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Taxonomic characteristics of novel *Flavobacterium* sp. B1 from a freshwater pond

Young-Min Bae†

Department of Life Science and Public Health, Changwon University, Changwon, Kyeongnam 51140, Korea

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요 약 : *Flavobacterium* 속(genus)은 *Bacteriodetes* 문(phylum), *Flavobacteriaceae* 과(family)의 대표 속(type genus)으로서, 이 속의 세균들은 황색 색소를 함유하는 그람 음성 간균이다. 이 속 세균들은 자연계의 다양한 환경에서 분리되고 있다. 황색색소를 함유하는 그람음성 간균이 경남 창원시 소재 창원대학교 교내의 연못에서 분리되었고, 이 세균은 균주 B1으로 명명되었다. 균주 B1을 생리학적, 생화학적 및 계통분석학적으로 분석한 결과, *Flavobacterium* 속에 속하는 것으로 결론지어졌다. 이 세균의 16S rRNA 유전자 염기서열을 BLAST로 분석해 본 결과, 다른 어떠한 세균과도 16S rRNA 유전자 염기서열의 상동성이 99.0%를 넘지 않았다. 균주 B1의 주된 지방산은 iso-$C_{15:0}$(19.6%), summed feature 3($C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c, 16.1%), iso-$C_{17:0}$ 3OH(10.2%), iso-$C_{15:0}$ 3OH(8.4%) 및 iso-$C_{15:1}$ G(6.6%)인 것으로 밝혀졌는데, 이는 다른 *Flavobacterium* 종들의 지방산 함량과 확연한 차이가 있는 것을 알 수 있었다. 이 세균의 16S rDNA 염기서열은 genbank에 accession number OP060681로 등록되었다.

Abstract : The genus *Flavobacterium*, type genus of the family *Flavobacteriaceae* and a member of the phylum *Bacteriodetes* includes gram-negative and yellow-pigmented rods. Those bacteria have been isolated from various environments of the earth. A yellow-pigmented, gram-negative rod was isolated from a pond in the campus of the Changwon University, Changwon, Kyeongnam and designated as strain B1. Strain B1 was further analyzed physiologically, biochemically and phylogenetically, and concluded to be a member of genus *Flavobacterium*. BLAST search of the 16S rRNA gene sequence of strain B1 shows homology no higher than 99.0% with those sequences of other bacteria. The major fatty acids of strain B1 are iso-$C_{15:0}$ (19.6%), summed feature 3($C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c, 16.1%), iso-$C_{17:0}$ 3OH(10.2%), iso-$C_{15:0}$ 3OH(8.4%) and iso-$C_{15:1}$ G(6.6%) showing significant differences in fatty acid compositions between strain B1 and the other known *Flavobacterium* species. DNA sequence of 16S rRNA gene of strain B1 was deposited in genbank under accession number OP060681.

Keywords : *bacteria, Flavobacterium, 16S rRNA gene, BLAST, phylogenetic analysis*

†Corresponding author
(E-mail: yominbae@changwon.ac.kr)

1. Introduction

The genus *Flavobacterium*, type genus of the family *Flavobacteriaceae* and a member of the phylum *Bacteriodetes*, is one of the most frequently isolated genera of this phylum[1, 2, 3]. Bacteria of the genus *Flavobacterium* are generally known as psychrophilic or psychrotropic and have been isolated from the arctic and antarctic regions[1]. *Flavobacterium* species have been also isolated from diverse environments such as freshwater[2, 4], seawater[5], soil[6, 7], algae[3], animal feces[8], plant surface[9] and oil-leaked soil[6, 7] from moderate to subtropical regions in addition to cold regions. These show extraordinary adaptation of those bacteria to the wide-range of harsh conditions including extreme temperatures, dryness, salt and hydrocarbons. Králová et al. isolated sevreal *Flavobacterium* strains from the Antarctica and studied their characteristics[1]. They proposed the psychrophilic and psychrotropic adaptation of those bacteria is due to the proteorhodopsins, ice-binding proteins, extracellular polysaccharides, and proteins removing reactive oxygen species or cold-shock proteins[1]. Plant surface is dry environment with occasional flooding. Ability of those bacteria to withstand high osmotic pressure such as high salt conditions or extreme dryness is worth of further research[9]. Although it has not been studied throughly about the influence of *Flavobacterium* bacteria on human life, some of them are known to be pathogenic to fish responsible for considerable economic losses to the aquaculture industry[10, 11, 12].

