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Cellulase Activity of Symbiotic Bacteria from Snails, *Achatina fulica*[1]

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ABSTRACT

Cellulase is the key enzyme in the use of cellulose-based biomaterials. Because of its structure, cellulose is difficult to be degraded by enzymes. In order to utilize cellulose-based biomaterials efficiently, evolutionary wisdom of how to use enzymes accurately and harmoniously in a biological system is needed, such as the cellulose digestive system in animals. In this study, the symbiotic bacteria from snails, *Achatina fulica*, were identified and their cellulase activity was evaluated. The 16S rRNA sequence analysis of 100 aerobic bacteria showed that they belonged to 9 genus and almost half of the bacteria were *Lactococcus* spp. Among 100 identified strains, only two *Aeromonas* sp. strains showed cellulase activity. *Aeromonas* sp. KMBS020 had both endo-β-glucanase and β-glucosidase activities but *Aeromonas* sp. KMBS018 had β-glucosidase activity only. None of the 100 bacterial colonies had any cellobiohydrolase activity.

Keywords : cellulase, symbiosis, *Achatina fulica*, *Aeromonas* sp.

1. INTRODUCTION

Cellulose is the most abundant glucose polymer in nature. Due to its abundance as well as that of its monomer glucose, cellulose is the best candidate as a resource for the production of both energy and bio-materials when it is hydrolyzed into glucose. However, converting cellulose into its monomer glucose is a critical barrier in using cellulose-based biomass. Cellulase is the enzyme that hydrolyzes cellulose and eventually converts it into its monomer glucose. While some cellulases that are secreted into the outside of biological systems are speculated to have evolved to degrade cellulose in a broad range of environmental conditions, other cellulases that are secreted into the biological system have evolved to function optimally in specific biological systems in order to maximize the efficiency of cellulose degradation. Because conditions of cellulose degradation within biological systems can be precisely controlled, these cellulases evolved without the pressure to adapt to unpredictable and diverse conditions.

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Many commercial cellulases from fungi belong to the former group. Examples of biological systems for cellulose degradation include the digestive tract of herbivorous animals and insects. Cellulose digestion in animals is a harmonious and orchestral process with many different cellulases from animals themselves and their symbiotic microbes. Understanding this harmonious reaction will provide insight on how cellulose is biologically degraded with maximized efficiency.

There are three types of cellulase; endo-β-glucanase (EG) which breaks β(1-4) glycosidic bonds internally, cellobiohydrolases (CBH) which breaks β(1-4) glycosidic bonds from the ends of cellulose chains and produces di- to tetrasaccharides, and β-glucosidase (BGL) which breaks β(1-4) glycosidic bonds from the ends of cellulose chains to produce glucose. All three cellulase types need to be produced at the proper location of the digestive tracts in proper amounts for the complete digestion of cellulose. In many cases, cellulase from the host animals or insects is often unavailable and is thus mostly produced by symbiotic microbes. The first step in understanding the harmonious cellulose degradation of the animal digestive tract is to identify the symbiotic microbes that produce cellulase.

Snails are herbivorous gastropod mollusks that have endogenous endo-β-glucanase (Maeda *et al*., 1996; Guo *et al*., 2008; Imjongjirak *et al*., 2008; Teng *et al*., 2010). The intestinal tracts of snails have a variety of bacteria (Ducklow *et al*., 1979; Ducklow *et al*., 1981; Charrier *et al*., 2006; Cardoso *et al*., 2012; Cardoso *et al*., 2012; Pawar *et al*., 2012; Van Horn *et al*., 2012; Nwiyi and Amaechi, 2013) but their cellulase production was evaluated in only a few studies. Gupta *et al*. (2012) isolated two kinds of bacteria which produce cellulase but did not identify these two bacteria. Oyeleke *et al*. (2012) isolated five Gram-positive bacteria and four fungi from the gut of snails. Cellulase activity was observed with *Bacillus subtilis* among the found bacteria and *Aspergillus niger* among the found fungi. Among the majority of Gram-negative bacteria in the intestinal tracts of snails, no cellulase producing strain has been reported as of yet. In the previous study with termites, sixteen symbiotic bacteria was identified (Cho *et al*., 2010). They produced CBH and BGL strongly but not EG. This unusually selective cellulase production was suggested as evidence of symbiotic adaptation. In this study, the symbiotic bacteria of the giant African snail, *Achatina fulica*, were identified and the cellulase activity of these symbiotic bacteria was analyzed.

