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Phylogenetic Analyses of the Genus *Hymenobacter* and Description of *Siccationidurans* gen. nov., and *Parahymenobacter* gen. nov

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Abstract

Phylogenetic analyses of 26 species of the genus *Hymenobacter* based on the 16S rRNA gene sequences, resulted in polyphyletic clustering with three major groups, arbitrarily named as Clade1, Clade2 and Clade3. Delineation of Clade1 and Clade3 from Clade2 was supported by robust clustering and high bootstrap values of more than 90% and 100% in all the phylogenetic methods. 16S rRNA gene sequence similarity shared by Clade1 and Clade2 was 88 to 93%, Clade1 and Clade3 was 88 to 91% and Clade2 and Clade3 was 89 to 92%. Based on robust phylogenetic clustering, less than 93.0% sequence similarity, unique *in silico* restriction patterns, presence of distinct signature nucleotides and signature motifs in their 16S rRNA gene sequences, two more genera were carved to accommodate species of Clade1 and Clade3. The name *Hymenobacter, sensu stricto,* was retained to represent 17 species of Clade2. For members of Clade1 and Clade3, the names *Siccationidurans* gen. nov. and *Parahymenobacter* gen. nov. were proposed, respectively, and species belonging to Clade1 and Clade3 were transferred to their respective genera. The genera *Hymenobacter* (*sensu stricto), Siccationidurans* gen. nov. and *Parahymenobacter* gen. nov. contained the signature motifs AAGGCTTTCTGAGTCGTAAA (414-432), TGACGGTACCTGAGGAATAA (480-499) and ATTAATACCGCATAACACT (168-185) in their 16S rRNA gene sequences, respectively. Further, the genus *Hymenobacter* was emended and proposed a more acceptable genus description.

Keywords: Phylogeny; 16S rRNA gene sequence; *Siccationidurans* gen. nov.; *Parahymenobacter* gen. nov.; *Hymenobacter*

Introduction

Analysis and validation of 16S rRNA gene sequence based phylogeny is the basis for prokaryotic systematics [1,2]. In this context, it is worth mentioning that, based on 16S rRNA gene sequence analyses, five distinct phylogenetic groups within the genus *Bacillus* [3], two novel orders, *Solirubrobacterales* and *Thermoleophilales* [4], and a new hierarchic classification structure for the actinomycete line of descent [5] were proposed and the phylogenetic affiliation of the genus pseudomonads was assessed [6]. In congruence with these, to take few examples, several novel genera, such as *Solibacillus* [7] and *Planomicrobium* [8], were created on the basis of their 16S rRNA gene sequence. Present work is focused on evaluating the internal features of the 16S rRNA gene sequences of the genus *Hymenobacter.*

The genus *Hymenobacter,* which belongs to the phylum *Bacteroidetes*, order *Sphingobacteriales* and family *Cytophagaceae*, was described by Hirsch et al. [9] and subsequently emended by Buczolits et al. [10]. The genus accommodates species that are strictly aerobic, Gram-negative, rod-shaped, non-motile, red-pigmented and contain menaquinone MK-7, fatty acids iso-$C_{15:0}$, anteiso-$C_{15:0}$, $C_{16:1}\omega5c$, summed feature 3 ($C_{16:1}$ ω7c/iso-$C_{15:0}$ 2-OH) and summed feature 4 (iso-$C_{17:1}$ I/anteiso-$C_{16:1}$ B) with high DNA G+C content of 55 to 65 (mol %). The genus presently contains 26 species, including recently described species [11,12], which were isolated from various ecological niches. Klassen and Foght [13] and Reddy and Garcia-Pichel [12], in their recent study, discussed the polyphyletic clustering of all species into three major clades, (Clade1, Clade2 and Clade3), based on 16S rRNA gene sequence. Clade1, Clade2 and Clade3 encompassed seven, seventeen and two species, respectively. Exploration of the internal features of the 16S rRNA gene sequences of 26 species of the genus *Hymenobacter* warranted the creation of two novel genera to accommodate species that belong to Clade1 and Clade3; for which the names *Siccationidurans* gen. nov. and *Parahymenobacter* gen. nov. are proposed. *Hymenobacter soli* PB17^T=LMG 24240^T=KCTC 12607^T, the oldest species belonging to Clade1, was elevated to the status of type species of the genus *Siccationidurans* and named as *Siccationidurans soli* PB17^T=LMG 24240^T=KCTC 12607^T. Similarly, *Hymenobacter ocellatus* Myx 2105^T=Txo1^T=DSM 11117^T=LMG 21874^T was transferred to the genus *Parahymenobacter* as *Parahymenobacter ocellatus* Myx 2105^T=Txo1^T=DSM 11117^T=LMG 21874^T and designated as the type species. Further, the genus *Hymenobacter* needs be emended as Hirsh et al. [9] and Buczolits et al. [10] had included strain specific characteristics, some of which were not characterized in all the species, as in the case of spermidines. With the identification of signature nucleotides and signature motifs, a more acceptable genus description is proposed.

Methods

Phylogenetic analyses

Almost full length 16S rRNA gene sequences belonging to species of the genera *Hymenobacter, Pontibacter, Adhaeribacter* and *Cytophaga,* were downloaded from the NCBI data base (http://www.ncbi.nlm.nih.gov). For phylogenetic analyses, all the sequences were aligned using CLUSTAL-W, the multiple alignment program option of MEGA5 [14]. Evolutionary distances between all species were computed using Kimura 2-model [15], present in the distance option of MEGA5. Phylogenetic trees were constructed using four different tree-making algorithms (Neighbor-Joining, Minimum Evolution, Maximum Likelihood and Maximum parsimony analysis), using MEGA5. Bootstrap analyses in all the phylogenetic trees were performed employing 1000 replicate data sets in order to assess the stability among clades recovered in the phylogenetic tree.