A *Flavobacterium* strain was isolated in Changwon in this study and physiological, biochemical and phylogenetic analyses were performed. Data obtained from those analyses suggest that the newly isolated strain to be a member of the genus *Flavobacterium*.

2. Experiments

2.1. Chemicals and kits

Nutrient broth(NB) used in this study was prepared with Difco™ Nutrient Broth(BD, Sparks, MD, USA). Nutrient agar diluted by one-half(1/2 NA) was prepared by addition of agar(USB Corporation, Cleveland, OH, USA) to the 1/2-strength NB. Genomic DNA was extracted by the NucleoSpin Microbial DNA Kit(Macherey-Nagel GmbH, Duren, Germany) and plasmid DNA by the NucleoSpin Plasmid EasyPure kit(Macherey-Nagel). *Pfu* DNA polymerase and dNTP mixture used for polymerase chain reaction(PCR) were from Promega Corporation (Madison, WI, USA).

2.2. Collection of bacteria

Water sample used in this study was collected from the pond in front of the dormitory buildings of the Changwon University(35° 14'49"N, 128° 41'16"E), Changwon, Kyeongnam, Korea. Collected sample was carried to the laboratory in a styrofoam box to minimize temperature change. Water sample was serially diluted and 0.1㎖ of the undiluted, 10-fold diluted and 100-fold diluted was plated on a 1/2 NA. Plated agar was incubated at 25℃ until distinct colonies were visible.

2.3. Morphological characterization

A flat, wide-spread and yellow-pigmented colony among the bacterial colonies was picked and named as strain B1. Bacterial cells of strain B1 was stained by the gram staining method and cell shape was observed by an optical microscope at 1,000-times magnification.

2.4. Isolation of genomic DNA

Strain B1 was grown in NB at 25℃ and 180 rpm agitation until sufficient growth was reached for genomic DNA extraction. NucleoSpin Microbial DNA kit(Macherey-

Nagel, Inc., Bethlehem, PA, USA) was used for genomic DNA extraction following the protocols included in the kit.

2.5. Amplification of the 16S rRNA gene

16S rRNA gene was amplified using the extracted genomic DNA as the template for PCR. PCR was performed in 100 μl reaction mixture including DNA template 200 ng, forward primer 2.5 pmol, reverse primer 2.5 pmol, dNTPs mixture(0.2 nM each) and *Pfu* DNA polymerase(6 U). Forward primer was fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') from the 5'-terminal conserved region of the bacterial 16S rRNA gene and reverse primer rP2 (5'-ACGGCTACCTTGTTACGACTT-3') from the 3'-terminal conserved region[13]. Each reaction consists of a cycle of 45 sec at 95℃, 45 sec at 55℃, 2 min at 72℃ and each cycle was repeated 30 times. Reaction mixture was incubated at 95℃ for 2min before starting the first cycle and 72℃ for 5min after the final cycle. *Pfu* DNA polymerase known to have proofreading activity was used for amplification to minimize errors produced during DNA synthesis.

