within the ice (Boetius *et al.* 2015).

Previous studies emphasize the cyanobacterial content of lift-off mats, primarily Oscillatoriales genera such as *Microcoleus*, as well as *Nostoc* (Taton *et al.* 2003, Jungblut *et al.* 2016). While most of our lift-off samples showed an abundance of cyanobacteria, one sample yielded cultures from which the majority of reads matched *R. antarcticus*. The finding of mat samples enriched for *R. antarcticus* indicates that portions of the lower layer of the *Rhodoferax* mat (Buffan-Dubau *et al.* 2001), along with the cyanobacterial upper layer, can break off and form lift-off patches that emerge from the ice. Our culture of *R. antarcticus* (*R. antarcticus* JLS) obtained from Lake Fryxell showed a mat-forming morphology that was very different from the motile single cells of *R. antarcticus* ANT.BR isolated from Cape Royds (Madigan *et al.* 2000, Baker *et al.* 2017). Despite the high genetic similarity, our cultured organism appears to represent a novel ecotype of *R. antarcticus*.

Our genomic reads from *R. antarcticus* JLS showed no matches to our ShortBRED antibiotic resistance markers, although the reference genome does indeed include various resistance genes including numerous resistance-nodulation-cell division (RND) and major facilitator superfamily (MFS) transporters as well as multidrug efflux components. Thus, many naturally occurring ARGs are likely to be missed by standard marker searches.

The prevalence of eukaryotic sequences in the lake water metagenomes is consistent with previous reports that protists play important roles in the Taylor Valley lake communities (Lizotte *et al.* 1996, Glatz *et al.* 2006, Bielewicz *et al.* 2011, Li *et al.* 2016). Microbial eukaryotes including phototrophs and mixotrophs provide prominent functions in the lake ecology (Li & Morgan-Kiss 2019). In our data, the eukaryotic communities of lakes Fryxell and Bonney showed three major taxa in common: *G. cryophila*, *M. rubrum* and *N. limnetica*. *G. cryophila* is a mixotrophic cryptophyte that feeds on bacteria but also conducts photosynthesis as a secondary endosymbiont alga (van den Hoff *et al.* 2020). *M. rubrum* is a ciliate that consumes cryptophytes but also uses the prey chloroplasts to conduct photosynthesis (kleptoplasty; Yih *et al.* 2004). *N. limnetica* is a heterokont alga, with red alga-derived chloroplasts, and is primarily a phototroph (Kong *et al.* 2012). Our Lake Bonney samples also showed sequences from *Chlamydomonas*, a green alga that dominates some parts of the Lake Bonney water column (Lizotte *et al.* 1996, Bielewicz *et al.* 2011).

We note that in the water samples, smaller aquatic phototrophs were probably missed by the 0.45 µm filter; 0.20 µm filters would have been preferable but were not available in the field at the time. Even 0.20 µm filters miss important microbial community members (Brown *et al.* 2015). The mat samples, however, underwent no filtration, so a broader spectrum of cell sizes was captured.

It is interesting that the Lake Fryxell water shows mainly eukaryotic phototrophs whereas the mat shows mainly cyanobacteria and proteobacterial phototrophs. The mat bacteria are likely to survive a wider range of light and temperature conditions than the eukaryotes. From the standpoint of drug resistance, cyanobacteria are more likely than eukaryotes to harbour and transfer ARGs of potential bacterial pathogens. Nevertheless, protists can regulate the bacterial ARG composition in terrestrial communities (Nguyen *et al.* 2020), so this factor may be of interest when assessing lake water ARG pools.

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Author contributions

ST designed the bioinformatic analysis of ARGs and drafted the manuscript. GH characterized the *Rhodoferax* enrichment culture and contributed to the

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manuscript. DB ran the bioinformatic pipelines and performed statistics. RMM-K led the field expedition and collected water samples. JLS conceived the central concept, collected the mat samples and isolated DNA, mentored students and completed the manuscript.

Availability of data and materials

Sequence read files from lake water and mat samples are deposited at NCBI under SRA accession numbers SRP104818, SRP104821, SRP098041, SRP098040, SRP098042, SRP104817, SRP098044, SRP104822, SRP098050, SRP104819, SRP104820, SRP104823. Sequence read files from cultured *Rhodoferax antarcticus* JLS are deposited at NCBI under SRA accession number PRJNA736311.

Supplementary material

Four supplemental tables will be found at https://doi.org/10.1017/S0954102022000360.

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