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Research Article

RESEARCH ARTICLE

Genomic and physiological characterization and description of *Marinobacter gelidimuriae* sp. nov., a psychrophilic, moderate halophile from Blood Falls, an antarctic subglacial brine

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One sentence summary: The ecology, physiology and genomic characteristics of a new species isolated from Blood Falls, an Antarctic subglacial environment.

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ABSTRACT

Antarctic subice environments are diverse, underexplored microbial habitats. Here, we describe the ecophysiology and annotated genome of a *Marinobacter* strain isolated from a cold, saline, iron-rich subglacial outflow of the Taylor Glacier, Antarctica. This strain (BF04_CF4) grows fastest at neutral pH (range 6–10), is psychrophilic (range: 0°C–20°C), moderately halophilic (range: 0.8%–15% NaCl) and hosts genes encoding potential low temperature and high salt adaptations. The predicted proteome suggests it utilizes fewer charged amino acids than a mesophilic *Marinobacter* strain. BF04_CF4 has increased concentrations of membrane unsaturated fatty acids including palmitoleic (33%) and oleic (27.5%) acids that may help maintain cell membrane fluidity at low temperatures. The genome encodes proteins for compatible solute biosynthesis and transport, which are known to be important for growth in saline environments. Physiological verification of predicted metabolic functions demonstrate BF04_CF4 is capable of denitrification and may facilitate iron oxidation. Our data indicate that strain BF04_CF4 represents a new *Marinobacter* species, *Marinobacter gelidimuriae* sp. nov., that appears well suited for the subglacial environment it was isolated from. *Marinobacter* species have been isolated from other cold, saline environments in the McMurdo Dry Valleys and permanently cold environments globally suggesting that this lineage is cosmopolitan and ecologically relevant in icy brines.

Keywords: subglacial; Antarctica; Blood Falls; psychrophile; halophile; Marinobacter

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INTRODUCTION

Greater than 80% of the Earth's biosphere is below 5°C and includes permafrost, sea ice, glaciers and much of the Earth's oceans. Despite this fact, a limited number of psychrophilic microorganisms have been characterized. Ice covers 10% of the Earth's terrestrial surface, with the majority (∼90%) being in Antarctica (Priscu and Christner 2004). Antarctic subglacial environments are permanently cold and dark due to ice covers up to 4.8 km thick (Fretwell *et al.* 2013). A diversity of subglacial ecosystems exist in Antarctica, ranging from shallow freshwater wetland systems (Fricker *et al.* 2013), deep closed basin lakes (Siegert *et al.* 2001) and saline brines (Mikucki *et al.* 2015). The microbial molecular diversity from the first direct sampling of an Antarctic subglacial lake, freshwater Subglacial Lake Whillans, was recently reported (Christner *et al.* 2014). Blood Falls, which is sourced from a highly saline (>8% salinity) subsurface aquifer (Mikucki *et al.* 2015), is, to date, the only other Antarctic subglacial environment to be sampled and characterized (Mikucki *et al.* 2004, 2009; Mikucki and Priscu 2007). While, little information is available on how microorganisms adapt to the cold, dark constraints imparted by subglacial environments, these ecosystems are now known to harbor diverse and metabolically active microbial communities (Mikucki and Priscu 2007; Christner *et al.* 2008, 2014). Because these features are difficult to access, microbial isolates from these systems offer important material for studying microbial ecophysiology of subglacial environments.

The challenges of living under icy conditions are numerous. Cells require a liquid milieu for metabolic processes, and at subzero temperatures, this can require living under high solute concentrations. Temperature and salinity extremes can alter both microbial biomolecules and normal cellular functions. For instance, low temperatures decrease membrane fluidity, inhibit transcription and translation, and inactivate proteins and ribosomes (reviewed in Phadtare 2004; D'Amico *et al.* 2006; Margesin and Miteva 2011; Piette, Struvay and Feller 2011; Siddiqui *et al.* 2013). Turgor pressure is essential for bacterial cell growth and division, but hypersalinity induces a decrease in the water activity and turgor pressure (Csonka 1989; Oren 2006; Overmann 2006). Thus, microorganisms living in cold, saline environments must evolve physiological and genomic adaptations to grow under such conditions.

Currently, forty-one species in the genus *Marinobacter* have been described. These isolates have been cultivated from diverse environments with saline properties including hydrothermal sediments, alkaline serpentine muds and an oil-producing well (Huu *et al.* 1999; Takai *et al.* 2005; Handley, Héry and Lloyd 2009; Papke *et al.* 2013). However, to our knowledge, no *Marinobacter* species from the McMurdo Dry Valleys, Antarctica have been fully characterized. The *Marinobacter* genus is within the class Gammaproteobacteria and includes Gram-negative, aerobic, motile, halotolerant or halophilic, rod-shaped bacteria (Grimaud 2010). The majority of isolated *Marinobacter* strains are mesophilic. To date, only one psychrophilic strain, *Marinobacter psychrophilus* 20041^T, originally isolated from sea ice in the Canadian Basin, has been characterized and is recognized as a formal microbial species (Zhang *et al.* 2008).

Here, we describe genomic and physiological characteristics and ecological context of the psychrophilic, moderately halophilic *Marinobacter* sp. strain BF04_CF4 that was isolated from Blood Falls, an iron-rich saline subglacial Antarctic outflow. Comparison of the annotated high quality draft genome with other closely related *Marinobacter* genomes, along with physiological characteristics indicated that BF04_CF4 represents a novel species within the *Marinobacter* genus that we name *Marinobacter gelidimuriae* sp. nov. We discuss how the physiological traits and genomic potential of strain BF04_CF4 provide insight into survival and growth in the conditions that prevail below the Taylor Glacier. Many of the liquid oases for life in McMurdo Dry Valleys of Antarctica are briny and occupied by *Marinobacter* spp., thus they appear to be cosmopolitan in the cold, dry valley deserts.

METHODS

Site description and organism isolation

Taylor Valley is located in the McMurdo Dry Valleys, the largest ice-free region in Antarctica (Levy *et al.* 2012). Taylor Glacier is an outlet glacier of the East Antarctic Ice Sheet, which terminates into Taylor Valley. There is evidence for widespread brine saturated sediments below Taylor Glacier spanning from the glacier snout to at least 5.75 km up glacier (Hubbard *et al.* 2004; Foley *et al.* 2015; Mikucki *et al.* 2015). Blood Falls, a feature at the glacier terminus, is the only known surface release point of this large subsurface aquifer (Mikucki *et al.* 2015). Subglacial outflow at Blood Falls has previously been characterized as cold, saline and ferrous (Lyons *et al.* 2005; Mikucki *et al.* 2009). The microbial diversity and ecology of Blood Falls has been described previously and was shown to contain numerous heterotrophic species, including representatives of the *Marinobacter* genus, and chemosynthetic organisms that utilize iron and sulfur compounds for growth (Mikucki *et al.* 2004; Mikucki and Priscu 2007, 2009). The brine from Blood Falls eventually flows into its proglacial lake, Lake Bonney. Lake Bonney has two lobes and a permanent ice cover that is ∼3.5 m thick (Fountain *et al.* 1998). The lake water is cold, ranging from approximately −5°C at ∼40 m depth to approximately 6°C in the upper depths. Lake Bonney is also meromictic with a halocline between 13 and 15 m in the west lobe and 17 and 20 m in the east lobe (Spigel and Priscu 1996; Ward and Priscu 1997). Salinity in Lake Bonney ranges from fresh surface waters to hypersaline (up to 15%) bottom waters (Spigel and Priscu 1996). The deeper waters (∼22–25 m) of the west lobe of Lake Bonney are geochemically similar to brine collected at Blood Falls (Lyons *et al.* 2005).

Samples of Blood Falls brine were collected during an active subglacial discharge event on 1 November 2004. Outflow was collected directly from the source for enrichment work and was saline (∼8% salinity), contained no detectable oxygen and had a $^{14}C_{DIC}$ value of −999.3‰ (essentially radiocarbon dead), indicating that the brine sampled for this study had limited contact with the atmosphere (Mikucki *et al.* 2009). The oxidation/reduction potential of the brine measured during sampling was 100 mV, which supported the oxygen measurements indicating the brine was suboxic. At the time of collection, the brine was cold (−5.2°C), circumneutral (pH = 6.2) and contained iron and sulfate at concentrations of 3.45 mM total Fe [>97% as Fe(II)] and 50 mM SO_4, respectively; no sulfides were detected (Mikucki *et al.* 2009). Measured dissolved species included inorganic nitrogen (94 μM; 100% as NH_4^+; i.e. no nitrate or nitrite was detected) and dissolved inorganic carbon (55 mM) and dissolved organic carbon (420 μM) (Mikucki *et al.* 2009). Brine was collected directly into precombusted 74 ml serum vials by holding vials under the outflow until overflowing. The serum vials were then crimp-sealed with an autoclaved butyl rubber stopper without headspace. Serum vials were kept in the dark at 4°C until returned to McMurdo Station (∼1 week). Brine (100 μl)

was removed from the serum vial with a sterile syringe, inoculated onto Marine Agar (Bacto) plates in a UV-sterilized laminar flow hood, and spread with a sterile cotton swab. Plates were then incubated at 2–4°C for ∼1 month until colonies appeared. Colonies were picked with a sterile pipette tip and restreaked through three passages to ensure purity. A single colony was then picked and gently vortexed in 1× TBE buffer. An aliquot was stained with SYBR gold nucleic acid stain per the manufacturer's protocol (Invitrogen, Thermo Scientific, Waltham, MA, USA), and a single morphotype was confirmed using epifluorescence microscopy; another aliquot was used for DNA sequencing of PCR-amplified 16S rRNA genes to further confirm purity. The same procedure was repeated for strain BF14_3D that was collected from Blood Falls on 30 November 2014. Whilethis isolate was not described in detail for this study, we have included it in our phylogenetic analyses and ecological discussion.