2.6. Cloning of the PCR product

PCR products were separated on a 0.7% agarose gel and purified by the Gel and PCR purification system of the Promega Corporation. Purified PCR product was phosphorylated on its 5' end with the ATP and T4 polynucleotide kinase. Plasmid pBluscript Ⅱ SK- was cleaved by a restriction enzyme, *Sma* Ⅰ and dephosphorylated by a bacterial alkaline phosphatase. Dephosphorylated vector and phosphorylated PCR product was mixed and incubated at 16℃ for ligation. Ligated DNA was used to transform competent *Escherichia coli* DH5α. Transformed *E. coli* cells were plated on a MacConkey agar containing 100 μg/ml ampicillin and incubated at 37℃ overnight. White colonies were picked and grown in a 2X YT liquid medium and plasmid DNA was extracted by the NucleoSpin Plasmid EasyPure kit. Extracted DNA was analyzed by an agarose gel electrophoresis. DNA sequencing of the insert DNA was performed by the Solgent(Daejeon, Korea) after the expected molecular weight of the insert DNA was confirmed.

2.7. Phylogenetic analysis of the 16S rRNA gene sequence

DNA sequence of the PCR product was sent to the genbank and the 16S rRNA gene sequences of the closely related bacteria were downloaded after BLAST search[14]. These sequences were used for multiple alignment by the Molecular Evolutionary Genetics Analysis (MEGA) software (version 11.0)[15]. A neighbor-joining phylogenetic tree was built by the Tamura-Nei model[15]. Percentage support values were obtained using a bootstrap procedure with 1,000 replications[15].

2.8. Physiological and biochemical characterization

To determine temperature range for growth of strain B1, bacterial cells were grown under agitation at 180 rpm in a 5㎖ NB at 1℃ intervals. Salt tolerance was measured by growing the cells in a 5㎖ NB at 25℃, 180 rpm at 0.1% intervals of NaCl concentration. Growth of cells lasted for one to five days and the optical density at 600nm was used to measure growth. Biochemical tests and carbon source utilization test was performed with the API 20NE and API ZYM strips(biomerieux) according to the manufacture's instructions. Composition of the cellular fatty acids was analyzed using the standard MIDI protocols by the AceEMzyme(Ansung, Kyeongkido, Korea).

3. Results and Discussion

3.1. Morphological, physiological and biochemical characteristics

Colonies of the strain B1 was smooth, flat and bright yellow on a 1/2 NA after 3-day

incubation at 25℃. Cells were gram-negative rods under microscopic observation. This strain grew at 2℃ to 34℃, but not at 1℃ or 35℃ (Table 1). However, *F. flabelliforme* isolated from antarctica grew at 1℃ and *F. jocheonensis* isolated from Jeju island of Korea grew at 35℃ indicating that temperature range for growth is dependent on each species in the *Flavobacterium* genus[1,3]. *F. jocheonensis* grew at NaCl concentration range of 0.5-5.0% indicating that *F. jocheonensis* is extremely salt-tolerant[3]. The upper limit of salt concentration for growth of the rest of *Flavobacterium* species is 3.0%[5]. Since biochemical or physiological data of the phylogenetically closest strains, *Flavobacterium* WB3.2-27 or *Flovobacterium* KD2009-28 were not available, I chose a strain from each of the Arctic and Antarctic, North Han River and East China Sea for biochemical and physiological comparisons(Table 1). Biochemical analyses show that there is no specific pattern for assimilation of carbon sources or enzyme activities indicating that genus *Flavobacterium* is a very diverse group physiologically or biochemically(Table 1). Since horizontal genetic exchange among bacteria is quite common, that result is not surprising. Strain B1 was able to grow at salt concentrations of 1.9% or below, but not at 2.0%(Table 1). It also reduced nitrate to nitrite, but not to nitrogen. It was able to assimilate glucose, arabinose, mannose, N-acetyl-glucosamine and maltose (Table 1). Activities of acid phosphatase, alkaline phosphatase, cystine arylamidase, esterase, esterase lipase, leucine arllamidase, N-acetyl-β-glucosaminidase, naphthol-AS-BI-phosphohydrolase, oxidase, protease, valine arylamidase, α-chymotrypsin, α-glucosidase, β-galactosidase and β-glucosidase were detected(Table 1).

