RESEARCH LETTER

The *gyrB* gene is a useful phylogenetic marker for exploring the diversity of *Flavobacterium* strains isolated from terrestrial and aquatic habitats in Antarctica

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Abstract

Within the phylum *Bacteroidetes*, the *gyrB* gene, encoding for the B subunit of the DNA gyrase, has been used as a phylogenetic marker for several genera closely related to *Flavobacterium*. The phylogenies of the complete 16S rRNA gene and the *gyrB* gene were compared for 33 Antarctic *Flavobacterium* isolates and 23 type strains from closely related *Flavobacterium* species. *gyrB* gene sequences provided a higher discriminatory power to distinguish between different *Flavobacterium* groups than 16S rRNA gene sequences. The *gyrB* gene is therefore a promising molecular marker for elucidating the phylogenetic relationships among *Flavobacterium* species and should be evaluated for all the other type strains of described *Flavobacterium* species. Combining the phylogeny of both genes, the new Antarctic *Flavobacterium* strains constitute 15 *Flavobacterium* groups, including at least 13 potentially new species together with one group of isolates probably belonging to the species *Flavobacterium micromati* and one group close to *Flavobacterium gelidilacus*.

Introduction

Heterotrophic bacterial communities in Antarctica are highly diverse in aquatic (Bowman *et al*., 2000; Van Trappen *et al*., 2002) as well as in terrestrial (Aislabie *et al*., 2006; Babalola *et al*., 2009) habitats. A genus that has been isolated often from these environments is *Flavobacterium* (Brambilla *et al*., 2001; Humphry *et al*., 2001; Van Trappen *et al*., 2002), and several novel *Flavobacterium* species were described from Antarctic habitats (*Flavobacterium gelidilacus*, *Flavobacterium gillisiae*, *Flavobacterium hibernum*, *Flavobacterium micromati*, *Flavobacterium psychrolimnae*, *Flavobacterium xanthum*) or other cold environments (*Flavobacterium xinjangense* and *Flavobacterium omnivorum*). Other *Flavobacterium* species have been mainly isolated from freshwater fish (*Flavobacterium branchiophilum*, *Flavobacterium columnare*, *Flavobacterium psychrophilum*), temperate freshwater (*Flavobacterium aquatile*, *Flavobacterium flevense*, *Flavobacterium saccharophilum*) and from soil (*Flavobacterium johnsoniae*, *Flavobacterium pectinovorum*). Most *Flavobacterium* species are psychrotolerant and as they are able to hydrolyse several carbohydrates and biomacromolecules such as gelatine, casein and starch, they might be of biotechnological importance (Bernardet & Bowman, 2006).

The family *Flavobacteriaceae* (phylum *Bacteroidetes*) as well as the genus *Flavobacterium* have been revised and added to repeatedly over the years (Vandamme *et al*., 1994; Bernardet *et al*., 1996, 2002). *Flavobacterium* was created in 1923 for all bacteria that formed yellow- or orange-pigmented colonies and weakly produced acid from carbohydrates (Bergey *et al*., 1923). This broadly defined and taxonomically heterogeneous group was further refined using phenotypic characteristics (Holmes *et al*., 1984) and the determination of guanine plus cytosine (G+C) content (Reichenbach, 1989). The introduction of the 16S rRNA gene oligonucleotide catalogue (Paster *et al*., 1985), DNA–rRNA hybridization data (Bauwens & De Ley, 1981; Segers *et al*., 1993; Vandamme *et al*., 1994) and sequence data (Woese *et al*., 1990; Gherna & Woese, 1992) changed the family and the genus further and provided the framework for the present classification. Currently, strains are assigned to the genus *Flavobacterium* (including 71 species to date) based on fatty acid analysis, the G+C content and a number of

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morphological and phenotypical characteristics following the proposal of Bernardet *et al.* (1996) in combination with 16S rRNA gene sequence analysis (Bernardet *et al.*, 2002; Bernardet & Bowman, 2006).

Although DNA–DNA hybridizations (DDH) are the gold standard for species identification (Stackebrandt *et al.*, 2002), these experiments are technically challenging, laborious and time consuming. Sequence analysis of 16S rRNA genes is used for prokaryotic classification (Rossello-Mora & Amann, 2001) to provide a tentative identification. It can often limit the number of DDH experiments required. Nevertheless, the 16S rRNA gene has a limited resolving power at the species level (Fox *et al.*, 1992; Probst *et al.*, 1998). Within the genus *Flavobacterium*, values of 97.2–98.7% 16S rRNA gene sequence similarity are found between distinct *Flavobacterium* species (Bernardet & Bowman, 2006). As protein-encoding genes evolve faster, they are considered more appropriate for the phylogenetic analysis of closely related species. Within the genus *Flavobacterium*, protein-encoding genes have not yet been used for detailed phylogenetic study. The *gyrB* gene was found to be a successful marker for phylogenetic analysis in several groups in other phyla, for example *Acinetobacter* (*Proteobacteria*) (Yamamoto & Harayama, 1996) and *Micromonospora* (*Actinobacteria*) (Kasai *et al.*, 2000), but also in the phylum *Bacteroidetes* in the genus *Marinilabilia* and related taxa (Suzuki *et al.*, 1999). In these studies, phylogenetic analysis based on the *gyrB* gene sequences was shown to be consistent with DDH and phenotypic comparison (Yamamoto & Harayama, 1996). Suzuki *et al.* (2001) applied *gyrB* gene sequencing to study the phylogenetic relationships of marine isolates within the phylum *Bacteroidetes* and included two *Flavobacterium* species. In addition, more *gyrB* sequences from *Flavobacterium* species are becoming available in the frame of genome projects (Duchaud *et al.*, 2007).

