Recovery and Identification of Bacteria from Polar and Non-polar Glacial Ice

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INTRODUCTION

Snowfall accumulates as glacial ice at both poles, and globally at high-altitudes in non-polar regions. Archived chronologically within these glaciers are samples of the atmospheric constituents at the time of snow deposition including particulates of inorganic and biological origin deposited originally on the surface of the snow, often by attachment to snow flakes. Studies of ice cores have established past climate changes and geological events, both globally and regionally, but rarely have these results been correlated with the insects, plant fragments, seeds, pollen grains, fungal spores and bacteria that also are present, and very few attempts have been made to determine the diversity and longevity of viable species entombed in such glacial ice. Fungi, algae, protists, bacteria and viruses have been

detected and recovered from polar ice cores (Abyzov *et al.*, 1982, 1998; Abyzov 1993; Dancer *et al.* 1997; Castello *et al.* 1999; Willerslev *et al*., 1999), but there are very few similar reports describing the microorganisms preserved in non-polar glacial iceof different age and from different locations. Fortunately, for such studies, we have access to ice cores archived at the Byrd Polar Research Center (BPRC) at The Ohio State University. These ice cores have been collected over many years, from globally-distributed sites, and many have already been subjected to extensive physical and chemical analyses. These, therefore, provide the opportunity to isolate and to characterize microorganisms from glacial ice formed at defined dates, under known climate conditions, at geographically very different locations (Figure 1). To avoid problems of surface contamination, we constructed an ice- core sampling system that melts the ice and collects the resulting meltwater from only the interior Here we review the results of bacterial isolations from meltwater generated using this system from the interiors of non-polar and polar glacial ice cores of different vintage, and from Lake Vostok accretion ice (Christner *et al*., 2000; 2001). These results document the longevity and features of bacteria that survive in terrestrial ice, and provide data for arguments that address the likelihood that microorganisms might survive frozen on Mars or Europa, or might travel through space frozen in ice water on comets, asteroids or spacecraft.

ICE CORE SAMPLING

Ice core exteriors are contaminated during drilling and transport, and a sampling system was designed and constructed to melt ice and collect the resulting meltwater aseptically only from the inside of an ice core (Figure 2). A thin section is first cut from one end of the core, using a dedicated dust-free bandsaw, and the newly-exposed flat surface is immersed for 2 min. in 95% ethanol. Exposure to ethanol does not cause the ice core to fracture and, in reconstruction experiments, such an ethanol treatment effectively killed all *Serratia marcesens* cells that were intentionally swabbed onto the saw blade and onto the resulting cut surface of the ice core before the ethanol treatment. However, this treatment may not kill all bacterial endospores, and it certainly would not destroy nucleic acids and

therefore, to monitor for such contamination, the cut surface of each ice core is swabbed after the ethanol treatment before initiating melting. These swabs are used to inoculate growth media and are evaluated for the presence of DNA by polymerase chain reaction (PCR) amplifications using universal 16S rDNA amplification primers. Only very rarely has growth occurred in a swab-inoculated culture, and no PCR product has yet been generated indicating that the levels of contamination on the ethanol-treated ice core surfaces are very low, and below those detectable by standard PCR procedures. Immediately after the exposure to ethanol, the ice core is positioned vertically in the sampling system with the ethanol-washed surface placed directly in contact with the sampling unit. The sampling unit is heated internally and as it melts the ice, it moves upwards through the ice core. The water generated passes through an orifice in the center of the sampling unit and is collected aseptically into sterile containers positioned outside the sampling system (Figure 2).

BACTERIA RECOVERED FROM GLACIAL ICE

The numbers and identities of bacteria that form colonies when meltwater is plated directly on solid media have been determined in ice from Sajama (Bolivia), Guliya (China), Greenland, and Antarctica. In general, meltwaters from non-polar, low-latitude, high-altitude glaciers contain greater number, as well as more diversity of colony-forming bacteria than melt waters from polar ice cores. For example, 180 colony- forming units per ml (cfu/ml) were present in melt water from a 200-year old sample of Guliya ice whereas water from an ~1,800-year old sample of polar ice from Taylor Dome (Antarctica) contained only ~10 cfu/ml. Even fewer cfus were present in meltwater from ice of a similar vintage from the Antarctic Peninsula and from the Summit and Dye 2 sites in Greenland. It is important to note that differences in the amount of annual snowfall, and in the subsequent rates of compression mean that equal volumes of meltwater from different cores do not necessarily represent equivalent time periods of microbial deposition. However, these results are consistent with those of Dancer *et al*. (1997) who recovered <5 cfu/ml from glacial ice from the Canadian high arctic after enrichment for coliform bacteria, and other reports of recovering even fewer bacteria (<1 cfu/ml) from melt waters from polar ice (Abyzov *et al*. 1982; Hardfield *et al*. 1992). Logically, these differences arise because non-polar glaciers are

closer to major sources of airborne microorganisms such as exposed soils, tropical and sub-tropical ecosystems. Consistent with this, meltwater from ice from a Taylor Dome site located at the head of the Taylor Valley in the dry valley complex of Antarctica contained relatively larger numbers of culturable bacteria (~10 cfu/ml), and microbiological surveys have documented the abundance of bacteria, fungi and algae in this area despite the very dry and cold climate (Priscu *et al*., 1998; Brambilla *et al*., 2001).

