Flavobacterium petrolei sp. nov., a novel psychrophilic, diesel-degrading bacterium isolated from oil-contaminated Arctic soil

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This study presents taxonomic description of two novel diesel-degrading, psychrophilic strains: Kopri-42T and Kopri-43, isolated during screening of oil-degrading psychrotrophs from oil-contaminated Arctic soil. A preliminary 16S rRNA gene sequence and phylogenetic tree analysis indicated that these Arctic strains belonged to the genus Flavobacterium, with the nearest relative being Flavobacterium psychrolimnae LMG 22018T (98.9% sequence similarity). The pairwise 16S rRNA gene sequence identity between strains Kopri-42T and Kopri-43 was 99.7%. The DNA-DNA hybridization value between strain Kopri-42T and Kopri-43 was 88.6 ± 2.1% indicating that Kopri-42T and Kopri-43 represents two strains of the same genomospecies. The average nucleotide identity and in silico DNA-DNA hybridization values between strain Kopri-42T and nearest relative F. psychrolimnae LMG 22018T were 92.4% and 47.9%, respectively. These values support the authenticity of the novel species and confirmed the strain Kopri-42T belonged to the genus Flavobacterium as a new member. The morphological, physiological, biochemical and chemotaxonomic data also distinguished strain Kopri-42T from its closest phylogenetic neighbors. Based on the polyphasic data, strains Kopri-42T and Kopri-43 represents a single novel species of the genus Flavobacterium, for which the name Flavobacterium petrolei sp. nov. is proposed. The type strain is Kopri-42T (=KEMB 9005-710T = KACC 19625T = NBRC 113374T).

Anthropological activities have increased the level of oil-based contaminants in Arctic and Antarctica regions1. Psychrophilic bacteria have been implemented to remediate these hazardous oil-based pollutants from coldest regions of the Earth. Native psychrotrophs with degradation capabilities can be employed for xenobiotic transformation and bioremediation of petroleum hydrocarbons from Polar regions2. As a remediating agent, several strains of the genus Flavobacterium have been also used in the field of environmental bioremediation3–5.

The genus Flavobacterium belonging to the family Flavobacteriaceae of the phylum Bacteroidetes was first established by Bergey et al.6 and later its emended description was provided by Bernardet et al.7. During manuscript preparation, this genus accommodates 208 species with valid names (http://www.bacterio.net/flavobacterium.html). The members of the genus Flavobacterium comprises diverse habitat and have been isolated from compost materials, diseased fish, algal mat, marine environments, rhizospheric niches, toxic circumstances (including petroleum products-contaminated soil), and psychrophilic regions (glacier, Antarctic lake, Tibetan Plateau, and Arctic soil)8–19. During the search of psychrophilic degraders of petroleum products, strains Kopri-42T and Kopri-43 were isolated from oil-contaminated Arctic soil. This study presents detail taxonomic investigation of strains Kopri-42T and Kopri-43 and briefly illustrated their diesel-degrading capacity. On the basis of polyphasic taxonomic results, both the strains are proposed as novel members of the genus Flavobacterium, with Kopri-42T as the type strain.

