Characterization of a novel *Acinetobacter bereziniae* subspecies harboring $bla_{\text{SIM-1}}$ metallo-ß-lactamase gene

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From the heart, I am thankful to my family, and always sorry for nursing my babies instead of me.

<TABLE OF CONTENTS>

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Characterization of novel *Acinetobacter bereziniae* subspecies *seouli* harboring the *bla*$_{SIM-1}$ metallo-ß-lactamase gene

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(Directed by Professor Dongeun Yong)

Acinetobacter species are aerobic, oxidase negative, non-motile, and non-fermentative gram-negative coccobacilli. They are widely distributed in nature and in the hospital environment and are the second most common non-fermenter among human clinical specimens. At present, 15 *Acinetobacter* nomenspecies have been isolated in humans. carbapenems are the last resort for the infection by nearly all *Acinetobacter* spp. isolates. However, carbapenem resistant *Acinetobacter* isolates that produce metallo-β-lactamases (MBLs) or OXA carbapenemases have been increasingly reported worldwide. We isolated an *Acinetobacter* strain YMC79 with the *bla*$_{SIM-1}$ gene, collected from an oral specimen of a patient diagnosed with acute myeloid leukemia, and it was unidentifiable using conventional biochemical tests. Phylogenetic analysis based on each targeted gene sequence, including 16S rRNA, RNA polymerase ß-subunit gene (*rpoB)*, gyrase B (*gyrB*) and 16S-23S rRNA gene intergenespacer (ITS) genes indicated that the strain YMC79 was closely related to *A. bereziniae*, with 98.9%, 96.8%, 91.0%, and 95.9% sequence similarity, respectively. In Matrix-Assisted Laser Desorption/Ionization Time-of-Flight

(MALDI-TOF) analysis, spectrums from the strain YMC79 showed specific signals at 5903.5, 6243.2 and 7176.3 m/z (the mass to charge ratio). Major fatty acids were $C_{18:1}$ w9c (34.1%), summed feature 3 (23.4%), $C_{16:0}$ (23.9%) and DNA G+C content 41.2 mol%. The strain YMC79 were clearly different from *A. bereziniae* and *A. guillouiae* species by phenotypical characteristics including utilization of D-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, D-mannose, D-melibiose, xylitol, D-fucose and malonate. In spite of the overall difference, the DNA–DNA relatedness between the strain YMC79 and *A. bereziniae* KTCT 1268 was 72%. Herein, we report a novel *seouli* subspecies with $bla_{\text{SIM-1}}$ gene and its phenotypical characteristics.

Key words: Novel *Acinetobacter* subspecies, SIM-1, metallo-β-lactamase

Characterization of a novel *Acinetobacter bereziniae* subspecies *seouli* harboring the bla_{SIM-1} metallo-ß-lactamase gene

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I. INTRODUCTION

Acinetobacter species are non-motile, non-fermentative, aerobic, gram-negative bacilli that are widely distributed in nature and in the hospital environment[1]. These organisms are isolated in human specimens. The genus *Acinetobacter* was proposed by Brisou and Prévot in 1954 and the species in this genus were divided into several DNA groups via DNA-DNA hybridization, because it is difficult to differentiate *Acinetobacter* species on the basis of phenotypic characteristics[3]. The dynamic changes of taxonomy in the genus *Acinetobacter* had been observed for decades with improvement of species identification techniques. Recently, the genus *Acinetobacter* was divided into 32 genomic species, including 21 species with validly published names by

continuous updated by nomenclature. For example, *Acinetobacter* genomic species 10 and 11 were given new valid species name as *Acinetobacter bereziniae* and *Acinetobacter guillouiae*, respectively[4]. Novel species have been reported, such as *Acinetobacter soli* isolated from forest soli[5], *Acinetobacter gyllenbergii* and *Acinetobacter beijerinckii*[6]. In addition, some *Acinetobacter* species were renamed as *Acinetobacter venetianus*[7]. From human specimens, 15 nomenspecies (*Acinetobacter baumannii*, *Acinetobacter calcoaceticus*, *Acinetobacter haemolyticus*, *Acinetobacter johnsonii*, *Acinetobacter junii*, *Acinetobacter lwoffii*, *Acinetobacter parvus*, *Acinetobacter radioresistens*, *Acinetobacter schindleri*, *Acinetobacter ursingii*, *Acinetobacter septicus*, *Acinetobacter beijerinckii*, *Acinetobacter gyllenbergii*, *Acinetobacter bereziniae* and *Acinetobacter guillouiae* have been isolated[4,6,8] .

Acinetobacter species are the second most common non-fermenters among nosocomial pathogens, and their distinctive characteristics have made it difficult to prevent their outbreak and clonal spreading in many geographical regions. Among the predominant traits, the first is the varied transmission modes of *Acinetobacter* species. They can survive either on patients and health care workers, or on potential fomites in a hospital environment, such as respiratory-therapy equipment, blood-pressure cuffs and suction waters[9]. The second is the ability to acquire multiple antimicrobial resistant mechanisms, including porin modifications, serine or metallo-β-lactamases (MBL) [1,9]. Therefore, term "multidrug resistance" and "pandrug-resistance" have been added to many *Acinetobacter* species and recently been introduced in many published

reports[10,11].

During the evaluation of *Acinetobacter* species with MBL genes isolated between 2003 and 2008, we found an *Acinetobacter* strain with a bla_{SIM-1} gene that could not be identified using conventional biochemical methods. Herein, we have identified a novel *Acinetobacter bereziniae* subspecies strain with SIM-1 type MBL at a tertiary-care hospital in Seoul, Korea.

II. MATERIALS AND METHODS

1. Collection of microorganism and specimen processing

In the screening of isolates from clinical samples for imipenem-resistant *Acinetobacter* with bla_{SIM-1} gene, the strain YMC79 was isolated from the oral specimen of a 44 year-old male patient diagnosed with acute myeloid leukemia in 2005 at a tertiary-care hospital in Seoul, Korea. The isolate was grown on MacConkey agar after 24h incubation at 37℃.

2. Antimicrobial susceptibility testing

Routine antimicrobial susceptibility testing was performed using the Clinical and Laboratory Standards Institute (CLSI) disk diffusion method[12] and VITEK N132 card (bioMérieux, Marcy l'Etoile, France).