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Restriction enzyme analysis

Fifteen Type II Restriction enzymes (Table S1) were considered for generating *in silico* restriction patterns. For this purpose, Restriction Mapper Version 3 (http://restrictionmapper.org) was used to map the restriction patterns of 26 *Hymenobacter* species (sequence length from 118 to 1460; with respect to *E. coli* 16S rRNA gene sequence with accession number J01695), employed for construction of phylogenetic framework. These restriction patterns were analyzed and a consensus pattern was determined for each species.

Cluster analysis for restriction profile

For cluster analyses NTSYSpcs, Numerical Taxonomy System and multivariate statistical package, software version 2.2 [16] was used. Initially, data for restriction patterns generated *in silico,* using different type II restriction enzymes, was entered in the form of 1 (presence of a band) and 0 (absence of a band) in NTedit 1.1. The similarity matrix was generated using SimQual of similarity, the dendogram was constructed with Shan of Clustering option and the trees were viewed with Graphics options present in NTSYSpc**.**

Signature nucleotides

Signature nucleotides that are highly conserved in every sequence or in a specific clade were identified in the alignment file that was generated using MEGA5 [14]. Every single signature nucleotide found was then positioned on the secondary structure of 16S rRNA molecule of *E. coli* (accession number J01695; obtained from (http://www.rna.icmb.utexas.edu/SIM/4C/mfold_Eval/accuracy/16s.acc.detailed). This analysis allowed interpretation of signatures found in terms of single or double compensatory mutations in helices of the secondary structure. Compensatory mutations are two nucleotides that stabilize a stem in the secondary structure (such as G–C or A-T), and are mutated (for example to C–G or T-A) in specific taxa.

Signatures motifs

Signatures motifs were identified in each of the species data set using the online MEME program [17]. Seven, seventeen and two sequence data sets of Clade1, Clade2 and Clade3, respectively, belonging to the genus *Hymenobacter* were submitted group wise in MEME program version 4.6.1 (http://meme.nbcr.net/meme/cgi-bin/meme.cgi). In order to obtain maximum number of motifs, the default setting was modified from 3 to 10 motifs. The default value of motif width was also modified and re-set between 10 and 20. Each of the 10 signatures was checked for its frequency of occurrence among a particular *Hymenobacter* species. The signatures which did not appear in other clades of the *Hymenobacter* species were considered as unique. BLAST sequence similarity search against the NCBI database http://www.ncbi.nlm.nih.gov [18] and EzTaxon [19], was carried out for these signatures to check their uniqueness.

Results and Discussion

Twenty six, seven and four species of the genera *Hymenobacter* [9,10], *Pontibacter* [20] and *Adhaeribacter* [21], respectively, were aligned using CLUSTAL W option of MEGA5 [14]. The aligned 16S rRNA gene sequences exhibited two hyper variable regions spanning from 72 to 115 (33 nucleotides long) and 180 to 195 (15 nucleotides long) (with respect to *E. coli* 16S rRNA gene sequence; accession number J01695) and mapped V1 and V2 regions, respectively [22,23]. Most of the variation in 16S rRNA gene sequences of species of the genera was contributed by these two regions and rest of the variation is randomly distributed in the entire region of rRNA gene sequences. To avoid the 'Felsenstein zone' (i.e. retrieving a wrong tree even if it has high bootstrap values), phylogenetic analyses [24] were performed with different phylogenetic methods. For this purpose, Neighbor joining (NJ), Minimum evolution (ME), Maximum likelihood (ML) and Maximum parsimony (MP) options were used to generate the trees. Topology of Neighbor joining (NJ) and Minimum evolution (ME) trees indicated that species of the genera *Pontibacter* [20] and *Adhaeribacter* [21] formed coherent monophyletic clusters with bootstrap values above 85% (Figure 1 and S1). In case of Maximum likelihood (ML) and Maximum parsimony analyses, species of the genera *Pontibacter* appeared as a monophyletic clade, but *Adhaeribacter* showed a split in clustering, wherein *Adhaeribacter terreus* [25] emerged as a separate branch from the main cluster represented by rest of the species (Figure S2 and S3). Species of the genus *Hymenobacter* [9] were highly divergent, polyphyletic in all the methods and formed three major clusters, named arbitrarily as Clade1, Clade2 and Clade3 (Figures 1 and S1-S3), which are deeply rooted from each other and are sister clades emerging from a common ancestor. Further, delineation of Clade1 and Clade3 from Clade2 was supported by robust clustering and high bootstrap values of more than 90% and 100%, respectively in all the phylogenetic methods (Figures 1 and S1-S3), and the present clustering of species of the genus *Hymenobacter* is consistent with previous studies [12,13, 26-29].

Evolutionary distances, based on 16S rRNA gene sequence, as calculated using Kimura 2-parameter model [15], were found to be 90 to 97%, 92 to 99% and 95%, respectively, among species belonging to Clade1, Clade2 and Clade3 of the genus *Hymenobacter.* 16S rRNA gene

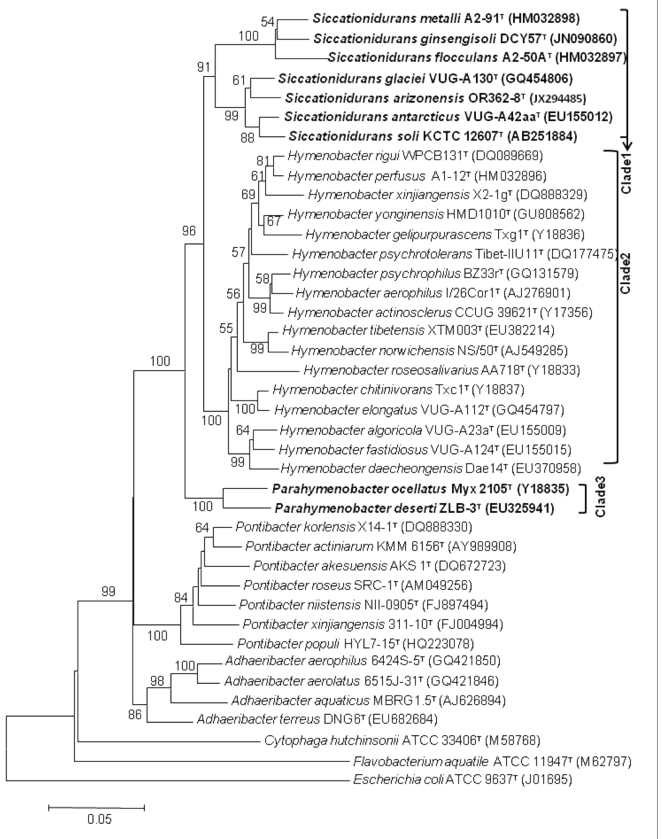

Figure 1: A Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationship between the genera *Siccationidurans* gen. nov., *Hymenobacter, Parahymenobacter* gen. nov., and other related genera of the family *Cytophagaceae*. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are indicated at the nodes.