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Results and Discussion

The nearly complete length of 16S rRNA gene sequences of the strains Kopri-42 T and Kopri-43 were 1,444 and 1,438 bps, respectively. The comparative analysis of 16S rRNA gene sequence using the EZBioCloud server revealed that the strains Kopri-42 T and Kopri-43 belong to the genus *Flavobacterium* and shared highest sequence similarity with *F. psychrolimnae* LMG 22018 T (98.9% and 99.3%, respectively). The pairwise 16S rRNA gene sequence identity between strains Kopri-42 T and Kopri-43 was found to be 99.7%. The high 16S rRNA gene sequence similarity between strains Kopri-42 T and Kopri-43 revealed that both the strains could be considered as a single novel species. In addition, DNA-DNA hybridization (DDH) values between strain Kopri-42 T and Kopri-43 was 88.6 ± 2.1% (reciprocal, 83.9 ± 1.5%), indicating that Kopri-42 T and Kopri-43 represents two strains of the same genomospecies. In addition, the DNA fingerprinting obtained by REP-PCR showed several similar bands between Kopri-42 T and Kopri-43 but differ with reference strains (see Fig. S1 in the supplementary material), suggesting that these two strains represent the same species. Based on the 16S rRNA gene sequence similarity, the other closest neighbors of the type strain Kopri-42 T were *F. limicola* DSM 15094 T (98.3%, sequence similarity); *F. tiangeerense* 0563 T (97.8%, sequence similarity); and *F. sinopsychrotolerans* 0533 T (97.7%, sequence similarity). The 16S rRNA gene sequence identities between the type strain Kopri-42 T and the closest phylogenetic relatives were in the range of 98.9–97.7%, which were below the threshold value of 98.7–99.0% used for species demarcation of prokaryotes. These 16S rRNA gene sequence similarities values along with average nucleotide identity and in silico DNA-DNA hybridization (described below) suggested to allocate strain Kopri-42 T as a new species of the genus *Flavobacterium*. Furthermore, the phylogenetic trees analyses obtained from 16S rRNA gene sequence alignments by maximum-likelihood (Fig. 1); neighbor-joining (Fig. S2); and maximum-parsimony (Fig. S3) also showed that strains Kopri-42 T and Kopri-43 were grouped within the genus *Flavobacterium* and formed a cluster which consists cold-adaptive *Flavobacterium* species.

The whole genome sequence of strain Kopri-42 T contains 3738413 bp (GenBank accession number: QNVY00000000). The full genome was assembled in 22 contigs with an N50 of 693296 bp and genome coverage of 609.5x. The genome-based similarity calculated based on OrthoANIu between strain Kopri-42 T and closest reference strain *F. psychrolimnae* LMG 22018 T was 92.4%. The average nucleotide identity (ANI) values with other reference strains were below 90.0% (Table 1). These values were below the threshold ANI value of
95.0–96.0% used for delineating prokaryotic species\textsuperscript{19}, suggesting strain Kopri-42\textsuperscript{T} is a novel strain of the genus Flavobacterium. In addition, \textit{in silico} DNA-DNA hybridization (DDH) value between strain Kopri-42\textsuperscript{T} and \textit{F}. \textit{psychrolimnae} LMG 22018\textsuperscript{T} was 47.9%. The \textit{in silico} DDH values with other reference strains were below 40.0% (Table 2). These values were significantly below the cut-off value of 70%, which clearly indicated that strain Kopri-42\textsuperscript{T} differs genetically from other type strains of genus Flavobacterium at the species level\textsuperscript{16,20}. The G+C content of the chromosomal DNA for strains Kopri-42\textsuperscript{T} and Kopri-43 were 34.8 and 35.1 mol %, which is in agreement with the range of 32.0–38.0 mol % for the genus Flavobacterium\textsuperscript{7,13}. Both strains were Gram-stain-negative, non-motile, and aerobic. The photomicrographs obtained from transmission electron microscopy revealed that cells of strains Kopri-42\textsuperscript{T} and Kopri-43 were rod-shaped without flagella (Fig. 2). The colonies of both strains on R2A agar were yellow, 1.0–2.0 mm in diameter, circular, convex, translucent, and glistening with entire margins. Strains Kopri-42\textsuperscript{T} and Kopri-43 grew well on R2A, TSA, and

Table 1. The average nucleotide identity (ANI) and \textit{in silico} DNA-DNA hybridization (DDH) values between strain Kopri-42\textsuperscript{T} and closely related reference strains.