The major fatty acids were iso-$C_{15:0}$(19.6%), summed feature 3($C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c, 16.1%), iso-$C_{17:0}$ 3OH(10.2%), iso-$C_{15:0}$ 3OH(8.4%) and iso-$C_{15:1}$ G(6.6%) (Table 2). The other related *Flavobacterium* species with some exceptions contained branched fatty acids iso-$C_{15:0}$ at the highest proportion(Table 2). Summed feature 3, $C_{15:1}$ ω6c, iso-$C_{15:0}$ 3OH, iso-$C_{15:1}$ G, iso-$C_{16:0}$ 3OH, iso-$C_{17:0}$ 3OH, anteiso-$C_{15:0}$ are also present at high percentages though the orders are variable depending on each species(Table 2).

Most bacterial species from the environment maintain viability under wide range of storage conditions and can be stored at 4℃ for several months on a solid medium. Strain B1, however, loses viability in the same conditions shortly after storage begins suggesting that this bacterium is not very hardy to the environmental changes in laboratories(data not shown). But, surprisingly, the members of *Flavobacterium* are isolated from various niches including extremely harsh conditions for bacteria to survive.

3.2. Phylogenetic analyses

DNA sequence of the 16S rRNA gene of strain B1 was determined. Total length of the obtained DNA sequence is 1,476-bp. *Flavobacterium* sp. WB3.2-27 strain WB3.2-27 appeared to be the most closely related with strain B1 with similarity of 98.6% after BLAST search of the 16S rDNA sequence. Phylogenetic analysis of the 16S rDNA sequence of strain B1 by the MEGA software(version 11.0) with those of closely related *Flavobacterium* species indicates *Flavobacterium* sp. KD2009-28 and *Flavobacterium* sp. WB3.2-27 are the most closely related species(Figure 1). This is unexpected because *Flavobacterium* sp. KD2009-28 was isolated in Greece and *Flavobacterium* sp. WB3.2-27 in Germany. Tens of *Flavobacterium* species have been isolated in Korea and their 16S rDNA sequences have been deposited in genbank but none of them show significant homology with that of strain B1 after BLAST search.

3.3. Taxonomic conclusion

Phylogenetic, biochemical and physiological

Table 1. Physiological characters of the strain B1. Symbols + mean positive and − negative. Strains : 1, strain B1 ; 2, *F. geliluteum* P7388 ; 3, *F. aquariorum* IMCC34762 ; 4, *F. petrolei* Kopi-42 ; 5, *F. zhairuonensis* A5.7. Data not available for the blank spaces

Chracteristics	1	2	3	4	5
Yellow pigment	+	+	+	+	+
Temperature range for growth(℃)	2-34	15-30	10-30	0-25	15-35
NaCl tolerance(%)	0-1.9	<1	0-1	0-1	0-2
Nitrate reduction	+	−	−		−
Assimilation of:					
L-Arabinose	+	+			
D-Mannose	+	+		+	
N-Acetyl-glucosamine	+	+			
Malic acid	−				−
Activities of					
Acid phosphatase	+			+	−
Alkaline phosphotase	+				−
Arginine dihydrolase	−				−
Cystine arylamidase	+		−	+	−
Esterase	+		+	+	−
Leucine arylamidase	+			+	
Lipase	−			−	−
N-Acetyl-β-glucosaminidase	+	−	−	+	
Naphthol-AS-BI-phosphohydrolase	+	+		−	
Oxidase	+			−	+
Trypsin	−		−	+	
Urease	−				+
Valine arylamidase	+	−			
α-Chymotrypsin	+		−	+	+
α-Fucosidase	−				+
α-Galactosidase	−		−		−
α-Glucosidase	+	−		+	
β-Galactosidase	+	−	+		−
β-Glucosidase(esculin hydrolysis)	+	+	+	+	−
β-Glucuronidase	−		+		−

