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ORIGINAL PAPER

Characterization of *Hymenobacter* isolates from Victoria Upper Glacier, Antarctica reveals five new species and substantial non-vertical evolution within this genus

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Abstract We isolated several *Hymenobacter*-like strains from Victoria Upper Glacier, Antarctica, basal ice that diverged substantially from currently defined *Hymenobacter* species according to their 16S rRNA and *gyrB* gene phylogenies. All strains were psychrotolerant, heterotrophic aerobes which grew preferentially on low salt and low nutrient strength agar. Further phenotypic and chemotaxonomic characterization of these isolates supported their assignment as five novel species: *H. algoricola* sp. nov., *H. antarcticus* sp. nov., *H. elongatus* sp. nov., *H. fastidiosus* sp. nov., and *H. glaciei* sp. nov. Remarkable among these data was the prevalence of horizontal gene transfers and phenotypic variation, even between apparently closely related strains. These results suggest extensive non-vertical evolution within the genus *Hymenobacter*, and may reflect evolutionary trajectories resulting from dormancy, e.g., during interment in glacial ice.

Keywords Systematics · Taxonomy · Psychrophiles · Psychrophile ecology · Psychrophile physiology

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Introduction

The genus *Hymenobacter* represents a deeply divergent and relatively poorly studied branch of the Flexibacteraceae (Bacteroidetes), including strains formerly and informally classified within the genus '*Taxeobacter*' (Reichenbach 1992; Buczolits et al. 2006). Representatives from this genus have been detected predominantly in extreme environments, including Antarctic and high-altitude soil and/or permafrost (Hirsch et al. 1998; Saul et al. 2005; Aislabie et al. 2006a, b; Yergeau et al. 2007; Zhang et al. 2007, 2008a, 2009; Costello et al. 2009; Dai et al. 2009), glacial ice (Zhang et al. 2006, 2008b; Klassen and Foght 2008), temperate desert soil (Rainey et al. 2005; Fredrickson et al. 2008), Antarctic lakes (Mojib et al. 2009), snow (Segawa et al. 2005; Fujii et al. 2010), the surface of plant leaves (Delmotte et al. 2009), aerosols (Buczolits et al. 2002, 2006; Osman et al. 2008; Polymenakou et al. 2008), irradiated meat (Collins et al. 2000) and sanitized clean-room facilities (Venkateswaran et al. 2003). Oxidative stress is common in all of these environments; oxidative stress resistance may therefore be a common and unifying characteristic of this genus.

Glacier ice is a well-established habitat for microbial life (Christner et al. 2008; Hodson et al. 2008; Miteva 2008). Recent efforts to obtain viable heterotrophic bacteria from Victoria Upper Glacier (VUG), Antarctica, resulted in the isolation of a suite of strains dominated by *Hymenobacter*-like organisms, particularly conspicuous by the bright red-pink pigmentation of their colonies (Klassen and Foght 2008; Klassen 2009). Because of the apparent phylogenetic novelty represented by these strains (Klassen and Foght 2008), the current study was undertaken to determine their precise taxonomic identifications. Here, we report five new *Hymenobacter* species isolated from VUG

and document substantial evidence for non-vertical modes of evolution within this genus. This research both expands the number of described *Hymenobacter* species and suggests the potential impact of dormancy on their evolution.

Methods

Strain isolation and genotypic selection

In total, 58 *Hymenobacter*-like strains were isolated from VUG basal ice as described previously (Klassen and Foght 2008). To ensure that only unique strains were further characterized, enterobacterial repetitive intergenic consensus (ERIC)- and repetitive extragenic palindromic (REP)-PCR analyses (De Bruijn 1992) were used to discriminate between highly related strains. Genomic DNA was extracted according to Foght et al. (2004) and used as templates for PCR reactions using the primers ERIC1R and ERIC2 for ERIC-PCR and REP1R-I and REP2-I for REP-PCR (see Table 1 for sequences). The reaction mixtures and PCR conditions used were those of De Bruijn (1992), and the resulting PCR products were visualized by staining with SYBR-Green (Molecular Probes). Unique strains isolated during this study were deposited in the JCM culture collection (strain numbers JCM 17213–17227). Reference strains were purchased from the DSMZ, CCUG and KCTC culture collections, or kindly donated by H.-J. Busse (University of Vienna), J. Aislabie (Landcare Research, New Zealand) and C. Fang (China Centre for Type Culture Collection).

Phylogenetic analysis

DNA was isolated from all strains and the 16S rRNA gene sequences were determined according to Foght et al. (2004) and Cheng and Foght (2007) (Table 1). Part of the *gyrB* gene encoding the DNA gyrase β subunit was also PCR amplified using the primers UP-1 and UP-2r and PCR protocol of Yamamoto and Harayama (1995; Table 1) and the same reaction mixture used for the 16S rRNA gene (Foght et al. 2004). Sequencing of *gyrB* amplicons was conducted using the Big Dye method (Applied Biosystems) according to standard protocols using the primers UP-1S, UP-2Sr (Yamamoto and Harayama 1995; Table 1), gyrBintF and gyrBintR (this study; Table 1). The GenBank accession numbers for the 16S rRNA and *gyrB* gene sequences determined here are GQ454797–GQ454840.

All sequences were assembled using the PREGAP v1.5 and GAP4 v4.10 programs of the Staden package (Dear and Staden 1991), ensuring that the *gyrB* sequence encoded an open reading frame. Assembled partial 16S rRNA gene sequences were aligned using CLUSTAL_X v2.0 (Larkin et al. 2007). Partial *gyrB* sequences were aligned as translated peptides using CLUSTALW (Thompson et al. 1994) as implemented in MEGA v4.0 (Tamura et al. 2007). All sequences were trimmed to eliminate primer sequences and to ensure homogeneous sequence starting and ending positions. Indels (1–5 nucleotides and codons for the 16S rRNA and *gyrB* sequences, respectively) were noted using GENDOC (http://www.nrbsc.org/gfx/genedoc/) but removed prior to tree construction.