Based on their small-subunit ribosomal RNA encoding sequences (16S rDNAs) most of the ice-core isolates are members of the non-sporulating Gram-positive, spore-forming *Bacillus, Paenibacillus* and *Actinobacterias*, α- and γ-proteobacterial lineages (Figure 3). Many form colored colonies, consistent with pigment production providing protection from solar irradiation during airborne transport and subsequent exposure on the glacier surface. Isolates with 16S rDNA sequences >95% similar to members of the bacterial genera *Acinetobacter, Arthrobacter, Aureobacterium, Bacillus, Cellulomonas, Clavibacter, Methylobacterium, Microbacterium, Nocardioides, Paenibacillus,* and *Sphingomonas* have been routinely recovered from both polar and non-polar glacial ices, and based on having 16S rDNA sequences >98% similar to the 16S rDNA sequences of the type strain, members of the following bacterial species have been isolated: *Acinetobacter radioresistans, Arthrobacter agilis, Bacillus macroides, Bacillus subtilis, Bacillus thuringiensis, Clavibacter michiganensis* and *Sphingomonas paucimobilis.*

ISOLATION OF BACTERIA FROM VERY OLD GLACIAL ICE

An ice core that extends over 300 meters below the surface (mbs), to the underlying bedrock was obtained from the Guliya Ice Cap in Tibet (Figure 1), and based on the abundance of ^{36}Cl (half life = 301,000 years) the ice at the bottom of this core is >500,000 years old (Thompson *et al.* 1997). This is the oldest glacial ice recovered to date and provides an opportunity to evaluate microbial survival in ice on a time scale potentially meaningful for inter-planetary transport. Aliquots of meltwater from this ice core from 296 mbs were inoculated into a variety of growth media and, after 30-60 days of aerobic incubation at 4^{o}C, growth was observed in very dilute nutrient and tryptic soy broths. These media were used at

1% of the concentration recommended by the manufacturer (Difco, Inc.). Despite the long period needed for initial growth, and the primary enrichment cultures being grown under oligotrophic conditions at 4°C, isolates were subsequently obtained from these cultures that grew and formed colonies in 2-7 days on nutrient-rich media at 25°C. Long-dormant cells must eliminate toxic metabolites, such as hydrogen peroxide, superoxide and free radicals, and repair macromolecular damage that has accumulated before they can grow and divide successfully (Dodd *et al.*, 1997). The results with the very old Guliya ice are consistent with this hypothesis, and indicate that successful recovery is facilitated by providing only a very low level of nutrients initially, sufficient for repair but insufficient to elicit an instant attempt at growth.

Fourteen 16S rDNA sequences, corresponding to nucleotides 27 through 1492 of the *Escherichia coli* 16S rDNA sequence have been determined from isolates from the very old Guliya ice (Figure 4). Based on these data, most of these belong to the same bacterial lineages as the isolates obtained from more recent polar and non-polar glacial ices, and ~50% are members of genera that form endospores known to facilitate long-term survival under non-growth conditions (Cano and Borucki, 1995; Vreeland *et al*., 2000). Light microscopy has revealed that some also have thick cell walls and form polysaccharide capsules that presumably also contribute to survival through the physical stresses imposed by freezing, compaction pressure, and thawing (Fogg, 1998).

ISOLATION OF BACTERIA FROM LAKE VOSTOK ACCRETION ICE

More than 70 sub-glacial lakes have been discovered in Antarctica. The largest, Lake Vostok, has been covered by a layer of glacial ice and isolated from direct surface input for at least 420,000 years (Petit *et al*., 1999). Glacial ice melts into Lake Vostok at the northern ice-water interface and water from Lake Vostok freezes and accumulates as accretion ice directly below the glacial ice over the central and southern regions (Kapitsa *et al*., 1996; Jouzel *et al*., 1999; Siegert *et al*., 2000). It seems very likely that viable bacteria are seeded into Lake Vostok as glacial ice melts into the lake. However, whether an active microbial community is established within Lake Vostok remains uncertain as concerns for contamination have

resulted in a moratorium on direct sampling of Lake Vostok water. Ice core drilling also was terminated above the ice-water interface although an ice core was retrieved in which the bottom ~150 meters are accretion ice and therefore represent a sample of Lake Vostok water. A section of this core from 3591.965 to 3592.445mbs, designated as core section 3593, was obtained from the National Ice Core Laboratory (Denver, CO), and has been subjected to microbiological investigation (Christner *et al*., 2001).

Scanning electron microscopy of materials filtered from core 3593 meltwater revealed the presence of particulates with size and morphology consistent with bacterial cells (Figure 5), and four different single-colony isolates were obtained from enrichment cultures inoculated with core 3593 meltwater. Based on their 16S rDNA sequences, these isolates are related to established species of *Brachybacterium, Sphingomonas, Paenibacillus* and *Methylobacterium* (Figure 6). Six bacterial 16S rDNAs also were amplified from core 3593 meltwater with sequences indicating that they originated from five different bacterial lines of descent. Interestingly, sequence pA419 originated from an $\alpha\alpha$-proteobacterium whose nearest 16S rRNA neighbors are isolates from Lake Baikal (Russia) (Benson *et al*., 2000). The closest 16S rRNA relatives of the remaining 16S rDNA amplicons obtained from Lake Vostok accretion ice meltwater also are from isolates from freshwater environments. Only very tenuous extrapolations can be made from 16S rDNA sequences, but the results obtained suggest that Lake Vostok is seeded, and probably inhabited by species with features similar to bacteria that inhabit other permanently-cold environments.