| Characteristic | 1            | 2            | 3            | 4            | 5            | 6            |
|----------------|--------------|--------------|--------------|--------------|--------------|--------------|
| Growth at 0°C/30°C | +/-          | +/-          | -/+          | +/-          | -/+          | -/+          |
| Highest salt tolerance (%, w/v) | 1.0          | 1.0          | 1.5          | 1.5          | 1.5          | 0.5          |
| pH range       | 6.0–10.5     | 6.0–10.5     | 6.5–11.0     | 5.5–10.5     | 6.5–10.0     | 6.5–9.5      |
| Catalase/oxidase | +/-          | +/-          | +/-          | +/-          | +/-          | +/-          | w/+          |
| Hydrolysis of Casein | -            | -            | +            | +            | +            | +            |
| Tween 40/60/80 | -/-/-/-     | -/-/-/-      | +/-/+/-/+    | +/-/-/+/-/+  | +/-/-/+/-/+  | +/-/-/+/-/+  |
| Starch         | -            | -            | -            | -            | -            | +            |
| DNA            | -            | -            | +            | +            | +            | -            |
| Enzyme activity |              |              |              |              |              |              |
| Esterase (C4)  | w            | w            | w            | +            | +            | +            |
| Lipase (C 14)  | -            | -            | w            | w            | w            | w            |
| Leucine arylamidase | +          | w            | +            | +            | +            | w            |
| Cystine arylamidase | w          | +            | +            | +            | +            | w            |
| Trypsin        | w            | w            | w            | w            | w            | w            |
| α-chymotrypsin | w            | w            | w            | w            | w            | w            |
| Acid phosphatase | +            | +            | +            | +            | +            | w            |
| Naphthol-AS-BI-phosphohydrolase | -          | -            | w            | w            | -            | -            |
| α-glucosidase  | +            | +            | w            | +            | w            |
| β-glucosidase  | +            | +            | w            | -            | -            | -            |
| N-acetyl-β-glucosaminidase | +      | +            | +            | +            | +            | -            |
| Assimilation from (API 20NE and API ID 32 GN) |              |              |              |              |              |              |
| d-Mannose      | +            | +            | +            | -            | -            | +            |
| Glycogen       | +            | +            | +            | +            | +            | -            |
| Salcin         | +            | w            | -            | -            | -            | -            |
| l-Proline      | +            | +            | -            | w            | +            | -            |
| DNA G + C content (mol %) | 34.8 (34.2)\textsuperscript{a} | 35.1          | 33.8–34.5\textsuperscript{a} | 35.0\textsuperscript{a} | 32.5\textsuperscript{a} | 34.8\textsuperscript{a} |

Table 2. Differentiating characteristics of strain Kopri-42\textsuperscript{T}, Kopri-43, and related species of the genus \textit{Flavobacterium}. Strains: 1, Kopri-42\textsuperscript{T}; 2, Kopri-43; 3, \textit{F}. \textit{psychrolimnae} KACC 11737\textsuperscript{T}; 4, \textit{F}. \textit{limicola} KACC 11965\textsuperscript{T}; 5, \textit{F}. \textit{sinopsychrotolerans} JCM 16398\textsuperscript{T}; 6, \textit{F}. \textit{tiangeerense} JCM 15087\textsuperscript{T}. All data presented in the table are generated from this study, unless marked. \textsuperscript{a}Data from Trappen et al.\textsuperscript{13}; \textsuperscript{b}Data from Tamaki et al.\textsuperscript{5}; \textsuperscript{c}Data from Xu et al.\textsuperscript{15}; \textsuperscript{d}Data from Xin et al.\textsuperscript{14}; \#value determined from genome sequence. +/-, positive; --, negative; w, weakly positive.