3. Detection of MBL gene

MBL production was screened by using a modified-Hodge test with imipenem disk on MacConkey agar and by using the double-disc synergy test (DDST) with imipenem (10 µg) and EDTA (750 µg) -sodium mercaptoacetic acid (2 mg) on Mueller-Hinton agar[13]. The type of MBL gene, including $bla_{\text{VIM-2}}$, $bla_{\text{IMP-1,}}$ and $bla_{\text{SIM-1}}$ was detected using PCR and sequencing (Table 1)[14].

4. Phenotypic characterization

Biochemical tests using ATB32 GN system, VITEK GNI card (bioMérieux, Marcy l'Etoile, France), the API 20NE and API50CH systems (bioMérieux, Durham, NC, USA) were performed according to manufacturer's instructions. *A. bereziniae* KTCT 12683 and *A. guillouiae* KTCT 12683 were used as control strains (*A. bereziniae* KTCT 12683 = *A. bereziniae* ATCC 17924^{T}, *A. guillouiae* KTCT 12683 = *A. guillouiae* ATCC11171^{T}).

5. Sequence analysis

The PCR and sequence analysis of 16S rRNA, RNA polymerase ß-subunit gene (*rpoB*), gyrase B (*gyrB*) and 16S-23S rRNA gene intergene spacer (ITS) genes were carried out on the strain YMC79. The primers used for gene amplification are listed in table 1.

16S rRNA gene amplification and sequencing were performed using primer set 8F and 1541R (corresponding *E. coli* 16S rRNA positions 8 to 1541)[15].

Amplification of the *rpoB* (corresponding *A. baumanii rpoB* positions

2916 to 3773) was performed using the primers Ac696F and Ac1598R. For sequencing, two additional primers, Ac1093R and Ac1055F, were also used[16].

CLSI recommends that identification of *Acinetobacter* species be confirmed using DNA sequencing of the *gyrB* gene as an alternative target[17]. UP-1E and AprU (nucleotide positions 316 to 1227 of the *Escherichia coli* K-12 sequence) were used for PCR and M13R and M13(-21)[18] were used for sequencing.

ITS amplifications and sequencings were performed using the bacterium-specific universal primers 1512F and 6R[19]. The 5' end of primer 1512F is located at position 1493 of the 16S rRNA gene, and the 5' end of primer 6R is located at position 108 downstream of the 5' end of the 23S rRNA gene of *E. coli*. We secured about 740 bp of ITS sequences for phylogenetic study.

The PCR and sequence analysis of 16S rRNA, ITS and *gyrB* genes were also carried out on *A. bereziniae* KTCT 12683 and *A. guillouiae* KTCT 12683. We were able to re-confirm that the 16S rRNA sequences of *A. bereziniae* KTCT 12683 and *A. guillouiae* KTCT 12683 were identical to those of *A. bereziniae* ATCC 17924 and *A. guillouiae* ATCC11171, respectively. We also compared ITS and *gyrB* gene sequences of *A. bereziniae* KTCT 12683 and *A. guillouiae* KTCT 12683 with the database sequences of genomic species 10 (AY601837; AB008689) and genomic species 11 (AY601838; AB008690), respectively.

Nucleotide sequences were compared with known sequences in the EMBL-EBI (http://www.ebi.ac.uk/Tools/fasta33/nucleotide.html) and EzTaxon server (http://www.eztaxon.org) databases (accessed 15 June 2010).

Table 1. Primers used for PCR amplification and sequencing of the 16S rRNA, *rpoB*, *gyrB* and *ITS* gens and detection of MBL gene

Primer	Use	Nucleotide sequence (5' to 3')	reference
16S rRNA-8F	PCR and Sequencing	AGA GTT TGA TCC TGG CTC AG	15
16S rRNA-1541R	PCR and Sequencing	AAG GAG GTG ATC CAG CCG CA	15
rpoB Ac696F	PCR and Sequencing	TAY CGY AAA GAY TTG AAA GAA G	16
rpoB Ac1598R	PCR and Sequencing	CGB GCR TGC ATY TTG TCR T	16
rpoB Ac1093R	Sequencing	CMA CAC CYT TGT TMC CRT GA	16
rpoB Ac1055F	Sequencing	GTG ATA ARA TGG CBG GTC GT	16
gyrB UP-1E	PCR	CAG GAA ACA GCT ATG ACC AYG SNG GNG GNA ART TYR A	18
gyrB APrU	PCR	TGT AAA ACG ACG GCC AGT GCN GGR TCY TTY TCY TGR CA	18
gyrB M13R	Sequencing	CAG GAA ACA GCT ATG ACC	18
gyrB M13(-21)	Sequencing	TGT AAA ACG ACG GCC AGT	18
ITS-1512F	PCR and Sequencing	GTC GTA ACA AGG TAG CCG TA	19
ITS-6R	PCR and Sequencing	GGG TTY CCC CRT TCR GAA AT	19
$bla_{\text{VIM-1}}$ F	Detection	ATG TTC AAA CTT TTG AGT AAG	14
$bla_{\text{VIM-1}}$ R	Detection	CTA CTC AAC GAC TGA GCG	14
$bla_{\text{IMP-1}}$ F	Detection	CAT GGT TTG GTG GTT CTT GT	14
$bla_{\text{IMP-1}}$ R	Detection	ATA ATT TGG CGG ACT TTG GC	14
$bla_{\text{SIM-1}}$ F	Detection and Sequencing	TAC AAG GGA TTC GGC ATC G	14
$bla_{\text{SIM-1}}$ R	Detection and Sequencing	TAA TGG CCT GTT CCC ATG TG	14

6. Phylogenetic analysis

Nucleotide sequence data of the 16S rRNA, *rpoB, gyrB* and ITS genes were included for phylogenetic analysis. Multiple alignments of the gene sequences were built by the CLUSTAL W computer program[20]. Neighbor-joining trees with bootstrap values (1,000 replicates) and Kimura 2-parameter distance were performed using MEGA 4.0 software in default settings except for pair-wise distance computation option[21].

7. Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry (MS)

A. bereziniae KTCT 12683, *A. guillouiae* KTCT 12684 and the strain YMC79 were grown on Mueller-Hinton agar for 24h at 37℃.

The cells (about 5 to 10 mg) were mixed with 300 µl water and 900 µl ethanol (100%) and centrifuged at maximum speed for about 2 min. We then removed the supernatant and re-suspended the pellet with 50 µl of 70% formic acid and acetonitrile (50 µl). The mixture was centrifuged at maximum speed for 1 minute. The supernatant was spotted on a MALDI target plate (Bruker Daltonik GmbH, Leipzig, Germany). The spot was overlaid with 2 µl matrix solution [α-cyano-4-hydroxy cinnamic was prepared in 50% acetonitrile, 2.5% trifluoroacetic acid], and the plate was dried on air at room temperature.