Table 1 Sequences of the primers used in this study

Primer name	Primer sequence (5′ → 3′)	Reference
ERIC1R	ATGTAAGCTCCTGGGGATTCAC	De Bruijn (1992)
ERIC2	AAGTAAGTGACTGGGGTGAGCG	De Bruijn (1992)
REP1R-I	IIIICGICGICATCIGGC	De Bruijn (1992)
REP2-I	ICGICTTATCIGGCCTAC	De Bruijn (1992)
PB36F	AGRGTTTGATCMTGGCTCAG	Foght et al. (2004)
PB36R	GKTACCTTGTTACGACT	Foght et al. (2004)
16S1F	ACTCCTACGGGAGGCAGCAG	Cheng and Foght (2007)
16S2R	GTATTACCGCGGCTGCTGGCA	Cheng and Foght (2007)
16S3F	GGATTAGATACCCKGGTAGTCC	Cheng and Foght (2007)
16S4R	GGTTAAGTCCCGCAACGAGC	Cheng and Foght (2007)
16S5R	GCTCGTTGCGGGACTTAACC	Cheng and Foght (2007)
UP-1	GAACTCATCATGACCGTTCTGCAY GCNGGNAARTTY	Yamamoto and Harayama (1995)
UP-2r	AGCAGGGTACGGATGTGCGAGCCRTCNGC RTCNGCRTCNGTC	Yamamoto and Harayama (1995)
UP-1S	GAACTCATCATGACCGTTCTGGA	Yamamoto and Harayama (1995)
UP-2Sr	AGCAGGGTACGGATGTGCGAGCC	Yamamoto and Harayama (1995)
gyrBintF	GCSGTSAGRCCYTCSCGGAAGTC	This study
gyrBinfR	AACTACAAYAAYTGCATC	This study

Bootstrapped maximum likelihood nucleotide trees were generated using RAxML (Stamatakis et al. 2008) as implemented through the CIPRES web portal (http://www.phylo.org/). Trees were rooted using *Cytophaga hutchinsonii* ATCC 33406 (genome accession number: NC_008255) and the number of bootstrap replicates was determined automatically. Because the maximum likelihood 16S rRNA gene tree had unusually low overall bootstrap values (typically <60%), a neighbor-joining tree for this gene was also constructed using PHYLIP v.3.65 (Felsenstein 1989) per Klassen and Foght (2008). Trees generated using either method were essentially congruent with each other.

Phenotypic characterization

All *Hymenobacter* strains were grown on R2 agar (R2A; Difco) at 18°C for 1 week in the dark. Whereas these conditions were near-optimal for growth of all VUG *Hymenobacter*-like strains, most reference *Hymenobacter* strains grew equally well or better at 28°C. Liquid culture was not used because of the poor growth of many *Hymenobacter* and related strains, particularly those isolated from VUG, in all liquid media tested (Klassen and Foght 2008).

Growth-permissive temperatures were assessed using R2A incubated in the dark at 4, 10, 18, 28 or 37°C. Growth on more nutrient-rich media was examined using Luria–Bertani agar (LB: 10 g/l tryptone, Difco; 10 g/l NaCl, EMD; 5 g/l yeast extract, Difco; and 15 g/l agar, Difco; pH 7.5) and trypticase soy agar (TSA: 30 g/l trypticase soy broth, BBL; and 15 g/l agar, Difco). Anaerobic and microaerobic growth was assayed using aerobically prepared R2A plates incubated in sealed jars containing Anaerocult A and C packages (BBH), respectively. Halotolerance was tested using R2A supplemented with 0.5, 1, 2, 3 or 4% (w/v) NaCl, and pH tolerance was tested using R2A agar adjusted following autoclaving to pH 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12 ± 0.2 using 0.22 μm Millipore filter-sterilized HCl or NaOH. Except for temperature tolerance experiments, all incubations were conducted at 18°C in the dark and scored after 1 and 2 weeks, using inocula cultures grown on R2A at 10°C in the dark for 1 week (by which time robust growth was just beginning to appear) to ensure their active growth.

Colony morphologies were determined on R2A, incubated and inoculated as above. These same cultures were used for microscopic examination using an Axiostar Plus microscope equipped with A-plan objective lenses and an AxioCam ICc 1 digital camera (Ziess). Images were recorded using the AxioVisionLE software v.4.6.1.0 (Ziess) with the white balance and image capture parameters determined automatically. Cell size was estimated using the length measurement tool in the AxioVisionLE software. Gram stains, KOH, catalase and oxidase tests were conducted according to standard methods (Hurst et al. 2007).

Metabolic profiling

Metabolic profiling was conducted using the Biolog GN2 MicroPlate assay (Biolog Inc.). Using a sterile cotton swab, colonies grown at 10°C in the dark for 1 week were resuspended in GN/GP-IF inoculating fluid (Biolog Inc.) by rubbing on the tube sides until a turbidity of approximately 50% was reached (recorded using a turbidimeter; Biolog Inc.). Three drops of sterile saturated sodium thioglycolate (Fisher) were added to each tube prior to inoculation to prevent metabolism of the endogenous bacterial capsule. Biolog plates were inoculated using 150 μl of these cell suspensions per well, avoiding clumps to ensure an even inoculation density. Plates were scored following 1 and 2 weeks incubation in the dark at 18°C using a Molecular Devices Emax plate reader (Biolog Inc.) by recording absorbance at 590 and 750 nm. Data were processed using default parameters using the proprietary Biolog database software attached to the plate reader.

Fatty acid and carotenoid determination

Cellular fatty acid composition was determined by Keystone Labs (Edmonton, Canada). Following growth on R2A at 18°C for 1 week in the dark, fatty acids were extracted, methylated and analyzed using the MIDI system according to the supplier's instructions (MIDI technical note #101, July 2009 revision). Carotenoids were analyzed from strains grown on R2A at 18°C for 1 week in the dark according to Klassen and Foght (2008) and identified by comparison to known standards (Klassen et al. 2009).

Results

Phylogenetic analysis of molecular marker genes

Twenty of the 58 *Hymenobacter*-like strains isolated from VUG possessed unique ERIC- and/or REP-PCR banding pattern types (data not shown) and were therefore characterized further. Partial 16S rRNA gene sequences from these VUG and reference *Hymenobacter* strains were determined and analyzed phylogenetically (Fig. 1). All analyzed VUG strains formed a single monophyletic clade with previously described *Hymenobacter* species sister to the related genera *Adhaeribacter*, *Effluviibacter* and *Pontibacter*. The monophyletic clustering of *Hymenobacter* relative to the other analyzed clades is confirmed by the

