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Taxonomic characterization of novel *Hymenobacter* sp. B2 isolated from a freshwater environment

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민물환경에서 분리된 novel Hymenobacter sp. B2의 분류학적 특성연구

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요 약 : *Hymenobacter* 속(genus)은 *Bacteroidota* 문(phylum), *Hymenobacteraceae* 과(family)의 대표 속(type genus)이다. 이 속에 속하는 세균들은 붉은색 색소를 함유하는 그람 음성 간균으로서, 자연계의 다양한 환경에서 분리되고 있다. 본 연구에서 붉은색 색소를 함유하는 그람 음성 간균이 경남 창원시 소재 창원대학교 교내의 연못에서 분리되었고, 이 세균은 균주 B2로 명명되었다. 균주 B2를 계통분석 및 생화학적으로 분석한 결과, *Hymenobacter* 속에 속하는 것으로 밝혀졌다. 이 세균의 16S rRNA 유전자 염기서열을 genbank의 BLAST로 분석해 본 결과, 다른 어떠한 세균과도 16S rRNA 유전자 염기서열의 상동성이 새로운 미생물로 인정되는 기준인 98.7%보다 낮은 것으로 나타났다. 균주 B2의 지방산을 분석해 본 결과, 주된 지방산은 summed feature 3($C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c, 22.8%), iso-$C_{15:0}$(16.2%), anteiso-$C_{15:0}$(12.9%), $C_{16:1}$ω5c(12.4%) 및 summed feature 4 (iso-$C_{17:1}$ I/anteiso-$C_{17:1}$)(9.5%)인 것으로 밝혀졌는데, 결과적으로 균주 B2의 지방산 함량은 다른 *Hymenobacter* 종들의 지방산 함량과 뚜렷한 차이가 있는 것을 알 수 있었다. 이 세균의 16S rRNA 유전자 염기서열은 genbank에 accession number OQ318247로 등록되었다.

주제어 : 박테리아, Hymenobacter, 16S rRNA 유전자, BLAST, 계통발생학적 분석

Abstract : The genus *Hymenobacter*, type genus of the family *Hymenobacteraceae* and a member of the phylum *Bacteroidota* includes gram-negative and red-pigmented rods. Those bacteria have been isolated from various environments of the earth. I isolated a red-pigmented, gram-negative rod from a pond in the campus of the Changwon University, Changwon, Kyeongnam and designated this bacterium as strain B2. Strain B2 was further analyzed phylogenetically and biochemically, and concluded as a member of genus *Hymenobacter*. BLAST search of the 16S rRNA gene sequence of strain B2 showed its homology lower than 98.7% with those sequences of

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the other bacteria whose 16S rRNA gene sequences have been reported. Fatty acid composition of the strain B2 was analyzed and its major fatty acids are summed feature 3($C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c, 22.8%), iso-$C_{15:0}$ (16.2%), anteiso-$C_{15:0}$(12.9%), $C_{16:1}$ ω5c(12.4%) and summed feature 4 (iso-$C_{17:1}$ I/anteiso-$C_{17:1}$)(9.5%) showing significant differences in fatty acid compositions between strain B2 and the other known *Hymenobacter* species. DNA sequence of 16S rRNA gene of strain B2 was deposited in genbank under accession number OQ318247.

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

1. Introduction

Bacteria of the genus, *Hymenobacter*, are Gram-negative, aerobic, non-motile, rod-shaped bacteria, and their colonies are red to pink in colour[1]. Bacteria of the genus *Hymenobacter*, member of the family *Hymenobacteraceae* are generally known as psychrophilic or psychrotropic and have been frequently isolated from the cold areas such as the arctic and antarctic regions[1,2,3]. Red-colored snow is observed occasionally in high-altitude or high-latitude areas and its red color is caused by bloom of cold-adapted phototrophic microorganisms including the *Hymenobacter* species[1]. Kojima *et al.* isolated bacteria from the 'red snow' in Antarctica and analysis of their 16S rRNA gene showed that the isolated bacteria belonged to the genus *Hymenobacter*[1].