DISCUSSION AND CONCLUSIONS

Microorganisms recovered from glacial ice are likely to have already endured desiccation, solar irradiation, freezing, a period of frozen dormancy, and thawing. It is not surprising therefore that many of the ice core isolates are pigmented and belong to bacterial groups that differentiate into spores that specifically confer resistance to such environmental abuse and facilitate long-term survival under non-growth conditions. Many also have thick cell walls and polysaccharide capsules and have been demonstrated to be more resistant to

repeated cycles of freezing and thawing than standard laboratory bacterial species. Interestingly, closely related bacteria have been recovered from glaciers separated by great distances, suggesting the possibility that some species may indeed have evolved features that help their survival and, conceivably, may even facilitate growth under freezing conditions. Thin films of liquid water may exist between ice crystals, even within apparently solid ice (Price, 2000 AND THIS VOLUME), and studies of permafrost (Rivkina *et al.*, 2000 AND THIS VOLUME), basal glacial ice (Skidmore *et al.*, 2000), and surface snow (Carpenter *et al*. 2000), have all demonstrated microbial activity under freezing conditions.

Ice cores from low-latitude, high-altitude glaciers generally contain more recoverable bacteria than polar ice cores, presumably because the Andes and Himalayas are closer to major sources of airborne biological materials. Similarly, polar ice from regions adjacent to the exposed soils and rock surfaces in the Taylor Valley (Antarctica) contains more recoverable bacteria than polar ice from remote regions. We have established that bacteria remain viable when frozen in glacial ice for >500,000 years and, based on other studies of *Bacillus* spore longevity (Cano and Borucki, 1995; Vreeland *et al*., 2000), this is almost certainly an underestimate. Therefore, it seems plausible that desiccation-resistant microorganisms, possibly cyptoendolithic microbial communities, could similarly be entombed and preserved in a frozen but viable state in ice on Mars. It is also possible that some microorganisms might even maintain some metabolic activity while apparently frozen within ice.

By identifying and counting the microorganisms present in glacial ice of very different age, we may be able to relate climate change and geography to local airborne microbial populations. Similarly by characterizing individual isolates, we can obtain information that contribute to discussions of the possibility that microorganisms might survive frozen in extra-terrestrial environments. These isolates should also provide data that are directly relevant to discussions of the prevalence of antibiotic resistance before the advent of antibiotic therapies, and the survival of life through "Snowball Earth" events (Hoffman *et al.,* 1998).

ACKNOWLEDGMENTS

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Figure 1. Locations of sampling sites and ice cores available for study at the Byrd Polar Research Center (BPRC). To date, bacteria have been isolated from ice cores sampled from glaciers at both poles, in the mountain ranges on the subtropical Tibetan plateau, and in the tropical Bolivian Andes. In each case, the nearest major ecosystem, and therefore most likely origin of airborne particulates, is very different.

Figure 2. The ice core sampling system. (A) The sampling system is assembled completely inside a laminar flow hood that is housed within a -10^0C walk-in freezer. All components of the system are autoclaved, dried and exposed to ethylene oxide for 12 h before use. An ice core is positioned vertically in the sampler with the cut end of the core contacting (B) the heated sampler head which melts upward (C) through the core and collects the resulting melt water. In (C), the sampler head is shown disassembled from the main unit to illustrate its movement through the interior of the ice core.

Figure 3. Bacterial genera represented most frequently by ice core isolates. The number of isolates from both polar and non-polar ice cores, obtained from each of the bacterial genera shown is listed in parentheses. The phylogenetic relationships illustrated are based on 16S rDNA sequences. They are not drawn to scale.

Figure 4. Phylogenetic position of 14 bacterial isolates from ice >500,000 years old from 296 meters below surface of the Guliya ice cap. 16S rDNA sequences (~1400 nucleotides) were obtained from the cells from a single colony of each isolate. They were aligned based on secondary structures using the ARB software package (Strunk *et al*. 1998) and a best fit neighbor-joining tree was constructed. Evolutionary distance is defined as the number of fixed nucleotide changes per position.

Figure 5. Scanning electron micrographs of materials filtered from meltwater from Lake Vostok deep ice core section 3593. The particulates shown, apparently bacteria, are retained on the surface of a 0.2 μm isopore (Millipore) filter.

Figure 6. Phylogenetic analysis of 16S rDNA sequences isolated from bacteria and directly amplified from meltwater from Lake Vostok core section 3593. Sequences that correspond to nucleotides 515 through 1392 of the *E. coli* 16S rDNA were obtained, aligned and used to construct the figure shown as in Figure 5 (Strunk *et al.*, 1998). A best fit tree was created using maximum likelihood with a 771 nucleotide mask of unambiguously aligned positions using fastDNAml (Olsen *et al.,* 1994).