sequence similarity shared by Clade1 and Clade2 is 88 to 93%, Clade1 and Clade3 is 88 to 91% and Clade2 and Clade3 is 89 to 92% (Table S2), significantly larger than the ~95% threshold typically used to split genera [30]. The robust clustering of three clades belonging to the genus *Hymenobacter* with high bootstrap values (in all the four phylogenetic methods employed) (Figures 1 and S1-S4), and aggregate sequence similarity of less than 93% among three clades (Clade1, Clade2 and Clade3), supported the proposal to assign species of Clade1 and Clade3 to a higher taxonomic rank [31]. The above proposal is significantly supported by the genera *Adhaeribacter* [21] and *Pontibacter* [20], as they also share a 16S rRNA gene sequence similarity of 88 to 92% and form a coherent cluster with high bootstrap values (Figures 1 and S1-S3).

In addition, the number of nucleotides conserved in the 16S rRNA gene of Gram-negative bacteria is more than that of Gram-positive bacteria. Gram-negative and Gram-positive bacteria contain 713/1542 and 568/1542 nucleotides, respectively, in the highly conserved regions [30], and a total of 145/1542 (9.4%) more nucleotides are conserved in Gram-negative bacteria. Under these circumstances, the difference of 88 to 93% among species of Clade1, Clade2 and Clade3, being Gram-negative by virtue, belonging to the genus *Hymenobacter,* is huge and demands the creation of new genera [30], since earlier the genera were carved with >92% 16S rRNA gene sequence similarity [7,8,32-34]. The main impediment is the lack of diagnostic phenotypic differences. However, Stackebrandt et al. [5], Ash et al. [3], Reddy and Garcia-Pichel [4] and Ivanova et al. [31] created the genera, families and orders based only on the presence of unique signatures in 16S rRNA gene sequences, further implicating that the phylogenetic evidence alone is sufficient to create a higher taxonomic rank. However, polyphasic taxonomy emphasizes the significance of consensus between phenotypic and genotypic characteristics [35-38], in delineation of taxa. But several phenotypic markers are variable and dependent on environmental cues. For instance, the expression of characteristics that serve in genus description, such as cell morphology [39-42], enzymes [43,44], fatty acids [45,46] menaquinones [47-49], lipids [50-53] and peptidoglycan [54-57], depend on the growth conditions. Further, discrepancies in the above traits were well documented among species of several genera [58,59], and thus hamper in drawing congruence between phylogeny and expressed characteristics. On the other hand, 16S rDNA sequence based phylogeny has been serving as a stable trait in delineation of several taxa, and is considered as the basis in creation of numerous taxanomic groups, as mentioned earlier [4,5]. However, the importance of other polyphasic characteristics cannot be discounted, but should be considered as significant auxiliary and descriptive, rather than distinctive markers *per se*. In the present study, it is unambiguously established by phylogenetic analyses that the genera, *Siccationidurans* gen. nov. and *Parahymenobacter* gen. nov., are distinctly different from already described nearest genera. Therefore, phenotypic traits were considered in the description of genera (please refer to the genus description).

Because of the low 16S rRNA sequence similarity of less than 93%, it was assumed that the restriction patterns would be different for the three clades of the genus *Hymenobacter*. In the present study, fifteen type II restriction enzymes (Table S1) were used *in silico* and they revealed differences in the fragmentation patterns. Restriction sites for *AluI, BfaI, BstUI, DpnI, HaeIII, HhaI, MboI, MseI*, *MspI, RsaI* and *Sau3AI* (11 enzymes) occurred with a frequency of 2-10, resulting in 3-11 fragments. The enzyme *SmaI* gave 2 restriction fragments of length 1280 and 48 (positions are with respect to *H. arizonensis*), restriction sites for the enzymes *BamHI* and *EcoRI* were not found in any of the 26 sequences studied and *HindIII* had a single cut in a single species; the *Hymenobacter psychrophilus*. Thus the enzymes, *BamHI, EcoRI, HindIII* and *SmaI* are less informative and serve no purpose. In spite of low frequency, *HindIII* can still be used to distinguish the species, *Hymenobacter psychrophilus* and *SmaI* can be combined with other enzymes to distinguish the members of *Hymenobacter* [9]. The enzymes, *AluI, BfaI, BstUI, HaeIII, HhaI, MseI*, *MspI, RsaI* and *SmaI* generated 31 out of 113 common fragments in all the 26 species and can serve as markers to identify the members of this group. Interestingly, the enzymes *DpnI, MboI, Sau3A1, HaeIII* and *RsaI* distinguish the species of Clade1 from other Clades in that *DpnI, MboI, Sau3A1* generated a fragment size of 209 in 19 species of Clade2 and Clade3, but not in Clade1. Similarly, *HaeIII* created a fragment size of 321 in species of Clade2 and Clade3, but not in Clade1 and *RsaI* produced a fragment of 556 in 6/7 species of Clade1 and lacks the fragment size of 645. Clade3 can be differentiated from Clade1 and Clade2 using *AluI,* which generates a fragment size of 515 in all, but Clade1 and in Clade2, the fragment is produced only in 6/17 species. Thus, the enzymes, *DpnI, MboI, HaeIII, Sau3A1* and *RsaI* can be used as markers to distinguish the species of the three clades. Approximately, 113 fragments generated among 26 species of the genus *Hymenobacter* were used in dendogram construction and the species of Clade1, Clade2 and Clade3 delineated at a similarity coefficient value of 80%, 81% and 86.5%, respectively (Figure S4). Thus, the *in silico* restriction patterns resulted in the differentiation of three clades belonging to the genus *Hymenobacter* and their robust clustering into three Clades, congruent to the 16S rRNA gene sequence based phylogeny further strengthened the need to create two more genera to accommodate the species of Clade1 and Clade3.