2. MATERIALS AND METHODS

2.1. Snails and their symbiotic bacteria screening

The snails were purchased from Bokdongine Snail Farm (Yeoju-gun, Gyeonggi-do, Korea). The nucleotide sequence of cytochrome oxidase subunit I region identified these snails as *Achatina fulica*, a species of land snails (data

not shown). After removing their hard shell, the intestine was separated. The length of removed intestine for bacterial screening was about 2 centimeters and 6.7 grams. The removed intestine was suspended in 10 mℓ of TYE media (1% tryptone and 0.5% yeast extract). After a serial dilution was made, 100 μℓ of each serial dilution was spread onto TYE agar plates (TYE media with 1.5% agar). The plates were incubated for 24 hours at 27℃. From a plate with the colony number from 25 to 250, 100 colonies in a region were picked in order to eliminate any biased selection. All colonies were streaked on TYE agar plates twice to get a single colony. The single colony were inoculated in 5 mℓ of TYE media, cultured for 24 hours at 27℃, and stored at -80℃ with 50% glycerol for a long term storage.

2.2. Identification of selected bacteria

The nucleotide sequences of 16S rRNA genes of all 100 selected strains were analyzed using two primers, 27F (5'-AGAGTTTGATCCTGGC TCAG-3') and 1492R (5'-GGTTACCTTGTTA CGACTT-3') by SolGent Co., Ltd. (Daejeon, Korea). The sequencing results were compared with a nucleotide database in National Center for Biotechnology Information (NCBI) using the BLAST search (http://www.ncbi.nlm.nih.gov/). The search results were listed in the Supplemental Data Table 1. The comparison of selected 16S rRNAs was performed by MEGA 6 using the neighbor-joining method with the maximum composite likelihood method (Tamura *et al*., 2013). The bacterial shape was observed using Zeiss AX10 Scope.A1 microscope with AxioCam HRm camera and ZEM 2012 blue edition software (Carl Zeiss Co., Ltd., Seoul, Korea).

2.3. Cellulase activity of selected bacteria

The stored 100 bacteria strains at −80℃ were streaked on TYE agar plates and incubated for 24 hours at 26℃. A single colony was inoculated in 5 mℓ of TYE media and incubated for 18 hours at 26℃ with 250 rpm shaking speed. The cell density at 600 nm was measured and subcultured with 0.05 absorbance value at 600 nm in 5 mℓ of TYE media with 1% carboxymethyl cellulose. After incubation for 24 hours at 26℃ with 250 rpm shaking speed, cells were removed by centrifugation. The cellulase activity of cell-free supernatants was measured. Endo-β-glucanase (EG) activity was evaluated by measuring the change of reducing power of sugar using Somogyi-Nelson method (Nelson, 1944) after a hydrolysis reaction of carboxylmethylcellulose (CMC). The detailed experimental procedure is as follows. Forty microliters of culture supernatant was mixed with 45 μℓ of 2% CMC in 100 mM sodium acetate buffer at pH 5.0. The mixtures were incubated for 4 hours at 50℃ for the EG enzymatic reaction. After finishing the EG reaction, 50 μℓ of the mixture of copper reagents was added. The mixture of copper reagents was made by mixing 25 mℓ of copper reagent A (25 g/ℓ of