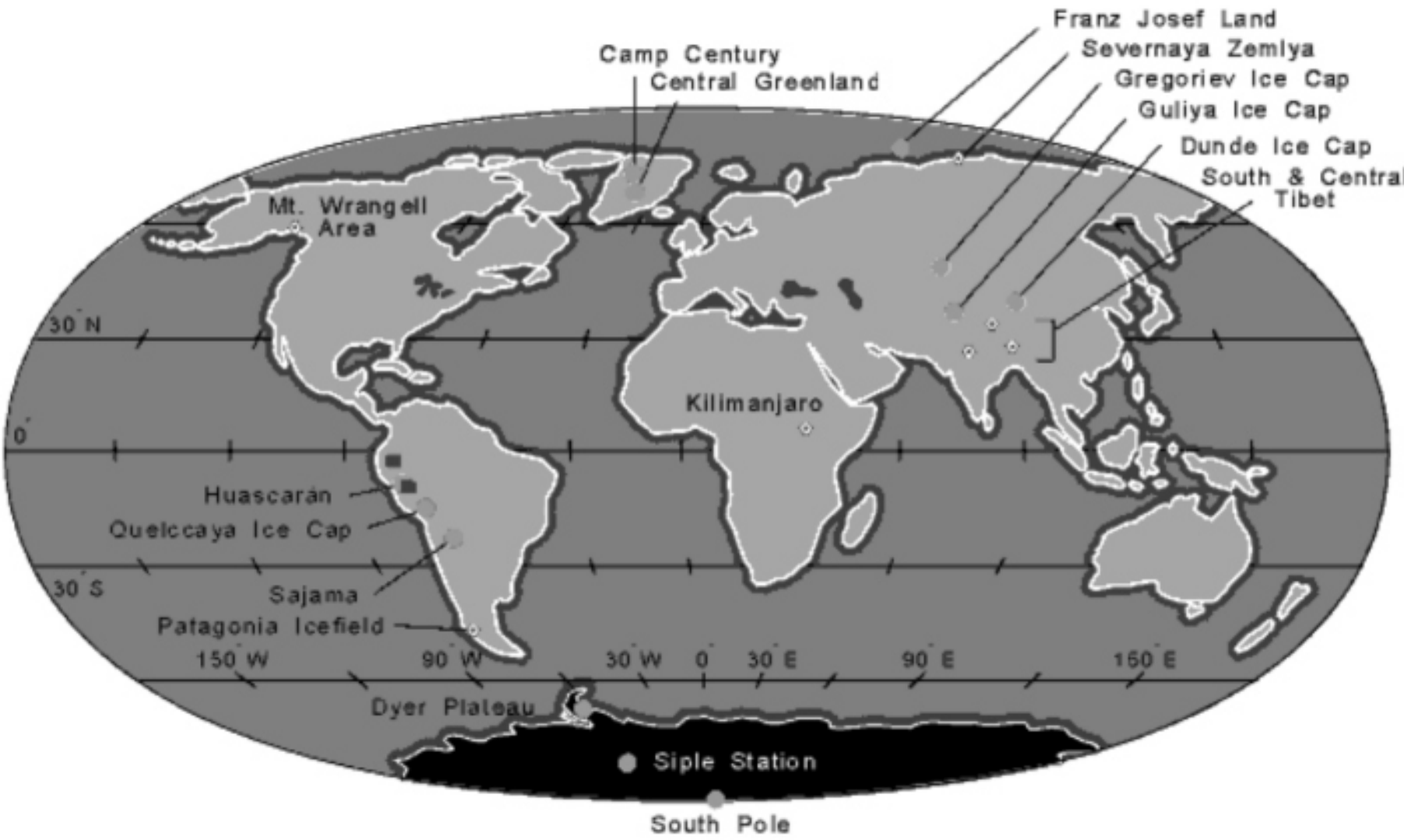

Franz Josef Land
Severnaya Zemlya
Camp Century
Central Greenland
Gregoriev Ice Cap
Guliya Ice Cap
Dunde Ice Cap
South & Central
Tibet
Mt. Wrangell
Area
30 N
Kilimanjaro
0
Huascarán
Quelccaya Ice Cap
30 S
Sajama
Patagonia Icefield
150 W
90 W
30 W
0
30 E
90 E
150 E
Dyer Plateau
Siple Station
South Pole
Present ● and Future * Ice Core Sites