Measurements were performed with a Microflex LT mass spectrometer (Bruker Daltonik) equipped with a 337 nm nitrogen laser. Calibration and parameter

optimization were performed with *E. coli* DH5α. Each spectrum was obtained after 200 shots in automatic mode at a variable laser power. Spectra were recorded in the positive linear mode (delay: 150 ns; ion source 1 voltage: 20 kV; ion source 2 voltage: 16.7 kV; lens voltage: 7 kV; mass range: 2 kDa to 20 kDa). Spectra were automatically acquired using AutoXecute acquisition control software and were imported into BioTyper™ version 2.0 software (Bruker Daltonik) and analyzed by standard pattern matching against the reference spectra of 3,290 species in the BioTyper™ database (in default settings).

The reliability of ID was represented by the scoring system as follows: Scores of < 1.7, not reliable identification; 1.7 - < 2.0, probable genus; 2.0 - < 2.3, identification to genus; > 2.3, highly probable species identification.

8. Chemotaxonomy

Fatty acid profiles of the strain YMC79 and phlyogenetically related type strains (*A. bereziniae* KTCT 12683 and *A. guillouiae* KTCT 12684) were determined under identical conditions. Cells grown on BSA for 24 h at 30℃ were prepared and analyzed with methyl esters using GLC according to the manufacturer's instructions for the Microbial Identification System (MIDI Inc., Newark, Delaware, USA).

The DNA G+C content (mol%) was determined using the thermal denaturation method of Marmur & Dorty(1962).

9. DNA-DNA hybridization

The DNA-DNA relatedness between strain YMC79 and type strains of *A. bereziniae* species and *A. guillouiae* species were evaluated using DNA-DNA hybridization. Whole genomic DNA extraction was done using the modified method of Boom, *et al*.

The strain was cultured on LB broth for 24 h at 35℃ (except A. *guillouiae* species, for 24 h at 30℃) and then was centrifuged at maximum speed for 5 min. The cell pellet was re-suspended in 5 mL Tris-EDTA solution with lysozyme and incubated for 5-10 min. After the mixture (0.5% SDS, 0.4 M EDTA, Proteinase K 1 mg/ml and 50 mM Tris-hydrochloric acid) was added, this sample was incubated in 50℃ water bath for 60 min. Samples were then extracted with phenol and chloroform/isoamyl alcohol (25:24:1) (Sigma-Aldrich, Seoul, Korea). Repeated extraction was carried out until there was no interface. An equal volume of chloroform was gently mixed with the aqueous phase, then centrifuged at 5,000 g for 20 min. We then carried out DNA precipitation with ethanol, washed with additional ethanol, and then re-dissolved in an appropriate buffer. DNA quality was determined by the ratio of absorbance at 260 and 280 nm ($A_{260\ nm}/A_{280\ nm}$) and all samples showed around 1.8 ratio[24]. DNA preparation was sheared using nebulizer (about 1 kb) in shearing buffer (20% glycerol in Tris-EDTA). The sheared DNA was re-suspended in 2x SSC and was used to carry out DNA-DNA hybridization. We calculated DNA-DNA similarity value using the equation by De Ley *et al.*

A threshold value of 70% DNA-DNA similarity was used for defining bacterial species according to the Ad Hoc Committee recommendations[24,26].

III. RESULTS

Initially, the isolate was identified as *A. lwoffii* by the ATB 32GN system and VITEK GNI card (bioMérieux), and showed imipenem resistance with the VITEK N132 card (bioMérieux). The phenotypic and genetic elements of carbapenemase in the strain YMC79 was confirmed by positive results on a modified Hodge test and DDS test with EDTA-SMA disk, and $bla_{\text{SIM-1}}$ type MBL gene was determined by PCR and nucleotide sequencing. The OXA-type genes ($bla_{\text{OXA23-like,}}$ $bla_{\text{OXA24-like,}}$ $bla_{\text{OXA51-1-like,}}$ and bla_{OXA58}) were not detected, so, we determined that the strain YMC79 was not a member of the *A. baumannii* specices.

Phylogenetic analyses

When the 16S rRNA gene sequences were compared with those of other bacteria in EzTaxon server databases, the strain YMC79 was most closely related to *A. bereziniae* ATCC 17924 ^{T} (98.9% of similarity), *A. guillouiae* ATCC 11171 ^{T} (98.8% of similarity) and *A. gerneri* 9A01 ^{T} (97.2% of similarity). The paired sequence similarity of the 16S rRNA gene varied from 94.4% (*A. towneri* AB1110^{T}) to 98.9% (*A. bereziniae* ATCC 17924 ^{T}). In the neighbor-joining tree,

the strain YMC79 was closely related to *A. bereziniae* ATCC 17924 ^{T} and *A. guillouiae* DSM 590^{T} (=ATCC 11171 ^{T}), and the genetic distances seemed to be farther than that between *A. bereziniae* ATCC 17924 ^{T} and *A. guillouiae* DSM 590^{T} (74% of bootstrap resamplings value, 99.0% of sequence similarity) (Figure 1).

In addition, the *rpoB*, *gyrB* and ITS gene sequences of the strain YMC79 were compared to those of strains in species of the *Acinetobacter* genus (Figure 2, 3, 4), because the 16S rRNA gene has a relatively lower resolution power for identifying *Acinetobacter* at the species level[17,18,27].