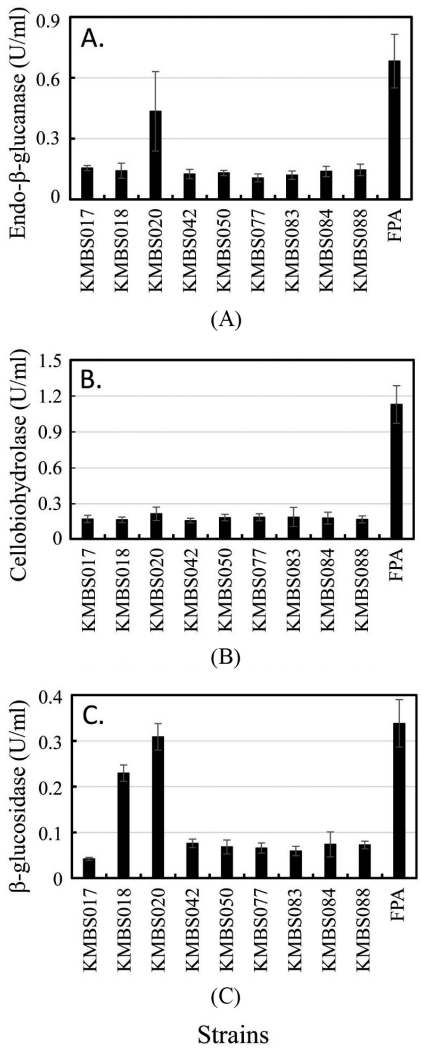

Fig. 1. Cellulase activity of nine symbiotic *Aeromonas* spp. from snails, *A. fulica*. Three types of cellulase, endo-β-glucanase (A), cellobiohydrolases (B), and β-glucosidase (C), were measured independently. The strain name on figures was the identification number of *Aeromonas* sp. in Supplementary Data Table 1.

Na_2CO_3, 25 g/ℓ of $KNaC_4H_4O_6 \cdot 4H_2O$, 20 g/ℓ $NaHCO_3$, and 20 g/ℓ Na_2SO_4) and 1 mℓ of copper reagent B (150 g/ℓ of $CuSO_4 \cdot 5H_2O$ and two drops of concentrated H_2SO_4 per 100 mℓ) just before the EG activity assay. Samples were boiled for 10 minutes and cooled down. Fifty microliters of the color reagent (50 g/ℓ of $(NH_4)_6Mo_7O_{24}$, 42 mℓ/ℓ of concentrated H_2SO_4, and 6 g/ℓ of NaH_2AsO_4) and 815 μℓ of distilled water were added and mixed well. After incubation at room temperature for 10 minutes, the absorbance at 650 nm of wave length light was measured using Optizen 2120UV spectrophotometer (Mecasys Co., Ltd., Daejeon, Korea). The glucose standard solutions were used to calculate the reducing power of samples. One unit was defined as the amount of activity that produced 1 μmol of reducing sugar (glucose equivalents)/min. Cellobiohydrolase (CBH) and β-glucosidase (BGL) were measured according to the previous study (Cho *et al*., 2010) with 2 hours of enzymatic reaction time instead of 15 minutes. Cellulase activities of *Fomitopsis palustris* FFPRI 0507 (FPA) were used as a control for the enzymatic activity assay in Fig. 1. One unit of CBH and BGL was defined as the amount of activity that generated 1 μmol of *p*-nitrophenol/min. The enzyme activity was independently assayed three times, of which the average and the standard deviation are shown in Fig. 1.

3. RESULTS and DISCUSSION

3.1. The intestinal bacterial community of *A. fulica*

After dissecting *Achatina fulica*, two centimeters of the intestine was suspended in 10 mℓ

of TYE media. After a serial dilution, colony numbers from spread plates showed that the intestine of snails had 7.8 (± 0.7 of the standard deviation) 10^6 cells/g of aerobic bacteria which were similar to that of a wild snail (Ducklow *et al*., 1981). One hundred colonies were selected in a region of a spread plate and their 16S rRNA was sequenced. The closest strains found by comparing with the 16S rRNA sequence with a nucleotide database of National Center for Biotechnology Information (NCBI) are listed in Supplementary Data Table 1. The summary of identified bacteria is shown in Table 1. The most common bacterium was *Lactococcus* spp. (49%), followed by *Aeromonas* spp. (23%), *Citrobacter* spp. (10%), and *Kluyvera* spp. (9%). The rest of the bacteria, *Buttiauxella* spp. (3%), *Enterobacter* spp. (3%), *Shewanella* spp. (2%), and *Klebsiella* spp. (1%), made up the remaining 9% of population. All bacteria except *Lactococcus* spp. were from the class Gammaproteobacteria.