Scanning electron microscopy

A 1 ml aliquot of cells grown in liquid culture was allowed to settle and attach to poly-l-lysine (Sigma, St. Louis, MO, USA; 300,000MW) coated cover slips for 30 min. A saturated solution of $HgCl_2$ (in double distilled H_2O) was added to a stock solution of 2% OsO_4 (in double distilled H_2O) in the ratio of six parts 2% OsO_4 to one part $HgCl_2$. Cells were fixed in this solution for 30 min at 4°C and rinsed three times with double distilled H_2O. Cells were then dehydrated with ethanol for 5 min each in 30%, 50%, 70% and 95% ethanol and washed three times with 100% ethanol for 10 min each. Cells were dehydrated by critical point drying with liquid CO_2. Cover slips were mounted onto Al stubs with silver paint and sputter coated with AuPd (http://www.dartmouth.edu/∼emlab/manuals/sempreps/pseudomonas.html). Images were obtained with an XL-30 ESEM-FEG (Environmental Scanning Electron Microscopy-Field Emission Gun) microscope (FEI Company, Hillsboro, OR, USA).

Genome sequence, assembly and annotation

A 50 ml volume of strain BF04_CF4 culture was obtained after growth to mid-log phase at 4°C for 2 weeks. Cells were pelleted at 12 000 rpm for 8 min and flash frozen. High-molecular weight DNA (∼500 ng) was extracted using a CTAB buffer/organic solvent extraction protocol. DNA was RNase treated, quality was confirmed on a 1% agarose gel and shipped to the Joint Genome Institute (JGI) for draft sequencing using Illumina sequencing technology. The draft genome sequences were assembled according to JGI SOP protocols (Mavromatis *et al.* 2009) and made available as a part of the Integrated Microbial Genomes and Microbiomes (IMG/M) data warehouse (Markowitz *et al.* 2014). An improved high-quality permanent draft genome was produced using the Velvet v. 1.1.04, ALLPATHS v. R41043 assembly method and Prodigal 2.5 gene calling. Hypothetical proteins were further analyzed and annotated with HHpred (probability cut-off: 85%) (Söding, Biegert and Lupas 2005). Amino acid analysis of shared proteins between strain BF04_CF4 and the mesophilic *Marinobacter lipolyticus* SM19^T (95% sequence identity) (Martín *et al.* 2003) was performed after reverse best hit Basic Local Alignment Search Tool (BLAST) analysis (Grzymski *et al.* 2008) to determine the core genome. Proteins were considered homologous between the two strains if they were top BLAST hit in an all against all BLAST at an *E*-value cut-off of E^{-50}. Substitutions were calculated from each aligned protein pair. Alignments were performed using Clustal (Larkin *et al.* 2007).

Phylogenetic analysis

The 16S rRNA gene sequence for BF04_CF4 generated previously by Mikucki and Priscu (2007) showed 100% identity to that obtained from the sequenced genome, and was used for the phylogenetic analysis. The 16S rRNA gene for strain BF14_3D was amplified by PCR from a genomic DNA sample prepared from a single isolated colony, using the MoBio PowerSoil® DNA Isolation Kit (Carlsbad, CA, USA). PCR reactions contained 1× AmpliTaq Gold 360 MasterMix (Applied Biosystems), 1 ng of template genomic DNA and 0.2 μM of the bacterial primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (CGGTTACCTTGTTACGACTT) under the following conditions: denaturation at 95°C for 10 min; 32 cycles at 95°C for 45 s, 50°C for 1 min and 72°C for 90 s; and a final extension at 72°C for 10 min. Sanger sequencing was performed by Eurofins Genomics (Huntsville, AL, USA) in both directions using primers 27F and 1492R. The raw sequences were processed using Geneious 5.5.6 (Kearse *et al.* 2012), with the ends of the reads containing low quality bases trimmed, and the resulting sequences aligned to generate a consensus sequence with a length of 1411 bp.

A nucleotide BLAST search was performed against the nonredundant nucleotide collection using the online version of BLAST (http://blast.ncbi.nlm.nih.gov). The top hits for BF04_CF4 and BF3D_14, along with selected sequences from representative isolates were aligned using the SILVA incremental aligner (SINA) v1.2.11 at http://www.arb-silva.de/aligner/ (Pruesse, Peplies and Glöckner 2012). The final alignment of 1375 bp, trimmed to the length of the shortest sequence, was imported into MEGA version 6.06 (Tamura *et al.* 2013) for phylogenetic analysis. A maximum-likelihood phylogenetic tree was constructed with the Tamura-Nei model with discrete Gamma distribution (TN93 + G), using the highest subtree-pruning-regrafting heuristic inference, and 1000 bootstrap replicates.

Genome comparisons between strain BF04_CF4 and other sequenced *Marinobacter* species were conducted using online software for pairwise average nucleotide identities (ANI) and DNA–DNA hybridization (DDH) estimates. Pairwise average nucleotide identities were computed using the genome comparison tools in the IMG/M microbial genome annotation and analyses software available through JGI. An additional parameter for species consideration is the DDH percentage between two closely related organisms. We used the genome to genome distance calculator (GGDC; version 2.1) for a genome-based species delineation (Auch *et al.* 2010a; Auch, Klenk and Göker 2010b; Meier-Kolthoff *et al.* 2013). GGDC calculates an intergenomic distance under three different distance formulas.

Growth rate experiments

The effects of temperature, NaCl concentration and pH on the growth of strain BF04_CF4 were measured using a modified marine broth. To prepare the marine broth basal medium (MBBM), the following chemicals were added to 1 l of Milli-Q water: ferric citrate (0.1 g), $MgCl_2$ (5.9 g), $MgSO_4$ (3.24 g), $CaCl_2$ (1.8 g), KCl (0.55 g), $NaHCO_3$ (0.16 g), KBr (0.08 g), $SrCl_2$ (34 mg), H_3BO_3 (22 mg), sodium silicate (4 mg), NaF (2.4 mg), NH_4NO_3 (1.6 mg) and Na_2HPO_4 (8 mg). MBBM (supplemented with 5 g peptone, 1 g yeast extract and 19.45 g NaCl) was used for the temperature experiments. The final pH of supplemented MBBM was approximately 7.6. Cells were grown to mid-log phase and transferred to supplemented MBBM (final cell concentration: 2% of total volume). Strain BF04_CF4 was then incubated at 0°C, 4°C, 10°C, 15°C,

20°C and 25°C . To test the effect of salinity, MBBM was supplemented with 5 g peptone, 1 g yeast extract and NaCl to final concentrations of 0%, 0.8%, 3%, 6%, 8%, 15% and 20%. For the pH experiments, the pH of MBBM (supplemented with 5 g peptone, 1 g yeast extract and 19.45 g NaCl) was adjusted to 5, 6, 7, 8, 9, 10 or 11 with 1 and 10 M HCl or NaOH. Strain BF04_CF4 was grown at 15°C for NaCl and pH experiments, the temperature at which fastest growth was observed. Growth was measured by absorbance at 600 nm with a GENESYS 20 visible spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Fatty acid analysis

Marinobacter lipolyticus SM19^T (DSM 15157^T) was obtained from DSMZ (German collection of microorganisms and cell cultures; Braunschweig, Germany) for fatty acid methyl ester (FAME) comparison. Strain BF04_CF4 and *M. lipolyticus* were grown in duplicate at 15°C in supplemented MBBM until mid-log phase. Cells were then pelleted by centrifugation at 5000 *g* and 15°C for 10 min. Pellets were sent to Microbial ID Inc. (Newark, DE, USA; http://www.microbialid.com/) for analysis of fatty acid methyl ester profiles.

Phenotypic analyses and carbon substrate utilization

The Gram stain was conducted on isolated colonies (Bartholomew 1962) and results were confirmed by the KOH test as detailed in (Buck 1982). Motility was determined by growth on BBL motility test medium (5% NaCl) according to the manufacturer's instructions.