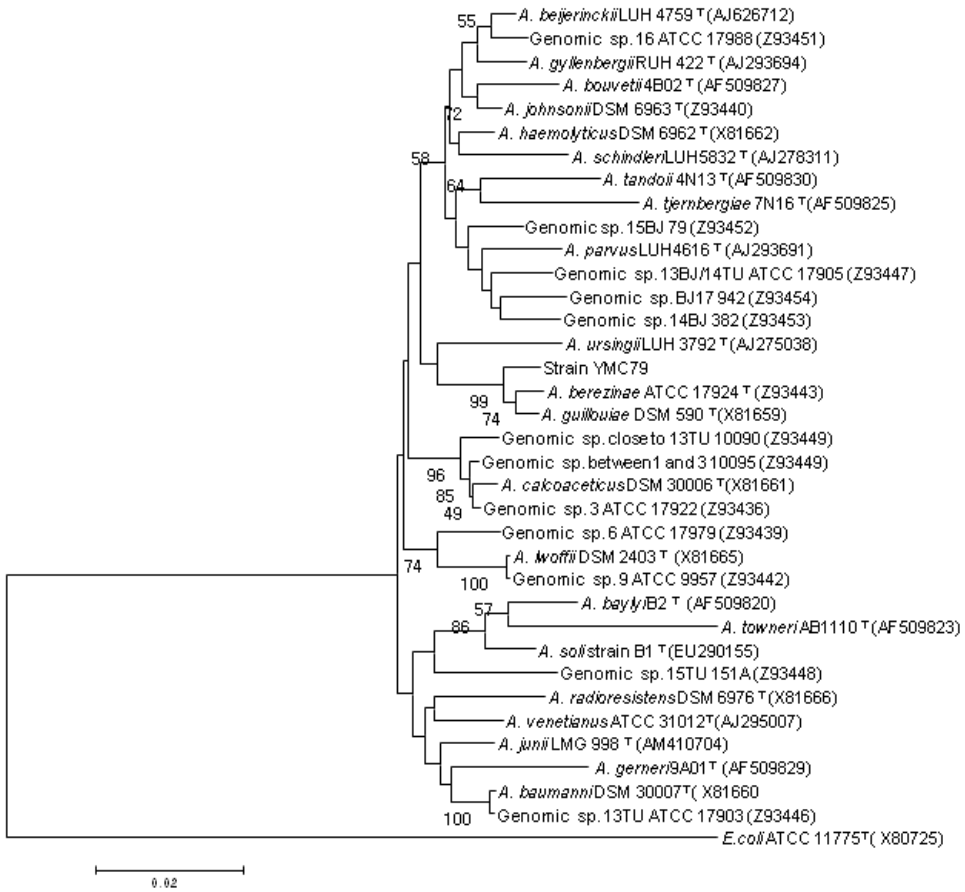

Figure 1. Rooted neighbor-joining tree based on 16S rRNA gene sequence (1,415 bp) of the strain YMC79 and almost all known representatives of the genus *Acinetobacter.* Cluster analysis was carried out using the CLUSTAL W computer program with *E. coli* ATCC 117755^{T} (X80725) as the out-group. Bootstrap values of $\geq$50% (based on 1,000 resamplings by MEGA 4.0 software) were shown. GeneBank accession numbers of sequences are given in parentheses.

Bar, 2% sequence divergence; ^{T}, type strain.

The Clustering and N-J analysis of the sequence was done based on the *rpoB* gene. In the tree, the novel strain exhibited the highest sequence similarity to *A. bereziniae* LMG 1003^{T} (99% of bootstrap resamplings value, 96.8% sequence similarity). This genetic distance was relatively long compared with that between *A. guillouiae* LMG 988 ^{T} and *A. baylyii* CCM 7195 ^{T} (51% of bootstrap resampling value, 98.9% sequence similarity) (Figure 2). However, some of *A. baylyi* strains such as C5 and CCM 7195^{T} were closely clustered with the *A. guillouiae* species (97.6–99.7 % similarity), but showed only 82.2-88.1% similarity to the others (*A. baylyi* ADP1, A7 and 93A2 that formed a relatively homogeneous cluster) according to a previous study (Nemec, *et al*. 2010). This may have resulted from the intragenic recombination of DNA fragments (partial *rpoB* gene, nucleotide positions 3140-3775) between *A. guillouiae* species and *A. baylyi* strains such as C5 and CCM 7195^{T}, so the *rpoB* gene sequences of *A. baylyi* ADP1, A7 were considered as that of an ancestral strain.

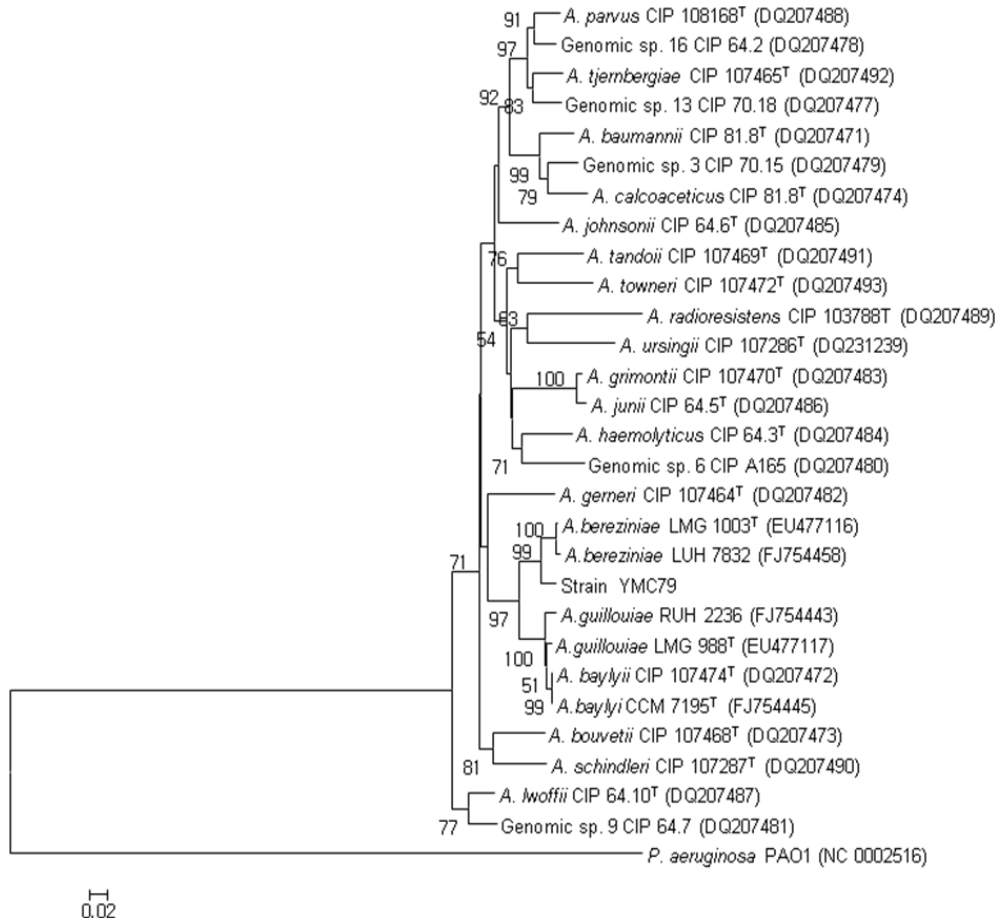

Figure 2. Rooted neighbor-joining tree based on a partial *rpoB* gene sequence (856 bp) showing the phylogenetic relationships among the genus *Acinetobacter*. Cluster analysis was carried out using the CLUSTAL W computer program with *P. aerusinosa* PAO1 (NC_0002516) as the out-group. Bootstrap values of ≥50% (based on 1,000 resamplings by MEGA 4.0 software) are shown. GeneBank accession numbers of sequences are given in parentheses.