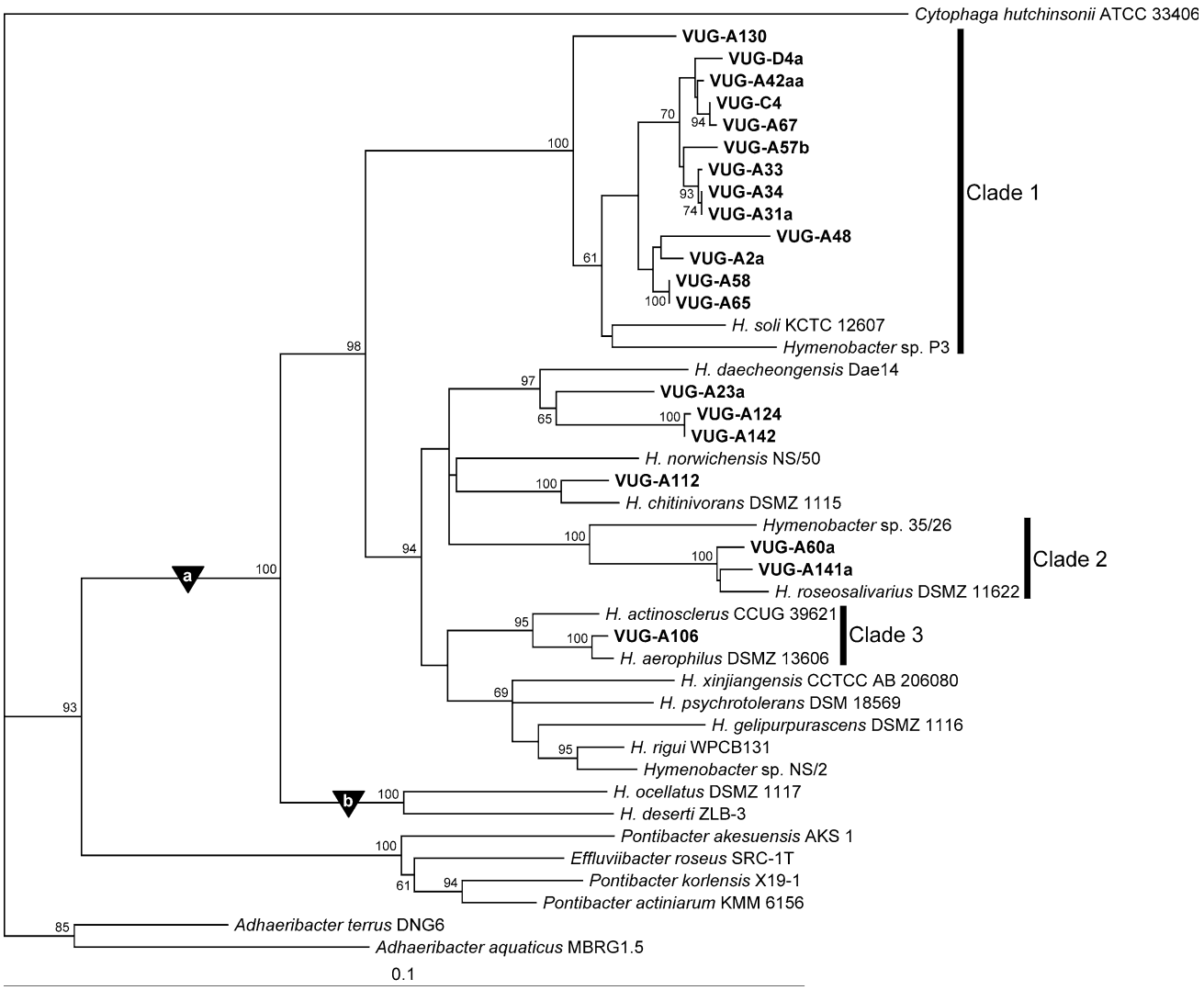

Fig. 1 Neighbor-joining phylogenetic tree of near full-length (≈1500 nt) 16S rRNA gene sequences for *Hymenobacter* and related VUG strains (*bolded*). *C. hutchinsonii* and species from the genera *Adhaeribacter*, *Effluviibacter* and *Pontibacter* were included as outgroups. Only bootstrap values ≥60 are shown and the *scale bar* represents 10% sequence divergence. Sequences containing phylogenetically conserved indels are shown by *triangles* on the indel-containing branch; *different letters* indicate unique indels. Those indels present in only one sequence are not shown, and all were excised from the alignment used to generate the phylogenetic tree

presence of 16S rRNA indel "a" present in these strains and absent in the outgroups.

Hymenobacter and related VUG strains formed several well-supported clades in the 16S rRNA gene phylogeny (Fig. 1). Clade 1 comprises strains VUG-A130, VUG-D4a, VUG-A42aa, VUG-C4, VUG-A67, VUG-A57b, VUG-A33, VUG-A34, VUG-A31a, VUG-A48, VUG-A2a, VUG-A58, VUG-A65, *H. soli* and strain P3. The 16S rRNA gene sequences from all VUG strains in this clade are 96–97% similar to that of *H. soli*. A second lineage comprises most of the currently described *Hymenobacter* species as well as strains VUG-A23a, VUG-A124, VUG-A142, VUG-A112, VUG-A60a, VUG-A141a and VUG-A106. Significant, well-supported clades within this lineage include strains 35/26, VUG-A60a, VUG-A141a and *H. roseosalivarius* (clade 2) and strains *H. actinosclerus*, VUG-A106 and *H. aerophilus* (clade 3). The 16S rRNA gene sequences from VUG-A23a, VUG-A124 and VUG-A142 are all 96–97% similar to that from *H. daecheongensis*, those from VUG-A112 and VUG-A106 are 99% similar to those from *H. chitinivorans* and *H. aerophilus*, respectively, and those from VUG-A60a and VUG-A141a are both 99% similar to that of *H. roseosalivarius*. Finally, a deep-branching clade contains solely *H. ocellatus* and *H. deserti*; this clade is further supported by the unique presence of 16S rRNA indel "b" within these organisms.

Surprisingly, the phylogeny generated using partial *gyrB* gene sequences differed substantially from those generated using the 16S rRNA gene (Fig. 2). Clades 1–3 were conserved in both trees, supported by the presence of both