Figure 1

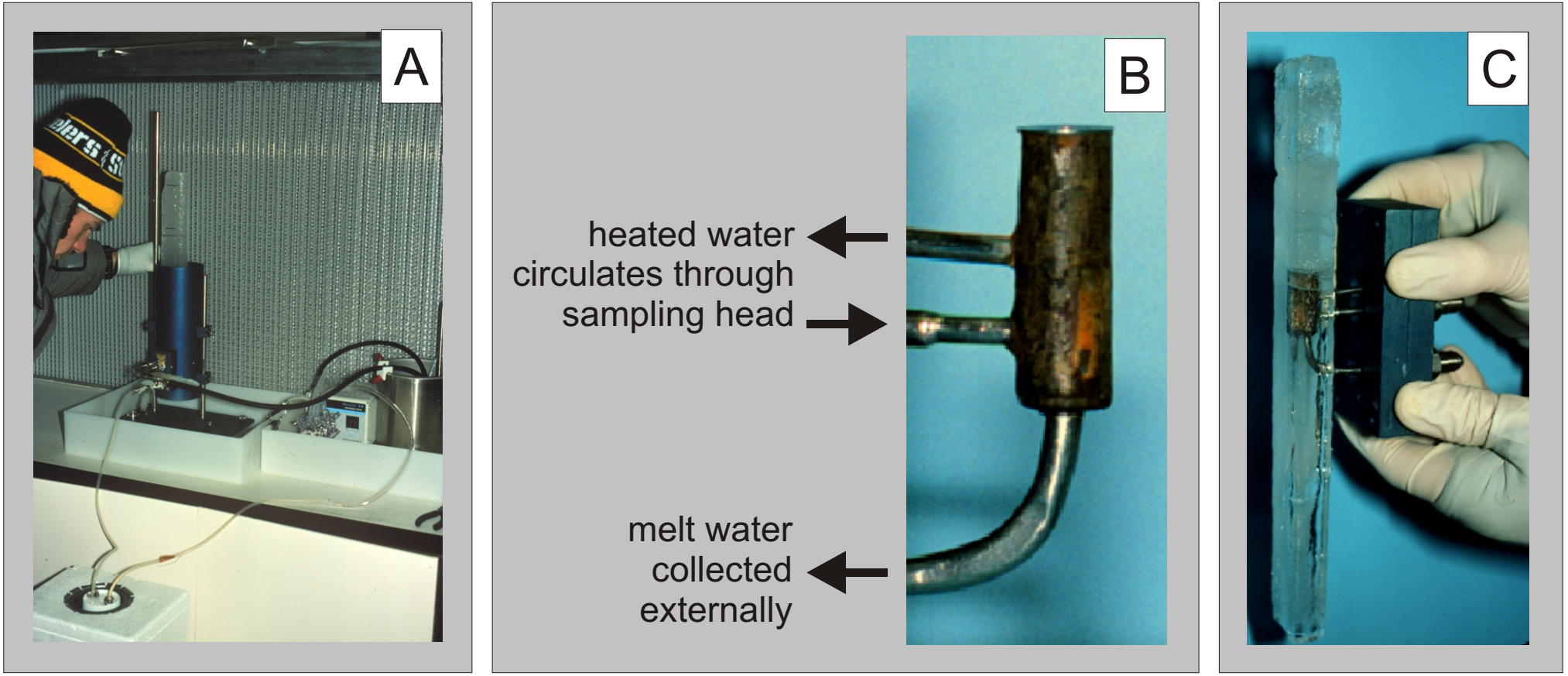

A
B
heated water
circulates through
sampling head
melt water
collected
externally
C