Organic carbon utilization profiles for BF04_CF4 were generated with Biolog PM1 and PM2A Microplates (BIOLOG Inc., Hayward, CA, USA). Briefly, BF04_CF4 cells were pelleted as above, washed and transferred to a minimal medium (MM) for inoculating Biolog Microplates according to Shivaji *et al.* (2005). MM was prepared with (per liter of MilliQ water): 1 g NH_4Cl, 0.075 g K_2HPO_4, 1.45 g $CaCl_2$, 60 g NaCl, 6.15 g $MgCl_2$, 0.75 g KCl and 0.028 g $FeSO_4$ without added carbon. Cell pellets were resuspended in MM, and Biolog Redox Dye mix D was added (1× final concentration) to the cell suspension. Cell suspensions (150 μl) were pipetted into each Biolog well (8×10^5 cells final concentration). Absorbance was measured at 530 nm with a Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA) every 12 h for 2 weeks. Carbon substrate wells corresponding to D- and L-alanine, L-arginine, glucose, L-glutamine, glycerol, L-isoleucine, leucine, L-methionine, L-ornithine, D-xylose, acetate and D-fructose showed color change in Biolog microplates after 2 weeks. BF04_CF4 was then grown in triplicate in MM with 10 mM of each carbon source that was positive via Biolog to confirm growth. BF04_CF4 was incubated at 15°C in 10 ml volumes with 5% inoculum. Growth was measured following two transfers. MM (with no added carbon source) was inoculated with strain BF04_CF4 as a negative control for substrate incubations.

Denitrification and ferrous iron (Fe(II)) oxidation

Cells were cultivated under anaerobic conditions [N_2/CO_2 80:20 (v/v)] at 15°C in a synthetic medium: (per liter of MilliQ water) 3.7 g NH_4Cl, 6.2 g $MgSO_4$ x $7H_2O$, 1.5 g $CaCl_2$ and 0.75 g KCl, supplemented with 60 g NaCl, 5 g peptone, 1 g acetate and 4 mM nitrate (KNO_3) (Gauthier *et al.* 1992). pH was adjusted to 7.0 with 1 M NaOH. Nitrate reduction was determined by a colorimetric assay as described by Lanyi (1987). Briefly, 0.1 ml of reagent A (sulphanilic acid, 8 g; 5 N acetic acid) and 0.1 ml of reagent B (N,N-dimethyl-l-naphthylamine, 6 ml; 5 N acetic acid, 1000 ml) were added to uninoculated controls and cells at mid-log phase. Change in media color to red was observed directly within 1 min. The reduction of nitrate forms nitrous acid in the aqueous medium. The nitrous oxide then reacts with sulphanilic acid and naphthylamine to form a visible red, compound. An absence of color change indicates either nitrate reduction did not occur or nitrite was further reduced to N_2. Following the 1 min observation, 25 mg of zinc dust was added to the culture to test for the absence of nitrate and therefore, complete reduction to N_2. *Pseudomonas stutzeri* strain DCP-Ps1 was used as a positive control for the nitrate reduction phenotype (Sanford *et al.* 2012). To test for enzymatic Fe(II) oxidation, BF04_CF4 cells were grown anaerobically [N_2/CO_2 80:20 (v/v)] in synthetic medium with the addition of 5 mM acetate and 10 mM Fe(II) ($FeSO_4 \cdot 7H_2O$) and nitrate. Cultures and abiotic controls were incubated at 15°C for approximately 2 weeks. Evidence for Fe(II) oxidation is a change in the media from clear to the formation of orange–red precipitate (ferric iron).

RESULTS AND DISCUSSION

Morphology

Strain BF04_CF4 cells are Gram-negative and a single, rod-shaped morphology (average length = ~1.7 μm; average width = ~0.5 μm) was observed using scanning electron microscopy (Fig. S1, Supporting Information). There was variation in cell length, with the shortest cell viewed as ~850 nm and the longest at ~2.3 μm. These observations are consistent with previous descriptions of the *Marinobacter* genus (Bowman and McMeekin 2005). Cells of BF04_CF4 did not have evidence of flagella and motility was not observed. The cell membrane appeared to contain pores under high magnification. Colonies were round, white to cream-colored and 1–1.5 mm in diameter when grown on Marine Agar (Difco, Thermo Scientific, Waltham, MA, USA).

Phylogenetic relationships

16S rRNA gene sequence analysis placed strain BF04_CF4 in the *Marinobacter* genus and comparative analysis in the NCBI nucleotide database using BLAST revealed highest sequence identity to both uncultured clones and cultured isolates collected from other cold and saline environments including other brines within the McMurdo Dry Valleys (Fig. 1). The top BLAST hits for BF04_CF4 were predominantly of Antarctic origin. BF04_CF4 shared the highest nucleotide identity (99.9%) to the uncultured clone 5III.9 isolated from sediments in the west lobe of Lake Bonney (Tang, Madigan and Lanoil 2013) and strain ELB17 (99.2% identity to BF04_CF4), which was originally isolated from 17 m depth in the east lobe of Lake Bonney. Blood Falls, or the subglacial source of brine to Blood Falls, supplies a significant amount of solutes to the west lobe of Lake Bonney (Lyons *et al.* 2005), so it is possible that members of the population represented by strain BF04_CF4 are transported from Blood Falls or below the Taylor Glacier into the west lobe and eventually into the east lobe. Water from the west lobe has been shown to migrate to the east lobe via a process termed 'chemocline leakage' (Doran *et al.* 2014).

Strain BF04_CF4 was collected from Blood Falls during the austral summer of 2004, yet showed high-sequence identity to environmental clones obtained from a sample collected during an active outflow event in 1999 (Mikucki and Priscu 2007). Clones BF_C65 and BF_C90 shared 98.4% and 98.2% sequence

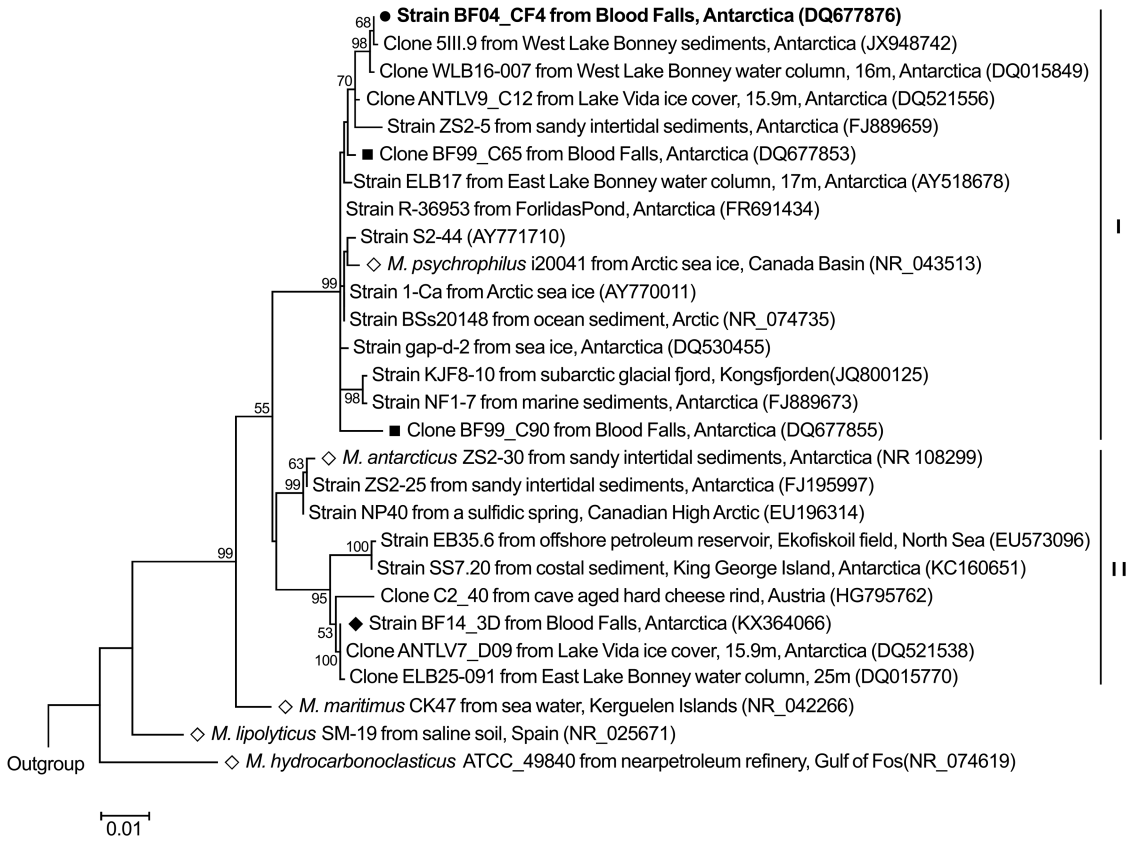

Figure 1. Maximum likelihood phylogenetic tree showing the relationship between 16S rRNA genes from strain BF04_CF4, closely related *Marinobacter* clones and isolates, and select *Marinobacter* type strains. Strain BF04_CF4 is indicated by the black circle; strain BF14_3D is indicated by the black diamond; *Marinobacter* clones isolated from Blood Falls are indicated by the black squares; *Marinobacter* type strains are indicated by the white diamonds. Bootstrap values greater than 50 (1000 replicates) are indicated at nodes. The scale bar represents 0.01× nucleotide substitutions per sequence position. The outgroup was *Shewanella sp.* strain BF02_Schw from Blood Falls, Antarctica (DQ677870).