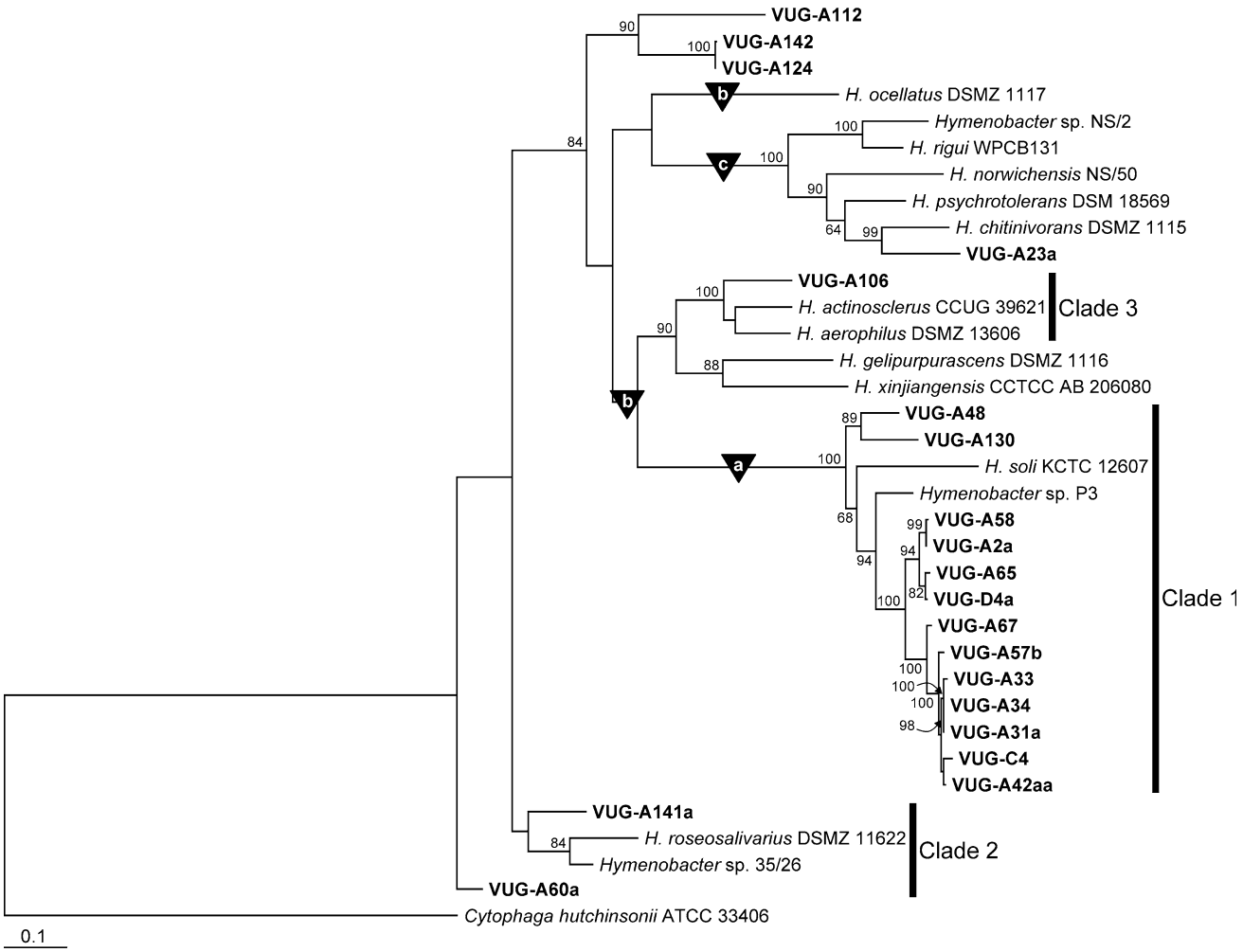

Fig. 2 Maximum-likelihood phylogeny of partial (≈1200 nt) gyrB nucleotide sequences. *C. hutchinsonii* was included as an outgroup; no outgroup gyrB sequences from *Adhaeribacter*, *Effluviibacter* or *Pontibacter* were available for analysis. Only bootstrap values ≥60 are shown and the *scale bar* represents 10% sequence divergence. Sequences containing phylogenetically conserved indels are shown by *triangles* on the indel-containing branch; *different letters* indicate unique indels. Those indels present in only one sequence are not shown, and all were excised from the alignment used to generate the phylogenetic tree

gyrB indels "a" and "b" in organisms of clade 1, solely *gyrB* indel "b" in organisms of clade 3, and the absence of any of *gyrB* indels "a", "b", or "c" in organisms of clade 2. Strongly supported clades present in the *gyrB* but not the 16S rRNA gene tree contained: (a) strain VUG-A112 with VUG-A124 and VUG-A142, all lacking any of *gyrB* indels "a", "b", or "c", but neither strain VUG-A23a nor *H. chitinivorans*; (b) strains NS/2, *H. rigui*, *H. norwichensis*, *H. psychrotolerans*, *H. chitinivorans* and strain VUG-A23a, further supported by the common presence of *gyrB* insertion "c"; (c) strains VUG-A106, *H. actinosclerus*, *H. aerophilus*, *H. gelipurpurascens*, *H. xinjiangensis* and strain VUG-A106, further supported by the common presence of *gyrB* insertion "b"; and (d) the relationship suggested by the common presence of *gyrB* insertion "b" between *H. ocellatus*, clades 1 and 3, *H. gelipurpurascens* and *H. xinjiangensis*. The deep branching of *H. ocellatus* suggested by the 16S rRNA gene phylogeny (Fig. 1) was not present in the *gyrB* tree (Fig. 2). These incongruencies suggest that either the true evolutionary history of *Hymenobacter* and related strains differs substantially from that suggested by 16S rRNA gene phylogenies or that *gyrB* has been frequently transferred horizontally within this genus.

Phenotypic characterization

A wide variety of growth phenotypes was assayed using all VUG and related *Hymenobacter* strains, especially including the type strains of all described species available at the time of this study, to complement the previously described genotypic characters (Table 2). All assayed strains were Gram negative by both staining and the KOH test, and oxidase positive. Most strains grew at both 4 and 18°C with some, particularly the previously described