Figure 2

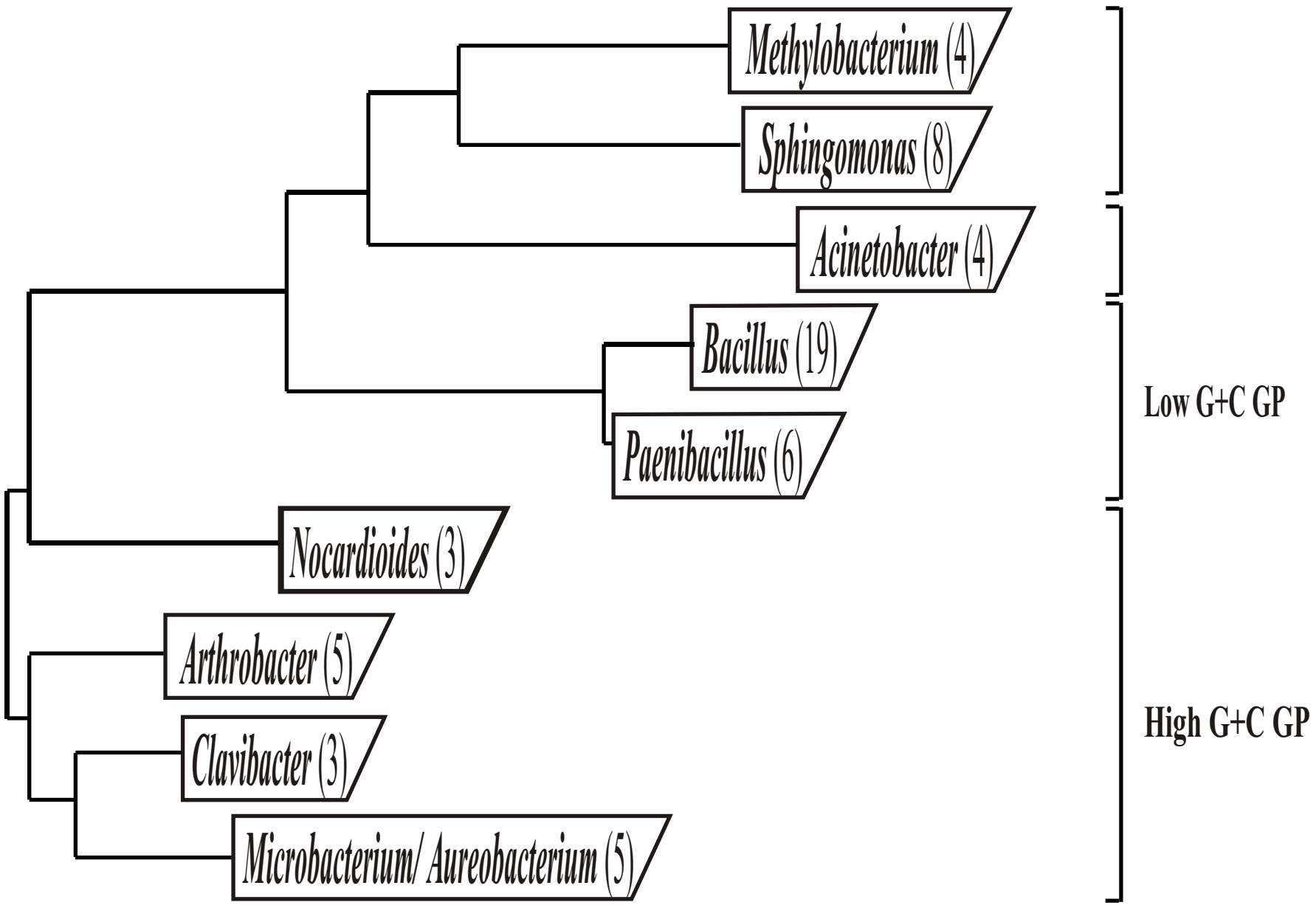
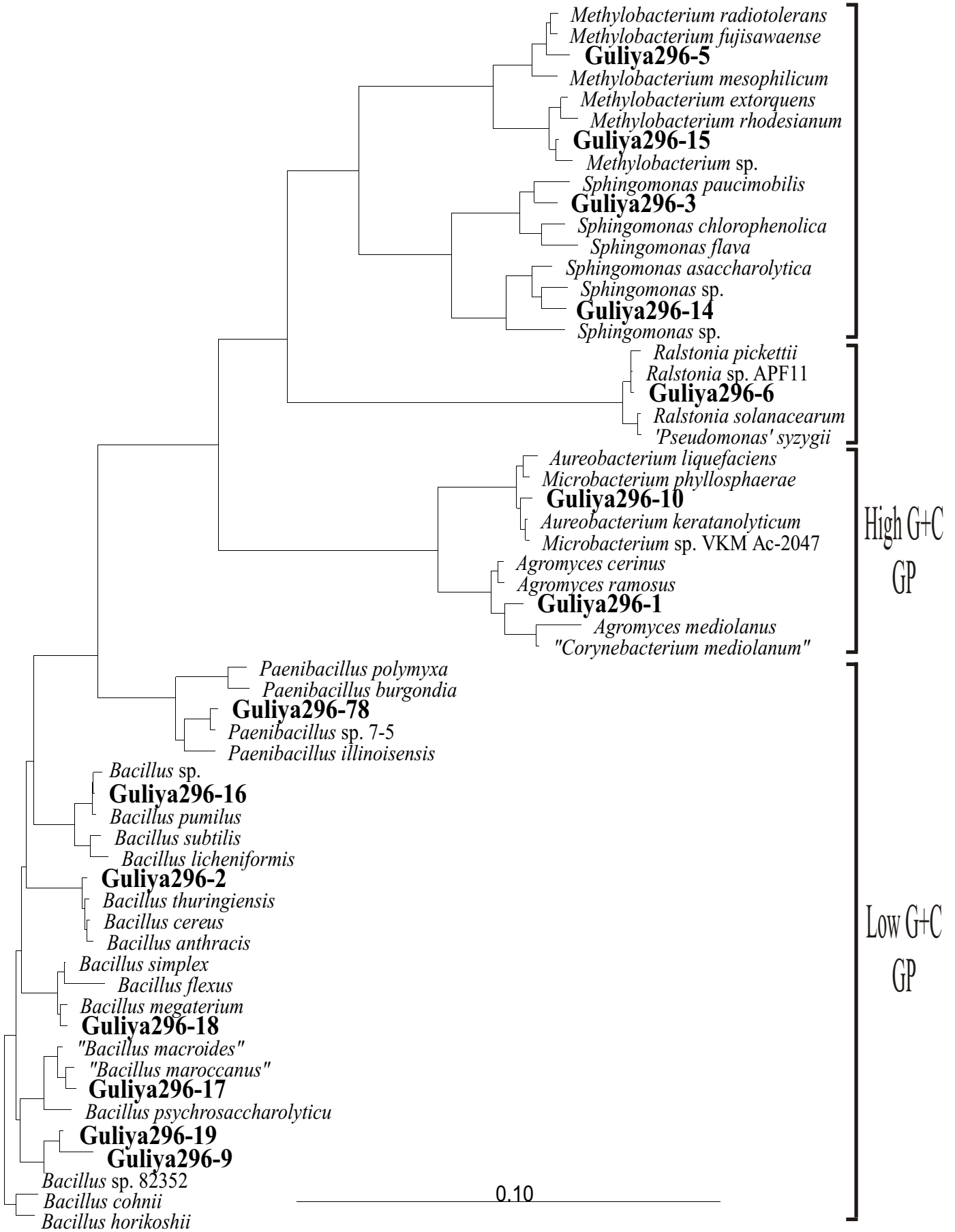