identity with BF04_CF4 respectively, and these clones comprised 3.7% of the clone library (Mikucki and Priscu 2007). BF04_CF4 showed somewhat lower sequence identity (96.4%) to the strain BF14_3D, which was isolated during an active outflow event in 2014. Another Antarctic strain (R-36953), sharing 99.1% sequence identity, was isolated from Forlidas Pond, located in the Ellsworth Land Region of Antarctica, which is a considerable distance from the dry valleys (Peeters *et al.* 2011). However, not all BLAST hits with high-sequence identity (>97%) were geographically constrained to Antarctica, and included isolates and clones of arctic and subarctic origin as well. Therefore, although the high identity in sequences between clones and isolates from Blood Falls and Lake Bonney may partially be explained by hydrologic connectivity, it does not fully explain the high identity to strains from more distant locations.

Our phylogenetic analysis also revealed that the two *Marinobacter* isolates from Blood Falls (BF04_CF4 and BF14_3D) belong to one of two apparent clades (Fig. 1). These clades contained clones and isolates derived from both Antarctica and elsewhere, including the Arctic and sub-Arctic. Such diverse *Marinobacter* species appear to be cosmopolitan in cold brines (Perreault *et al.* 2007, 2008; Niederberger *et al.* 2010). Collectively this suggests that, the diversity of *Marinobacter* in Blood Falls and other briny dry valley lakes may not be the result of local speciation, but rather a result of diverse *Marinobacter* species present in the ancient marine waters that originally formed the brines.

The closest characterized species for which BF04_CF4 shared high 16S rRNA gene sequence similarity were the psychrophilic *M. psychrophilus* 20041^T (97.8%) and psychrotolerant *Marinobacter antarcticus* ZS2–30^T (96.3%), originally isolated from coastal sediments near Larsemann Hills, Antarctica (Liu *et al.* 2012). These rRNA gene sequence identities indicate that strain BF04_CF4 is a distinct species from *M. antarcticus* and is close to the species cut-off (97% or greater) with *M. psychrophilus*. Although >97% 16S rRNA gene sequence similarity is widely used to classify different species (i.e. Stackebrandt and Goebel 1994), recent studies have shown that strains with less than 99% 16S rRNA gene sequence similarity benefit from additional comparisons such as DDH or determination of an ANI (i.e. Meier-Kolthoff *et al.* 2013). *In silico*-based estimates of DDH between BF04_CF4 and *M. psychrophilus* was 44–48.4% (Table S1, Supporting Information), which is far below the >70% DDH species delineation cut-off. The genome of strain BF04_CF4 had an ANI of 92.95% to bidirectional best hits of the strain *M. psychrophilus* (Table S1, Supporting Information).

For decades, the 'gold standard' for delineating microbial species has been >97% 16S rRNA gene sequence similarity (Stackebrandt and Goebel 1994). Even before the broad accessibility of microbial genome sequencing, scientists have debated this threshold as potentially deficient (Stackebrandt *et al.* 2002). With the growing database of sequenced microbial genomes, ANI has emerged as a highly robust metric, with a threshold of 95%–96% delineating species (e.g. Konstantinidis and Tiedje 2005; Konstantinidis, Ramette and Tiedje 2006; Chan *et al.* 2012). A recent meta-analysis of available genomes corroborates this finding and determined that 95–96% ANI corresponds to

98.65% 16S rRNA gene sequence similarity (Kim *et al.* 2014). The authors conclude that >98% is a better standard for species designation when examining high quality, full-length 16S rRNA gene sequences. We contend that the differences in 16S rRNA sequence, ANI and DDH values indicate that BF04_CF4 is a distinct species from *M. psychrophilus*.

Genome summary

The BF04_CF4 genome is composed of 3,633,797 base pairs with a G + C content of 54.63%, which is comparable to other closely related *Marinobacter* species (Table 1); for example, *M. psychrophilus* contains 53.84% G + C content. The permanent high-quality draft genome contained 136 scaffolds and 139 contigs. The genes encode for 3753 proteins, 2784 of which have been associated with a predicted function. The RNA genes were re-annotated because they were broken between contigs and incomplete in length. There are likely a total of 92 RNA genes with 6 total rRNA in 2 rRNA operons, 45 tRNA genes, 11 other miscellaneous RNAs and 30 group II catalytic introns with high identities to integrases from other bacteria, fragments and un-annotated parts of the deep sea, cold-adapted *Photobacterium profundum* SS9 genome (Vezzi *et al.* 2005). The BF04_CF4 genome contains at least 153 transposases or insertion sequence elements, 6 resolvases and 59 phage-related genes or integrases. The presence of insertion sequence elements has been implicated in genetic mobility and versatility, which may allow for rapid adaptations to new environmental conditions (Touchon and Rocha 2007). Several transposase flanking-genes encoded for other transposases, phage-related proteins, nucleases, nucleic acid-binding domains, chemotaxis proteins, and proteins of unknown function. Phage-related proteins include site-specific recombination integrases, another factor in genome flexibility and modification (Argos *et al.* 1986; Hochhut *et al.* 2006; Ogier *et al.* 2010). Other putative phage-related genes in strain BF04_CF4 include a major coat protein Gp8, phage tail, capsid, and maturation and terminase proteins (Table S1, Supporting Information). The majority of the nucleotide sequences from the phage-related proteins did not produce sequence identities except a phage integrase (IMG gene ID: 2515470055). The nucleotide sequence identity of the phage integrase was 93% identical to the sequence of plasmid pMAQU02 in *M. aquaeolei* VT8. Presence of both insertion sequence elements and phage genes in the genome may indicate horizontal gene transfer events in strain BF04_CF4.

Our survey of the BF04_CF4 genome and comparisons made to its close relative, *M. psychrophilus* (Fig. S2, Supporting Information), further support the differences between these two strains and reveal distinct attributes in BF04_CF4 that may reflect its unique ecological history. A comparison of cluster of orthologous genes (COG) gene abundances between the two strains showed that, overall, BF04_CF4 has a higher percentage of genes that are not in COG databases (42.8% vs. 26.4%).

The genome BF04_CF4 has a greater number of defense mechanisms (3.71%) and mobilome (1.88%) related genes (Fig. S3, Supporting Information) compared to *M. psychrophilus* (1.93% and 0.48% respectively), including genes for restriction modification systems not found in *M. psychrophilus*. BF04_CF4 also appears to have a complete Type I-C/Dvulg CRISPR/cas system (Nam *et al.* 2012 and Makarova *et al.* 2015). *M. psychrophilus* lacks any CRISPR/cas genes, as do most other sequenced *Marinobacter* species. The CRISPR/cas gene set found in BF04_CF4 was also found in strain ELB17, where these genes share between 90–99% protein identity. CRISPR/cas systems act as an immune system for bacteria, providing resistance against phages (Hille and Charpentier 2016). Often CRISPR/cas systems are acquired by horizontal gene transfer events, which could explain the lack of a homologous CRISPR/cas system in other *Marinobacter* strains. The presence of a CRISPR/cas system in BF04_CF4, as well as numerous mobile elements, may be indicative of higher levels of phage predation for this strain.

Marinobacter psychrophilus has a higher abundance of cell motility genes (3.4%) compared to BF04_CF4 (1.7%), which likely correlates with their phenotypes; *M. pscyhrophilus* was motile (Zhang *et al.* 2008), whereas we did not observe motility in BF04_CF4. Of the currently 52 sequenced *Marinobacter* genomes, only strains BF04_CF4 and two strains originally isolated from Lake Vida (strains LV10MA510–1, and LV10R520–4) contain genes for gas vesicles (Table S1, Supporting Information). Gas vesicles are typically used by microbes for buoyancy in stratified water columns (Walsby 1994) and provide an advantage to aerobic halophilic microorganisms because oxygen is less soluble in salt-concentrated water (Oren 2002; Pfeifer 2006). Strain BF04_CF4 may use gas vesicles to float to the surface of the Blood Falls brine conduit for access to oxygen; it has also been speculated that in slow-growing organisms, utilization of gas vesicles to maintain buoyancy actually decreases energy costs in comparison to flagella utilization (Walsby 1994; Jung *et al.* 2004). However, little is known about the subglacial structure of the brine or how gas vesicles might be an adaptive advantage in brine-saturated sediments, which is significantly deeper in the subsurface (>450 m deep). Chivian *et al.* (2008) reported the presence of genes for the formation of gas vesicles in '*Candidatus Desulforudis audoxyiator*,' whose genome was constructed from an extremely low diversity sample of fracture water of a South African gold mine at 2.8 km depth. Perhaps there are yet unknown functions of gas vesicles that convey a growth advantage in the deep subsurface.