Further evidence in support of awarding genus status to members of Clades1 and 3 comes from the analyses based on comparison of base-to-base 16S rRNA gene sequences. Species of Clade1 are characterized by the presence of nucleotides G-C (294-303), G (306), G-C (317-336), A (408), T-A (419-424), C (427), T (434), G-C/C-G (462-476), T (477), A (658), A (728), T (747), C-G/G-C (897-902), G (1285) and A (1286) (20 nucleotides), of which nucleotides at positions 306, 317-336, 408, 419-424, 427, 658, 728, 747 are unique (Table 1). Species of Clade2 contain C (256), C (268), C-G (294-303), T-A (317-336), C-G (419-424), C (441), G (493), C-G (897-902), A (903), A (1285) (14 positions), of which nucleotides at positions 294-303, 441, 493 are distinctive. Similarly Clade3 possesses G (127), G (128), T (131), T (140), T (165), A (206), A (215), C (219), A (231), C (233), C (234), G-C (294-303), C (304), T-A (317-336), G (490), T (492), C (594), T (833), C (854), G-C (897-902), G (1019), C (1174), A (1275), C (1364) (27 positions). Positions 127, 128, 131, 140, 1364 and the absence of nucleotide at 462 differentiate Clade3 from Clade1 and Clade2 (Table 1). Close to 48 signature nucleotides were identified at various positions (Table 1), of which 20, 14 and 27 were highly conserved among Clade1, Clade2 and Clade3, respectively. There is no limit to the number of signature nucleotides in carving new genera as Dai et al. [60], identified a difference of just 2 signature nucleotides between the genera *Planococcus* and *Planomicrobium*. Further, based on the difference of 13 unique nucleotides, the genus *Sphingosinicella,* without strong phenotypic support, was dissected from *Sphingomonas* [61]. Thus, the presence of above signature nucleotides not only distinguishes between the three Clades, but also serves as additional evidence in carving the genera.

Splitting of the genus *Hymenobacter* [9] into three genera was further substantiated by unique signature motifs. Out of 19 signatures motifs identified among the three clades of the genus *Hymeno-*

		Position of the nucleotide															
	Species	127	128	131	140	165	206	215	219	223	231	233	234	256	268	294	303
Clade1	*E. coli* (J01695)	G	G	A	T	G	C	C	T	A	T	C	C	T	T	T	A
	S. arizonensis (JX294485)	A	C	C	T	C	G	T	T	A	G	G	T	G	C	**G**	**C**
	S. glaciei (GQ454806)	G	C	C	A	C	G	T	T	T	G	G	C	C	C	**G**	**C**
	S. antarcticus (EU155012)	A	C	C	A	C	T	T	T	T	G	G	T	A	T	**G**	**C**
	S. soli (AB251884)	A	C	C	A	C	T	T	T	T	G	G	T	C	C	**G**	**C**
	S. metalli (HM032898)	A	C	C	A	C	T	T	T	T	G	G	T	T	T	**G**	**C**
	S. flocculans (HM032897)	A	C	C	G	C	G	T	G	C	G	G	T	T	T	**G**	**C**
	S. ginsengisoli (JN090860)	A	C	C	A	C	A	T	T	T	G	G	T	T	T	**G**	**C**
Clade2	*H. actinosclerus* (Y17356)	A	C	C	A	C	T	T	T	T	G	G	T	**C**	**C**	***C***	***G***
	H. aerophilus (AJ276901)	A	C	T	A	T	T	T	T	T	A	G	T	**C**	**C**	***C***	***G***
	H. algoricola (EU155009)	A	C	C	A	C	A	T	T	T	G	G	T	**C**	**C**	***C***	***G***
	H. chitinivorans (Y18837)	A	C	C	A	C	T	T	T	T	G	G	T	**C**	**C**	***C***	***G***
	H. daecheongensis (EU370958)	A	C	C	A	C	G	T	T	T	G	G	T	**C**	**C**	***C***	***G***
	H. elongatus (GQ454797)	A	C	C	A	C	T	T	T	T	G	G	T	**C**	**C**	***C***	***G***
	H. fastidiosus (EU155015)	A	C	C	A	C	G	T	T	T	G	G	T	**C**	**C**	***C***	***G***
	H. gelipurpurascens (Y18836)	A	C	C	G	C	G	T	T	C	G	G	T	**C**	**C**	***G***	***C***
	H. norwichensis (AJ549285)	A	C	C	A	C	T	T	T	T	G	G	T	**C**	**C**	***C***	***G***
	H. roseosalivarius (Y18833)	A	C	C	C	C	T	T	T	G	G	G	T	**C**	**C**	***C***	***G***
	H. rigui (DQ089669)	A	C	C	G	C	G	T	T	C	G	G	T	**C**	**C**	***C***	***G***
	H. psychrophilus (GQ131579)	A	C	C	A	C	T	T	T	T	G	G	T	**C**	**C**	***C***	***G***
	H. psychrotolerans (DQ177475)	A	C	C	G	C	G	T	T	C	G	G	T	**C**	**C**	***C***	***G***
	H. perfusus (HM032896)	A	C	C	G	C	T	T	T	C	G	G	T	**C**	**C**	***C***	***G***
	H. xinjiangensis (DQ888329)	A	C	C	G	C	T	T	T	C	G	G	T	**C**	**C**	***C***	***G***
	H. yonginensis (GU808562)	A	C	C	A	C	T	T	T	T	G	G	T	**C**	**C**	***C***	***G***
	H. tibetensis (EU382214)	A	C	C	G	C	T	T	T	C	G	G	T	**C**	**C**	***C***	***G***
Clade3	*Ph. deserti* (EU325941)	**G**	**G**	**T**	**T**	**T**	**A**	**A**	**C**	**A**	**A**	**C**	**C**	C	C	**G**	**C**
	Ph. ocellatus (Y18835)	**G**	**G**	**T**	**T**	**T**	**A**	**A**	**C**	**A**	**A**	**C**	**C**	T	T	**G**	**C**