Table 2 Phenotypic characterization of *Hymenobacter* and related VUG strains

Strain	Gram reaction	KOH test	Oxidase test	Catalase test	Growth-permissive temperatures (°C)[a]	Relative growth rate[b]	Microaerobic growth	Anaerobic growth	Maximum % NaCl tolerated[c]	Growth-permissive pH range[d]	Growth on Luria–Bertani agar	Growth on TSA
VUG-A130	−[e]	+	+	+	4, 10, 18, 28	Slow	+	−	<0.5	5–11	−	−
VUG-D4a	−	+	+	−	4, 10, 18	Slow	+	−	0.5 (w)	6–12	−	−
VUG-A42aa	−	+	+	−	4, 10, 18	Slow	+	−	<0.5	6-12	−	−
VUG-C4	−	+	+	−	4, 10, 18	Slow	+	−	0.5 (w)	6–12	−	−
VUG-A67	−	+	+	−	4, 10, 18	Slow	−	−	<0.5	6–10	−	−
VUG-A57b	−	+	+	−	4, 10, 18	Slow	+	−	<0.5	5–12	−	−
VUG-A33	−	+	+	−	4, 10, 18	Slow	+	−	0.5	6–11	−	−
VUG-A34	−	+	+	−	4, 10, 18	Slow	−	−	<0.5	6–11	−	−
VUG-A31a	−	+	+	−	4, 10, 18	Slow	−	−	0.5 (w)	6–12	−	−
VUG-A48	−	+	+	−	4, 10, 18	Slow	+	−	0.5	5–11	−	−
VUG-A2a	−	+	+	−	4, 10, 18	Slow	+	−	<0.5	5–11	−	−
VUG-A58	−	+	+	−	4, 10, 18	Slow	+	−	<0.5	6–11	−	−
VUG-A65	−	+	+	−	4, 10, 18	Slow	+	−	<0.5	6–11	−	−
H. soli	−	+	+	+	4, 10, 18, 28	Fast	ND[f]	−	0.5	5–11	−	−
Hymenobacter sp. P3	−	+	+	+	4, 10, 18	Slow	−	−	<0.5	5–11	−	−
H. daecheongensis[g]	−	NR[g]	+	+	4–30	Fast	NR	−	<1	5–10	NR	−
VUG-A23a	−	+	+	−	4, 10, 18	Slow	−	−	<0.5	6–11	−	−
VUG-A124	−	+	+	−	4, 10, 18	Slow	−	−	<0.5	6–10	−	−
VUG-A142	−	+	+	−	4, 10, 18	Slow	−	−	<0.5	7–10	−	−
H. norwichensis	−	+	+	+	4, 10, 18, 28	Fast	+	−	1	5–12	−	+
VUG-A112	−	+	+	−	4 (w), 10, 18	Slow	−	−	<0.5	6–11	−	−
H. chitinivorans	−	+	+	+	4, 10, 18, 28	Fast	+	−	1 (w)	5–12	w	−
Hymenobacter sp. 35/26	−	+	+	+	4, 10, 18, 28 (w)	Slow	−	−	0.5 (w)	7–11	−	−
VUG-A60a	−	+	+	+	4 (w), 10, 18, 28 (w)	Slow	−	−	1	7–9	−	−
VUG-A141a	−	+	+	+	4, 10, 18, 28	Slow	−	−	2 (w)	6–12	−	w
H. roseosalivarius	−	+	+	+	10, 18 (w)	Slow	−	−	<0.5	6–12	−	−
H. actinosclerus	−	+	+	+	4, 10, 18, 28	Fast	+	−	2	5–12	+	−
VUG-A106	−	+	+	−	4, 10, 18, 28	Slow	−	−	3 (w)	6–12	+	+
H. aerophilus	−	+	+	+	4, 10, 18, 28	Fast	+	−	2	5–12	+	+
H. xinjiangensis	−	+	+	+	4, 10, 18, 28	Fast	+	−	1	5–11	+	+
H. psychrotolerans	−	+	+	+	4, 10, 18, 28	Fast	ND	−	1 (w)	5–11	−	−
H. gelipurpurascens	−	+	+	+	4, 10, 18, 28	Fast	+	−	1	5–12	−	w
H. rigui	−	+	+	−	4, 10, 18, 28	Fast	+	−	1	5–12	−	+
Hymenobacter sp. NS/2	−	+	+	w	4, 10, 18, 28	Fast	+	−	1	5–12	w	−

Table 2 continued

Strain	Gram reaction	KOH test	Oxidase test	Catalase test	Growth-permissive temperatures (°C)[a]	Relative growth rate[b]	Microaerobic growth	Anaerobic growth	Maximum % NaCl tolerated[c]	Growth-permissive pH range[d]	Growth on Luria–Bertani agar	Growth on TSA
H. ocellatus	–	+	+	+	10, 18, 28	Fast	+	–	1	5–12	–	–
H. deserti[g]	–	NR	+	+	4–40	NR	NR	NR	<2	5–11	NR	–

The order in which strains are presented is the same as that in Fig. 1. All strains were grown on R2A agar at 18°C in the dark for 1 week, except where indicated

NR not reported, *ND* not detected

[a] Temperatures tested: 4, 10, 18, 28 and 37°C

[b] Slow and fast growth required approximately 7 and 3 days for robust growth to appear, respectively

[c] Concentrations tested: 0.5, 1, 2, 3, and 4% (w/v)

[d] Values reflect the ability of each strain to commence growth at pH 3–12. The degree to which the cell alters the pH of the medium during growth is unknown

[e] (−): negative result, (w): weak positive result, (+): positive result

[f] Manufacture of the Anaerocult C system used for these tests was discontinued before these strains could be assayed

[g] Phenotypic characterization of *H. daecheongensis* and *H. deserti* were taken from Xu et al. (2009) and Zhang et al. (2009), respectively. Note that the assay conditions used by these authors differ somewhat from those used here

species and related VUG isolates, also able to grow at 28°C. These latter strains were also more likely to produce catalase. In contrast to previously reported results (Reichenbach 1992; Buczolits et al. 2006), no strains were able to grow at 37°C; the reason for this discrepancy is unknown. Most strains were poorly tolerant of NaCl and rich media but could commence growth at high pH values. At extreme pH values, many strains grew only poorly or changed colony morphology, e.g., due to increased capsule production (data not shown). Whether observation of growth truly reflects different optimal pH values or variable protection from extreme pH values is uncertain. No strains could grow anaerobically and microaerobic growth was variable (Table 2).

Most *Hymenobacter* and related VUG strains were morphologically similar single bacilli or slightly vibriod cells approximately 1.5–2 μm × 0.5–0.8 μm. Inclusions were visible in strains VUG-A31a, VUG-A60a, VUG-A67, VUG-A130, VUG-A141a, *H. gelipurpurascens* and *H. xinjiangensis*. Several strains (VUG-C4, *H. gelipurpurascens*, *H. rigui*, NS/2) also clustered along their long axes as palisades and some cells were noticeably longer than their relatives (VUG-A48: 4.2 ± 1.2 μm × 1.0 ± 0.2 μm; VUG-A112: 4.0 ± 2.0 μm × 0.7 ± 0.2 μm). Whereas most VUG *Hymenobacter*-like strains formed small, smooth, shiny colonies, most reference strains exhibited much larger colony sizes, especially emphasized by their ability to spread into uninoculated regions of the plate.

Despite some variability, the phenotypes (Table 2), cell and colony morphologies determined here can be clustered relative to the gene phylogenies discussed above (Figs. 1, 2). The phylogenetic split between clade 1 (Figs. 1, 2) and all other *Hymenobacter* strains is particularly evident. Compared to the latter, the former have greater tolerance of NaCl, rich media and higher growth temperatures, more commonly possess catalase and exhibit faster relative growth rates and greater propensity to form large, spreading colonies on R2A incubated at 18°C in the dark. Minor phenotypic differences between VUG-A130, P3 and *H. soli* and the other strains comprising clade 1 (Figs. 1, 2) support their relationship to this cluster as outliers. The unusually high (for *Hymenobacter*) tolerance to salt and high nutrient concentrations of the strains comprising clade 2 is also congruent with their phylogenetic relatedness (Figs. 1, 2).