Cold adaptation

Strain BF04_CF4 grew at 0°C (0.15 cell division per day) with fastest growth observed at 15°C (0.8 cell divisions per day); no growth was observed at 25°C, indicating the bacterium can be described as psychrophilic (Table 1). Psychrophiles grow optimally at temperatures of 15°C or lower, tolerate a maximum temperature of approximately 20°C, and have a minimum growth temperature at or below 0°C (Morita 1975). The laboratory-observed growth temperature range for BF04_CF4 was 0–20°C (Table 1) that was comparable to the range reported for *M. psychrophilus* (range = 0°C–22°C) (Zhang *et al.* 2008). However, Blood Falls outflow was approximately −5°C when samples were collected which was considerably lower than the strain's optimal (fastest) growth temperature (T_{opt}). Using the Weibull distribution (Fig. 2A), the growth rate of BF04_CF4 was predicted to be approximately 0.07 divisions per day at −4.5°C suggesting BF04_CF4 could be active and growing slowly in Blood Falls or the subglacial source to Blood Falls, rather than merely surviving in a dormant state. Laboratory-determined optimal growth temperatures are generally higher than *in situ* temperatures, and this trend is commonly found in microorganisms isolated from low-temperature environments (Ward and Priscu 1997; Bakermans and Nealson 2004). Deviations can be explained by psychrophiles maximizing growth yield as growth rates decrease at low temperatures by lowering rates of RNA and protein synthesis (Ward and Priscu 1997; Feller and Gerday 2003; Bakermans and Nealson 2004), another possible mechanism for cold adaptation. In fact, increased growth rates at higher temperatures,

Table 1. Comparison of phenotypic characteristics between strain BF04_CF4 and closely related cultured *Marinobacter* species.

	Isolation source	Geographic location	Cell size (μm)	Nitrate utilization	Temperature optimum and range (°C)	%NaCl optimum and range (w/v)	pH optimum and range	GC content (%)
Marinobacter sp. strain BF04_CF4	Subglacial outflow	Blood Falls, Antarctica	Average: 0.5 × 1.7	Yes	15 and 0–20	6.0 and 0.8–15.0	7.0 and 6.0–10.0	54.63
Marinobacter sp. strain ELB17 [99.2%] (Ward and Priscu 1997)	Perennially ice-covered lake	Lake Bonney, Antarctica	ND	Yes	12–15* and ND	1.8–3.5 and ND	ND	54.34
Marinobacter psychrophilus 20041^T [97.8%] (Zhang *et al.* 2008)	Sea-ice	Canadian Basin in Arctic Ocean	0.3–0.4 × 1.0–2.5	Yes	16–18 and 0–22	ND and 2.0–8.0	6.0–9.0 and 5.0–10.0	53.84
Marinobacter antarcticus ZS2–30^T [96.3%] (Liu *et al.* 2012)	Antarctic intertidal sandy sediment	Chinese Antarctic Zhongshan Station on the Larsemann Hills	0.5–0.8 × 1.5–2.3	Yes	25 and 4–35	3.0–4.0 and 0.0–25.0	7.0 and 5.0–10.5	54.86
Marinobacter lipolyticus SM19^T [94.5%] (Martin *et al.* 2003)	Saline soil	Cádiz, Spain	0.3–0.5 × 2.5–3.5	No	37 and 15–40	7.5 and 1.0–15.0	7.5 and 5.0–10.0	56.76

Percent 16S rRNA gene sequence identity to strain BF04_CF4 are in brackets. *Resolution (variation) of temperatures did not allow for optimum temperature identification. G + C number of bases determined from genome statistics in the Integrated Microbial Genomes & Microbiomes (IMG/M) system. ND: no data available.

including T_{opt}, could be an indication of heat stress (Siddiqui *et al.* 2013).

Several key cold adaptation traits are common among psychrophilic genomes including that of BF04_CF4. These traits include genes encoding for cold shock proteins (Csps), chaperones, and RNA helicases (Lauro *et al.* 2011), all of which have possible roles in maintaining translation capacity. Translation is affected by low temperature-induced changes in mRNA secondary structures because secondary structures prevent ribosome movement (Phadtare and Inouye 2008). There are five copies of RNA chaperone *cspA* cold shock protein genes in strain BF04_CF4. This is similar to other *Marinobacter* species including *M. psychrophilus*, which also contains 5 copies of this gene. Cold shock protein homologues have also been found in the genomes of other well-characterized psychrophiles however at different abundances, including *Colwellia psychrerythraea* 34H (4 *csps*) (Methé *et al.* 2005), *Desulfotalea psychrophila* Lsv54 (8 *csps*) (Rabus *et al.* 2004), *Psychrobacter arcticus* 273–4 (3 *csps*) (Ayala-del-Río *et al.* 2010), and *Psychromonas ingrahamii* 37 (12 *csps*) (Riley *et al.* 2008). The *csp* sequences of BF04_CF4 ranged from 48 to 81% identity to the aforementioned psychrophilic *csps*. However, within the *Marinobacter* genus, all five BF04_CF4 *csp* sequences shared highest identity (>90%) to the *csps* of *M. psychrophilus*, ELB17, BSs20148 and two isolates from Lake Vida (LV10R520–4 and LV10R510–11A) (Table S1, Supporting Information). Strain BF04_CF4 has three *cspA* thermoregulators which are 5′ untranslated (UTR) RNAs that regulate the expression of *cspA* and likely other protein-encoding genes during stress response. Again, the BF04_CF4 *cspA* thermoregulator sequences were most similar (>95%) to sequences of *M. psychrophilus* and strain BSs20148. These thermoregulators and the low *in situ* temperature of Blood Falls could induce an increase in the number of stable mRNA secondary structures. BF04_CF4 also contains four DEAD-box containing genes homologous to *csdA*, *dbpA*, *rhlB* and *rhlE* that encode proteins responsible for ATP-dependent RNA helicase activity (Prud'homme-Généreux *et al.* 2004; Kuhn 2012). The DEAD-box containing genes are all most closely related to cold-adapted *M. psychrophilus* and strains ELB17 and BSs20148 (Table S1, Supporting Information). In addition, strain BF04_CF4 possesses genes for protein chaperones, which are associated with assisting in three-dimensional protein structure formation (Hoffmann, Bukau and Kramer 2010). Protein chaperone genes in strain BF04_CF4 encode for the trigger factor protein, DnaK, DnaJ, GrpE, GroEL, and GroES, most of which are also most closely related to *M. psychrophilus* and strains ELB17 and BSs20148 (Table S1, Supporting Information). The trigger factor is a molecular chaperone that has peptidyl-prolyl cis-trans isomerase activity (Kandror and Goldberg 1997) that accelerates folding of growing polypeptide chains (Nagradova 2008). The protein chaperones DnaK, DnaJ, GrpE, GroEL and GroES also assist in protein folding, and studies have shown upregulation of these genes in psychrotolerant and psychrophilic microorganisms (Suzuki *et al.* 2004; Gao *et al.* 2006; Qiu, Kathariou and Lubman 2006; Hartl and Hayer-Hartl 2009; Ting *et al.* 2010).

FAME analysis of strain BF04_CF4 incubated at 15°C revealed the presence of mostly unsaturated fatty acids (Table 2). The percentage of unsaturated fatty acids in BF04_CF4 was higher than the mesophile *M. lipolyticus* (which has an optimal growth temperature = 37°C). However, when grown at 15°C palmitoleic, oleic and vaccenic acids were the most abundant unsaturated fatty acids produced by both strains (Table 2; Russell 2008). This is not unexpected since most organisms increase the concentration of unsaturated fatty acids in their cell membranes at lower temperatures (Marr and Ingraham 1962; Gounot 1991; Hébraud and Potier 1999). All the genes necessary for palmitoleic acid biosynthesis via the type II fatty acid synthetic system, including *fabB* and *fabF*, which encode for β-ketoacyl-ACP synthase I and II respectively, and are enzymes involved in fatty acid unsaturation, are present in the BF04_CF4 genome (Table S1). *M. psychrophilus* and ELB17 were among the few *Marinobacter* strains that do not contain a copy of *fabF* (6 out of the 52 *Marinobacter* genomes in IMG lacked *fabF*). The *fabF* gene in BF04_CF4, showed highest identity to beta-ketoacyl-ACP synthase II from *Shewanella* species including a gene (WP_014609653) from *S. putrefaciens* (70% identity) and a gene (WP_011635827) from *S. frigidimarina* (68%). A strain closely related to *S frigidimarina* (98% 16S rRNA gene sequence similarity) has been previously isolated from Blood Falls (Mikucki and Priscu 2007). In contrast, all other *Marinobacters* from cold locales, showed highest sequence identity (92–93%) to *fabF* from other *Marinobacter* species. While

Table 2. Cellular fatty acid composition (%) of strain BF04_CF4 and related species of the *Marinobacter* genus.