In a previous study of aquatic and terrestrial microbial mats in Antarctica, several *Flavobacterium* strains were isolated that showed a low similarity to described *Flavobacterium* species, based on the partial or the full 16S rRNA gene sequences (Peeters *et al.*, submitted). In the present study, we determined the *gyrB* gene sequence of 33 of these new Antarctic isolates and of the type strains of related *Flavobacterium* species to study the diversity of our isolates in more detail and to elucidate the usefulness of *gyrB* as a phylogenetic marker for phylogeny in the genus *Flavobacterium*. We also compared with the phylogeny based on the near-complete 16S rRNA gene sequences.

Materials and methods

Strains used

The *Flavobacterium* strains studied here (Table 1) were obtained as part of a large study into the diversity of heterotrophic bacteria in microbial mats from Antarctica (Peeters *et al.*, submitted). The samples used in that study originated from a terrestrial sample, taken in the close neighbourhood of the Princess Elisabeth Station in Utsteinen, Dronning Maud Land (Peeters *et al.*, 2011a), and microbial mat samples from lakes in the Transantarctic Mountains (Peeters *et al.*, 2011b), the Schirmacher Oasis and on Pourquoi-Pas Island (Antarctic Peninsula) (for details, see Table 1). In these previous studies, isolates were first grouped by rep-PCR fingerprinting and representatives of all rep-types were tentatively identified by full or partial 16S rRNA gene sequencing (Peeters *et al.*, 2011a; Peeters *et al.*, 2011b; Peeters *et al.*, submitted). Several of these strains were identified as *Flavobacterium* and 33 of these were used in this study (Table 1). To elucidate their phylogenetic relationships, type strains of closely related *Flavobacterium* species were also included (Table 2).

16S rRNA gene sequence analysis

The complete 16S rRNA gene sequences of four Antarctic *Flavobacterium* isolates were available from previous studies (Peeters *et al.*, 2011a, 2011b). The 16S rRNA genes of the remaining 29 Antarctic *Flavobacterium* isolates were only partially sequenced (400 bp) (Peeters *et al.*, submitted). These sequences were completed in this study (accession numbers listed in Table 1) using the same method as that described before (Vancanneyt *et al.*, 2004). A multiple sequence alignment of all complete 16S rRNA gene sequences was performed using the BIONUMERICS (version 5.1.) software package (Applied-Maths) and a region of 912 bp, containing good sequence data for all strains, was delimited for further analysis. After visual inspection, distances were calculated using the Kimura-2 correction. A neighbour-joining dendrogram (Saitou & Nei, 1987) was constructed and bootstrapping analysis was performed using 500 bootstrap replicates. A maximum likelihood dendrogram was calculated using the program PHYML (Guindon & Gascuel, 2003). The reliability of the tree was checked using the approximate likelihood ratio test (aLRT) method (Anisimova & Gascuel, 2006).

gyrB gene sequence analysis

For *F. johnsoniae*, *F. aquatile* and *Myroides odoratus* the *gyrB* sequences were available in the EMBL database (Table 2). For the other strains used, the *gyrB* sequences were determined in this study. DNA preparation was carried out as described by Baele *et al.* (2003). Primers were designed in KODON 3.5 using all available *gyrB* sequences from *Flavobacterium* and species from closely related genera (*Bacteroides*, *Cytophaga*, *Flexibacter*, *Terrimonas*, *Porphyrobacter*, *Parabacteroides*, *Salinibacter* and *Prevotella*) in the EMBL database (September 2009). A *gyrB* segment about 1200 bp

Table 1. Strain numbers, accession numbers and isolation source of the Antarctic *Flavobacterium* isolates used