Lactococcus spp. were also found from the intestine of *Helix pomatia*, the Burgundy snail that is more commonly known as escargot (Charrier *et al*., 2006). Pawar *et al*. (2012) analyzed the bacterial community of the intestine of *A. fulica* and also found that *Lactococcus* spp. were the major intestinal bacteria. *Aeromonas* spp. were the second major population among identified bacteria in this study (Table 1). The pathogenicity of the genus *Aeromonas* is under debate (Igbinosa *et al*., 2012). While two of the previous studies (Charrier *et al*., 2006; Pawar *et al*., 2012) found *Lactococcus* spp. but failed to find *Aeromonas* spp. from the intestine of snails, other studies found *Aeromonas* spp. from the intestine of snails, *A. fulica* (Dean *et al*., 1970), *H. aspersa* (Kodjo *et al*., 1997; Kiebre-Toe *et al*., 2005), *Biomphalaria glabrata* (Ducklow *et al*., 1979), *Achatina achatina* (Obi and Nzeako, 1980), and *Ampullaria* spp. (Nwiyi and Amaechi, 2013). *Citrobacter* spp. and *Kluyvera* spp. were also found together from the intestine of snail in previous studies (Charrier *et al*., 2006; Pawar *et al*., 2012) and independently *Citrobacter* spp. from *B. glabrata* (Ducklow *et al*., 1979) and *Kluyvera* spp. from snails (Müller *et al*., 1996). Recently, Cardoso *et al*. (2012) analyzed the intestinal bacterial communities of *A. fulica* using culture-independent molecular analysis. They found six genus out of nine identified bacteria in Table 1, *Lactococcus* spp., *Aeromonas* spp., *Citrobacter* spp., *Klebsiella* spp., *Enterobacter* spp., and *Shewanella* spp.

3.2. *Aeromonas* spp. has cellulase activities among the intestinal bacteria from *A. fulica*

Three cellulase activities, endo-β-glucanase (EG), cellobiohydrolase (CBH), and β-glucosidase (BGL), of all 100 identified strains were measured (data not shown). Among them, only nine colonies had a detectable cellulase activity. All nine strains were *Aeromonas* spp. Among them, two strains had notable cellulase activities (Fig. 1). *Aeromonas* sp. KMBS018 had more BGL activity only and *Aeromonas* sp. KMBS020 had more activities of both EG and BGL. When

Table 1. The summary of 100 identified bacteria based on the 16S rRNA homology search

Identified bacterial name	Number of colonies	List of references which identified from snails
Aeromonas spp.	23	Dean *et al*., 1970 Ducklow *et al*., 1979 Obi and Nzeako, 1980 Kodjo *et al*., 1997 Kiebre-Toe *et al*., 2005 Cardoso *et al*., 2012 Nwiyi and Amaechi, 2013
Buttiauxella spp.	3	Müller *et al*., 1996 Charrier *et al*., 2006
Citrobacter spp.	10	Ducklow *et al*., 1979 Charrier *et al*., 2006 Cardoso *et al*., 2012 Pawar *et al*., 2012
Enterobacter spp.	3	Ducklow *et al*., 1979 Charrier *et al*., 2006 Cardoso *et al*., 2012 Nwiyi and Amaechi, 2013
Klebsiella spp.	1	Cardoso *et al*., 2012 Nwiyi and Amaechi, 2013
Kluyvera spp.	9	Müller *et al*., 1996 Charrier *et al*., 2006 Pawar *et al*., 2012
Lactococcus spp.	49	Charrier *et al*., 2006 Cardoso *et al*., 2012 Pawar *et al*., 2012
Shewanella spp.	2	Cardoso *et al*., 2012
Total number	100	

their cellulase activities were compared to that of *Fomitopsis palustris* FFPRI 0507, which is well known as cellulose degrading fungus (Yoon and Kim, 2005; Yoon *et al*., 2007), the EG activity of *Aeromonas* sp. KMBS020, the BGL activity of *Aeromonas* sp. KMBS018 and the BGL activity of *Aeromonas* sp. KMBS020 were shown to be 64%, 68%, and 91%, respectively. These results showed that *Aeromonas* spp. were the symbiotic bacteria in the intestine of *A. fulica* that helped the cellulose digestion of snails. Several previous studies showed that *Aeromonas* spp. were cellulase producers (Ohya *et al*., 1976; Kubata *et al*., 1994; Jiang *et al*., 2011; Ahmad *et al*., 2013; Muñoz *et al*., 2014). Among these previous studies, some *Aeromonas* spp. were found in the intestine of grass carp (Jiang *et al*., 2011), moths (Kubata *et al*., 1994), and mollusks (Muñoz *et al*., 2014). However this is the first study to show the symbiotic cellulase producing *Aeromonas* spp. in snails.