Bar, 2% sequence divergence; ^{T}, type strain.

Genomic species 10 and 11 were renamed *A. bereziniae* and *A. guillouiae*, respectively (Nemec *et al.,* 2010) but *gyrB* and ITS gene sequence database were not updated. Thus, *A. bereziniae* and *A. guillouiae* were still called 'genomic species 10' and 'genomic species 11', respectively (Figures 3, 4). The highest concurrence of the *gyrB* gene sequence was shown between the strain YMC79 and genomic species 10 CIP 70.12 (91.0% sequence similarity and 100% of bootstrap resampling value). Furthermore, the sequence similarity between genomic species 10 CIP 70.12 and genomic species 11 CIP 63.46 was 87.5% (Figure 3).

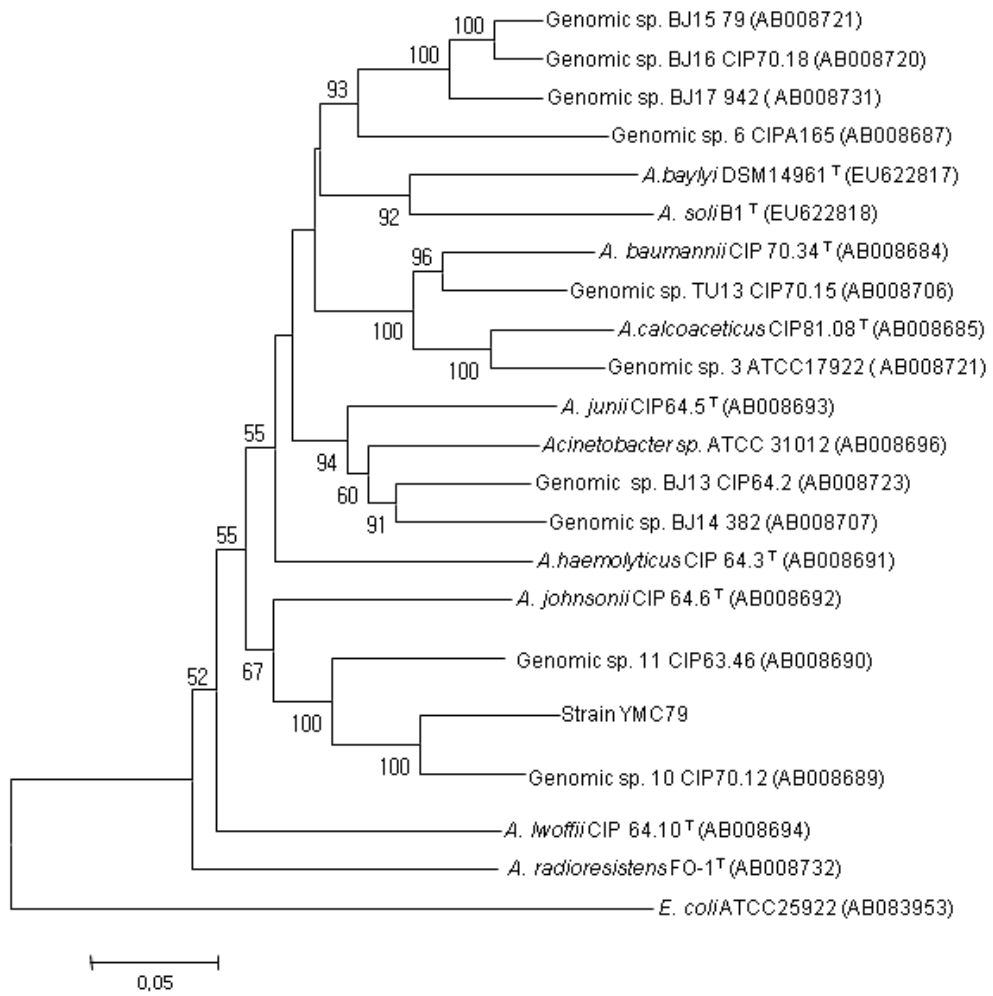

Figure 3. Rooted neighbor-joining tree based on *gyrB* gene sequence (897 bp) showing the phylogenetic relationships among the genus *Acinetobacter*. Cluster analysis was carried out using the CLUSTAL W computer program with *E. coli* ATCC 25922^{T} (AB083953) as the out-group. Bootstrap values of ≥50% (based on 1,000 resamplings by MEGA 4.0 software) are shown. GeneBank accession numbers of sequences are given in parentheses.

Bar, 2% sequence divergence; ^{T}, type strain.

According to the phylogentic tree (Figure 4), the strain YMC79 was closely related to the genomic species 10 BCRC 15423 and 11BCRC 15424 (98% and 100% of bootstrap resampling values, showing 95.9% and 89.2% sequence similarities, respectively). *A. junii* BCRC 14854^{T}and *A. venetianus* CCUG 45561^{T} (98% of bootstrap resampling value, 92.3% sequence similarity) also showed relatively close genetic relationship but it was not smaller.

The cluster groups including the strain YMC79 did not show a significant distance from the clusters composed with the rest of type strains in dendrogram of cluster analysis with *rpoB*, *gyrB* and ITS gene sequences (Figure 2-4). So, we could not qualify strain YMC79 as a novel type strain in the genus *Acinetobacter*.