		Accession no.		
Species	Strain no.	16S rRNA gene	*gyrB* gene	Isolation source
Flavobacterium sp. 1	R-40838	FR682718*	FR772324	Terrestrial microbial mat, Utsteinen nunatak, Antarctica
	R-40949	FR772055	FR772296	Terrestrial microbial mat, Utsteinen nunatak, Antarctica
Flavobacterium sp. 2	R-36233	FR682719*	FR772292	Terrestrial microbial mat, Utsteinen nunatak, Antarctica
	R-36668	FR772052	FR772293	Terrestrial microbial mat, Utsteinen nunatak, Antarctica
	R-36669	FR772053	FR772294	Terrestrial microbial mat, Utsteinen nunatak, Antarctica
	R-36523	FR772054	FR772295	Terrestrial microbial mat, Utsteinen nunatak, Antarctica
Flavobacterium sp. 3	R-41499	FR772077	FR772318	Aquatic microbial mat, Schirmacher Oasis, Antarctica
Flavobacterium sp. 4	R-38377	FR772072	FR772313	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
	R-37599	FR772073	FR772314	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
	R-38423	FR772067	FR772308	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
	R-40835	FR772071	FR772312	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
	R-36964	FR691441*	FR772322	Aquatic microbial mat, Forlidas Pond, Antarctica
Flavobacterium sp. 5	R-38388	FR772056	FR772297	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
Flavobacterium sp. 6	R-38274	FR772058	FR772299	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
	R-38352	FR772069	FR772310	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
Flavobacterium sp. 7	R-38477	FR772059	FR772300	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
Flavobacterium sp. 8	R-40837	FR772060	FR772301	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
	R-38313	FR772065	FR772306	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
	R-38503	FR772061	FR772302	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
	R-41504	FR772062	FR772303	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
Flavobacterium sp. 9	R-38294	FR772063	FR772304	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
	R-38296	FR772064	FR772305	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
Flavobacterium sp. 10	R-38392	FR772074	FR772315	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
Flavobacterium sp. 11	R-37608	FR772076	FR772317	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
Flavobacterium sp. 12	R-38474	FR772057	FR772298	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
	R-38373	FR772070	FR772311	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
Flavobacterium sp. 13	R-40832	FR772078	FR772319	Aquatic microbial mat, Forlidas Pond, Antarctica
	R-36976	FR772080	FR772323	Aquatic microbial mat, Forlidas Pond, Antarctica
	R-36963	FR691440*	FR772321	Aquatic microbial mat, Forlidas Pond, Antarctica
	R-36961	FR772079	FR772320	Aquatic microbial mat, Forlidas Pond, Antarctica
Flavobacterium sp. 14	R-38349	FR772068	FR772309	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
Flavobacterium sp. 15	R-38420	FR772066	FR772307	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
	R-37612	FR772075	FR772316	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica

The 16S rRNA gene sequences marked with an asterisk were determined in previous studies (Peeters *et al.*, 2011a, 2011b).

long was obtained with the primers *gyrB*-241F (5′-GA YACCGGWCGTGGTATTCC-3′) and *gyrB*-1588R (5′-TC DAYATCGGCATCACACAT-3′), which were used both for amplification and for sequencing reactions. For amplification, the reaction mix (50 μL) consisted of 5 μL GeneAmp® 10 × PCR buffer (Applied Biosystems), 5 μL dNTP's (2 mM), 0.5 μL of the forward and reverse primer (50 μM), 1 μL Taq polymerase (1 U μL^{-1}), 33 μL MilliQ water and 5 μL template DNA. After an initial denaturation step (95 °C for 5 min), three cycles of preamplification (95 °C for 1 min, 55 °C for 2 min 15 s and 72 °C for 1 min 15 s) and 25 cycles of amplification (95 °C for 35 s, 55 °C for 1 min 15 s and 72 °C for 1 min 15 s) were performed, finishing with 72 °C for 7 min. PCR products were purified using a Nucleofast 96 PCR cleanup membrane system (Machery-Nagel, Germany) and a Tecan Workstation 200. The sequencing PCR was performed as described before (Vancanneyt *et al.*, 2004). Sequence assembly and phylogenetic analysis was performed with the BIONUMERICS (version 5.1) software package (Applied-Maths) using a region of 1006 bp, containing good sequence data for all strains. The multiple alignment was verified by comparison with an alignment of the corresponding amino acids. After visual inspection of the sequence alignments, distances were calculated using the Kimura-2 correction. A neighbour-joining dendrogram (Saitou & Nei, 1987) was constructed and bootstrapping analysis was performed using 500 bootstrap replicates. A maximum likelihood dendrogram was calculated using the program PHYML (Guindon & Gascuel, 2003). The reliability of the tree was checked using the aLRT method (Anisimova & Gascuel, 2006). Accession numbers of the *gyrB* gene sequence of the *Flavobacterium* strains and the type strains of the *Flavobacterium* species are listed in Tables 1 and 2, respectively.