Hymenobacter species have been also isolated from various niches other than the Arctic or Antarctic regions such as desert[4,5], deep seawater[6], high-altitude soil[7], mineral ores[8] and processed meat[9] as well as water and soil of moderate regions[10, 11,12]. These show excellent adaptation of the *Hymenobacter* bacteria to the wide-range of harsh conditions including extreme temperatures, dryness, salt and high water pressure. Those bacteria are also known as resistant to ionic and ultraviolet irradiations[5,7]. Ability of those bacteria to withstand high amount of UV or ionic irradiation is worth of further research including the mechanism of resistance.

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

2. Experiments

2.1. Chemicals and kits

Nutrient broth(NB) used in this study was prepared with Difco^{TM} 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), and T4 DNA ligase were purchased from Promega Corporation (Madison, WI, USA). Restriction enzyme *Sma* I, bacterial alkaline phosphatase and T4 polynucleotide kinase were from Takara-Bio Inc., Shiga, Japan.

2.2. Collection of bacteria

Water sample used in this study was obtained from the pond in front of the dormitory buildings of the Changwon University(35° 14'49"N, 128° 41'16"E), Changwon, Kyeongnam, Korea. Obtained

sample was brought to the laboratory in a styrofoam box to keep temperature unchanged. Water sample was serially diluted and 0.1㎖ of the undiluted, 10-fold diluted and 100-fold diluted was plated on an NA. Plated agar was incubated at 25℃ until distinct colonies were observable.

2.3. Morphological characterization

A flat, wide-spread and red to pink-pigmented colony among the bacterial colonies was picked and named as strain B2. Bacterial cells of strain B2 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 B2 was grown in 5㎖ NB at 25℃ and 180 rpm agitation until sufficient growth was observed for genomic DNA extraction. NucleoSpin Microbial DNA kit(Macherey-Nagel, Inc., Bethlehem, PA, USA) was used for genomic DNA extraction following the protocols provided by the manufacturer.

2.5. Amplification of the 16S rRNA gene

16S rRNA gene of the strain B2 was amplified using the extracted genomic DNA as the template for PCR. PCR was performed in 100㎕ volume 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 used was fD1 (5'-AGAGTTTGATCCTGGCT CAG-3') from the 5'-terminal conserved region of the bacterial 16S rRNA gene and reverse primer used rP2 (5'-ACGGCTACCTTG TTACGACTT-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 reaction cycle was repeated 30 times. Reaction mixture was incubated at 95℃ for 2 min before starting the first cycle and 72℃ for 5 min after the final cycle. *Pfu* DNA polymerase is known to have proofreading activity, therefore was used for amplification to minimize errors produced during amplification.

2.6. Cloning of the PCR product

PCR products were analyzed 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 cells. 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]. Obtained DNA sequences were used for multiple alignment(Clustal W) 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 B2, bacterial cells were grown under agitation at 180 rpm in a 5㎖ NB at 1℃ intervals from 2℃ to 34℃. 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 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 B2 was smooth, flat and pink to red on an NA after 3-day incubation at 25℃. Cells were gram-negative rods under microscopic observation. This strain grew at 3℃ to 33℃, but not at 2℃ or 34℃ (Table 1) indicating that strain B2 is relatively psychrophilic or psychrotrophic. It has been reported *Hymenobacter* species were able to grow as low as at 4℃ and *H. actinosclerus* was even reported to grow as high as at 42℃ indicating that temperature range for growth is dependent on each species of the *Hymenobacter* genus[16]. The lower limit for growth at 3℃ of the strain B2 might be the lowest ever reported for this genus. Sun et al. reported that *H. profundi* $M2^T$ and *H. chitinovorans* $Txc1^T$ grew at 3.0% NaCl[6] indicating that those two species are extremely salt-tolerant [6]. Four *Hymenobacter* species closely related to the strain B2 phylogenetically were chose for biochemical and physiological comparisons (Table 1). Biochemical analyses show that the five *Hymenobacter* species compared are quite similar with each in their enzyme activities except trypsin, β-galactosidase and β-glucosidase indicating that genus *Hymenobacter* is a very conserved group physiologically or biochemically(Table 1). Strain B2 was able to grow at salt concentrations of 1.1% or below, but not at 1.2%(Table 1). Activities of acid phosphatase, alkaline phosphatase, cystine arylamidase, esterase, esterase lipase, leucine arylamidase, N-acetyl-β-glucosaminidase, naphthol-AS-BI-