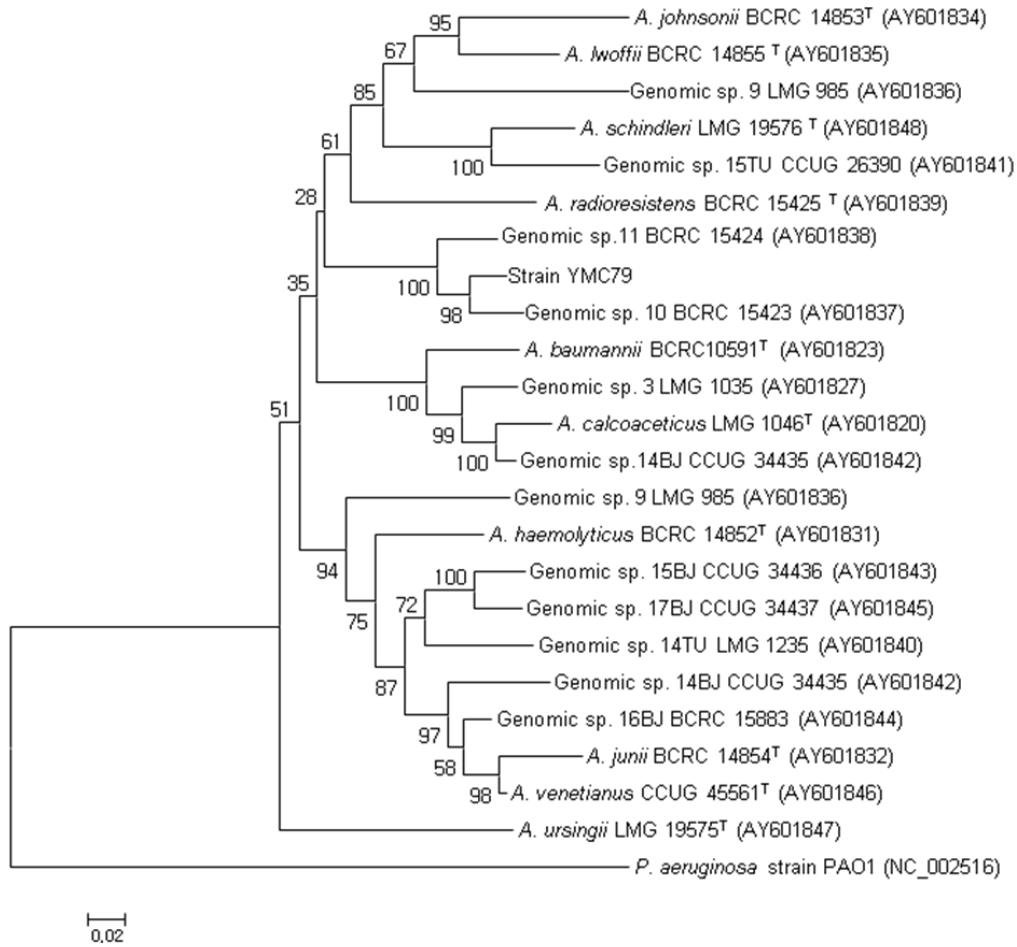

Figure 4. Rooted neighbor-joining tree based on ITS gene sequence (743 bp) showing the phylogenetic relationships among the genus *Acinetobacter*. Cluster analysis was carried out using the CLUSTAL W computer program with *P. aerusinosa* PAO1 (NC_0002516) as the out-group. Bootstrap values of ≥50% (based on 1,000 resamplings by MEGA 4.0 software) are shown. GeneBank accession numbers of sequences are given in parentheses.

Bar, 2% sequence divergence; ^{T}, type strain.

Phenotypic characteristics

The strain YMC79 did not show hemolytic ability on sheep's blood agar and was not grown at 42℃, but was able to grow on some of the carbon sources.

Grown-up colonies were circular, entire margin, opaque and 1-2 mm in diameter. Phenotypic characteristics of the strain YMC79 and two phlyogenetically related type strains (*A. bereziniae* KTCT 12683 and *A. guillouiae* KTCT 12684) were described in Table 2. After 2 days of incubation, strain YMC79 was differentiated from these two strains by its ability to utilizing of D-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, D-mannose, D-melibiose, xylitol, D-fucose and malonate.

Chemotaxonomy

The fatty acid profiles of the strain YMC79, *A. bereziniae* KTCT 12683 and *A. guillouiae* KTCT 12684 are arranged in Table 3. The experiment was carried out under identical condition for all three species. Strain YMC79 was mainly composed of $C_{18:1}$ w9c (34.1%), summed feature 3 (23.4%) and $C_{16:0}$ (23.9%). The major quinine of the novel strain was $C_{18:1}$ w9c (34.1%).

The DNA G+C content (mol%) of strain YMC79 was 41.2 mol%, and *A. bereziniae* KTCT 12683 and *A. guillouiae* KTCT 12684 were 40.8% and 39.4%, respectively.

Table 2. Characteristics of strain YMC79 from closely related type strains

Characteristic	Strain YMC79	*A. bereziniae*[a]	*A. guillouiae*[b]
Growth at 42℃	−	−	−
Growth at 38℃	+	+	w
Growth at 30℃	w	w	+
Lyse sheep erythocytes	−	−	−
Assimilation of (API 20NE)			
Malate	+	+	+
Caprate	+	+	+
Phenyl acetate	w	w	+
Utilization of (API 50CH)			
L-Arabinose	w	−	−
D-Ribose	+	−	−
D-Xylose	+	−	−
L-Xylose	−	+	−
D-Adonitol	−	+	−
D-Galactose	+	w	−
D-Mannose	+	−	−
D-Melibiose	+	−	w
D-Sucrose	−	−	w
Inulin	−	−	w
Xylitol	−	+	w
Gentiobiose	+	−	w
D-Fucose	+	−	−
Utilization of (ID card[c])			
Malonate	+	−	−
DNA G+C content (mol%)	41.2	40.8	39.4

A. bereziniae KTCT 12683 (= *A. bereziniae* ATCC 17924^T); [b], *A. guillouiae* KTCT 12683 (= *A. guillouiae* ATCC 11171^T); [c], VITEK GN ID card. +, positive reaction; w, weakly positive; −, negative reaction.