Table 2. *Flavobacterium* species included in this study

Species	Strain no.	Accession no.		Isolation source	References
		16S rRNA gene	*gyrB* gene		
Flavobacterium antarcticum	LMG 25319^T	FM163401	**FR774016**	Terrestrial sample from the Antarctic	Yi *et al*. (2005)
Flavobacterium aquatile	LMG 4427^T	AM230485	AB034225	Deep well, Kent, England	Bernardet *et al*. (1996)
Flavobacterium degerlachei	LMG 21915^T	AJ557886	**FR774017**	Microbial mats in Antarctic lakes	Van Trappen *et al*. (2004)
Flavobacterium flevense	LMG 8328^T	D12662	**FR774018**	Freshwater lake, the Netherlands	Bernardet *et al*. (1996)
Flavobacterium frigidarium	LMG 21010^T	AF162266	**FR774019**	Marine sediment, Antarctica	Humphry *et al*. (2001)
Flavobacterium frigoris	LMG 21922^T	AJ557887	**FR850657**	Microbial mats in Antarctic lakes	Van Trappen *et al*. (2004)
Flavobacterium fryxellicola	LMG 22022^T	AJ811961	**FR774020**	Microbial mats in Antarctic lakes	Van Trappen *et al*. (2005)
Flavobacterium gelidilacus	LMG 21477^T	AJ440996	**FR774021**	Microbial mats in Antarctic lakes	Van Trappen *et al*. (2003)
Flavobacterium gillisiae	LMG 21422^T	U85889	**FR774014**	Antarctic coastal sea ice	McCammon & Bowman (2000)
Flavobacterium glaciei	LMG 25320^T	DQ515962	**FR774022**	China No.1 glacier	Zhang *et al*. (2006)
Flavobacterium hibernum	LMG 21424^T	L39067	**FR774023**	Freshwater Antarctic lake	McCammon *et al*. (1998)
Flavobacterium johnsoniae	LMG 1340^T	AM230489	AB034222	Soil or mud, Rothamsted or Cambridge, England	Bernardet *et al*. (1996)
Flavobacterium limicola	LMG 21930^T	AB075230	**FR774015**	Freshwater sediments	Tamaki *et al*. (2003)
Flavobacterium micromati	LMG 21919^T	AJ557888	**FR774024**	Microbial mats in Antarctic lakes	Van Trappen *et al*. (2004)
Flavobacterium omnivorum	LMG 21986^T	AF433174	**FR774025**	China No. 1 glacier	Zhu *et al*. (2003)
Flavobacterium psychrolimnae	LMG 22018^T	AJ585428	**FR774026**	Microbial mats in Antarctic lakes	Van Trappen *et al*. (2005)
Flavobacterium psychrophilum	LMG 13179^T	AB078060	**FR774027**	Kidney of salmon	Bernardet *et al*. (1996)
Flavobacterium reichenbachii	LMG 25512^T	AM177616	**FR774028**	Hard water rivulet, Germany	Ali *et al*. (2009)
Flavobacterium succinicans	LMG 10402^T	AM230492	**FR774029**	Eroded fin of salmon, Washington	Bernardet *et al*. (1996)
Flavobacterium swingsii	LMG 25510^T	AM934651	**FR774030**	Hard water rivulet, Germany	Ali *et al*. (2009)
Flavobacterium tegetincola	LMG 21423^T	U85887	**FR774031**	Antarctic cyanobacterial mat	McCammon & Bowman (2000)
Flavobacterium xanthum	LMG 8372^T	AF030380	**FR774032**	Pool mud, Syowa, Antarctica	McCammon & Bowman (2000)
Flavobacterium xinjiangense	LMG 21985^T	AF433173	**FR774033**	China No. 1 glacier	Zhu *et al*. (2003)
Myroides odoratus	NBRC 14945^T	M58777	AB034239	Urine and serum specimen	Vancanneyt *et al*. (1996)

Accession numbers for newly determined sequences are shown in bold.

Results and discussion

This study was carried out to resolve the relationships of 33 Antarctic *Flavobacterium* strains that were previously characterized by partial 16S rRNA gene sequencing and found to represent several potentially novel groups. We completed the 16S rRNA gene sequences for all the strains and performed a phylogenetic analysis including also the type strains of 23 related or Antarctic *Flavobacterium* species. Neighbour-joining and maximum likelihood trees (Fig. 1 and Supporting Information, Fig. S1) showed a similar topology with the *Flavobacterium* isolates forming 15 groups, labelled *Flavobacterium* sp. 1–15. *Flavobacterium* sp. 13 and *Flavobacterium* sp. 5 were located close to, respectively, *F. micromati* and *F. gelidilacus*, with 99.8% and 99.0% sequence similarity to the respective type strain. It is well known that because of its high conservation, the 16S rRNA gene sequence has limited resolving power at the species level (Rossello-Mora & Amann, 2001). Indeed, there are examples of distinct species with identical or nearly identical 16S rRNA gene sequences (Fox *et al*., 1992; Probst *et al*., 1998), microheterogeneity of the 16S rRNA genes within one species (Bennasar *et al*., 1996) or single organisms with two or more 16S rRNA genes with a relatively high sequence divergence (Nübel *et al*., 1996). In the genus *Flavobacterium*, several new species have been described with a rather high 16S rRNA gene sequence similarity, for example the type strains of *Flavobacterium weaverense* and *Flavobacterium segetis* share 98.9% 16S rRNA gene sequence similarity, and yet, they have a DDH value of only 34% (Yi & Chun, 2006). Because protein-encoding genes are generally less conserved (Ochman & Wilson, 1987), they may be more appropriate for phylogenetic analysis of closely related species. Several protein-encoding genes such as *glnA*, *recA* and *hsp60* have been used for typing and taxonomical purposes within genera in the *Bacteroidetes* (Gutacker *et al*., 2002; Sakamoto *et al*., 2010). In this study, the *gyrB* gene, encoding for the B subunit of the DNA gyrase, was selected because it was previously used successfully to distinguish between closely related taxa affiliated with the genus *Flavobacterium* (Suzuki *et al*., 1999, 2001). Izumi *et al*. (2003) reported on the use of *gyrB* primers in a PCR-restriction fragment length