Table 2. Composition of the cellular fatty acids of strain B1. Values are percentages of total fatty acids. *Summed features represent groups of two fatty acids that are not separated by the MIDI system. Summed feature 2 contains $C_{14:0}$ 3OH and/or iso-$C_{16:1}$ I, feature 3 $C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c, feature 9 iso-$C_{17:1}$ ω6c/$C_{16:0}$ 10-methyl. Strains : 1, strain B1 ; 2, *F. geliluteum* P7388 ; 3, *F. aquariorum* IMCC34762 ; 4, *F, petrolei* Kopi-42 ; 5, *F. zhairuonensis* A5.7. Abbreviations: ND, not detected; TR, trace amount. Data not available for the blank spaces

Fatty acid	1	2	3	4	5
Saturated					
$C_{13:0}$	0.1				
$C_{14:0}$	0.9			0.5	TR
$C_{15:0}$	4.0				ND
Unsaturated					
$C_{15:1}$ ω6c	4.1	7.4	7.4	11.6	7.8
$C_{16:1}$ ω5c	0.3			0.3	
$C_{17:1}$ ω6c	3.3	4.5	4.6	6.1	4.9
$C_{17:1}$ ω8c	0.9	TR	3.7	1.0	2.4
Branched					
iso-$C_{13:0}$	0.3		3.4		
iso-$C_{14:0}$	0.7	1.0	4.9	3.0	
iso-$C_{15:0}$	19.6	24.8	15.0	9.8	33.8
iso-$C_{15:0}$ 3OH	8.4	9.1	3.2	4.7	6.3
iso-$C_{15:1}$ G	6.6	6.0	9.0	5.8	5.9
iso-$C_{16:0}$	2.0	1.8	5.3	5.7	1.3
iso-$C_{16:0}$ 3OH	4.1	9.1	1.5	9.2	1.4
iso-$C_{16:1}$ H	0.5	1.7	2.6	5.4	
iso-$C_{17:0}$	0.2			0.7	
iso-$C_{17:0}$ 3OH	10.2	8.3	3.6	5.3	9.6
anteiso-$C_{15:0}$	6.3	6.4	4.0	8.0	2.1
anteiso-$C_{15:1}$ A	0.7	TR		1.0	
Hydroxylated					
$C_{15:0}$ 2OH	0.8	TR	1.7	0.4	
$C_{15:0}$ 3OH	1.7	ND	1.7	1.7	ND
$C_{16:0}$ 3OH	4.1	1.5	0.9	0.8	TR
$C_{17:0}$ 2OH	0.9	TR		0.9	
$C_{17:0}$ 3OH	0.8			0.4	
Summed features*					
2	0.7				
3	16.1	9.6	17.3	10.1	6.6
9	1.8	5.3	3.3	4.4	8.2