The possible loss of EG activity by symbiotic bacteria due to the lack of exposure to large size cellulose polymers in the distalmost organs of the cellulose digestive track of termites had

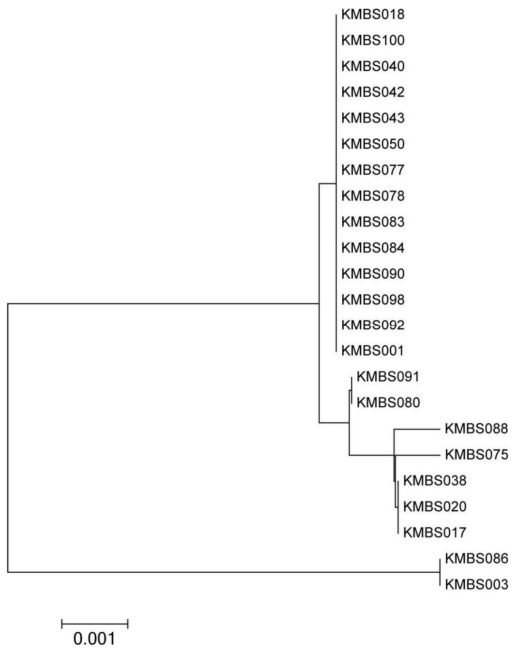

Fig. 2. Phylogenetic analysis of 23 identified *Aeromonas* spp. using the MEGA 6 program. The strain name was the identification number of *Aeromonas* sp. in Supplementary Data Table 1.

previously been proposed (Cho *et al*., 2010). In the case of two *Aeromonas* strains, *Aeromonas* sp. KMBS018 had only BGL activity, which was similar to the symbiotic bacteria in the gut of termites while *Aeromonas* sp. KMBS020 had additional EG activity. This observation can be explained by the different diets of snails and termites. Cellulose is the main food for termites, *Reticulitermes speratus*, but snails can consume most parts plants including cellulose. Therefore, the efficiency of cellulose degradation by snails should be lower than that of termites. Because the population of cellulase producing bacteria will be lower than that of termites, the bacteria in the distalmost intestine of snails may be exposed to large sized cellulose polymers.

In this study, the only bacteria producing cellulases from the intestine of snails were the genus *Aeromonas*. Among the original 100 selected colonies, twenty three colonies were the genus *Aeromonas*. Among them, only 9 colonies had a detectable amount of cellulase activities. Finally, two *Aeromonas* colonies, KMBS018 and KMBS020, had distinct cellulase activities. The 16S rRNA sequences of all 23 *Aeromonas* colonies were compared and analyzed using a bioinformatic program, MEGA 6 (Tamura *et al*., 2013). The phylogenetic tree of the genus *Aeromonas* is shown in Fig. 2. Because some colonies showed the same sequence of 16S rRNA, only one example of the same sequence group was used for phylogenetic analysis in Fig. 2. KMBS017 had the identical 16S rRNA sequence with KMBS020. The 16S rRNA sequence of KMBS042, KMBS050, KMBS077, KMBS083, and KMBS084, was identical to that of KMBS018. Although the 16S rRNA sequences were identical, the cellulase activity varied from colony to colony. The BLAST search in a nucleotide database of NCBI using the 16S rRNA sequence of *Aeromonas* sp. KMBS020 found three different strains, *Aeromonas caviae*, *Aeromonas hydrophila*, and *Aeromonas punctate*, with 100% match in identity without a single nucleotide mismatch. This BLAST search result implied that the homology search using 16S rRNA sequence was not enough to identify the cellulase producing strain in the genus *Aeromonas*. Even the cell shapes of nine *Aeromonase* spp. which