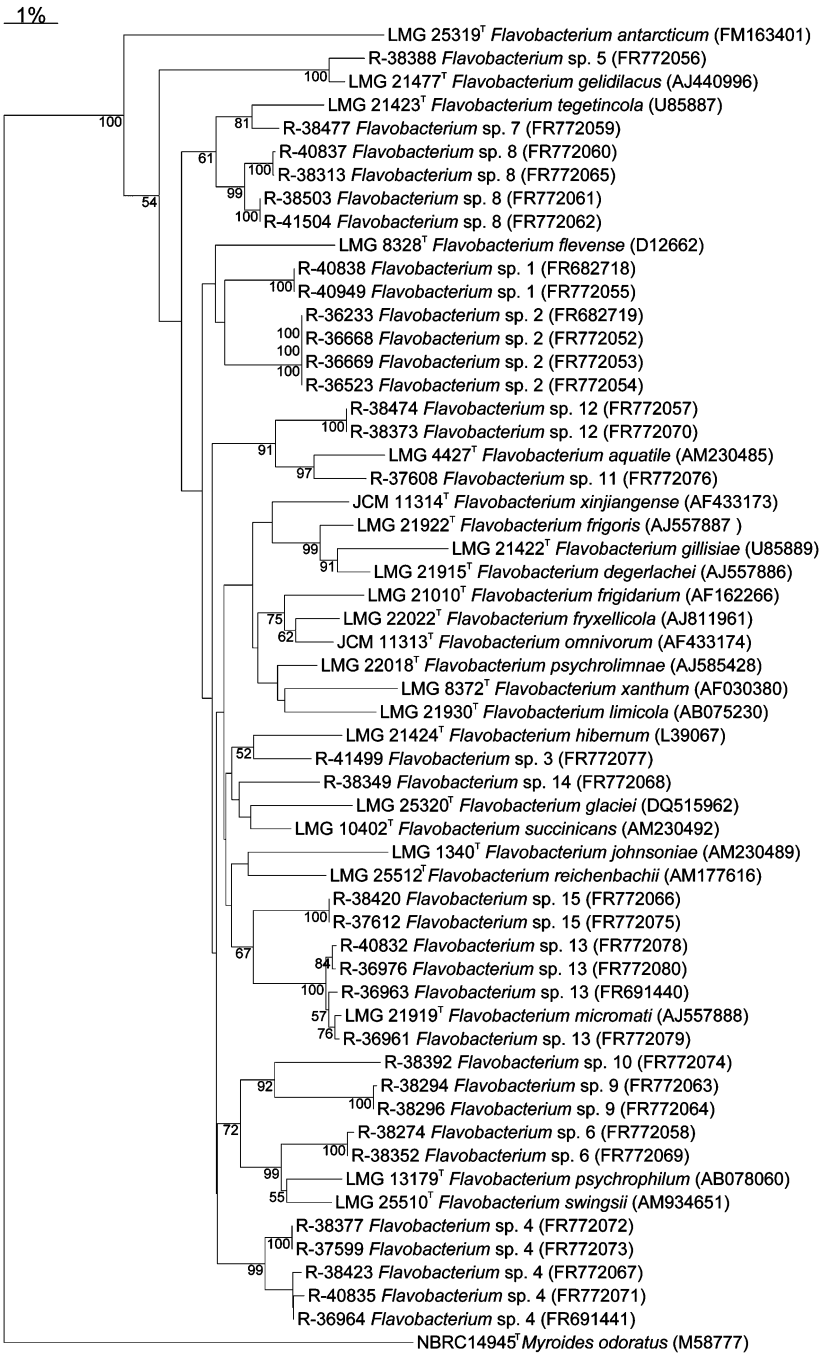

Fig. 1. Phylogenetic tree based on neighbour-joining analysis of the 16S rRNA gene sequence similarities of the *Flavobacterium* strains and closely related species. New *Flavobacterium* isolates are marked as *Flavobacterium* sp., followed by a number. The numbers at branch nodes are bootstrap values shown as percentages of 500 bootstrap replicates (only values > 50% are shown). Scale bar represents 1% estimated substitutions.

polymorphism analysis for the genotyping of *F. psychrophilum*, and Suzuki *et al.* (1999) designed *gyrB* primers to study the phylogenetic relationship for the genus *Marinilabilia* (*Bacteroidetes*) and related taxa. We tested all primers reported in these studies *in silico* on the *gyrB* sequences available from related genera and from the complete genome of *F. johnsoniae* DSM 2064 and found considerable mismatches with all groups included in the comparison. Therefore, more general primers were designed based on the available sequence information.