	Marinobacter sp. strain BF04_CF4	*Marinobacter psychrophilus* 20041^T (97.8%)	*Marinobacter antarcticus* ZS2–30^T (96.3%)	*Marinobacter lipolyticus strain* SM19 (95.1%)
16:1 ω7c	33.1	22.2	–	22.0
18:1 ω9c	27.5	13.4	14.8	18.3
16:0	13.0	14.0	15.9	15.7
12:0 3OH	6.5	10.2	7.5	7.5
18:1 ω7c	5.9	1.6	–	11.4
18:0	4.4	1.2	1.2	3.0
12:0	3.0	4.4	5.2	5.9
17:1 ω8c	1.1	8.6	2.5	0.8
17:0	0.6	7.8	0.9	0.3
16:1 ω9c	0.0	8.8	10.1	6.3

Percent 16S rRNA gene sequence identities to strain BF0F_CF4 are in parentheses. The mean of two replicate fatty acid profiles from BF04_CF4 and SM19 grown at 15°C grown to mid log phase is presented. *M. psychrophilus* 20041^T was grown at 15°C for 5 days (Zhang *et al.* 2008) and ^{C}*M. antarcticus* ZS2–30^T was grown at 16°C for 5 days (Liu *et al.* 2012). -: No data reported.

the exact role of *fabF* in the BF04_CF4 genome is unknown, it appears to have been acquired via horizontal gene transfer from the *Shewanella* and may provide a unique advantage in Blood Falls. All the genes required for oleic acid production are present in BF04_CF4 except stearoyl-CoA desaturase (Table S1), which is responsible for the first step of oleic acid production. It is possible that strain BF04_CF4 does contains stearoyl-CoA desaturase, but that the sequence data is absent in our analysis due to the draft genome status; alternatively oleic acid production could proceed via a different pathway in BF04_CF4. Regardless, the FAME results (Table 2) demonstrate BF04_CF4 is capable of synthesizing palmitoleic and oleic acids, which may help maintain cell membrane homeoviscosity to optimize transfer of nutrients and waste at colder temperatures in environments such as Blood Falls.

Our analysis shows only subtle amino acid usage differences between strains BF04_CF4 and its mesophilic relative, *M. lipolyticus*. The mesophilic strain uses more negatively charged amino acids than the psychrophile in the shared genome and in its overall predicted proteome (Fig. S4). Changes in amino acid usage between mesophiles and psychrophiles are common, but not always indicative of cold adaptation (Feller and Gerday 1997; Grzymski *et al.* 2008), as there are multiple factors involved in tolerating low temperatures. Charged amino acids form salt bridges that provides rigidity to protein folds. Minimizing these folds could be an important mechanism in a cold, saline subglacial environment. Given the role of helicases as cold shock proteins in cold adaptation and abiotic stress response, the amino acid composition and substitutions in this class of proteins were specifically examined. Between strains BF04_CF4 and *M. lipolyticus* helicases, the substitutions D → N, D → A/S, E → Q, S → A, and E → A were more commonly found in the psychrophile than the mesophile (Fig. S5). These patterns are also well defined when the entire shared predicted proteome is calculated by reciprocal BLAST and examined (Fig. S6). For example, there are ~1% fewer D and E amino acids in the shared proteome of BF04_CF4 compared *M. lipolyticus*. This reduction in negatively charged, salt-bridge forming amino acids is likely an adaptation to cold (Feller and Gerday 1997; Grzymski *et al.* 2008).

Overall, BF04_CF4 possesses genomic traits found among other psychrophiles; including numerous genetic mobile elements, increased membrane lipid unsaturation, a reduction of charged amino acids, and the presence of genes encoding for cold shock proteins, chaperones, and helicases. Our analysis did not bring to light significant differences in gene abundances for proteins known to be involved in psychrophilic adaptations. Psychrophilic genomes have also been demonstrated to contain redundant genes involved in cold adaptation (reviewed in De Maayer *et al.* 2014). A high number of tRNA genes have been associated with psychrophilic genomes (reviewed in De Maayer *et al.* 2014) because diffusion rates are slower at low temperatures, and translation rates are dependent on tRNA diffusion rates (Bulmer 1991). However, strain BF04_CF4 has at least 45 tRNA genes, which is comparable to all other *Marinobacter* species with sequenced genomes, including mesophiles, suggesting that the number of tRNA genes may not necessarily indicate a psychrophilic lifestyle.

Salt adaptation

Strain BF04_CF4 grew optimally (fastest) in 6% (60 g L^{-1}) NaCl and did not reproduce at 0% NaCl, and therefore can be characterized as a moderate halophile (Fig. 2B). Optimal growth rates for moderate halophiles fall between ~2.9 and ~14.6% NaCl concentration (Kushner 1978). Growth was observed in cultures up to 15% NaCl concentrations, thus strain BF04_CF4 could grow under *in situ* brine salinity (~8%) (Mikucki and Priscu 2007). The genome of BF04_CF4 contains genes important for adaptation to saline environments. Cells maintain osmotic balance by two main strategies, which include concentrating non-ionic solutes or actively pumping protons (Oren 2008). These approaches prevent water loss and provide a neutral solvent environment within the cell. Because salts can act to prevent or depress the freezing of water, many psychrophiles reside in halophilic habitats (Kelly 2011) and thus have strategies that help the cell contend with both conditions.

The initial response to high osmolarity in a bacterium is to increase intracellular concentrations of K^+ and pump out protons (White 2007). Genes for Trk transport systems, one of the most conserved K^+ uptake systems in bacteria (Heermann and Jung 2012), are present in BF04_CF4 (Table S1). The BF04_CF4 genome contains genes for glutamate and trehalose synthesis and glycine betaine, proline, and carnitine transport (Table S1). The accumulation of these molecules in the cytoplasm can serve as both osmo- and cryoprotectants for microbial cells. Genes for ATP-binding and permease components of an ectoine/hydroxyectoine ABC transporter for exogenous ectoine import and an ectoine synthase gene involved in ectoine biosynthesis were identified in BF04_CF4 (Table S1). BF04_CF4

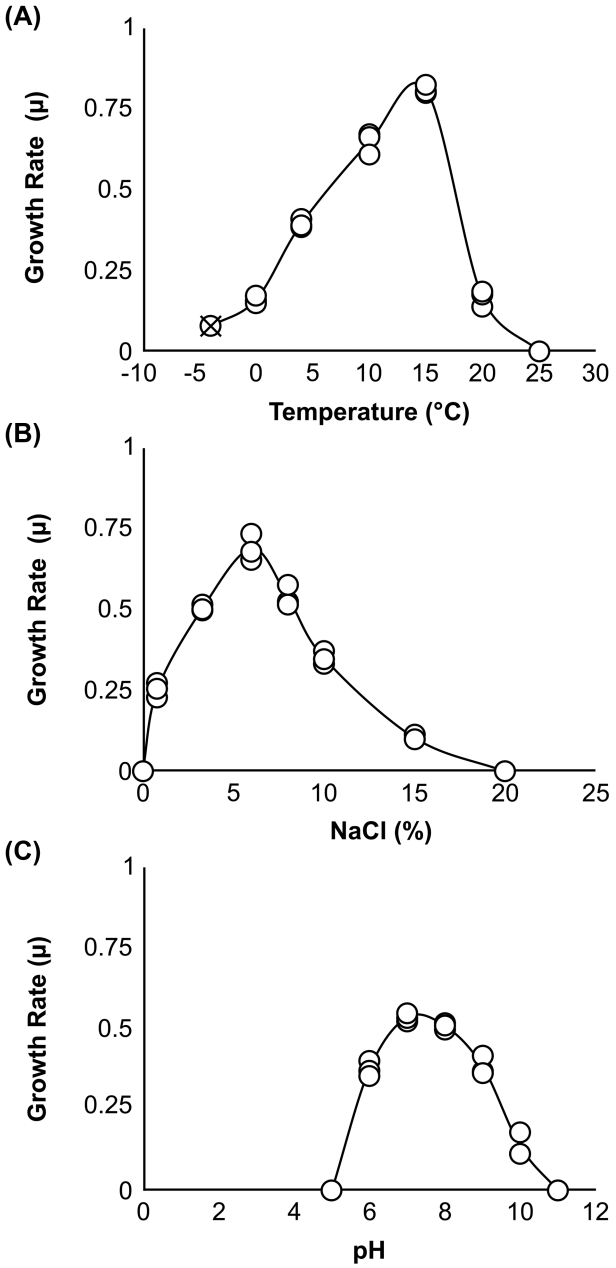

Figure 2. Effect of temperature (A), NaCl concentration (B), and pH (C) on the growth rates of strain BF04_CF4. Fitted curves were created from averages of specific growth rates under each condition, and open circles represent three replicates of the growth rates. Subzero temperature growth rates were predicted using the Weibull distribution (4 parameter) in SigmaPlot. Modeled temperature value is indicated by an X.