		Position of the nucleotide															
	Species	304	306	317	336	408	419	424	427	**434**	441	462	476	477	490	492	493
	E. coli (J01695)	T	A	T	A	**A**	C	G	T	**T**	A	G	T	**C**	C	C	A
Clade1	*S. arizonensis* (JX294485)	T	**G**	**G**	**C**	**A**	**T**	**A**	**C**	**T**	G	**G**	**C**	**C**	T	G	T
	S. glaciei (GQ454806)	T	**G**	**G**	**C**	**A**	**T**	**A**	**C**	**T**	G	**G**	**C**	**T**	T	G	T
	S. antarcticus (EU155012)	T	**G**	**G**	**C**	**A**	**T**	**A**	**C**	**T**	A	**G**	**C**	**T**	T	A	T
	S. soli (AB251884)	T	**G**	**G**	**C**	**A**	**T**	**A**	**C**	**T**	G	**G**	**C**	**T**	T	G	T
	S. metalli (HM032898)	T	**G**	**G**	**C**	**A**	**T**	**A**	**C**	**T**	G	**C**	**G**	**T**	T	C	A
	S. flocculans (HM032897)	T	**G**	**G**	**C**	**A**	**T**	**A**	**C**	**T**	G	**C**	**G**	**T**	T	C	A
	S. ginsengisoli (JN090860)	T	**G**	**G**	**C**	**A**	**T**	**A**	**C**	**T**	G	**C**	**G**	**T**	T	C	A
Clade2	*H. actinosclerus* (Y17356)	T	A	***T***	***A***	C	***C***	***G***	T	G	**C**	-	A	C	T	A	**G**
	H. aerophilus (AJ276901)	T	D	***T***	***A***	C	***C***	***G***	T	G	**C**	-	A	C	T	A	**G**
	H. algoricola (EU155009)	T	A	***T***	***A***	C	***C***	***G***	T	G	**C**	-	A	A	T	A	**G**
	H. chitinivorans (Y18837)	T	A	***T***	***A***	C	***C***	***G***	T	G	**C**	-	A	A	T	A	**G**
	H. daecheongensis (EU370958)	T	A	***T***	***A***	C	***C***	***G***	T	G	**C**	-	A	A	T	A	**G**
	H. elongatus (GQ454797)	T	A	***T***	***A***	A	***C***	***G***	T	T	**C**	-	A	A	T	A	**G**
	H. fastidiosus (EU155015)	T	A	***T***	***A***	C	***C***	***G***	T	G	**C**	-	A	A	T	A	**G**
	H. gelipurpurascens (Y18836)	T	A	***T***	***A***	C	***C***	***G***	T	G	**C**	-	A	C	T	A	**G**
	H. norwichensis (AJ549285)	T	A	***T***	***A***	C	***C***	***G***	T	G	**C**	-	A	C	T	A	**G**
	H. roseosalivarius (Y18833)	T	A	***T***	***A***	C	***C***	***G***	T	G	**C**	-	A	A	T	A	**G**
	H. rigui (DQ089669)	T	A	***T***	***A***	C	***C***	***G***	T	G	**C**	-	A	C	T	A	**G**
	H. psychrophilus (GQ131579)	T	A	***T***	***A***	C	***C***	***G***	T	G	**C**	-	A	C	T	A	**G**
	H. psychrotolerans (DQ177475)	T	A	***T***	***A***	C	***C***	***G***	T	G	**C**	-	A	A	T	A	**G**
	H. perfusus (HM032896)	T	A	***T***	***A***	.	***C***	***G***	T	T	**C**	-	A	A	T	A	**G**
	H. xinjiangensis (DQ888329)	T	A	***T***	***A***	C	***C***	***G***	T	G	**C**	-	C	A	T	A	**G**
	H. yonginensis (GU808562)	T	A	***T***	***A***	C	***C***	***G***	T	G	**C**	-	A	A	T	A	**G**
	H. tibetensis (EU382214)	T	A	***T***	***A***	C	***C***	***G***	T	G	**C**	-	A	C	T	A	**G**