As expected for a more variable housekeeping gene, the distance between the *Flavobacterium* groups and the type strains is significantly higher in the *gyrB* gene dendrogram (Figs 2 and S2) in comparison with the 16S rRNA gene dendrogram (Figs 1, S1 and Table 3). The threshold for species definition has been suggested to be 98.7–99.0% 16S

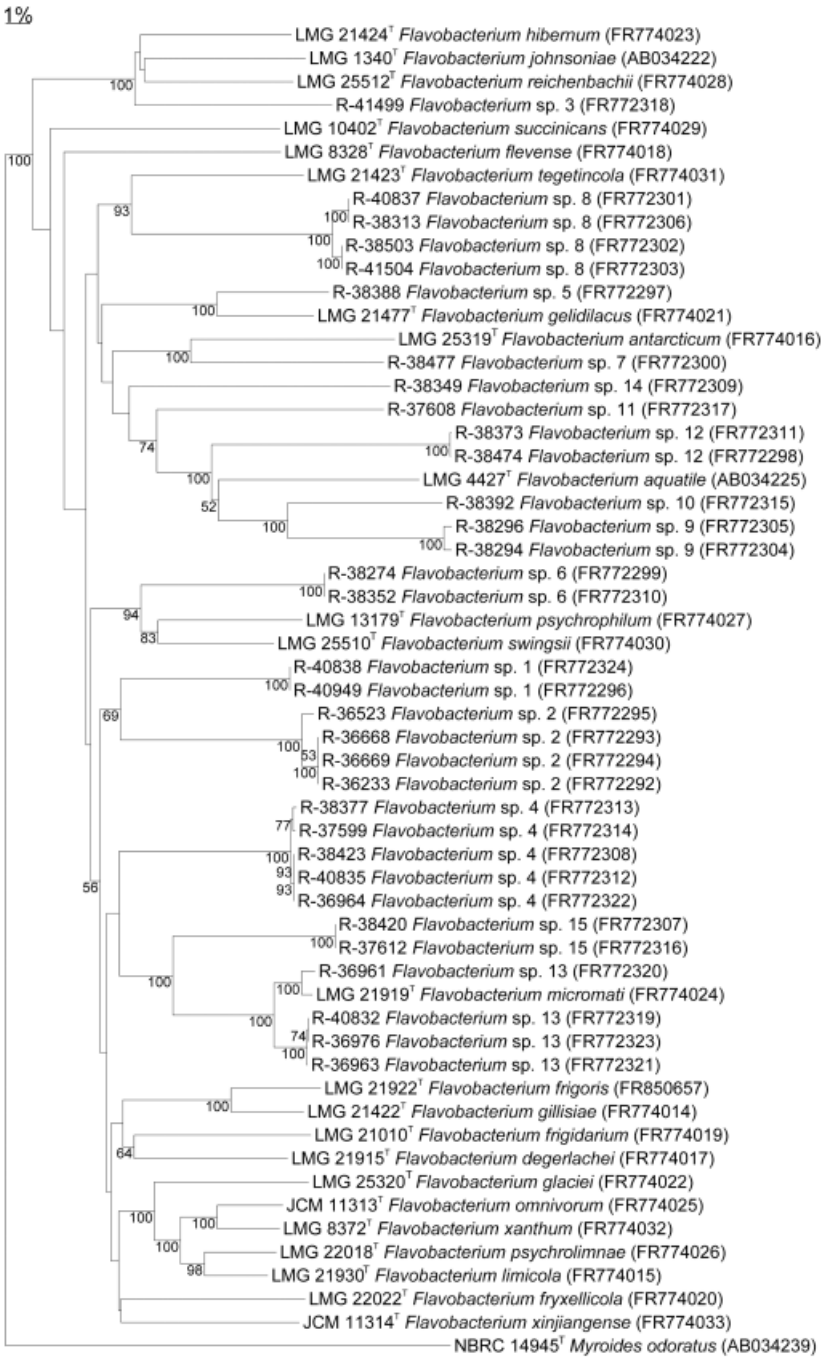

Fig. 2. Phylogenetic tree based on neighbour-joining analysis of the *gyrB* gene sequence similarities of the *Flavobacterium* strains and closely related species. New *Flavobacterium* isolates are marked as *Flavobacterium* sp., followed by a number. The numbers at branch nodes are bootstrap values shown as percentages of 500 bootstrap replicates (only values > 50% are shown). Scale bar represents 1% estimated substitutions.