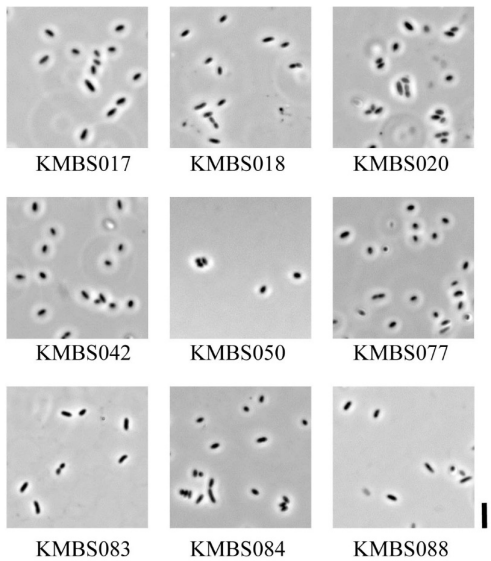

Fig. 3. The microscopic shape of isolated bacteria from *A. fulica*. The pictures were taken by a phase contrast microscopy with the magnification of 1,000 times. The strain name under the picture was the identification number of *Aeromonas* sp. in Supplementary Data Table 1. The scale bar on the right of the images represents 5 μm.

produced cellulases were indistinguishable (Fig. 3).

4. CONCLUSION

In this study, we found that *Aeromonas* spp. were symbiotic and cellulase producing bacteria in the intestine of *A. fulica*, a species of land snails. *Aeromonas* spp. were the second populous genus after *Lactococcus* spp. among symbiotic aerobic bacteria of snails. One strain of *Aeromonas* spp. had only BGL activity similar to that of the symbiotic bacteria of termites and another *Aeromonas* strain had both EG and BGL activities. However, both strains did not have any CBH activity. Such information induced the proposal that these specific cellulase activities may represent the consequence of symbiotic adaptation to the snail diet.

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Supplemental Data

Table 1. List of the closest bacterial strains of 100 isolated colonies from the intestine of *Achatina fulica* by searching a nucleotide database of National Center for Biotechnology Information using their 16S rRNA sequences

Identification number	Strains	Score (bits)	Identities	Gaps
KMBS001	*Aeromonas* sp.	2590	1402/1402 (100%)	0/1402 (0%)
KMBS002	*Enterobacter* sp.	2514	1389/1405 (99%)	0/1405 (0%)
KMBS003	*Aeromonas* sp.	2556	1396/1402 (99%)	0/1402 (0%)
KMBS004	*Lactococcus* sp.	2588	1404/1405 (99%)	1/1405 (0%)
KMBS005	*Lactococcus* sp.	2562	1409/1418 (99%)	8/1418 (1%)
KMBS006	*Lactococcus* sp.	2564	1410/1419 (99%)	8/1419 (1%)
KMBS007	*Lactococcus* sp.	2564	1410/1419 (99%)	8/1419 (1%)
KMBS008	*Lactococcus* sp.	2632	1428/1429 (99%)	1/1429 (0%)
KMBS009	*Lactococcus* sp.	2571	1414/1423 (99%)	8/1423 (1%)
KMBS010	*Kluyvera* sp.	2527	1398/1413 (99%)	2/1413 (0%)
KMBS011	*Lactococcus* sp.	2560	1409/1419 (99%)	8/1419 (1%)
KMBS012	*Lactococcus* sp.	2569	1414/1424 (99%)	8/1424 (1%)
KMBS013	*Lactococcus* sp.	2566	1414/1424 (99%)	9/1424 (1%)
KMBS014	*Lactococcus* sp.	2566	1414/1424 (99%)	9/1424 (1%)
KMBS015	*Lactococcus* sp.	2621	1422/1423 (99%)	1/1423 (0%)
KMBS016	*Kluyvera* sp.	2519	1376/1382 (99%)	1/1382 (0%)
KMBS017	*Aeromonas* sp.	2590	1402/1402 (100%)	0/1402 (0%)
KMBS018	*Aeromonas* sp.	2590	1402/1402 (100%)	0/1402 (0%)
KMBS019	*Lactococcus* sp.	2628	1425/1427 (99%)	1/1427 (0%)
KMBS020	*Aeromonas* sp.	2590	1402/1402 (100%)	0/1402 (0%)
KMBS021	*Lactococcus* sp.	2588	1406/1408 (99%)	2/1408 (0%)
KMBS022	*Lactococcus* sp.	2521	1394/1408 (99%)	1/1408 (0%)
KMBS023	*Lactococcus* sp.	2573	1415/1424 (99%)	8/1424 (1%)
KMBS024	*Lactococcus* sp.	2560	1413/1424 (99%)	9/1424 (1%)
KMBS025	*Buttiauxella* sp.	2625	1432/1438 (99%)	0/1438 (0%)
KMBS026	*Lactococcus* sp.	2630	1427/1428 (99%)	1/1428 (0%)
KMBS027	*Kluyvera* sp.	2516	1374/1381 (99%)	1/1381 (0%)
KMBS028	*Citrobacter* sp.	2547	1397/1406 (99%)	0/1406 (0%)
KMBS029	*Kluyvera* sp.	2490	1368/1382 (99%)	1/1382 (0%)
KMBS030	*Citrobacter* sp.	2527	1408/1427 (99%)	4/1427 (0%)
KMBS031	*Lactococcus* sp.	2573	1414/1423 (99%)	7/1423 (0%)
KMBS032	*Lactococcus* sp.	2573	1415/1424 (99%)	8/1427 (1%)
KMBS033	*Lactococcus* sp.	2627	1426/1428 (99%)	1/1428 (0%)
KMBS034	*Lactococcus* sp.	2638	1428/1428 (100%)	0/1428 (0%)
KMBS035	*Citrobacter* sp.	2599	1407/1407 (100%)	0/1407 (0%)