95.0–96.0% used for delineating prokaryotic species\textsuperscript{19}, suggesting strain Kopri-42\textsuperscript{T} is a novel strain of the genus \textit{Flavobacterium}. In addition, \textit{in silico} DNA-DNA hybridization (DDH) value between strain Kopri-42\textsuperscript{T} and \textit{F}. \textit{psychrolimnae} LMG 22018\textsuperscript{T} was 47.9%. The \textit{in silico} DDH values with other reference strains were below 40.0% (Table 2). These values were significantly below the cut-off value of 70%, which clearly indicated that strain Kopri-42\textsuperscript{T} differs genetically from other type strains of genus \textit{Flavobacterium} at the species level\textsuperscript{16,20}. The G+C content of the chromosomal DNA for strains Kopri-42\textsuperscript{T} and Kopri-43 were 34.8 and 35.1 mol %, respectively, which is in agreement with the range of 32.0–38.0 mol % for the genus \textit{Flavobacterium}\textsuperscript{7,13}. Both strains were Gram-stain-negative, non-motile, and aerobic. The photomicrographs obtained from transmission electron microscopy revealed that cells of strains Kopri-42\textsuperscript{T} and Kopri-43 were rod-shaped without flagella (Fig. 2). The colonies of both strains on R2A agar were yellow, 1.0–2.0 mm in diameter, circular, convex, translucent, and glistening with entire margins. Strains Kopri-42\textsuperscript{T} and Kopri-43 grew well on R2A, TSA, and
NA; poorly grew on LB, marine agar, and veal infusion agar; and did not grow on MacConkey agar. Growth was detected at temperatures 0–25 °C, pH 6.0–10.5, and 0–1% (w/v) NaCl concentration (Table 2). The optimum temperature for strain Kopri-42T was 10–15 °C as revealed from the growth curve (Fig. S4). Based on the growth temperature data, both strains are concluded to be psychrophilic bacteria. The presence of higher amount of cellular unsaturated fatty acids may contribute to the cold adaptation of these strains (Table 3). It has been documented that presence of higher proportion of unsaturated fatty acids content in the cell membrane favors bacteria to grow at low temperature21,22. Both strains were positive for catalase test but negative for oxidase test. However, oxidase test was positive for all the reference strains considered in this study. Production of flexirubin-type pigment was absent from both strains. Hydrolyze aesculin and CM-cellulose but cannot hydrolyze Tween (40/60/80), starch, casein, DNA, and tyrosine. Strain Kopri-42T was sensitive to novobiocin, tetracycline, nalidixic acid, rifampicin, cyclohexamide, trimethoprim, sulfamethoxazole and chloramphenicol but resistant to kanamycin, neomycin, gentamycin, penicillin, ampicillin, and streptomycin. Other differentiating properties and phenotypic traits resulted from API ZYM, API 20NE, and API ID 32GN test kits of strains Kopri-42T and Kopri-43 are depicted in the digital protologue table and presented along with other nearest phylogenetic members (Tables 2 and S1).

Both strains comprise menaquinone-6 (MK-6) (Fig. S5) as sole respiratory quinone which is typical to menaquinone system of the genus Flavobacterium. Strains Kopri-42T and Kopri-43 contain phosphatidylethanolamine (PE) as the major polar lipid. The unidentified aminolipids (AL1–AL6) and unidentified lipids (L1–L4) were also detected in minor amount (Fig. S6a,b). The major polar lipid profile of both strains shared similar pattern with F. psychrolimnae KACC 11737T (Fig. S6c). However, some proportional difference exists in the minor polar lipid profile between strains Kopri-42T, Kopri-43 and F. psychrolimnae KACC 11737T (Fig. S6). The major cellular fatty acids present in Kopri-42T were C15:0 3-OH (11.6%), summed feature 3 (C16:1ω7c and/or C16:1ω6c; 10.1%), iso-C15:0 3-OH (9.8%), iso-C16:1ω3 (9.2%), anteiso-C15:0 (8.0%), iso-C15:1 G (5.8%), iso-C16:1, H (5.4%), and iso-C17:0 3-OH (5.3%). The observed patterns of fatty acids are similar to closest neighbors. Despite of the comprehensive similarities, a differentiating pattern exhibited with minor amount of fatty acids. Both strains detected iso-C17:0 which was absent from closest members. C15:0 3-OH was present in strains Kopri-42T and Kopri-43 but not detected from F. psychrolimnae KACC 11737T and F. limicola KACC 11965T. Most of the reference strains had anteiso-C17:0 and C18:1ω7c but absent from strains Kopri-42T and Kopri-43 (Table 3).