Table 1. Physiological characters of the strain B2 and related *Hymenobacter* species

Chracteristics	1	2	3	4	5
Red pigment	+	+	+	+	+
Temperature range for growth(℃)	3-33	10-30	4-30	10-30	4-37
NaCl tolerance(%)	0-1.1	0-3.0	0-1.0	0-0.5	0-2.0
Activities of enzymes					
Trypsin	+	+	+	-	-
β-Galactosidase	+	-	+	+	-
β-Glucosidase	+	-	+	+	-

Symbols + mean positive and - negative. Strains: 1, strain B2; 2. *H. yonginensis* $HMD1010^T$; 3, *H. perfusus* $A1-12^T$; 4, *H. metallilatus* $9PBR-2^T$; 5, *H. rigui* $WPCB131^T$.

Table 2. Composition of the cellular fatty acids of strain B2 and related *Hymenobacter* species

Fatty acid	1	2	3	4	5
Saturated					
$C_{16:0}$	1.5	2.4	TR	3.3	6.4
Unsaturated					
$C_{16:1}\,\omega 5c$	12.4	21.9	9.7	13.4	15.0
Branched					
iso-$C_{15:0}$	16.2	14.5	19.4	20.3	34.8
iso-$C_{15:0}$ 3OH	2.8	5.3	2.1	2.6	ND
iso-$C_{16:0}$	1.3	1.3	1.1	ND	5.9
iso-$C_{16:1}$ H	1.4	ND	1.3	1.2	ND
iso-$C_{17:0}$	1.7	1.6	3.3	3.1	5.0
iso-$C_{17:0}$ 3OH	4.3	4.3	3.6	4.7	3.1
anteiso-$C_{15:0}$	12.9	3.0	19.8	10.5	5.9
anteiso-$C_{16:0}$	ND	ND	ND	ND	1.9
anteiso-$C_{17:0}$	TR	TR	1.5	ND	ND
Hydroxylated					
$C_{15:0}$ 2OH	0.8	ND	1.0	ND	ND
$C_{16:0}$ 3OH	1.1	1.5	TR	ND	ND
Summed features*					
1	1.9	2.2	1.6	1.2	ND
2	ND	3.2	ND	ND	ND
3	22.8	17.7	11.1	18.5	ND
4	9.5	11.9	12.4	12.2	13.8
5	ND	ND	ND	ND	14.4

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 1 contains iso-$C_{15:1}$ H and/or $C_{13:0}$ 3OH, feature 2 iso-$C_{15:1}$ I/$C_{13:0}$ 3OH, feature 3 $C_{16:1}\ \omega 7c$ and/or $C_{16:1}\ \omega 6c$, feature 4 iso-$C_{17:1}$ I/anteiso-$C_{17:1}$ B, feature 5 anteiso-$C_{17:1}$ B/iso I. Numbers of 1 to 5 represent the strain B2, *H. yonginensis* HMD1010^{T}, *H. perfusus* A1-12^{T}, *H. metallilatus* 9PBR-2^{T}, and *H. rigui* WPCB131^{T}, respectively. Abbreviations: ND, not detected; TR, trace amount(<1.0%). Data not available for the blank spaces.

phosphohydrolase, oxidase, protease, valine arylamidase, α-chymotrypsin, α-glucosidase, β-galactosidase and β-glucosidase were detected(Table 1). Strain B2 was negative for activities of lipase, α-Chymotrypsin, α-fucosidase, α-Galactosidase, β-Glucuronidase and α-Mannosidase.