Clade3	*Ph. deserti* (EU325941)	**C**	A	***T***	***A***	C	***C***	***G***	T	G	G	-	A	A	**G**	**T**	T
	Ph. ocellatus (Y18835)	**C**	A	***T***	***A***	C	***C***	***G***	T	G	G	-	A	A	**G**	**T**	T
		Position of the nucleotide															
	Species	594	658	728	747	833	854	896	**897**	**902**	903	1019		1275	1285	1286	1364
	E. coli (J01695)	T	C	A	A	G	T	C	**C**	**G**	G	A	G	A	A	T	T
Clade1	*S. arizonensis* (JX294485)	T	**A**	**A**	**T**	C	T	C	***C***	***G***	G	A	T	**C**	**G**	**A**	T
	S. glaciei (GQ454806)	T	**A**	**A**	**T**	G	T	T	***C***	***G***	A	A	T	**C**	**G**	**A**	T
	S. antarcticus (EU155012)	T	**A**	**A**	**T**	G	T	T	***C***	***G***	A	A	T	**C**	**G**	**A**	T
	S. soli (AB251884)	T	**A**	**A**	**T**	G	T	C	***C***	***G***	G	A	T	**C**	**G**	**A**	T
	S. metalli (HM032898)	T	**A**	**A**	**T**	C	T	C	***G***	***C***	G	A	T	T	**G**	**A**	T
	S. flocculans (HM032897)	T	**A**	**A**	**C**	C	T	C	***G***	***C***	G	A	T	T	**G**	**A**	T
	S. ginsengisoli (JN090860)	T	**A**	**A**	**T**	C	T	C	***G***	***C***	G	A	T	T	**G**	**A**	T
Clade2	*H. actinosclerus* (Y17356)	T	C	T	G	G	T	**T**	***C***	***G***	**A**	A	T	T	**A**	C	T
	H. aerophilus (AJ276901)	T	C	T	G	G	T	**T**	***C***	***G***	**A**	A	T	T	**A**	C	T
	H. algoricola (EU155009)	T	C	T	G	C	T	**T**	***C***	***G***	**A**	A	T	T	**A**	C	T
	H. chitinivorans (Y18837)	T	C	T	G	C	A	**T**	***C***	***G***	**A**	A	T	T	**A**	C	T
	H. daecheongensis (EU370958)	C	C	T	G	C	T	**T**	***C***	***G***	**A**	A	T	T	**A**	C	T
	H. elongatus (GQ454797)	T	C	T	G	C	T	**T**	***C***	***G***	**A**	A	T	T	**A**	C	T
	H. fastidiosus (EU155015)	C	C	T	G	C	T	**T**	***C***	***G***	**A**	A	T	T	**A**	C	T
	H. gelipurpurascens (Y18836)	T	G	T	C	G	T	**T**	***C***	***G***	**A**	A	T	T	**A**	C	T
	H. norwichensis (AJ549285)	T	C	T	G	G	T	**T**	***C***	***G***	**A**	A	T	T	**A**	G	T
	H. roseosalivarius (Y18833)	T	G	T	C	C	T	**T**	***C***	***G***	**A**	A	T	C	**A**	C	T
	H. rigui (DQ089669)	T	C	T	G	G	T	**T**	***C***	***G***	**A**	A	T	T	**A**	C	T
	H. psychrophilus (GQ131579)	T	C	T	G	G	T	**T**	***C***	***G***	**A**	A	T	T	**A**	A	T
	H. psychrotolerans (DQ177475)	T	C	T	G	G	T	**T**	***C***	***G***	**A**	G	T	T	**A**	C	T
	H. perfusus (HM032896)	T	C	T	G	G	T	**T**	***C***	***G***	**A**	A	T	T	**A**	C	T
	H. xinjiangensis (DQ888329)	T	C	T.	G	G	T	**C**	***C***	***G***	**G**	A	T	T	**A**	A	T
	H. yonginensis (GU808562)	T	C	T	G	G	T	**T**	***C***	***G***	**A**	G	T	T	**A**	C	T
	H. tibetensis (EU382214)	T	C	T	G	G	T	**T**	***C***	***G***	**A**	A	T	T	**A**	G	T
Clade3	*Ph. deserti* (EU325941)	**C**	C	T	G	**T**	**C**	C	***G***	***C***	G	**G**	**C**	**A**	G	**A**	**C**
	Ph. ocellatus (Y18835)	**C**	C	T	G	**T**	**C**	C	***G***	***C***	G	**G**	**C**	**A**	A	**A**	**C**

Table 1: Signature nucleotides of the 16S rRNA gene sequences that differentiate the genera *Siccationidurans* gen. nov.; *Hymenobacter* [9] and *Parahymenobacter* gen. nov**.** The numbering of nucleotide position is with respect to *Escherichia coli* 16S rRNA gene sequence (Acc. No. J01695); *S. Siccationidurans; H. Hymenobacter; Ph, Parahymenobacter;* -,absence of a nucleotide; Positions in bold are the signature nucleotides of the genera.

bacter using MEME [17], only 1-2 unique signatures motifs were found to be unique. Out of 19, 11 motifs (ATGTGGTTTAATTC-GATGAT [949-968], ATTTATTGGGTTTAAAGGGT [559-578], AGTAGGGAATATTGGGCAAT [356-375], AGCGGTGAAAT-GCATAGATA [687-706] and AACAGGATTAGATACCCTGG [781-800], GTGAAACTCAAAGGAATTGA [905-923], TAATA-CATGCAAGTCGAA [50-66], AACCTTACCTAGGCTAGAAT [977-995], ACAAAGCAAGGTGCTGCATG [1037-1057], AAGGCCTTCTGGGTTGTAAA [414-432]) were non-specific and identified other than *Hymenobacter* genera in BLAST. Motif TGAAAT-GCATAGATACCAT [692-710] was non-specific and restricted to 7 species of Clade1 to 3 of *Hymenobacter.* Four motifs were specific and identified the genus *Hymenobacter* in the EzTaxon and BLAST, of which motifs, AAGCTGGAATCACTAGTAAT [1332-1351], GTAAAC-GATGGATACTCGCT [812-831] and TTAGCGAAAGCGTTAAG-TAT [858-877] were common to all three clades, and AAAGGCGGAT-TAATACCGCA [160-179] was present in Clade1 and Clade2. Motifs AAGGCTTTCTGAGTCGTAAA [414-432], TGACGGTACCTGAG-GAATAA [480-499] and ATTAATACCGCATAACACT [168-185] and TAGTTAAAGAATTT [205-218] were uniquely identified among the species of Clade1, Clade2 and Clade3 respectively.

Based on the robust phylogenetic clustering of the genus *Hymenobacter* into three clades, Clade1, Clade2 and Clade3, 16S rRNA gene sequence similarity of less than 93.0%, unique *in silico* restriction fragmentation pattern, signature nucleotides and signature motifs, two more genera were created to accommodate species of Clade1 and Clade3, retaining the name *Hymenobacter, sensu stricto,* to represent 17 species of the Clade2. For members of Clade1 and Clade3, the names *Siccationidurans* gen. nov. and *Parahymenobacter* gen. nov. are proposed and species belonging to Clade1 and Clade3 are transferred to their respective genera. In addition, Clade1 species are different from Clade2 and Clade3 in that they are negative for nitrate reduction and do not contain the fatty acid iso-$C_{17:0}$ 3-OH (Table 2). The other diagnostic characteristics of the genera are listed in Table 2.