Metabolic profiling

Metabolic profiles for *Hymenobacter*-like VUG strains (as determined using the Biolog GN system) were highly heterogeneous. Whereas in total the substrates used were typical of described *Hymenobacter* species (Hirsch et al. 1998; Collins et al. 2000; Buczolits et al. 2002, 2006; Baik et al. 2006; Zhang et al. 2007, 2008a, 2009; Kim et al.

2008; Xu et al. 2009), even strains which were closely related according to gene phylogenies (Figs. 1, 2) differed substantially in their substrate utilization patterns (e.g., compare VUG-A33 and VUG-A34; Supplementary Table S1). Whereas most assayed strains could metabolize organic acids and keto acids, various sugars and/or amino acids could only be used by some. Strains VUG-A48 and VUG-A130 differed substantially from their closest relatives VUG-A2a, VUG-A33, VUG-A34, VUG-A42aa, VUG-A57b, VUG-A58 and VUG-A67, particularly in their inability to utilize α-keto acids and L-glutamate as sole carbon sources (Supplementary Table S1); this is congruent with the phylogenetic divergence between these strains (Figs. 1, 2). Differences between VUG-A23a, VUG-A112, VUG-A124 and VUG-A142 and all other assayed strains were also apparent (Supplementary Table S1); whether these differences are phylogenetically informative or strain-specific is unclear.

Fatty acid composition

Total cellular fatty acids were extracted from representative *Hymenobacter*-like VUG strains, methylated and identified using the MIDI system. As is typical of *Hymenobacter* species (Hirsch et al. 1998; Collins et al. 2000; Buczolits et al. 2002, 2006; Baik et al. 2006; Zhang et al. 2007, 2008a, 2009; Kim et al. 2008; Xu et al. 2009), the major FAMEs identified in all VUG strains were C15:0 iso and anteiso, C16:1 *ω*5*c* and "Sum In Features" 3 and 4 (Supplementary Table S2). The percentages of these FAMEs differed somewhat from those reported for reference *Hymenobacter* species; however, it remains unknown whether these differences are phylogenetically informative or are the result of the different growth conditions used in other studies. Whereas many other FAMEs were also present in the analyzed VUG strains at relatively low abundances (<5% total FAME content), their abundances varied considerably between closely related strains and were therefore not considered taxonomically significant. Also present were several FAMEs which were phylogenetically conserved but not identifiable using the MIDI database ("unknown ECL"; Supplementary Table S2), comprising 11–17% of the total FAME contents of VUG-A23a, VUG-A124, VUG-A142 and VUG-A112. Although their phylogenetic conservation suggests that these FAMEs are indeed fatty acids produced by these strains and not experimental artifacts, their nature remains otherwise unknown.

The current dataset was generated using consistent growth conditions and can therefore be validly used to delimit phylogenetic boundaries. Accordingly, strains VUG-A42aa, VUG-A67, VUG-A57b, VUG-A33, VUG-A34, VUG-A2a and VUG-A58 all exhibit highly similar FAME profiles, in accordance with their close relatedness in 16S rRNA and *gyrB* gene phylogenies (Figs. 1, 2) and common phenotypes (Table 2). Whereas the FAME profile of VUG-A48 is similar to these former strains, that of strain VUG-A130 differs from them considerably, especially in its higher and lower content of C15:1 iso G and C16:1 *ω*5*c*, respectively. The FAME profiles of strains VUG-A23a, VUG-A112 and the pair of VUG-A124 and VUG-A142 also contain unique features, especially the presence C18:1 *ω*9*c* and the unknown FAMEs having ECLs of 15.76, 16.35 and 17.20. These results further support the taxonomic structure of these strains derived from their phenotypes (Table 2) and gene phylogenies (Figs. 1, 2), as discussed above.

Carotenoid composition

The carotenoid complement of *Hymenobacter*-like VUG strains is composed primarily of 2′-hydroxy- and 2′-methoxyflexixanthin and, in lesser amounts, 3′-deoxy-2′-methoxyflexixanthin, plectaniaxanthin and 2′-methyl-plectaniaxanthin (Supplementary Table S3). These results are congruent with those determined previously (Klassen and Foght 2008; Klassen et al. 2009) and are consistent with the common inclusion of these VUG strains in the genus *Hymenobacter*. None of the glycosylated carotenoids identified previously in *H. ocellatus*, *H. gelipurpurascens* and strains VUG-A60a and VUG-A141a (Klassen and Foght 2008; Klassen et al. 2009) were detected in the strains assayed here. The lower amounts of 2′-methoxyflexixanthin in strains VUG-A2a and VUG-A58 compared to strains VUG-A33, VUG-A34, VUG-A42aa, VUG-A57b, VUG-A58 and VUG-A67 (Supplementary Table S3) are consistent with their inclusion in two separate 16S rRNA and *gyrB* subclades (Figs. 1, 2). Similarly, the higher abundance of 2′-methoxyflexixanthin in both VUG-A124 and VUG-A142 is also congruent with their unique phylogenetic position.

Discussion

Based upon gene phylogenies (Figs. 1, 2), phenotype (Table 2), metabolic profiling (Supplementary Table S1), fatty acid and carotenoid composition (Supplementary Tables S2 and S3), all VUG strains analyzed in this study belong to the genus *Hymenobacter* as currently defined (Buczolits et al. 2006). Strains VUG-A60a and VUG-A141a are most closely related to *H. roseosalivarius* and VUG-A106 to *H. aerophilus* and *H. actinosclerus*; whether these strains truly belong to these taxa requires more detailed study (e.g., DNA:DNA hybridization). Many differences were apparent between the other *Hymenobacter*-like

VUG strains studied here and those taxa described previously. This is especially evident for strains VUG-A2a, VUG-A31a, VUG-A33, VUG-A34, VUG-A42aa, VUG-A57b, VUG-A58, VUG-A65, VUG-A67, VUG-C4 and VUG-D4a, which are clearly distinct from all other previously described taxa, especially considering their 16S rRNA and *gyrB* gene phylogenies, phenotypic properties and fatty acid composition. Therefore we propose the species name *Hymenobacter antarcticus* sp. nov. for these organisms. Whereas this proposed species forms two subclades in the 16S rRNA and *gyrB* gene phylogenies, supported by differences in their carotenoid composition, subclade membership differs between these two genes (e.g., compare the location of VUG-D4a in Figs. 1, 2) and is not clearly supported by differences in metabolic profiles, fatty acid composition or any of the phenotypes assayed. Therefore, although we consider these strains to belong to the same species, defined as the lowest reproducibly recoverable phylogenetic clade (Staley 2006), we recognize that future data may provide grounds for further subdivision. Strain VUG-A48 is similar to these strains in many, but not all respects. Therefore we hesitate to apply the epithet *H. antarcticus* to this strain, pending future studies using techniques with higher phylogenetic resolution than those applied here.