The major fatty acids were summed feature 3($C_{16:1}\ \omega 7c$ and/or $C_{16:1}\ \omega 6c$, 22.8%), iso-$C_{15:0}$(16.2%), anteiso-$C_{15:0}$(12.9%), $C_{16:1}\,\omega 5c$ (12.4%), summed feature 4(9.5%), iso-$C_{17:0}$ 3OH(4.3%), and iso-$C_{15:0}$ 3OH(2.8%) (Table 2). The proportion of the cellular fatty acids between theother related *Hymenobacter* species shows large differences(Table 2). This phenomenon might be due to the different environment where each species were isolated.

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 B2, 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 *Hymenobacter* are isolated from various niches including extremely harsh conditions for bacteria to survive.

Another interesting feature of the *Hymonobacter* species is their exceptional resistance to UV or ionic irradiations. Lee et al. reported *Hymenobacter aquaticus* survived after exposure to 3 kGy gamma irradiation [17]. It is known that UV irradiation promotes formation of cyclobutane pyrimidine dimers (CPDs) and ionic radiation breakage of the phosphodiester bonds of the DNA molecules. Marizcuerrena et al. cloned the gene of a 50.8 kDa photolyase from the UVC-resistant Antarctic bacterium *Hymenobacter* sp. UV11 [18]. That enzyme was produced by recombinant DNA technology, purified using immobilized metal affinity chromatography and its activity was analyzed using different approaches: detection of cyclobutane pyrimidine dimers by immunochemistry, high-performance liquid chromatography and comet assays using Chinese Hamster Ovary (CHO) and immortalized nontumorigenic human epidermal (HaCat) cells[18]. They concluded that their recombinant protein has the ability to repair the formation of CPDs, on both double- and single-stranded DNA[18, 19]. It is still unclear whether photolyase is the major tool for repair of damages caused by UV irradiation. The fact that those bacteria are also resistant to ionic irradiation suggests they might have highly efficient nucleotide excision repair(NER) in addition to the photolyase or other repair systems. The celluar pigments might also play an important role to protect bacterial cells from the harmfull effect of the irradiations.

3.2. Phylogenetic analyses

DNA sequence of the 16S rRNA gene of strain B2 was determined. Total length of the obtained DNA sequence is 1,476-bp. *Hymenobacter perfusus* A1-12^T appeared to be the most closely related with strain B2 with similarity of 98.36% after BLAST search of the 16S rRNA gene sequence[14]. Phylogenetic analysis of the 16S rRNA gene sequence of strain B2 by the MEGA software(version 11.0) with those of closely related *Hymenobacter* species indicates *Hymenobacter yonginensis* HMD1010^T is the most closely related species ahead of *Hymenobacter perfusus* A1-12(Fig. 1)[15]. Tens of *Hymenobacter* species have been isolated in Korea and their 16S rRNA gene sequences have been deposited in genbank but none of them show significant homology with that of strain B2 after BLAST search.

3.3. Taxonomic conclusion

Phylogenetic, biochemical and physiological characteristics of the strain B2 suggested that this bacterium is a member of the genus *Hymenobacter*. It has been suggested that a bacterial species whose 16S rRNA gene sequence shares less than 98.7% similarity with any other known bacteria should be considered a new species[20, 21, 22]. The stain B2, therefore, might be proposed a new species if further analyses have been done. DNA sequence of the 16S rRNA gene of *Hymenobacter* sp. B2 is available at the genbank under accession number OQ0318247.

4. Conclusion

A bacterial strain was isolated from a freshwater pond, and this bacterium forms smooth, flat and yellow colonies on a nutrient agar after 3-day incubation at 25℃. This strain was designated strain B2 and cells of strain B2 were observed as gram-negative rods under optical microscopy. This bacterium grew