also contained a gene for choline and glycine betaine aldehyde dehydrogenase (IMG gene ID: 2515470750), an enzyme required for glycine betaine biosynthesis from choline, which is common in psychrophilic genomes (reviewed in Bakermans *et al.* 2012). A gene from BF04_CF4 coding for glutamate synthase (IMG Gene ID: 2515472203) was in the top ten BLAST hits and had 64% sequence identity for a glutamate synthase gene (Accession: ABM04053) in *Psychromonas ingrahamii* 37, a well-characterized psychrophile from sea ice (Riley *et al.* 2008). The symport of proline and Na + are commonly found in the genomes of psychrophiles (Methe *et al.* 2005; Bowman 2008) and we detected all the genes of the ABC-type proline/glycine betaine transport system (*opuA*) and genes for the high-affinity proline-specific uptake sodium:solute symporter family (*opuE*) were also detected. These genes shared highest identity with *M. psychrophilus* (97–98%) but also showed low identity to *Paenibacillus darwinianus* genes (41–51%). *P. darwinianus* was originally isolated from irradiated Antarctic soils (Dsouza *et al.* 2014a) and shows similar genomic features (Dsouza *et al.* 2014b) suggesting the accumulation of compatible solutes for osmoprotection.

The BF04_CF4 genome also contains *capD* (capsular polysaccharide biosynthesis protein) genes which are involved in the production of exopolymeric substances (EPS), extracellular organic molecules that can act as cryoprotectants at subzero temperatures (Table S1). This is similar to *Colwellia psychrerythraea* 34H, *Psychrobacter arcticus* 273–4, and *P. ingrahamii*, isolates capable of growth at subzero temperatures. In experiments, *C. psychrerythraea* showed increased EPS production in response to lower temperatures and increased salinity (Marx *et al.* 2009) suggesting EPS production can provide an advantage to cells in both cold and saline environments. Strain BF04_CF4 can grow at *in situ* Na^+ concentrations (~8%) and contains genes involved in a variety of strategies for tolerating high salt concentrations, including proteins for maintaining cell osmotic homeostasis and EPS production. These factors indicate BF04_CF4 has adaptations that would support growth in the salinity of Blood Falls.

Metabolic features

BF04_CF4 grew on a limited number of carbon substrates including D- and L- alanine, a small amino acid, acetate and L-leucine (Table 3). Analysis of the BF04_CF4 genome revealed incomplete versions of pathways typically found in heterotrophs. Consistent with BF04_CF4's inability to utilize glucose, fructose bisphosphatase and the oxaloacetate-decarboxylating malate dehydrogenase genes of the glycolysis and gluconeogenesis pathways were notably absent. The non-oxidative branch of the pentose phosphate pathway is complete in strain BF04_CF4, but only genes encoding for glucose-6-phosphate 1-dehydrogenase for the oxidative branch were found in the genome (IMG gene ID: 2515470888). The complete TCA cycle, common in most microbes, is also present in strain BF04_CF4. This is consistent with the ability of strain BF04_CF4 to utilize acetate as a sole carbon source. *M. psychrophilis* and *M. antarcticus* have different carbon utilization profiles compared to strain BF04_CF4 (Table 3), although *M. antarcticus* also utilizes L-alanine as an energy source (Zhang *et al.* 2008; Liu *et al.* 2012). *M. psychrophilus* and *M. antarcticus* metabolize D-serine and L-serine (Zhang *et al.* 2008; Liu *et al.* 2012), respectively, while strain BF04_CF4 lacks genes for D-serine ammonia-lyase and L-serine deaminase, which are required to convert serine to pyruvate and ammonium. Acetate has been proposed as a preferable carbon source for microorganisms in cold environments because transporters are not required for acetate uptake, and acetyl coenzyme A is produced in at most, a two-step reaction (Ayala-del-Río *et al.* 2010). Alanine conversion to pyruvate only requires alanine racemase and/or D-alanine:quinoneoxidoreductase, enzymes present in BF04_CF4 (Table S1, Supporting Information). These traits suggest energetic efficiency in BF04_CF4 and could help with growth in the subglacial environment below Taylor Glacier.

Nitrate reduction

Strain BF04_CF4 coupled acetate metabolism to nitrate reduction under anaerobic conditions. Replicates of BF04_CF4 and the

Table 3. Carbon sources utilized by strain BF04_CF4 and its closest characterized relatives.

	Marinobacter sp. strain BF04_CF4	*Marinobacter psychrophilus* 20041^T (97.8%) (Zhang *et al.* 2008)	*Marinobacter antarcticus* ZS2–30^T (96.3%) (Zhang *et al.* 2010)
Acetate	+	ND	ND
L-alanine	+	-	+
D-alanine	+	-	-
D-arabinose	-	+	-
Glycerol	-	+	-
L-lactate	-	-	+
L-leucine	+	-	-
D-serine	-	+	-
L-serine	-	-	+
Sucrose	-	+	-

Marinobacter psychrophilus 20041^T and *antarcticus* ZS2–30^T were isolated from the Arctic and Antarctic, respectively. Percent 16S rRNA gene sequence identities to strain BF05.4 are in parentheses. +: positive for growth; -: negative for growth. ND: No data available.

positive control showed no color change after the addition of denitrification reagents and zinc, indicating nitrate had been reduced beyond nitrite to N_2. The BF04_CF4 genome contained genes encoding for nitrate, nitrite, nitric oxide, and nitrous oxide reductases (Table S1, Supporting Information), all proteins necessary for denitrification. Nitrate and nitrite are low to below detection in Blood Falls brine (Mikucki *et al.* 2009) and may be a consequence of an active nitrate reducing population.

Close relatives of BF04_CF4 that are capable of denitrification have been isolated from other brines in the dry valleys. For example, strain ELB17, was capable of denitrification in laboratory experiments (Ward and Priscu 1997). Using indirect immunofluorescence staining, the maximum concentration of ELB17 cells was found between 17 and 35 m depth in the east lobe and 13 and 22 m depth in the west lobe, which corresponds with the highest concentrations of nitrous oxide production (Ward and Priscu 1997, Lee *et al.* 2004), an intermediate of microbial denitrification under low oxygen concentrations. Similar to Lake Bonney, there are high levels of inorganic nitrogen including nitrous oxide in Lake Vida brine (58.8–86.6 μmol/L N_2O) (Murray *et al.* 2012). These authors concluded that the nitrate and ammonium detected were of atmospheric origin; however, isotopic measurements of the nitrous oxide were inconclusive. *Marinobacter* sp. strains from Lake Vida ice (15.9 m) were capable of nitrous oxide production (Trubl 2013); therefore, *Marinobacter* species may use nitrate as a terminal electron acceptor and contribute to nitrous oxide concentrations under *in situ*, suboxic conditions in Antarctic brines. While our genomic evidence from strain BF04_CF4 and that of strain ELB17 supports the notion that the nitrous oxide profiles in Lake Bonney are of biogenic origin, both biotic and abiotic reactions could be responsible for the concentrations observed. Alternatively, the dissolved iron (up to 55 μM Fe [total]) in Lake Bonney could react with nitrite (up to ~50 μM NO_2^-) in the water column (Ward *et al.* 2003) to form nitrous oxide (Picardal 2012), an abiotic process similar to what has been demonstrated between nitrite-rich (16.1–34.5 μM) Don Juan Pond brine and Fe(II)-bearing minerals (Samarkin *et al.* 2010).