Both the strains degraded substantial amount of diesel in liquid media and soil environment. In MSM liquid media, strains Kopri-42T and Kopri-43 degraded 60.0% and 58.3% of diesel oil, respectively (Fig. 3a). Statistical analysis indicates that the rates of diesel degradation by strains Kopri-42T and Kopri-43 were not significantly different (p > 0.05). In diesel contaminated soil, the degradation rate was improved with various treatment conditions. When the diesel contaminated soil was treated with only bacterial strain Kopri-42T, the remediation efficiency was 44.8%. The degradation rate was found to be enhanced (64.8%) when the contaminated soil was treated with Kopri-42T + nutrients + biosurfactants (Figs 3b and S7). Statistical analysis showed that the rates of diesel degradation between various treatment conditions were significantly different (p < 0.05). This result indicates that strain Kopri-42T and Kopri-43 can be used as biological agent for remediating diesel oil from cold environments.

In conclusion, 16S rRNA gene sequence analysis, phylogenetic trees, and chemotaxonomic data indicate both strains clearly belong to the genus Flavobacterium. The differentiating phenotypic properties and chemotaxonomic data presented in Tables 2 and 3 preliminary distinguish the two strains as members of a new species in the genus Flavobacterium. The genome sequence characteristics, OrthoANsu and DDH values, and REP-PCR confirm that both strains represent single novel species. Furthermore, both psychrophilic strains can degrade diesel oil and able to thrive in oil-contaminated cold environments indicating their significance in the bioremediation field. Considering the above mentioned genotypic, phylogenetic, phenotypic, biochemical, and chemotaxonomic data, both strains are concluded to represent a novel species in the genus Flavobacterium, with the proposed name Flavobacterium petrolei sp. nov. The type strain is Kopri-42T (=KEMB 9005-710T = KACC 19625T = NBRC 113374T). The formal proposal of the new species name Flavobacterium petrolei sp. nov. is given in the Table S1 with the TaxonNumber TA00628 (http://imedea.uib-csic.es/dprotologue/edit_entryForm.php?form_id=10891&entry_id=628).
**Materials and Methods**

**Bacterial isolation, growth conditions, and maintenance of strain.** For isolation, soil samples were collected during August and September 2012 from different corners of the base station near the DASAN, Korean Arctic Station, N-9173 Ny-Alesund, Norway (GPS location: 78°55′30.13″N 11°55′20.21″E). The diesel oil used in this study was purchased from petroleum station (GS Caltex, Suwon, South Korea). The composition of mineral salt medium (MSM) used for isolation of oil-degrading bacteria is given in Table S2. An enrichment cultivation technique employing Transwell plate (Corning) was implemented for the isolation of psychrophilic oil-degrading bacteria as described previously9,23. Pure isolates were obtained after multiple streaking bacterial colonies on R2A (MB Cell) agar incubating at 10 °C. All the strains isolated during this study are listed in Table S3. The pure culture of strains Kopri-42T and Kopri-43 grown in R2A agar was temporarily stored at 4 °C and subcultured regularly at the interval of two weeks until the taxonomic study was completed. For long-term storage both strains were stored at −80 °C in R2A broth with 20% (v/v) glycerol and later deposited permanently in culture collection center.

**Genotypic characterization and phylogenetic analysis.** Two strains Kopri-42T (=KEMB 9005-710T = KACC 19625T = NBRC 113374T) and Kopri-43 (=KEMB 9005-7101 = KACC 19626 = NBRC 113411) isolated from Arctic soil were characterized in this study. Genomic DNA from strains Kopri-42T and Kopri-43 was isolated using a DNA isolation kit following the exact protocol provided with the kit (InstaGene Matrix kit, Bio-Rad, USA). The conditions of PCR amplification, primers information, purification of PCR products,