Methylobacterium (4)
Sphingomonas (8)
Acinetobacter (4)
Bacillus (19)
Paenibacillus (6)
Nocardioides (3)
Arthrobacter (5)
Clavibacter (3)
Microbacterium/ Aureobacterium (5)
Low G+C GP
High G+C GP

Figure 3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Methylobacterium fujisawaense
Guliya296-5
Methylobacterium mesophilicum
Methylobacterium extorquens
Methylobacterium rhodesianum
Guliya296-15
Methylobacterium sp.
Sphingomonas paucimobilis
Guliya296-3
Sphingomonas chlorophenolica
Sphingomonas flava
Sphingomonas asaccharolytica
Sphingomonas sp.
Guliya296-14
Sphingomonas sp.
Ralstonia pickettii
Ralstonia sp. APF11
Guliya296-6
Ralstonia solanacearum
'Pseudomonas' syzygii
Aureobacterium liquefaciens
Microbacterium phyllosphaerae
Guliya296-10
Aureobacterium keratanolyticum
Microbacterium sp. VKM Ac-2047
Agromyces cerinus
Agromyces ramosus
Guliya296-1
Agromyces mediolanus
"Corynebacterium mediolanum"
Paenibacillus polymyxa
Paenibacillus burgondia
Guliya296-78
Paenibacillus sp. 7-5
Paenibacillus illinoisensis
Bacillus sp.
Guliya296-16
Bacillus pumilus
Bacillus subtilis
Bacillus licheniformis
Guliya296-2
Bacillus thuringiensis
Bacillus cereus
Bacillus anthracis
Bacillus simplex
Bacillus flexus
Bacillus megaterium
Guliya296-18
"Bacillus macroides"
"Bacillus maroccanus"
Guliya296-17
Bacillus psychrosaccharolyticu
Guliya296-19
Guliya296-9
Bacillus sp. 82352
Bacillus cohnii
Bacillus horikoshii
0.10
High G+C GP
Low G+C GP