Seven species of Clade1, *H. arizonensis* [12], *H. glaciei* and *H. antarcticus* [13], *H. soli* [62], *H. metalli, H. flocculans* [63] and *H. ginsengisoli* [11] were transferred to the genus *Siccationidurans* gen. nov. as *Siccationidurans arizonensis comb. nov., Siccationidurans glaciei comb. nov., Siccationidurans antarcticus comb. nov., Siccationidurans soli comb. nov., Siccationidurans metalli comb. nov., Siccationidurans flocculans comb. nov.,* and *Siccationidurans ginsengisoli comb. nov. Siccationidurans soli* PB17^T=LMG 24240^T=KCTC 12607^T was designated as the type species of the genus, first described species among Clade1. Clade2 contains 17 species; *H. actinosclerus* [64], *H. aerophilus* [65], *H. algoricola, H. elongatus* and *H. fastidiosus* [13], *H. chitinivorans, H. gelipurpurascens* and *H. norwichensis* [10], *H. daecheongensis* [66], *H. roseosalivarius* [9], *H. rigui* [67], *H. psychrophilus* [68], *H. psychrotolerans* [69], *H. perfusus* [63], *H. tibetensis* [70], *H. xinjiangensis* [71] and *H.*

Characteristic	1	2	3	4	5
Motility	-	-	-	+	-
Pigment	Red-pink	Pink or Brick-red	Pink-red or Brick red or Pink	Pink	Pink
Biochemical characteristics					
Catalase	V	+	V	+	+
Oxidase	V	+	V	+	+
Urease	-	-	V	ND	-
α-galactosidase	V	-	V	-	+
β-galactosidase	V	-	V	+	+
Alkaline phosphatae	+	+	V	+	ND
Nitrate reduction	-	+	V	-	-
Starch hydrolysis	V	+	V	-	+
Aesculin	V	-	V	+	ND
Trypsin	V	-	V	+	ND
Tween 80	V	-	V	-	ND
Casein	+	+	V	-	ND
Gelatin	V	+	V	+	ND
Fatty acid methyl esters					
iso-$C_{15:0}$	+	+	+	+	+
anteiso-$C_{15:0}$	+	+	+	+	+
$C_{16:1}$ω5c	+	+	+	-	+
iso-$C_{15:0}$ 2OH	-	-	-	-	+
iso-$C_{15:0}$ 3-OH	-	+	+	<5.0%	<5.0%
iso-$C_{17:0}$ 3-OH	-	+	+	+	+
Summed feature 3*	+	+	+	+	-
Summed feature 4*	+	+	+	+	+
Menaquinone	MK-7	MK-7	MK-7	MK-7	MK-7
Polar lipids	PE	PE	PE	PE	ND
Mole % DNA content	58-70	58-65	54-65	45-60	40-48

Table 2: Phenotypic characteristics that differentiate the genera *Siccationidurans* gen. nov., and *Parahymenobacter* gen. nov., from other related genera 1. *Siccationidurans* gen. nov.[1,2]; 2. *Parahymenobacter* gen. nov.[3,4]; 3. *Hymenobacter*[3,5]; 4. *Pontibacter*[6]; 5. *Adhaeribacter*[7]. *+, positive; -, negative; ND, not determined; V, variable; PE, phosphatidyl ethanolamine; Summed feature 3 (iso-$C_{15:0}$ 2-OH; and/or $C_{16:1}$ω7c); Summed feature 4 (iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B). Data from[1]Reddy and Garcia-Pichel [12]; [2]Hoang et al. [11]; [3]Buczolits et al. [10]; [4] Zhang et al. [73]; [5]Hirsch et al. [9]; [6]Nedashkovskaya et al. [20]; [7]Rickard et al. [21].

yonginensis [72]. Clade3 containing two species, *H. deserti* [73] and *H. ocellatus* [10] were transferred to the genus *Parahymenobacter* gen. nov. as *Parahymenobacter ocellatus comb. nov.,* and *Parahymenobacter deserti* comb. nov. The type species of the genus is *Parahymenobacter ocellatus* Myx 2105^T=Txo1^T=DSM 11117^T=LMG 21874^T.

Emended description of the genus *Hymenobacter* Hirsch et al. [9], emend. Buczolits et al. [10]

(Gr. n. *humen*, pellicle, thin layer; N.L. masc. n. *bacter*, rod; N.L. masc. n. *Hymenobacter*, a rod growing in thin layers). Members of the genus *Hymenobacter* are Gram-negative, strictly aerobic, pigmented, non-motile, non-spore forming and rod shaped. They are negative for indole production, arginine dihydrolase and urease, but positive for leucine arylamidase. Major polar lipids present are phosphatidylethanolamine (PE) and an unknown aminophospholipid (APL3), while the quinone system present is MK-7. Fatty acid profile consists of iso- and anteiso $C_{15:0}$, $C_{16:1}$ω5c, iso-$C_{15:0}$ 3-OH, iso-$C_{17:0}$ 3-OH, summed feature 3 ($C_{16:1}$ω7c/iso-$C_{15:0}$ 2OH) and summed feature 4 (iso-$C_{17:1}$ I/anteiso-$C_{17:1}$ B). Species of the genus contain C (256), C (268), C-G (294-303), T-A (317-336), C-G (419-424), C (441), G (493), C-G (897-902), A (903), A (1285) as the signature nucleotides and TGACGGTACCTGAGGAATAA (480-499) as the signature motif in the 16S rRNA gene sequences. The DNA G+C content of the genus ranges from 54 to 65 mol%. The genus currently comprises of seventeen species and *Hymenobacter roseosalivarius* AA-718^T=CIP 106397^T=DSM 11622^T is the type species.