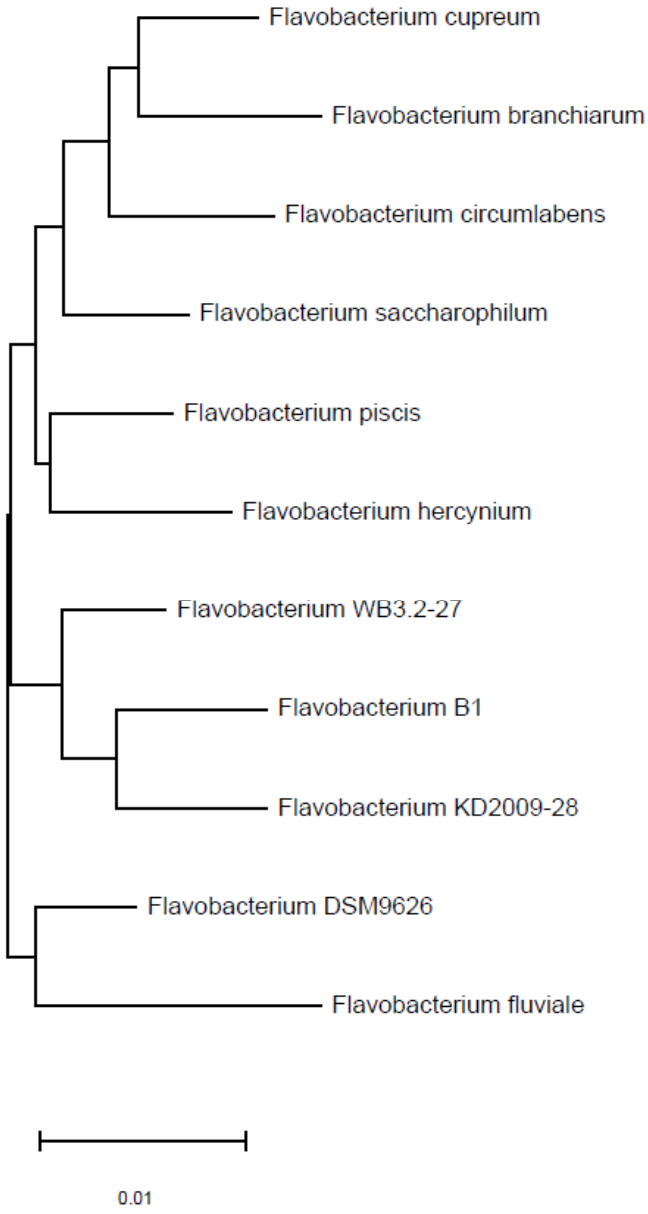

Fig. 1. Phylogenetic tree based on 16S rDNA sequences showing the phylogenetic position of the *Flavobacterium* strain B1 among its closely related species within the genus *Flavobacterium*. Scale bar denoted 0.01 represents 0.01 substitutions per nucleotide position.

characteristics of the strain B1 suggested that this bacterium is a member of the genus *Flavobacterium*. It has been suggested that a bacterial species whose 16S rRNA gene sequence shares less than 99.0% similarity with any other known bacteria should be considered a new species[16, 17, 18]. The stain B1, therefore, might be proposed a new species if further analyses have been done. DNA sequence of the 16S rRNA gene of *Flavobacterium* sp. B1 is available at the genbank under accession number OP060681.

4. Conclusion

A bacterial strain was isolated from a freshwater pond, and this bacterium forms smooth, flat and yellow colonies on a 1/2 nutrient agar after 3-day incubation at 25℃. This strain was designated strain B1 and cells of strain B1 were observed as gram-negative rods under optical microscopy. This bacterium grew at 2℃ to 34℃, but not at 1℃ or 35℃. It was able to grow at salt concentration of 1.9% or below, but not at 2.0%. It also reduced nitrate to nitrite, but not to nitrogen. It was also able to assimilate glucose, arabinose, mannose, N-acetyl-glucosamine and maltose. Activities of acid phosphatase, alkaline phosphatase, cystine arylamidase, esterase, esterase lipase, leucine arllamidase, N-acetyl-β-glucosaminidase, Naphthol-AS-BI-phosphohydrolase, oxidase, protease, valine arylamidase, α-chymotrypsin, α-glucosidase, β-galactosidase and β-glucosidase were detectable.

The major fatty acids were iso-$C_{15:0}$(19.6%), summed feature 3($C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c, 16.1%), iso-$C_{17:0}$ 3OH(10.2%), iso-$C_{15:0}$ 3OH(8.4%) and iso-$C_{15:1}$ G(6.6%).

The 16S rRNA gene sequences of hundreds of *Flavobacterium* species were deposited in genbank, but none of them showed homology with that of strain B1 at or higher than 99.0%. The strain B1 is, therefore, qualified to be a new species.

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