| Fatty acid | 1   | 2   | 3   | 4   | 5   | 6   |
|-----------|-----|-----|-----|-----|-----|-----|
| Saturated |     |     |     |     |     |     |
| C14:0     | 0.5 | 0.6 | 1.0 | 0.5 | 0.8 | 0.6 |
| C16:0     | 1.6 | 1.8 | 0.8 | 2.8 | 0.9 | 1.6 |
| iso-C15:1 G | 5.8 | 5.3 | 9.2 | 2.6 | 8.3 | 3.6 |
| iso-C16:1 H | 5.4 | 5.1 | 2.3 | 9.3 | 5.1 | 6.6 |
| anteiso-C14:0 | —   | —   | 1.5 | —   | —   | —   |
| anteiso-C15:1 A | 1.0 | 0.9 | 2.5 | 0.8 | 0.6 | 1.3 |
| anteiso-C16:0 | 8.0 | 9.3 | 19.0 | 7.4 | 4.9 | 9.1 |
| iso-C15:0 | —   | —   | 1.3 | —   | —   | —   |
| iso-C16:0 | 3.0 | 2.8 | 2.2 | 4.3 | 4.7 | 4.0 |
| iso-C17:0 | 9.8 | 10.1 | 15.4 | 4.3 | 11.8 | 4.8 |
| iso-C18:0 | 5.7 | 5.2 | 4.6 | 11.8 | 4.6 | 6.9 |
| iso-C19:0 | 0.7 | 0.6 | —   | —   | —   | —   |

**Unsaturated**

| C11:0 ω6c | 11.6 | 11.5 | 11.3 | 5.4 | 14.5 | 11.1 |
| C14:1 ω5c | 0.3 | 0.3 | — | 0.3 | 0.5 | 0.4 |
| C15:0 ω7c | 6.1 | 5.8 | 0.6 | 3.5 | 5.9 | 7.4 |
| C16:0 ω9c | 1.0 | 1.0 | 0.2 | 0.5 | 0.5 | 1.2 |
| anteiso-C17:1 ω9c | — | — | 3.4 | 1.1 | — | — |
| C18:1 ω7c | — | — | 1.3 | 0.6 | — | 0.6 |

**Hydroxy**

| iso-C14:2-OH | 0.4 | 0.3 | 0.5 | 0.9 | 0.5 | 0.9 |
| C15:2-OH | 0.4 | 0.4 | 1.9 | 0.7 | 0.3 | 0.7 |
| C16:3-OH | 1.7 | 1.7 | — | — | 1.9 | 1.5 |
| iso-C15:3-OH | 4.7 | 4.5 | 6.1 | 2.9 | 6.8 | 3.9 |
| C16:3-OH | 0.8 | 0.7 | 2.4 | 1.3 | 1.2 | 1.3 |
| iso-C16:3-OH | 9.2 | 9.7 | 2.8 | 12.6 | 7.8 | 10.7 |
| C17:2-OH | 0.9 | 1.0 | 2.1 | 0.8 | 0.3 | 0.7 |
| C17:3-OH | 0.4 | 0.3 | 0.2 | — | 0.2 | 0.5 |
| iso-C17:3-OH | 5.3 | 5.9 | 4.3 | 2.9 | 3.6 | 3.5 |

**Summed features**

| 3 | 10.1 | 10.3 | 8.7 | 19.0 | 10.2 | 12.8 |
| 9 | 4.4 | 4.0 | 2.5 | 2.1 | 5.1 | 2.4 |

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Table 3. Cellular fatty acid profile of strain Kopri-42T, Kopri-43, and related species of the genus *Flavobacterium*. Strains: 1, Kopri-42T; 2, Kopri-43; 3, *F. psychrolimnae* KACC 11737T; 4, *F. limicola* KACC 11965T; 5, *F. sinopsychrotolerans* JCM 16398T; 6, *F. tiangeerense* JCM 15087T. All data presented below in the table are generated from the present study. Values are percentages of total fatty acids. Values with < 0.2% of the total fatty acids are not presented; —, not detected or < 0.2%. *Summed features represent groups of two or three fatty acids that could not be separated using the MIDI system. Summed feature 3 contained C16:1 ω7c and/or C16:1 ω6c, Summed feature 9 contained iso-C17:0 ω9c and/or C16:0 10-methyl.*
and sequencing and analysis of 16S rRNA gene were performed following a previously described protocol\textsuperscript{19,24}. For DNA fingerprinting, repetitive extragenic palindromic (REP) PCR was performed using BOXA1R primer (5′-CTACGGCAAGGCGACGCTGACG-3′) following the conditions as mentioned previously\textsuperscript{25}. The gel-electrophoresis image of PCR product was analyzed and the dendrogram was generated using PyElph 1.4 software by UPGMA method, with a bootstrap of 100. The similarity matrix was computed using Dice coefficient\textsuperscript{26}.