rRNA gene sequence similarity by Stackebrandt & Ebers (2006), whereas for the *gyrB* phylogeny, this is less well documented. Suzuki *et al.* (2001) reported that the proposed limit for species identity, the 70% DNA reassociation value, corresponds to 88.8% *gyrB* sequence similarity in the subset of the *Bacteroidetes* they studied, whereas several other studies revealed a wide range of interspecies similarity values [60.0–89.0% *gyrB* gene sequence similarity within the genus *Helicobacter* (*Epsilonproteobacteria*) (Hannula & Hanninen, 2007), 75.4–95.0% within the genus *Bacillus* (*Firmicutes*) (Wang *et al.*, 2007), 85.0–97.5% within the genus *Aeromonas* (*Gammaproteobacteria*) (Yanez *et al.*, 2003), 77.5–97.6% within the genus *Gordonia* (*Actinobacteria*) (Kang *et al.*, 2009), 89.5–98.2% within the genus *Kribbella* (*Actinobacteria*) (Kirby *et al.*, 2010) and 70.1–98.7% within the genus *Streptococcus* (*Firmicutes*) (Itoh *et al.*, 2006)]. Among the

Table 3. Within-group similarity, closest related species and corresponding sequence similarity for the different Antarctic *Flavobacterium* groups based on the 16S rRNA and the *gyrB* gene phylogeny

16S rRNA gene				*gyrB* gene			
Antarctic species	Within group similarity (%)	Nearest neighbour	Similarity (%)	Antarctic species	Within group similarity (%)	Nearest neighbour	Similarity (%)
Flavobacterium sp. 1	100	*Flavobacterium psychrolimnae* LMG 22018^T	97.3	*Flavobacterium* sp. 1	100	*Flavobacterium limicola* LMG 21930^T	86.1
Flavobacterium sp. 2	100	*Flavobacterium succinicans* LMG 10402^T	96.4	*Flavobacterium* sp. 2	98.9–98.8	*Flavobacterium psychrolimnae* LMG 22018^T	86.6–86.4
Flavobacterium sp. 3		*Flavobacterium succinicans* LMG 10402^T	97.5	*Flavobacterium* sp. 3		*Flavobacterium hibernum* LMG 21424^T	87.2
Flavobacterium sp. 4	99.5–99.2	*Flavobacterium succinicans* LMG 10402^T	97.9–97.8	*Flavobacterium* sp. 4	99.8–99.7	*Flavobacterium degerlachei* LMG 21915^T	86.9–86.7
Flavobacterium sp. 5		*Flavobacterium gelidilacus* LMG 21477^T	99.0	*Flavobacterium* sp. 5		*Flavobacterium gelidilacus* LMG 21477^T	91.9
Flavobacterium sp. 6	99.9	*Flavobacterium swingsii* LMG 25510^T	97.9–97.8	*Flavobacterium* sp. 6	100	*Flavobacterium swingsii* LMG 25510^T	88.6
Flavobacterium sp. 7		*Flavobacterium tegetincola* LMG 21423^T	98.2	*Flavobacterium* sp. 7		*Flavobacterium antarcticum* LMG 25319^T	85.5
Flavobacterium sp. 8	99.1–100	*Flavobacterium tegetincola* LMG 21423^T	97.5–96.9	*Flavobacterium* sp. 8	100–99.0	*Flavobacterium tegetincola* LMG 21423^T	85.8–85.7
Flavobacterium sp. 9	99.9	*Flavobacterium swingsii* LMG 25510^T	95.6–95.5	*Flavobacterium* sp. 9	99.4	*Flavobacterium aquatile* LMG 4008^T	84.6
Flavobacterium sp. 10		*Flavobacterium swingsii* LMG 25510^T	96.1	*Flavobacterium* sp. 10		*Flavobacterium aquatile* LMG 4008^T	84.2
Flavobacterium sp. 11		*Flavobacterium aquatile* LMG 4427^T	97.8	*Flavobacterium* sp. 11		*Flavobacterium swingsii* LMG 25510^T	82.9
Flavobacterium sp. 12	100	*Flavobacterium aquatile* LMG 4427^T	97.1	*Flavobacterium* sp. 12	99.9	*Flavobacterium aquatile* LMG 4008^T	84.1–83.7
Flavobacterium sp. 13	99.6–99.4	*Flavobacterium micromati* LMG 21919^T	99.8–99.4	*Flavobacterium* sp. 13	99.9–97.2	*Flavobacterium micromati* LMG 21919^T	99.0–96.9
Flavobacterium sp. 14		*Flavobacterium succinicans* LMG 10402^T	97.7	*Flavobacterium* sp. 14		*Flavobacterium swingsii* LMG 25510^T	84.1
Flavobacterium sp. 15	100	*Flavobacterium succinicans* LMG 10402^T	97.2	*Flavobacterium* sp. 15	100	*Flavobacterium micromati* LMG 21919^T	88.5

Antarctic *Flavobacterium* groups for which no within-group similarity is listed consist of one strain.

type strains of the *Flavobacterium* species investigated in this study, the interspecies *gyrB* sequence similarity values varied from 79.1% between *F. aquatile* and *Flavobacterium reichenbachii* to 94.9% between *F. xanthum* and *F. omnivorum*.