Table 1. Continued

Identification number	Strains	Score (bits)	Identities	Gaps
KMBS036	*Lactococcus* sp.	2571	1421/1433 (99%)	10/1433 (1%)
KMBS037	*Lactococcus* sp.	2573	1415/1424 (99%)	8/1424 (1%)
KMBS038	*Aeromonas* sp.	2590	1402/1402 (100%)	0/1402 (0%)
KMBS039	*Lactococcus* sp.	2573	1415/1424 (99%)	8/1424 (1%)
KMBS040	*Aeromonas* sp.	2590	1402/1402 (100%)	0/1402 (0%)
KMBS041	*Citrobacter* sp.	2547	1381/1382 (99%)	0/1382 (0%)
KMBS042	*Aeromonas* sp.	2590	1402/1402 (100%)	0/1402 (0%)
KMBS043	*Aeromonas* sp.	2590	1402/1402 (100%)	0/1402 (0%)
KMBS044	*Kluyvera* sp.	2616	1374/1382 (99%)	1/1382 (1%)
KMBS045	*Lactococcus* sp.	2566	1414/1424 (99%)	9/1424 (1%)
KMBS046	*Citrobacter* sp.	2508	1387/1403 (99%)	1/1403 (0%)
KMBS047	*Lactococcus* sp.	2573	1415/1424 (99%)	8/1424 (1%)
KMBS048	*Lactococcus* sp.	2573	1415/1424 (99%)	8/1424 (1%)
KMBS049	*Kluyvera* sp.	2579	1406/1411 (99%)	1/1411 (0%)
KMBS050	*Aeromonas* sp.	2590	1402/1402 (100%)	0/1402 (0%)
KMBS051	*Lactococcus* sp.	2575	1414/1422 (99%)	7/1422 (0%)
KMBS052	*Lactococcus* sp.	2566	1414/1424 (99%)	9/1424 (1%)
KMBS053	*Lactococcus* sp.	2573	1415/1424 (99%)	8/1424 (1%)
KMBS054	*Lactococcus* sp.	2555	1408/1420 (99%)	8/1420 (1%)
KMBS055	*Lactococcus* sp.	2571	1414/1423 (99%)	8/1423 (1%)
KMBS056	*Lactococcus* sp.	2562	1409/1418 (99%)	8/1418 (1%)
KMBS057	*Lactococcus* sp.	2566	1414/1424 (99%)	9/1424 (1%)
KMBS058	*Lactococcus* sp.	2573	1415/1424 (99%)	8/1424 (1%)
KMBS059	*Lactococcus* sp.	2627	1426/1428 (99%)	1/1428 (0%)
KMBS060	*Lactococcus* sp.	2571	1424/1437 (99%)	12/1437 (1%)
KMBS061	*Lactococcus* sp.	2571	1414/1423 (99%)	8/1423 (1%)
KMBS062	*Lactococcus* sp.	2579	1419/1428 (99%)	8/1429 (1%)
KMBS063	*Lactococcus* sp.	2586	1400/1400 (100%)	0/1400 (0%)
KMBS064	*Lactococcus* sp.	2621	1422/1423 (99%)	1/1423 (0%)
KMBS065	*Lactococcus* sp.	2569	1424/1438 (99%)	11/1438 (1%)
KMBS066	*Lactococcus* sp.	2562	1409/1418 (99%)	8/141 (1%)8
KMBS067	*Lactococcus* sp.	2593	1404/1404 (100%)	0/1404 (0%)
KMBS068	*Lactococcus* sp.	2569	1418/1429 (99%)	10/1429 (1%)
KMBS069	*Kluyvera* sp.	2562	1398/1404 (99%)	1/1404 (0%)
KMBS070	*Lactococcus* sp.	2564	1410/1419 (99%)	8/1419 (1%)
KMBS071	*Lactococcus* sp.	2625	1422/1423 (99%)	0/1423 (0%)
KMBS072	*Shewanella* sp.	2591	1410/1415 (99%)	0/1415 (0%)
KMBS073	*Citrobacter* sp.	2558	1403/1412 (99%)	0/1412 (0%)
KMBS074	*Enterobacter* sp.	2516	1400/1419 (99%)	4/1419 (0%)