The nearest phylogenetic members were determined by comparing 16S rRNA gene sequences of strains Kopri-42\textsuperscript{T} and Kopri-43 with the sequences available in the GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and EZBioCloud server\textsuperscript{27}. For phylogenetic analysis, multiple alignment with the sequences of closest neighbors was carried out using CLUSTAL X 2.1\textsuperscript{26}. Gaps between the 5′ and 3′ ends of the aligned sequences were deleted utilizing the BioEdit program\textsuperscript{29}. Phylogenetic trees were inferred with the help of software package MEGA6\textsuperscript{30} by using three different algorithms: the neighbor-joining\textsuperscript{31}, maximum-parsimony\textsuperscript{32} and maximum-likelihood\textsuperscript{33}. Kimura two-parameter model\textsuperscript{32} was used to calculate evolutionary distances and the bootstrap values were calculated based on 1000 replicates\textsuperscript{34,35}.

For whole genome sequencing, genomic DNA from strain Kopri-42\textsuperscript{T} was extracted using the commercial kit (Genomic DNA Purification Kit, Wizard, Promega) following the manufacturer’s protocol. The whole genome sequence of strain Kopri-42\textsuperscript{T} was obtained using an Illumina MiSeq sequencer and assembled utilizing an assembly toolkit software SPAdes v3.10.1 at the ChunLab (Seoul, South Korea). The genome sequence was annotated using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP, 2013). The genomic relatedness of the novel strain Kopri-42\textsuperscript{T} with the whole genome shotgun sequences of closely related species of \textit{Flavobacterium} was determined based on the Average Nucleotide Identity using USEARCH software tool (OrthoANIu)\textsuperscript{36}.

In silico DDH was calculated using Genome-to-GenomeDistance Calculator (GGDC 2.1; http://ggdc.dsmz.de/ggdc.php) with recommended BLAST+ alignment and formula 2 (identities/HSP length)\textsuperscript{20}.

The G + C content of the chromosomal DNA for strains Kopri-42\textsuperscript{T} and Kopri-43 was determined by real-time PCR analysis\textsuperscript{37} and also genomic G + C content for the type strain was calculated based on the whole genome sequence data. DNA-DNA hybridization (DDH) was conducted between strain Kopri-42\textsuperscript{T} and Kopri-43 as described previously\textsuperscript{38}. Salmon sperm was considered as the negative control and photobiotin was used as probes to label genomic DNA of strain Kopri-42\textsuperscript{T}. The values of DDH were determined fluorometrically in microplate wells using a 1420 Multilabel Counter (Perkin Elmer). Additionally, for reverse hybridization DNA of strain Kopri-43 was labeled with photobiotin and used as a probe to strain Kopri-42\textsuperscript{T}. All the experiments were performed in triplicate.

**Selection of reference strains.** Based on preliminary 16S rRNA gene sequence and the phylogenetic analysis, \textit{Flavobacterium psychrolimnae} KACC 11737\textsuperscript{T}, \textit{Flavobacterium limicola} KACC 11965\textsuperscript{T}, \textit{Flavobacterium sinopsychrotolerans} JCM 16398\textsuperscript{T}, and \textit{Flavobacterium tiangeerense} JCM 15087\textsuperscript{T} were considered for comparative morphological, physiological, biochemical, and chemotaxonomic studies. In addition to the above type strains, \textit{Flavobacterium piscis} CCUG 60099\textsuperscript{T} and \textit{Flavobacterium xueshanense} sr22\textsuperscript{T} were also selected for genomic relatedness (ANI and in silico DDH) study.