Table 3. Cellular fatty acid composition of strain YMC79 and *Acinetobacter* species

Isolates	Strain YMC79	*A. bereziniae* KTCT 12683[a]	*A. guillouiae* KTCT 12684[b]
Saturated fatty acids:			
$C_{10:0}$	ND	tr	0.5
$C_{12:0}$	6.4	8.8	7.1
$C_{13:0}$	ND	tr	tr
$C_{14:0}$	0.9	0.9	0.8
$C_{16:0}$	23.9	20.5	16.5
$C_{17:0}$	0.4	0.5	0.6
$C_{18:0}$	0.9	0.6	0.5
Branched fatty acids:			
iso-$C_{19:1}$	0.1	0.1	0.2
anteiso-$C_{17:1}$	ND	0.1	ND
Unsaturated fatty acids:			
$C_{17:1}$ w8c	0.4	0.6	1.1
$C_{18:1}$ w9c	34.1	33.5	36.0
Hydroxy fatty acids:			
$C_{12:0}$ 2-OH	1.9	1.9	2.3
$C_{12:0}$ 3-OH	3.9	5.4	5.8
Summed feature 2	0.7	0.2	0.1
Summed feature 3	23.4	24.1	25.0
Summed feature 8	3.1	2.6	3.4

The summed feature represents the group of two or three fatty acids that could not be separated by GLC with the MIDI system. The values are percentages of total fatty acid. Summed feature 2 contained $C_{14:0}$ 3-OH/iso-$C_{16:1}$, Summed feature 3 contained $C_{16:1}$ w7c and/or $C_{16:1}$ w6c, and Summed feature 8 contained $C_{18:1}$ w7c and/or $C_{18:1}$ w6c. [a], *A. bereziniae* KTCT 12683 (= *A. bereziniae* ATCC 17924^T); [b], *A. guillouiae* KTCT 12684 (= *A. guillouiae* ATCC 11171^T). ND, not detected; tr, <0.1%.

MALDI-TOF MS identifications

The duplicated spectra of three strains of the genus *Acinetobacter* were imported to BioTyper™ version 2.0 software (Bruker Daltonik) and analyzed by standard pattern matching against the reference spectra of 3,290 species in the BioTyper™ database (in default settings). According to the BioTyper™ database, *A. bereziniae* KTCT 12683 and strain YMC79 were incorrectly identified as Genomic species 3 serovar 15 DSM or *Acinetobacter* species DSM 30013 (score of <2.3). These erroneous identifications of two strains might have been influenced by the amount of the reference strain in database and by the bias such as culture medium, culture condition, sample handling and variant properties of objects, etc[28]. Whereas, *A. guillouiae* KTCT 12684 was identified as *Acinetobacter* genomosp 11 DSM 590 (score of 2.31). Among the spectra of these species, the specific signals of strain YMC79, i.e., 5903.5, 6243.2 and 7176.3 m/z, distinguished this strain from the others (Figure 5).

DNA-DNA relatedness

The DNA-DNA relatedness between strain YMC79 and *A .bereziniae* KTCT 1268 was 72%, and 47 % between strain YMC79 and *A. guillouiae* KTCT 12684.

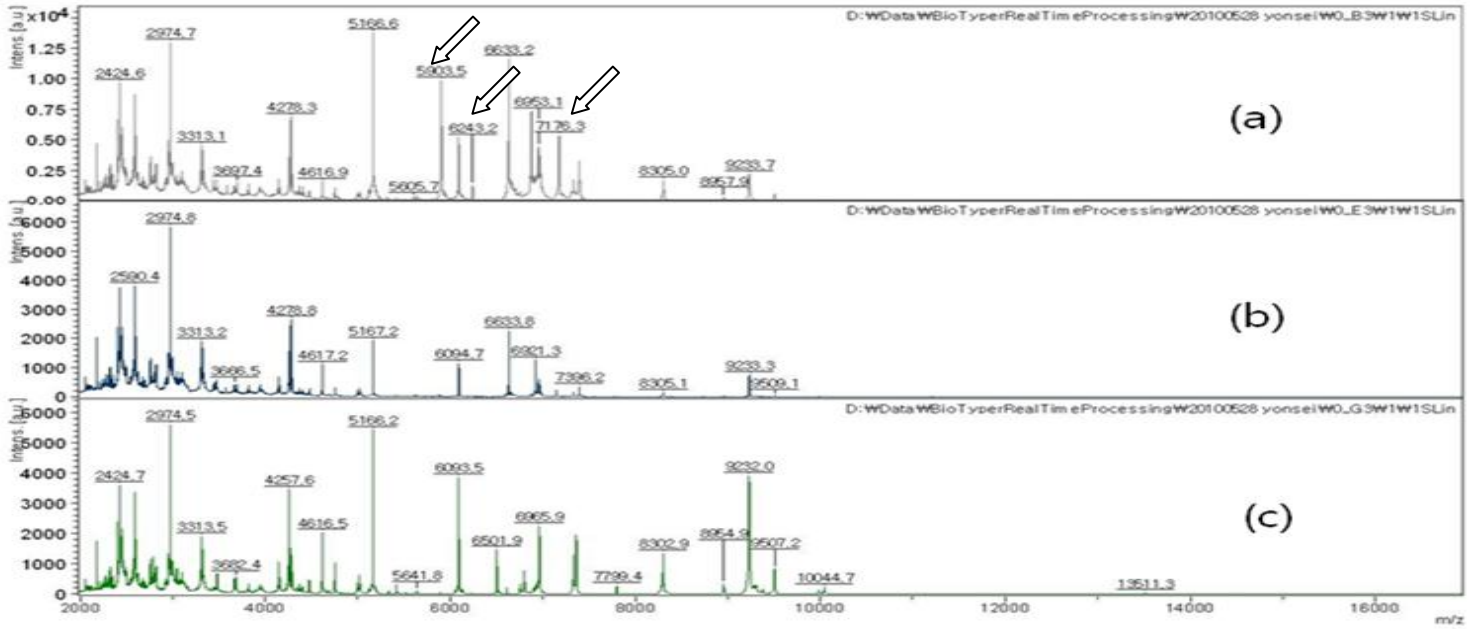

Figure 5. MALDI-TOF MS of whole-cell extracts from the novel strain and the reference strain. (a), strain YMC79; (b), *A. bereziniae* KTCT 12683 (= *A. bereziniae* ATCC 17924); (c), *A. guillouiae* KTCT 12684 (= *A. guillouiae* ATCC 11171).

IV. DISCUSSION

Dynamic changes in taxonomy have been observed after "genus *Acinetobacter*" was proposed by Brisou and Prévot in 1954[2]. Recently, the genus *Acinetobacter* has been divided into 32 genomic species, including 17 species with a valid name[4] and 15 nomenspecies have been isolated in human specimens[4,6,8]. Nowadays, *Acinetobacter* species are highly regarded as critical pathogens in nosocomial infection. In a nationwide survey in 2005, 144 carbapenem-resistant *Acinetobacter* species were isolated, of which 19.4% and 74.3% were respectively MBL and OXA producers[29]. As such, these multidrug resistant *Acinetobacter* species have brought forth limitations on available antibiotics and have been a warning to nosocomial infection control[10,11]. Among metallo-β-lactamases including $bla_{\text{VIM-2}}$, $bla_{\text{IMP-1,}}$ and $bla_{\text{SIM-1}}$ types, SIM-1 producer has been found only in Korea[14,30] and strain YMC79 also produced SIM-1 type metallo-β-lactamases.