Figure 4

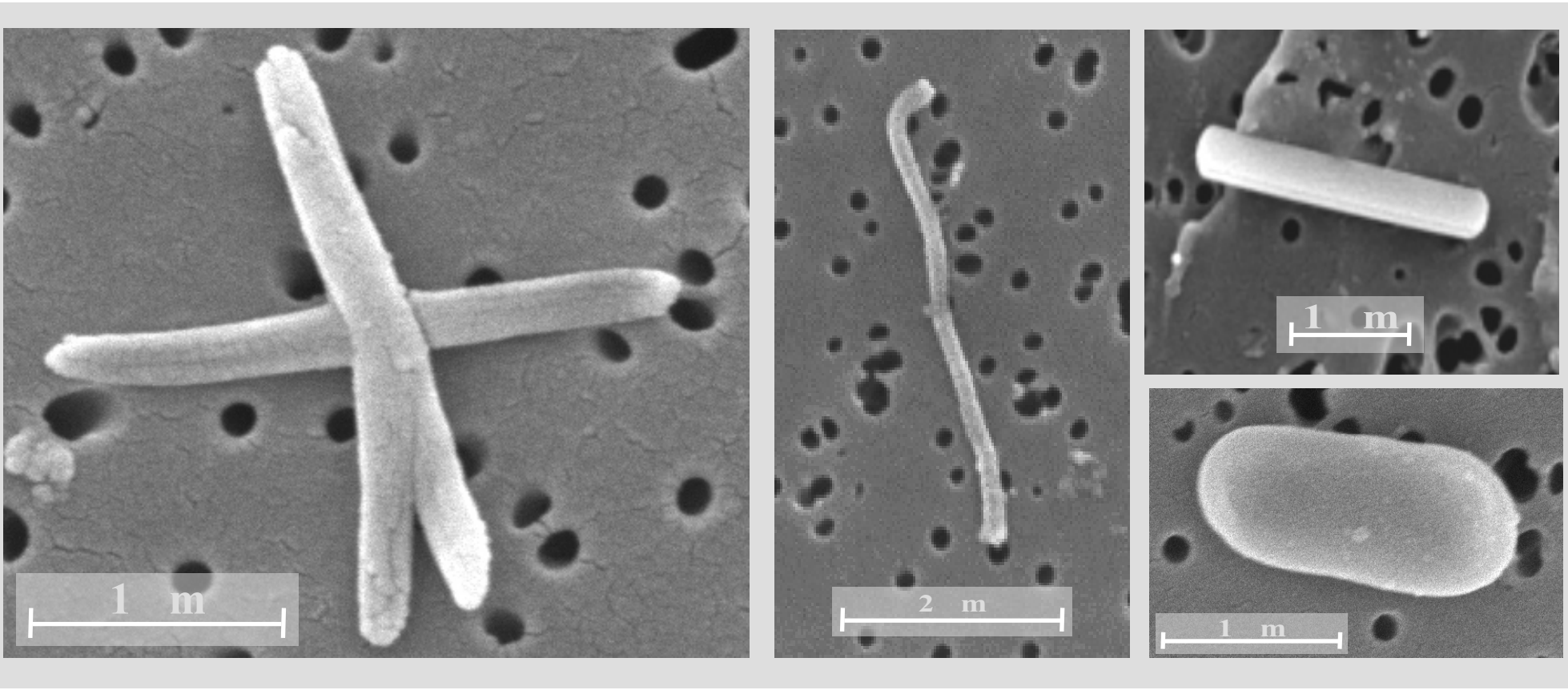
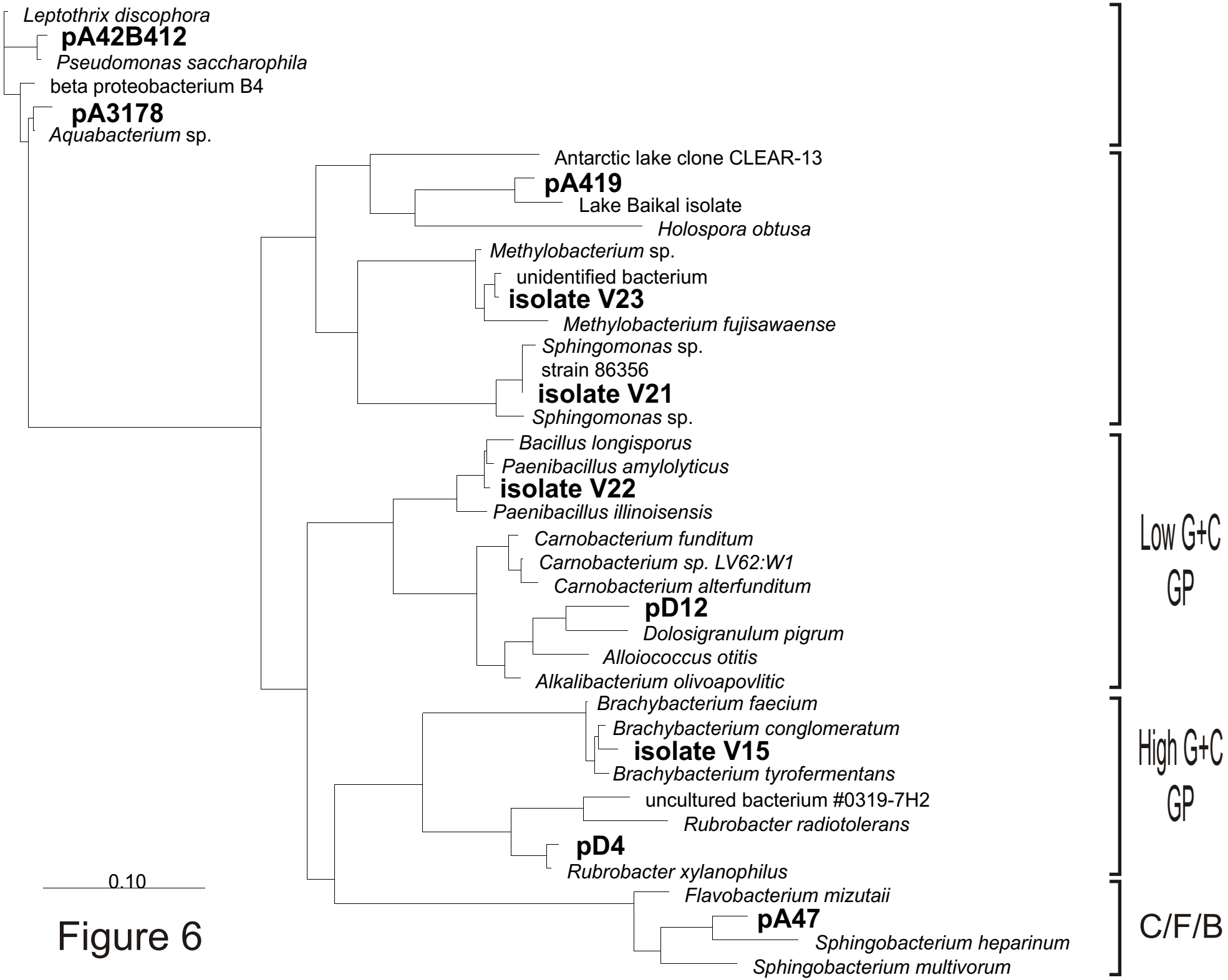


Figure 5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Pseudomonas saccharophila
beta proteobacterium B4
pA3178
Aquabacterium sp.
Antarctic lake clone CLEAR-13
pA419
Lake Baikal isolate
Holospora obtusa
Methylobacterium sp.
unidentified bacterium
isolate V23
Methylobacterium fujisawaense
Sphingomonas sp.
strain 86356
isolate V21
Sphingomonas sp.
Bacillus longisporus
Paenibacillus amylolyticus
isolate V22
Paenibacillus illinoisensis
Carnobacterium funditum
Carnobacterium sp. LV62:W1
Carnobacterium alterfunditum
pD12
Dolosigranulum pigrum
Alloiococcus otitis
Alkalibacterium olivoapovlitic
Brachybacterium faecium
Brachybacterium conglomeratum
isolate V15
Brachybacterium tyrofermentans
uncultured bacterium #0319-7H2
Rubrobacter radiotolerans
pD4
Rubrobacter xylanophilus
Flavobacterium mizutaii
pA47
Sphingobacterium heparinum
Sphingobacterium multivorum
0.10
Low G+C GP
High G+C GP
C/F/B

Figure 6