The phylogenetic trees based on the *gyrB* sequences (Figs 2 and S2) show that the groups found in the 16S rRNA gene dendrogram (Figs 1 and S1) were confirmed. The Antarctic *Flavobacterium* groups generally showed lower *gyrB* gene sequence similarity to neighbouring groups and species, which confirmed their status as potentially new species. *Flavobacterium* sp. 13 and sp. 5, which, in the 16S rRNA gene phylogeny, were closely related to *F. micromati* and *F. gelidilacus*, respectively, also group with these species in the *gyrB* phylogeny. Both groupings are well supported; however, the *gyrB* similarity of *Flavobacterium* sp. 13 to *F. micromati* LMG 21919 (97.0%) is higher than that of *Flavobacterium* sp. 5 to *F. gelidilacus* LMG 21477 (91.9%). *Flavobacterium* sp. 13 probably belongs to *F. micromati* that was originally isolated from microbial mats in Antarctic lakes (Van Trappen *et al.*, 2004) as were the isolates of *Flavobacterium* sp. 13 (Table 1). *Flavobacterium* sp. 5 probably represents a new species in view of the rather low *gyrB* gene sequence similarity to *F. gelidilacus* in comparison with the higher similarity values obtained between some type strains. Nevertheless, the precise relation to *F. gelidilacus*, another species from Antarctic microbial mats (Van Trappen *et al.*, 2003), remains to be investigated further.

The similarities within the delineated *Flavobacterium* groups are generally very high for the 16S rRNA gene sequences (Table 3). The *gyrB* sequences were mostly also very similar within groups and ranged from 97.2% to 100% (Table 3). In *Flavobacterium* sp. 2, sp. 8 and sp. 13 (Figs 2 and S2) subclusters were observed with 97.2–99.0% sequence similarity. In other genera, comparable high intraspecies *gyrB* gene sequence similarities were observed, for example 98.5–100% *gyrB* gene sequence similarity within the genus *Streptomyces* (*Actinobacteria*) (Hatano *et al.*, 2003), 97.4–100% within the genus *Aeromonas* (*Gammaproteobacteria*) (Yanez *et al.*, 2003), 95.0–100% within the genus *Bacillus* (*Firmicutes*) (Wang *et al.*, 2007) and 94.6–100% within the genus *Helicobacter* (*Epsilonproteobacteria*) (Hannula & Hanninen, 2007).

It should be noted that all *Flavobacterium* groups studied here comprised several rep-types (Peeters *et al.*, submitted) and the strains were chosen to represent this diversity. The topologies of the neighbour-joining and the maximum likelihood dendrogram were slightly different for the 16S rRNA gene compared with the *gyrB* gene (Figs 1, 2, S1 and S2), as has also been observed for other groups (Yamamoto & Harayama, 1996). However, overall, the phylogenies of the 16S rRNA (Figs 1 and S1) and *gyrB* (Figs 2 and S2) gene were similar and confirmed the division of the Antarctic strains into 15 groups, one probably belonging to *F. micromati* and one close to *F. gelidilacus*. The other 13*Flavobacterium* groups formed separate groups in both the 16S rRNA gene and the *gyrB* gene phylogeny and probably represent new species. However, additional characterization is necessary to confirm this and to describe them as new species.

In conclusion, this study showed that within the genus *Flavobacterium*, the *gyrB* gene has a higher discriminatory power than the 16S rRNA gene. In comparison with the 16S rRNA gene sequence, the sequence similarities for the *gyrB* gene between the delineated groups are significantly lower whereas within the different groups they are still very high. Although there are differences in topology in the dendrograms based on either gene, the same groups of Antarctic *Flavobacterium* strains were recovered. Thus, the *gyrB* gene is a promising molecular marker to elucidate the phylogenetic relationships among *Flavobacterium* species and should be evaluated for all the other *Flavobacterium* species described. The phylogeny of both the 16S rRNA gene and the *gyrB* gene showed that the Antarctic *Flavobacterium* isolates studied here represent at least 13 potentially new species. These will be studied in more detail using various methods to confirm this and describe these groups appropriately.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Phylogenetic tree calculated using the maximum likelihood method based on the 16S rRNA gene sequences of the *Flavobacterium* strains and closely related species.
Fig. S2. Phylogenetic tree calculated using the maximum likelihood method based on the *gyrB* gene sequences of the *Flavobacterium* strains and closely related species.

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