Related to the strains now defined as *H. antarcticus* is strain VUG-A130. This strain differs substantially from all other described *Hymenobacter* species according to gene phylogenies, phenotypes and fatty acid composition (Figs. 1, 2; Supplementary Tables S1 and S2). Therefore we suggest that this strain represents a second novel species, for which we propose the name *Hymenobacter glaciei* sp. nov. Phenotypic, genotypic and chemotaxonomic evidence also suggests that strains VUG-A23a, VUG-A112 and the pair of VUG-A124 and VUG-A142 are distinct from all previously recognized taxa. Therefore we suggest that these strains also represent novel *Hymenobacter* species, and propose the names *Hymenobacter algoricola* sp. nov. for VUG-A23a, *Hymenobacter elongatus* sp. nov. for VUG-A112 and *Hymenobacter fastidiosus* sp. nov. for VUG-A124 and VUG-A142.

Although we feel that our data justify the creation of *H. algoricola*, *H. elongatus*, *H. fastidiosus* and *H. glaciei* as novel species, we acknowledge that these species are described based upon the characters of very few studied strains. This is also true of most other described *Hymenobacter* species. The assignment of a novel species name to an organism depends not only upon demonstrating its phylogenetic and phenotypic uniqueness but also that this uniqueness is phylogenetically conserved; this is especially troubling considering the high level of variation observed between strains of *H. antarcticus* (e.g., substrate utilization; Supplementary Table S1). Poor recognition of both these criteria has been noted previously as a widespread and misleading taxonomic practice (Christensen et al. 2001; Felis and Dellaglio 2007), albeit unavoidable when only limited numbers of strains are available to study, such as in this case. This concept is especially represented by the proposal of "*species proponenda*", i.e., species clearly distinct from their taxonomic relatives yet described based on characterization of a very few strains (Felis and Dellaglio 2007), and clearly holds for all currently described *Hymenobacter* species except *H. antarcticus*. Therefore we expect the taxonomy of this genus to continually evolve with increased availability of new strains, especially regarding which characters are phylogenetically informative at the species level.

A particularly surprising result from the current research is the large number of phylogenetic characters which have apparently evolved non-vertically, i.e., by mechanisms other than diversification by descent, as represented in an organismal phylogenetic tree. This does not invalidate the classification of these strains as novel species, but instead likely reflects peculiarities in their evolution and ecology. Particularly notable are the apparent horizontal transfer of either 16S rRNA or *gyrB* genes, strain-specific metabolic patterns, and heterogeneous distribution of carotenoids. We suggest three possible explanations for these patterns. First, *Hymenobacter* species may have a low capacity for allopatric divergence. They have been commonly detected in aerosols (Buczolits et al. 2002, 2006; Osman et al. 2008; Polymenakou et al. 2008), suggesting a potentially cosmopolitan distribution, and may have a significant capacity for recombination (e.g., horizontal transfer of the 16S rRNA or *gyrB* genes). In this case, theory suggests genetic coalescence between strains because of recombination during co-habitation within the same ecological niche (Berg and Kurland 2002). In this scenario, species boundaries may be "fuzzy", as observed for genera such as *Streptococcus* and *Neisseria* (Hanage et al. 2006). Secondly, ice-bound organisms, such as *Hymenobacter* (Zhang et al. 2006, 2008b; Klassen and Foght 2008), might harbor genotypes which are preserved during their interment in the ice matrix for re-introduction into the more temperate biosphere despite becoming extinct elsewhere (Rogers et al. 2004). Evolution in this manner may allow for the survival of less-fit genotypes through an evolutionary bottleneck experienced by their global population until that genotype is again fit enough to persist. This would allow the co-existence of both modern and ancient genotypes, which, following recombination, would lead to reticulate (i.e., web-like) instead of tree-like patterns of evolution. Thirdly, cells entrapped in VUG are forced through an evolutionary bottleneck due to the requirement for relatively long-term survival in the ice matrix. Stresses in this environment presumably differ substantially from those

that the cells encounter in their more typical habitats, e.g., desert soil or aerosols. Passage through this bottleneck might therefore discriminate between phenotypes that are selectively advantageous for cellular survival in ice and those required for survival prior to interment. Correspondingly, genes non-advantageous for survival in ice may suffer genetic decay in this habitat, even if previously these were selectively favorable. Because mutation is a stochastic process, different genes in each cell would be expected to accumulate different sets of mutations. If strains were isolated as descendants from single cells (as expected during colony formation on Petri plates), these different sets of mutations would result in apparently random phenotypic differences between closely related strains. This is precisely the pattern observed in metabolic profiles of the assayed *H. antarcticus* strains. Which (if any) of these evolutionary scenarios is most relevant to the evolution of *Hymenobacter* and other species commonly experiencing dormancy in related niches remains a topic for future study.

Description of *Hymenobacter algoricola* sp. nov.

Hymenobacter algoricola: al.go.ri'co.la. L. masc. n. algor -oris, cold; L. suff. -cola (from L. n. incola), inhabitant, dweller; N.L. n. algoricola, cold-dweller. Cells are rods or slightly vibriod (typically 1–2 μm long × 0.5–0.8 μm wide) and do not form endospores. Slimy, red-pink colonies form readily on R2A medium but not on higher strength media such as Luria–Bertani or tryptic soy agar. Aerobic growth occurs in the range of 4–18°C but not at 28°C. Aerobic growth at 18°C occurs on plates containing <0.5% NaCl and a pH between 6 and 11. Growth does not occur microaerobically or anaerobically. Cells are Gram negative according to staining and the KOH test, catalase negative and oxidase positive. At 18°C, the type strain uses dextrin, Tween 40, α-D-glucose, α-ketobutyrate, α-ketoglutarate and α-ketovalerate as metabolic substrates. The major fatty acids in the type strain (>5%, in descending order) grown on R2A at 18°C include: Sum In Feature 3 (contains C16:1 *ω7c* and C16:1 *ω6c*), C16:1 *ω5c*, Sum In Feature 4 (contains C17:1 iso I and C17:1 anteiso B), C15:0 iso and C15:0. 2′-Hydroxyflexixanthin is the major carotenoid produced. The type strain VUG-A23a (=JCM 17214) was isolated from VUG, Antarctica, basal ice.