According to our data, strain YMC79 was most closely related to *A. bereziniae* type strain, with 98.9% similarity in the 16S rRNA sequence. In partial *rpoB*, *gyrB* gene and ITS sequences, strain YMC79 showed 96.8%, 91.0% and 95.9% similar identities with those of *A. bereziniae* type strain, respectively. The genetic distance between strain YMC79 and *A. bereziniae* type strain was relatively closer in phylogenetic tree studies based on *gyrB* gene and ITS. However, *A. bereziniae* and *A. guillouiae* type strain in 16S rRNA phylogenetic

trees and *A. guillouiae* LMG 988 ^{T} (=ATCC 11171^{T}) and *A. baylyii* CCM 7195^{T} in *rpoB* phylogenetic tree were more closely clustered than strain YMC79 and *A. bereziniae* LMG 1003^{T}(=ATCC 17924^{T})(Figure 1, 2). However, according to a previous study [4], some of *A. baylyi* strains such as C5 and CCM 7195^{T} were closely clustered with the *A. guillouiae* species (97.6–99.7 % similarity) by intragenic recombination events in partial region of *rpoB* gene.

The DNA–DNA reassociation value between strain YMC79 and *A. bereziniae* KTCT 1268(=ATCC 1171^{T}) was 72%. If we synthesized the cluster analysis results and DNA-DNA relatedness value, strain YMC79 seem to be considered as an *A. bereziniae* like species. However, two unique features represent strain YMC79 as a novel *A. bereziniae* subspecies. The first is that strain YMC79 showed distinctive appeareances in phenotypic characteristics (described in table 2). The second is the specific signals 5903.5, 6243.2 and 7176.3 (*m/z*) of its MALDI-TOF spectrum.

V. CONCLUSION

After all the analysis, strain YMC79 (= novel subspecies *seouli*) is considered as belonging to *Acinetobacter bereziniae,* but representing novel subspecies by unique phenotypic characteristics and MALDI-TOF spectrum.

A novel subspecies *seouli* with *bla*$_{\text{SIM-1}}$ gene was isolated from a 44-year old male patient diagnosed with acute myeloid leukemia at a tertiary-care hospital in

Seoul, Korea.

Description of *Acinetobacter bereziniae* subspecies *seouli* nov.

Colonies on MacConkey agar after 24 h incubation at 37℃ are 1.0–2.0 mm in diameter,circular, convex and slightly opaque with entire margins. This strain shows common trait with the genus *Acinetobacter* such as being gram-stain-negative, strictly aerobic, oxidase-negative and non-motile coccobacillus, Hemolysis was not observed on sheep blood agar media. A novel subspecies *seouli* utilized D-arabinose, D-ribose, D-xylose, D-galactose, D-mannose, D-melibiose, gentiobiose, D-fucose and malonate in the API 50CH tests and/or VITEK2 ID card. Utilization of L-xylose, D-adonitol, D-sucrose, inulin, xylitol was not observed. Its the DNA G+C content (mol%) was 41.2 mol% and major fatty acids are $C_{18:1}$ w9c (34.1%), summed feature 3 (23.4%) and $C_{16:0}$ (23.9%). The DNA–DNA reassociation value between a novel subspecies *seouli* and *A. bereziniae* was 72%.

This novel *Acinetobacter bereziniae* subspecies *seouli* with $bla_{\text{SIM-1}}$ gene was isolated from a human clinical specimen in Seoul, Korea.

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ABSTRACT (IN KOREAN)

SIM-1 형의 MBL 을 생성하는 새로운 *Acinetobacter bereziniae* 의 아종의 규명

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Acinetobacter species는 포도당 비발효 그람음성 구간균으로 토양과 물 속등의 환경에 널리 존재하고 동시에 임상검체에서도 흔히 분리되고 있다. 현재까지 *Acinetobacter* 균속은 명명된 21 균종과 11 유전적 균종(genomic spp.)이 속해있다. 명명된 21 균종 중 15 균종이 임상검체에서 분리 보고되었다. *Acinetobacter* species 중에는 획득성 Metallo-β-lactamases (MBLs)생성으로 carbapenemases에 내성인 균주가 흔하다. 이들에 대한 연구 중 SIM-1 효소를 생성하고 전통적 생화학 시험과 16s rRNA 분석법으로 동정이 어려운 균주를 발견하였고 추가 시험을 통하여 이를 새로운 아종으로 명명하고자 한다. 대상 균주는 급성백혈병으로 진단된 환자의 입안 검체에서 분리되었다. 16s rRNA, *rpoB*, *gyrB*와 ITS 유전자 염기서열 분석을 시행한 결과 *A. bereziniae*의 것과 각각 98.9%, 96.8%, 91.0% 와 95.9% 의 유사성을 보였고 계통분석 시행 결과 *A. bereziniae* KTCC 12683(= ATCC 172924^{T})과 *A. guillouiae* KTCC 12684(= ATCC 11171^{T})이 유전적으로 가장 가까운 균종이었다.

유전자 유사성(DNA-DNA relatedness)검사 결과 특히 YMC79와 *A. bereziniae* KTCC1268는 72%의 유사성을 보였다. 하지만, 대상 균주가 계통학적으로 근접한 비교 균주들(*A. bereziniae* KTCC12683, *A. guillouiae* KTCC 12684)과 다르게 D-arabinose, D-ribose, D-xylose, D-mannose, D-melibiose, D-fucose, malonate를 이용하고 L-xylose, D-adonitol, xylitol을 이용하지 못하는 생화학적 특성을 보였고 균동정을 위한 MALDI-TOF 시행 결과상 5903.5, 6243.2 와 7176.3 (*m/z*) 위치에서 두 비교균주(*A. bereziniae* KTCC12683, *A. guillouiae* KTCC 12684)와 상이한 단백 peak가 관찰되었다. 이에 본 연구는 YMC79를 *Acinetobacter bereziniae*의 새로운 아종 *seouli*로 보고하는 바이다.

핵심되는 말: *Acinetobacter* 새로운 아종, SIM-1, metallo-β-lactamase