Table 1. Continued

Identification number	Strains	Score (bits)	Identities	Gaps
KMBS075	*Aeromonas* sp.	2584	1401/1402 (99%)	0/1402 (0%)
KMBS076	*Citrobacter* sp.	2501	1382/1398 (99%)	0/1398 (0%)
KMBS077	*Aeromonas* sp.	2590	1402/1402 (100%)	0/1402 (0%)
KMBS078	*Aeromonas* sp.	2590	1402/1402 (100%)	0/1402 (0%)
KMBS079	*Citrobacter* sp.	2495	1381/1398 (99%)	0/1398 (0%)
KMBS080	*Aeromonas* sp.	2590	1402/1402 (100%)	0/1402 (0%)
KMBS081	*Kluyvera* sp.	2433	1338/1349 (99%)	0/1349 (0%)
KMBS082	*Lactococcus* sp.	2573	1415/1424 (99%)	8/1424 (1%)
KMBS083	*Aeromonas* sp.	2590	1402/1402 (100%)	0/1402 (0%)
KMBS084	*Aeromonas* sp.	2590	1402/1402 (100%)	0/1402 (0%)
KMBS085	*Kluyvera* sp.	2501	1371/1381 (99%)	2/1381 (0%)
KMBS086	*Aeromonas* sp.	2556	1396/1402 (99%)	0/1402 (0%)
KMBS087	*Bacterium* sp.	2497	1387/1405 (99%)	2/1405 (0%)
KMBS088	*Aeromonas* sp.	2590	1402/1402 (100%)	0/1402 (0%)
KMBS089	*Enterobacter* sp.	2566	1419/1437 (99%)	0/1437 (0%)
KMBS090	*Aeromonas* sp.	2590	1402/1402 (100%)	0/1402 (0%)
KMBS091	*Aeromonas* sp.	2590	1402/1402 (100%)	0/1402 (0%)
KMBS092	*Aeromonas* sp.	2590	1402/1402 (100%)	0/1402 (0%)
KMBS093	*Klebsiella* sp.	2579	1400/1403 (99%)	0/1403 (0%)
KMBS094	*Buttiauxella* sp.	2529	1390/1404 (99%)	1/1404 (0%)
KMBS095	*Lactococcus* sp.	2573	1415/1424 (99%)	8/1424 (1%)
KMBS096	*Buttiauxella* sp.	2532	1390/1402 (99%)	0/1402 (0%)
KMBS097	*Shewanella* sp.	2586	1410/1415 (99%)	1/1415 (0%)
KMBS098	*Aeromonas* sp.	2590	1402/1402 (100%)	0/1402 (0%)
KMBS099	*Citrobacter* sp.	2460	1364/1381 (99%)	1/1381 (0%)
KMBS100	*Aeromonas* sp.	2590	1402/1402 (100%)	0/1402 (0%)