Description of *Hymenobacter antarcticus* sp. nov.

Hymenobacter antarcticus: an.tarc'ti.cus. L. masc. adj. antarcticus, southern, by extension pertaining to the Antarctic, referring to its isolation source. Cells are rods or slightly vibriod (typically 1–2 μm long × 0.5–1 μm wide) and do not form endospores. Slimy, red-pink colonies form readily on R2A medium but not on higher strength media such as Luria–Bertani or tryptic soy agar. Aerobic growth occurs in the range of 4–18°C but not at 28°C. Aerobic growth occurs at 18°C on plates containing <0.5% NaCl and a pH between 6 and 11. Growth also occurs microaerobically but not anaerobically. Cells are Gram negative according to staining and the KOH test, catalase negative and oxidase positive. At 18°C, all tested strains use dextrin, pyruvate methyl ester, α-ketobutyrate, α-ketovalerate, L-glutamate and L-proline as metabolic substrates. Other carbon sources can also be used, but the ability to do so differs between strains. The major fatty acids in all strains (>5%, in descending order) grown on R2A at 18°C include: Sum In Feature 3 (contains C16:1 *ω7c* and C16:1 *ω6c*), C16:1 *ω5c*, C15:0 iso and C15:0 anteiso. 2′-Hydroxyflexixanthin is the major carotenoid produced in all strains; some strains also produce substantial amounts of 2′-methoxyflexixanthin. The type strain, VUG-A42aa (=JCM 17217), was isolated from VUG, Antarctica, basal ice. Other strains include VUG-A2a (=JCM 17213), VUG-A33 (=JCM 17215), VUG-A34 (=JCM 17216), VUG-A57b (=JCM 17219), VUG-A58 (=JCM 17220) and VUG-A67 (=JCM 17222).

Description of *Hymenobacter elongatus* sp. nov.

Hymenobacter elongatus: e.lon.ga'tus. L. masc. part. adj. elongatus, elongated, referring to the pronounced elongation of the cells. Cells are rods and slightly vibriod (typically 2–6 μm long × 0.5–0.8 μm wide) and do not form endospores. Slimy, red-pink colonies form readily on R2A medium but not on higher strength media such as Luria–Bertani or tryptic soy agar. Aerobic growth occurs in the range of 4–18°C but not at 28°C. Aerobic growth at 18°C occurs on plates containing <0.5% NaCl and a pH between 6 and 11. Growth does not occur microaerobically or anaerobically. Cells are Gram negative according to staining and the KOH test, catalase negative and oxidase positive. At 18°C, the type strain uses dextrin, D-galactose, α-D-glucose, maltose, L-glutamate and glycyl-L-glutamate as metabolic substrates. The major fatty acids in the type strain (>5%, in descending order) grown on R2A at 18°C include: Sum In Feature 4 (contains C17:1 iso I and C17:1 anteiso B), C15:0 iso, Sum In Feature 3 (contains C16:1 *ω7c* and C16:1 *ω6c*), C16:1 *ω5c*, unknown ECL 16.35, C17:0 iso 3-OH and C16:0. 2′-Hydroxyflexixanthin is the major carotenoid produced. The type strain, VUG-A112 (=JCM 17223), was isolated from VUG, Antarctica, basal ice.

Description of *Hymenobacter fastidiosus* sp. nov.

Hymenobacter fastidiosus: fas.ti.di.o'sus. L. masc. adj. fastidiosus, fastidious, referring to its fastidious character.

Cells are rods or slightly vibriod (typically 1–3 μm long × 0.5–1 μm wide) and do not form endospores. Slimy, red-pink colonies form slowly on R2A medium but not on any other tested medium, including diluted or concentrated R2A. Aerobic growth occurs in the range of 4–18°C but not at 28°C. Aerobic growth at 18°C occurs on plates containing <0.5% NaCl and a pH between 7 and 10. Growth does not occur microaerobically or anaerobically. Cells are Gram negative according to staining and the KOH test, catalase negative and oxidase positive. At 18°C, both tested strains use dextrin, Tween 40, α-ketobutyrate, α-ketoglutarate and α-ketovalerate as metabolic substrates. Utilization of α-D-glucose, maltose (weak), pyruvate methyl ester, L-glycyl-L-aspartate is variable between strains. The major fatty acids in both tested strains (>5%, in descending order) grown on R2A at 18°C include: Sum In Feature 3 (contains C16:1 *ω7c* and C16:1 *ω6c*), C16:1 *ω5c*, Sum In Feature 4 (contains C17:1 iso I and C17:1 anteiso B), C15:0 anteiso and unknown ECL 16.35. 2′-Methoxyflexixanthin is the major carotenoid produced, and 2′-hydroxyflexixanthin is also present in substantial amounts. The type strain, VUG-A124 (=JCM 17224), was isolated from VUG, Antarctica, basal ice. A second strain is VUG-A142 (=JCM 17227).

Description of *Hymenobacter glaciei* sp. nov.

Hymenobacter glaciei: gla.ci'e.i. L. gen. n. glaciei, of ice, referring to its isolation from a glacier. Cells are rods or slightly vibriod (typically 1–2 μm long × 0.5–0.8 μm wide) and do not form endospores. Slimy, red-pink colonies form readily on R2A medium but not on higher strength media such as Luria–Bertani or tryptic soy agar. Aerobic growth occurs in the range of 4–28°C but not at 37°C. Aerobic growth at 18°C occurs on plates containing <0.5% NaCl and a pH between 5 and 11. Growth also occurs microaerobically but not anaerobically. Cells are Gram negative according to staining and the KOH test and catalase and oxidase positive. At 18°C, the type strain uses dextrin, *p*-hydroxyphenylacetate, α-ketovalerate (weakly), glycyl-L-aspartate (weakly), L-proline (weakly) and L-serine (weakly) as metabolic substrates. The major fatty acids in all strains (>5%, in descending order) grown on R2A at 18°C include: Sum In Feature 3 (contains C16:1 *ω7c* and C16:1 *ω6c*), C15:0 anteiso, C15:0 iso, C15:1 iso G and Sum In Feature 4 (contains C17:1 iso I and C17:1 anteiso B). 2′-Hydroxyflexixanthin is the only carotenoid produced. The type strain, VUG-A130 (=JCM 17225), was isolated from VUG, Antarctica, basal ice.

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