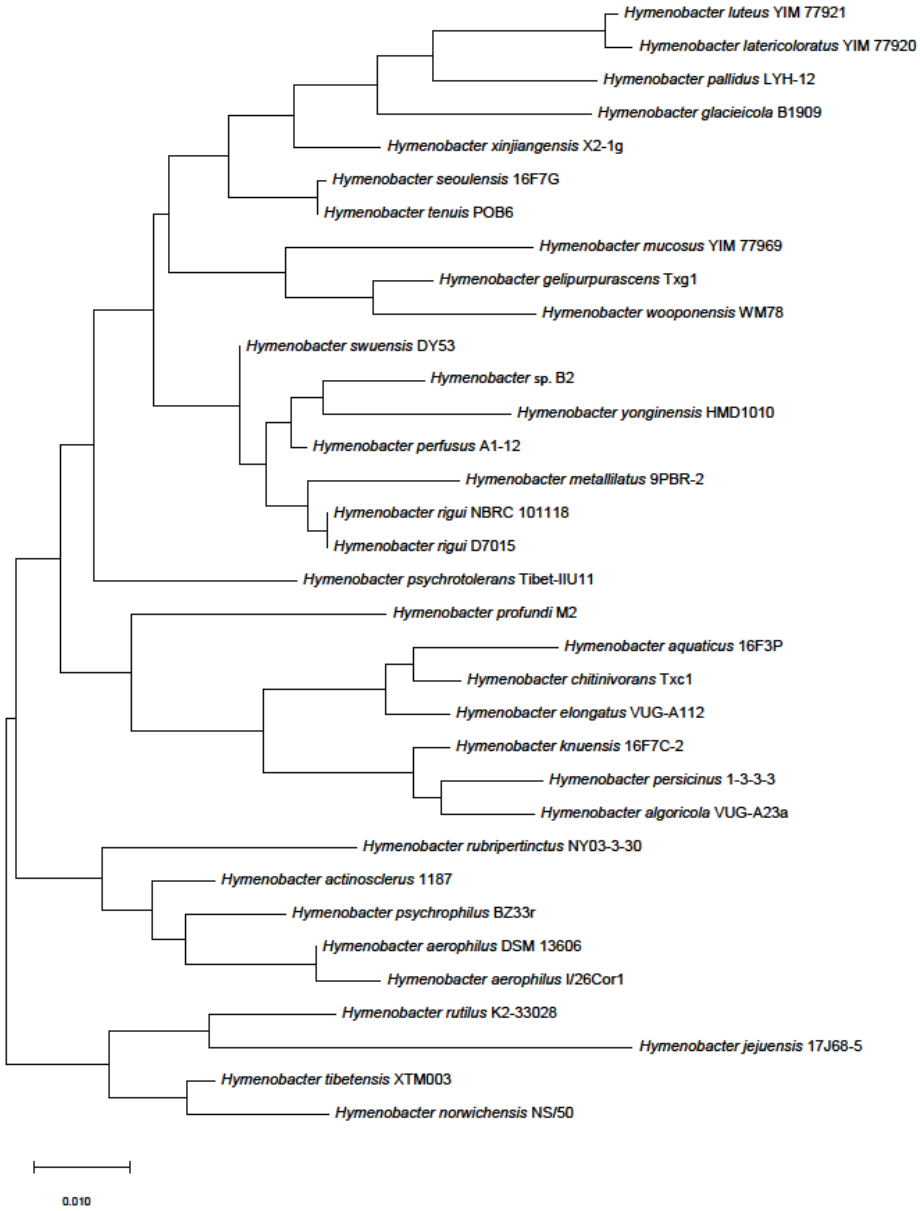

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the phylogenetic position of the *Hymenobacter* sp. strain B2 among its closely related species within the genus *Hymenobacter*. Scale bar denoted 0.010 at the bottom represents 0.01 substitutions per nucleotide position.

at 3℃ to 33℃, but not at 2℃ or 34℃. It was able to grow at salt concentration of 1.1% or below, but not at 1.2%. 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 summed feature 3($C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c, 22.8%), iso-$C_{15:0}$(16.2%), anteiso-$C_{15:0}$(12.9%), $C_{16:1}$ω5c (12.4%), summed feature 4(9.5%), iso-$C_{17:0}$ 3OH(4.3%), and iso-$C_{15:0}$ 3OH(2.8%).

The 16S rRNA gene sequences of hundreds of *Hymenobacter* species were deposited in genbank, but none of them showed homology with that of strain B2 at or higher than 98.7%. The strain B2 is, therefore, qualified to be a new species.

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