Iron oxidation

BF04_CF4 has genes encoding for enzymes involved in iron acquisition and dissimilatory iron oxidation, but not iron reduction (Table S1, Supporting Information). Genes in BF04_CF4 encode for ABC-type cobalamin/Fe^{3+}-siderophore transport systems used in iron acquisition for assimilatory cellular needs such as enzyme production. During energy acquisition from iron oxidation, electrons from Fe(II) are transported through one of two pathways. One pathway involves electron transfer to cytochrome c oxidase, which then transfers the electrons to oxygen. Alternatively, electrons are shuttled from the bc_1 complex, ubiquinone, and then to the NADH-ubiquinone reductase to regenerate NADH for biosynthesis and carbon fixation (Valdés *et al.* 2008). BF04_CF4 has genes encoding for both pathways including two operons with cytochrome c oxidase genes (*coxA*, *coxB* and *coxC*); there is one operon in the genome that includes genes for the bc_1 complex (*petA*, *petB* and *petC*). These genes are also found in the representative iron-oxidizing bacterium *Acidithiobacillus ferrooxidans* (Accession: AF220499). The concentration of iron in the Blood Falls outflow was high (0.4-3.45 mM) and primarily in the reduced form (>97% as Fe(II)); theoretically, iron oxidation coupled to nitrate reduction could be thermodynamically favorable under the brine E_h (100 mV) and pH (6.2) conditions if nitrate is available (Straub *et al.* 1996; Straub, Benz and Schink 2001). However, the iron is thought to be oxidized abiotically when the brine is exposed to the atmosphere at the glacier surface (Mikucki *et al.* 2009); both pH and E_h of the brine increase rapidly when exposed to air, making iron oxides the more favorable form. BF04_CF4 grew best at neutral pH with a range of 6–10 (Fig. 2C), which indicates it is capable of growth at the *in situ* brine pH as well as the higher values measured when brine is exposed to the surface (Mikucki *et al.* 2004). It is currently unknown if the Fe(II) is microbially oxidized at the surface as well, but it is possible BF04_CF4 is involved in this process. In fact, several *Marinobacter* species are capable of dissimilatory iron oxidation (Handley and Lloyd 2013); *Marinobacter santoriniensis* NKSG[1], *koreensis* DSM 17924^T, and *aquaeolei* DSM 11845^T were shown to chemoheterotrophically oxidize iron coupled to nitrate reduction (Handley, Héry and Lloyd 2009). The ability of BF04_CF4 to oxidize Fe(II) enzymatically was also tested, and when compared to the abiotic control, a red-orange precipitate formed in the BF04_CF4 cultures after approximately two weeks. Thus, it is possible that BF04_CF4 is biotically oxidizing Fe(II) to Fe(III); but the biotic process can be difficult to discern due to the rapid abiotic Fe(II) oxidation by nitrite and to a lesser extent nitrate (Picardal 2012). It is more likely that BF04_CF4 is reducing nitrate to nitrite, thus allowing for abiotic Fe(II) oxidation, a reaction that might also occur in Blood Falls. Perhaps a similar process is responsible for the lack of measureable nitrite in Blood Falls, however further experimentation would be required to confirm this.

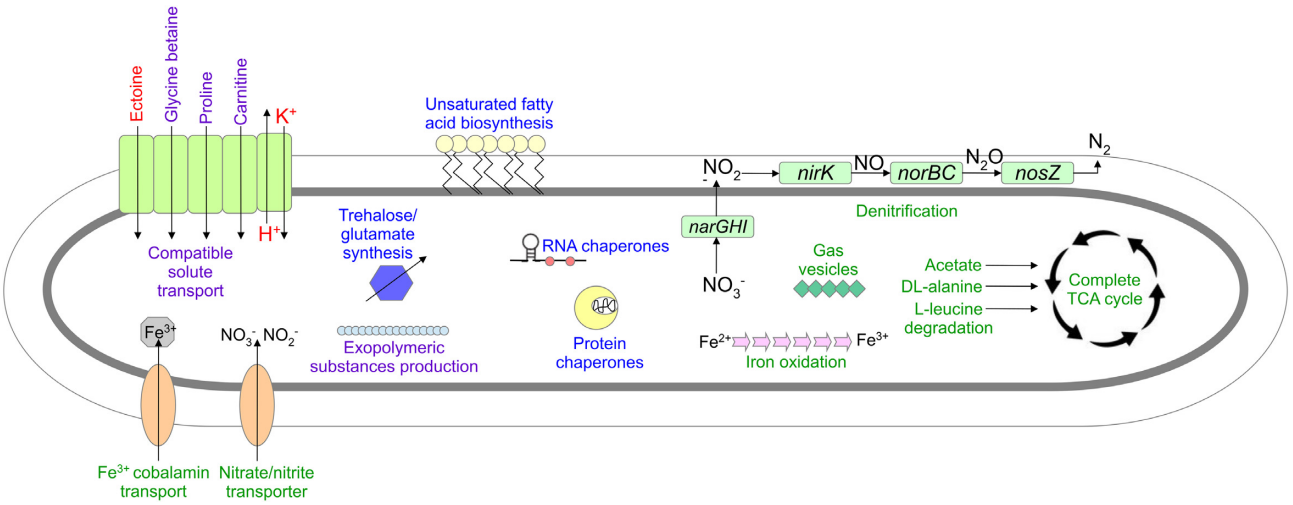

Figure 3. Summary of the main features discussed in this text from the genomic analysis of BF04_CF4. Text color indicates the potential involvement with the following processes. Blue: cold adaptation; red: salt adaptation; purple: cold and salt adaptations; green: metabolic capabilities.

Concluding remarks

Strain BF04_CF4 was isolated from a cold, saline subglacial brine and coinciding with its isolation source, exhibits genomic and physiological adaptations to low temperatures and high salt concentrations (Fig. 3). BF04_CF4 shares similar genomic characteristics to psychrotrophs and psychrophiles, including essential genes in the fatty acid synthase II system. The isolate also has the capability to produce compatible solutes usually involved in osmotic stress response. The isolate grows optimally (fastest) under cold and salty conditions and contains genes for energy efficiency and metabolic pathways potentially useful for growth in the geochemical conditions that exist below the Taylor Glacier and in McMurdo Dry Valley lakes. The metabolic activities of BF04_CF4 demonstrate the strain could contribute to nitrogen and iron cycling in Lake Bonney and Blood Falls. Close relatives of BF04_CF4 have been detected in subsequent brine collections from the conduit feeding Blood Falls, other brine lakes in the McMurdo Dry Valleys and elsewhere in Antarctica and globally that have similar ecological characteristics suggesting the *Marinobacter* lineage may be cosmopolitan to cold salty brines. These data support the notion that BF04_CF4 is not a transient interloper at Blood Falls, rather that the *Marinobacter* lineage, represented by the strain BF04_CF4 we characterized here, is a versatile, ecologically relevant organism in the Blood Falls microbial community and many of the briney lakes in the McMurdo Dry Valleys.

Phylogenetic distance, unique genomic elements and phenotypic distinctions all support strain BF04_CF4 is a unique species. BF04_CF4 contained genes for a CRISPR/cas system and gas vesicles that were not found in its closest characterized relative, *M. psychrophilus*. BF04_CF4 showed a higher salt tolerance (up to 15% NaCl) compared to *M. psychrophilus* (maximum 8% NaCl (Zhang *et al.* 2008) and utilized distinct carbon substrates (Table 3). Collectively, based on genomic analyses and phenotypic characterization presented here, strain BF04_CF4 meets the recommendations for placement of an organism into a new species (Stackebrandt *et al.* 2002; Tindall *et al.* 2010). Therefore, we propose a new species, *Marinobacter gelidimuriae* with strain BF04_CF4 as the type strain.

Marinobacter gelidimuriae (ge.li.di.muÀri.ae. L. adj. *gelidus* icy; L. fem. n. *muria* brine; N.L. gen. n. *gelidimuriae* from icy brine)

Cells are rods of an average 0.5 μm diameter and 1.7 μm length, occurring singly and sometimes in pairs. Cells stain Gram negative and are nonmotile with no noted flagella. Good growth occurs on marine agar plates at 15°C; white, off-white and beige colonies with circular, raised colonies with entire edges form within 10 to 14 days. Acetate, L-alanine, D-alanine and L-leucine can be utilized as organic nutrients for required cell carbon; growth can be coupled to nitrate reduction under anaerobic conditions. Fastest growth (0.8 day^{-1}) occurs in organic rich media at 15°C, within a salinity range of 0.8 to 15% NaCl and a pH range of 6.0 to 10. The predominant fatty acids are unsaturated and included 16:1 ω7c (33%), 18:1 ω9c (27.5%) and 18:1 ω7c (5.9%); saturated fatty acids included 16:0 (13%) and 18:0 (4.4%); as well as the hydroxy fatty acid 12:0 3OH (6.5%) indicative of Gram negative organisms and lesser amounts of 17:1 ω8c (1.1%) and 17:0 (0.6%). The genome is ~3.63 Mb with 54.6% G + C content. The genome contains genes for cold and salt adaptation, gas vesicles and a Type I-C/Dvulg CRISPR/cas system. Originally isolated from saline (8% NaCl), subzero (−5 to −7°C), ferrous, subglacial brine released at Blood Falls in the McMurdo Dry Valleys, Antarctica. Type strain is BF04_CF4 (=ATCC TSD-107 = DSMZ 106148).

Nucleotide sequence accession numbers

The whole genome sequence from *Marinobacter* sp. strain BF04_CF4 has been deposited in GenBank under accession numbers KB889827 to KB889962. The 16S rRNA gene of strain BF14_3D has been deposited to GenBank under accession number KX364066.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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Conflict of interest. None declared.

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