Description of *Siccationidurans* gen. nov.: *Siccationidurans*

Sic.ca.ti.o.ni.du'rans. L. n. siccatioonis, drying, desiccation; L. part. adj. durans, resisting; N.L. masc. n. (N.L. masc. part. adj. used as a substantive) *siccationidurans*, desiccation-resisting. Cells of the genus *Siccationidurans* gen. nov. are Gram-negative, strictly aerobic, pigmented, non-motile, non-spore forming and rod shaped. Major fatty acids present (above 5.0%) are iso-$C_{15:0}$, anteiso-$C_{15:0}$, $C_{16:0}$, $C_{16:1}$ω5c, summed feature 3 ($C_{16:1}$ω7c/iso-$C_{15:0}$ 2OH), summed feature 4 (anteiso-$C_{17:1}$ B/iso-$C_{17:1}$ I) and MK-7 is the sole respiratory quinine. They contain G-C (294-303), G (306), G-C (317-336), A (408), T-A (419-424), C (427), T (434), G-C/C-G (462-476), T (477), A (658), A (728), T (747), C-G/G-C (897-902), G (1285), A (1286) as signature nucleotides and lacks restriction sites for enzymes *DpnI, MboI, Sau3A1* at position 209 and *HaeIII* at position 321 (positions with respect to *Siccationidurans arizonensis*). The genus possesses a unique 20 nucleotide long signature motif AAGGCTTTCTGAGTCGTAAA. Mole % G+C DNA content of the genus ranges from 58.0 to 70.0%. *Siccationidurans soli* PB17^T=KCTC 12607^T=LMG 24240^T is the type species.

Description of *Siccationidurans soli* comb. nov.

Siccationidurans soli (L. gen. n. *soli*, of soil, the source of the type strain)

Basonym: *Hymenobacter soli* [62].

The species description is the same as that described by Kim et al. [62].

Description of *Siccationidurans arizonensis* comb. nov.

Siccationidurans arizonensis (*arizonensis*: a.ri.zo.nen'sis.N.L. masc. adj. *arizonensis*, of or belonging to Arizona, one of the states of United States of America).

Basonym: *Hymenobacter arizonensis* [12].

The species description is the same as that described by Reddy and Garcia-Pichel [12].

Description of *Siccationidurans antarcticus* comb. nov.

Siccationidurans antarcticus (L. masc. adj. *antarcticus*, southern, by extension pertaining to Antarctic, referring to its isolation source).

Basonym: *Hymenobacter antarcticus* [13].

The species description is the same as that described by Klassen and Foght [13].

Description of *Siccationidurans glaciei* comb. nov.

Siccationidurans glaciei (L. gen. n. *glaciei*, of ice, referring to its isolation from a glacier).

Basonym: *Hymenobacter glaciei* [13].

The species description is the same as that described by Klassen and Foght [13].

Description of *Siccationidurans flocculans* comb. nov.

Siccationidurans flocculans (N.L. part. adj. *flocculans*, flocculating, referring to the organism's trait to flocculate in liquid cultures).

Basonym**:** *Hymenobacter flocculans* [63].

The species description is the same as that described by Chung et al. [63].

Description of *Siccationidurans metalli* comb. nov.

Siccationidurans metalli (L. gen. n. *metalli*, of a mine, from a mining area with metals).

Basonym**:** *Hymenobacter metalli* [63].

The species description is the same as that described by Chung et al. [63].

Description of *Siccationidurans ginsengisoli* comb. nov.

Siccationidurans ginsengisoli (gin.sen.gi.so'li. N.L. n. *ginsengum* ginseng; L. n. *solum* soil; ginseng field, the source of the type strain)

Basonym**:** *Hymenobacter ginsengisoli* [11].

The species description is the same as that described by Hoang et al. [11].

Description of *Parahymenobacter* gen. nov.

Parahymenobacter: *Pa.ra.hy.me.no.bac'ter.* Gr. prep. para, beside, near, like; N.L. masc. n. *Hymenobacter*, a bacterial genus name; N.L. masc. n. *Parahymenobacter*, beside *Hymenobacter.*

The genus *Parahymenobacter* gen. nov. contain the cells that are Gram-negative, strictly aerobic, pigmented, non-motile, non-spore forming and rod shaped. Catalase, oxidase, leucine arylamidase and alkaline phosphatase positive, urease, arginine dihydrolase and indole production negative, hydrolyzes casein, gelatin and starch. MK-7 is the major menaquinone, while phosphatidylethanolamine (PE) and an unknown aminophospholipid (APL3) are the dominating lipids. Major fatty acids (above 5.0%) are iso-$C_{15:0}$, anteiso-$C_{15:0}$; $C_{16:1}\omega5c$, iso-$C_{15:0}$ 3-OH, iso-$C_{17:0}$ 3-OH, summed feature 3 ($C_{16:1}\omega7c$/ iso-$C_{15:0}$ 2OH) and summed feature 4 (anteiso-$C_{17:1}$ B/ anteiso-$C_{17:1}$ I). Signature nucleotides present are G (127), G (128), T (131), T (140), T (165), A (206), A (215), C (219), C (233), C (234), G-C (294-303), C (304), T-A (317-336), T (492), T (833), G-C (897-902), G (1019), C, A (1275), C (1364) and the signature motifs consists of the sequence ATTAATACCGCATAACACT (168-185) and TAGTTAAAGAATTT (205-218). Mole % G+C DNA content of the genus ranges from 58 to 65. *Parahymenobacter ocellatus* Myx 2105^T=Txo1^T=DSM 11117^T=LMG 21874^T is the type species of the genus.

Description of *Parahymenobacter ocellatus* comb. nov.

Parahymenobacter ocellatus (L. masc. adj. *ocellatus*, showing little eyes, referring to the bright granules at the cell poles).

Basonym**:** *Hymenobacter ocellatus* [10].

The species description is the same as that described by Buczolits et al. [10].

Description of *Parahymenobacter deserti* comb. nov.

Parahymenobacter deserti (L. gen. n. *deserti*, of a desert).

Basonym**:** *Hymenobacter deserti* [73].

The species description is the same as that described by Zhang et al. [73].

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