**Morphological, physiological and biochemical characterization.** Cellular morphologies of the strains Kopri-42\textsuperscript{T} and Kopri-43 cultivated on R2A for 5 days at 10°C were observed by transmission electron microscopy (Libra 120 ETEM; Zeiss). Colony morphologies of both strains on R2A agar were determined using a Zoom Stereo Microscope (SZ61; Olympus Japan) after incubation at 10°C for 5 days on TSA. Gram staining type was studied following the protocol described by Doetsch\textsuperscript{39}. Cellular motility, growth ability on various commercial culture media, temperature range, pH range, NaCl tolerance, catalase, and oxidase tests, spore staining, and
DNA degradation assay was determined as illustrated previously. To determine psychrophilic optimum temperature, the growth curve was determined at 4, 10, 15, 20, and 25 °C by measuring growth absorbance at 600 nm using a spectrophotometer (Biochrom Libra 54). Anaerobic growth was assessed after 14 days incubation at 10 °C using an anaerobic jar (BBL, Becton Dickinson) with generating Pouch System (GasPak™ EZ). Indole test and H₂S production were evaluated in SIM medium. MR-VP broth was used to perform the MR-VP test. Hydrolysis of Tween 40, Tween 60, Tween 80, starch, gelatin, aesculin, casein, tyrosine, and CM-cellulose was performed as previously described. The presence of flexirubin-type pigments was analyzed with 20% (w/v) KOH solution. Antibiotic susceptibility test was performed on R2A plates by the paper disc method with the following commercial antibiotics: tetracycline (30 μg), kanamycin (30 μg), rifampicin (10 μg), nalidixic acid (30 μg), novobiocin (30 μg), neomycin (30 μg), streptomycin (10 μg), gentamycin (10 μg), ampicillin (30 μg), penicillin (10 μg), chloramphenicol (100 μg), cycloheximide (30 μg), trimethoprim (30 μg), and sulfamethoxazole (30 μg). API ZYM, API 20NE, and API ID 32GN test kits (bioMérieux) were used to determine other enzymatic, physiological, biochemical, and assimilation properties following the manufacturer's instructions.

### Chemotaxonomic characterization

The respiratory quinone and polar lipids were extracted and analyzed from freeze-dried cells following the method as presented previously. The TLC chromatograms of polar lipids were sprayed with appropriate detection reagents for visualization of various spots as described previously.

### Determination of diesel degradation ability

The preliminary diesel degrading ability of strains Kopri-42 and Kopri-43 was assessed in MSM liquid media containing 3000 ppm diesel oil at 10 °C as described previously. Furthermore, the degrading ability was analyzed in standard soil contaminated with diesel oil. The diesel-contaminated soil was prepared in the lab using sand, kaolin, peat moss, and diesel oil at 600 mg kg⁻¹. All the components were mixed thoroughly and sterilized for 1 month prior to conducting the experiment. The physio-chemical parameters of the prepared soil were studied after stabilization and have been presented in Table S4. The diesel degradation experiment was performed in the plastic vessel (size: 24 cm × 17 cm × 16 cm) each containing 2.5 Kg of prepared soil. Diesel-remediation was evaluated in five different treatment conditions (Table S5). The bacterial inoculums, nutrients, and biosurfactants were delivered to the experimental vessels.

The preliminary diesel degrading ability of strains Kopri-42 and Kopri-43 was determined using GC-FID (HP 6890, Agilent, USA) following the procedure as explained previously. The data of diesel degradation were subjected for statistical analysis to determine mean, standard deviation (SD), and standard error. The p-value between different treatments were calculated using two-way ANOVA in the Microsoft Office Excel 2013. The p-value less than 0.05 was used to conclude significant differences between various treatment conditions.

### Accession numbers

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence of strains Kopri-42 and Kopri-43 are MH019220 and MH019221, respectively. This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession QNVY00000000. The version described in this paper is version QNVY02000000.

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Author Contributions
D.K.C. and D.-U.K. conceived, designed and conducted all the experiments. D.K. involved in sample collection and data interpretation. J.K. coordinated and supervised the study. D.K.C. and D.-U.K. analyzed all the data and wrote the manuscript. All authors read, discussed, edited and approved the final draft of the manuscript.

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