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Original Article

Elizabethkingia miricola BM10, a New Symbiotic Bacterium Isolated from the Hindgut of the Termite *Reticulitermes speratus* KMT001[1]

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ABSTRACT

Elizabethkingia miricola BM10, a symbiotic bacterium, has been isolated from the hindgut of *Reticulitermes speratus* KMT001, a termite which occurs on Bukhan Mountain in Seoul, Korea. This strain demonstrated a symbiotic characteristic, in that it lacked endo-β-1,4-glucanase activity, in a previous study. The major fatty acids of *E. miricola* BM10 were iso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH, and summed feature 3 (iso-$C_{16:1}\omega7c$/$C_{16:1}\omega6c$). The content of iso-$C_{17:0}$ 3-OH was higher, while those of ECL 13.566, iso-$C_{17:1}1\omega9c$, and summed feature 4 were lower than the other three type-strains of the *Elizabethkingia* genus. The 16S rRNA phylogenetic analysis confirmed that *E. miricola* BM10 is a new species. The whole genome of *E. miricola* BM10 was sequenced. The average nucleotide identity of strain BM10 as evaluated by pairwise comparison with *E. anophelis* R26, *E. meningoseptica* ATCC 13253, and *E. miricola* GTC 862 was shown to be 91.5%, 81.2%, and 94.29%, respectively. Based on our study results, *E. miricola* BM10 appears to represent a new strain of the genus *Elizabethkingia*.

Keywords: *Elizabethkingia miricola*, termite, symbiotic bacteria, hindgut, new strain

1. INTRODUCTION

Termites are pests that cause a great damage to wood by consuming cellulose as a main food. Generally, termites are known to inhabit subtropical and tropical regions. Two species of lower termites occur in Korea (Wonhoon *et al.*, 2015), and they can be observed at Bukhan Mountain in Seoul, Korea (Cho *et al.*, 2010). During the winter in Korea, the temperature can drop below zero degrees Celsius, so even if termites can avoid freezing in their shelters, they must be physiologically adapted to these low temperatures. Lower termites rely on symbiotic microbes in their gut to assist their digestion (Brune and Ohkuma, 2011). Therefore, some of these symbiotic microorganisms in the intestine need to support the metabolic activity of termites at low temperatures. In a previous study, we found that one of the bacterial species that forms a symbiotic relationship with the termite species *Reticulitermes speratus* KMT001, known as *Elizabethkingia* sp. BM10, produces β-glucosidase at 10°C (Lee *et al.*, 2018). *Elizabethkingia* sp. BM10 has since been re-named

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Elizabethkingia miricola BM10. Because of the unique characteristics that help termites by producing cellulases at low temperatures, further physiological studies of the strains were necessary. In the study we report here, we investigated the physiological characteristics of *E. miricola* BM10.

The genus *Elizabethkingia* is found in a variety of places including people, plants, insects, and water. It is well known to pathogenic bacteria in human. The genus *Elizabethkingia* currently consists of three species (Bernardet *et al.*, 2006; Ceyhan and Celik, 2011). Two species, *E. meningoseptica* and *E. miricola*, were first classified when the genus *Elizabethkingia* was reclassified from the genus *Chryseobacterium*, while the third species, *E. anophelis*, was classified following its isolation from the midgut of the mosquito *Anopheles gambiae* (Kämpfer *et al.*, 2011; Kim *et al.*, 2005). *E. meningoseptica* was first reported more than five decades ago, whereas *E. miricola* was isolated and classified from condensation water of the space station Mir, in 2003 (King, 1959; Li *et al.*, 2003). An additional new species, *E. endophytica*, was suggested based on DNA–DNA hybridization results and some biochemical properties, but a later, whole-genome sequencing comparison suggested that the new species was a subjective synonym of *E. anophelis* (Doijad *et al.*, 2016; Kämpfer *et al.*, 2015). Therefore, the controversial species *E. endophytica* was eliminated from comparisons with the new strain, *E. miricola* BM10. In this study, genetic analysis including genome, fat content analysis, and various physiological metabolism analysis were performed and compared with three type strains in the genus *Elizabethkingia* to determine the taxonomic location of *E. miricola* BM10.

2. MATERIALS and METHODS

2.1. *E. miricola* BM10 culture conditions

E. miricola BM10 was isolated from the hindgut of the termite species *Reticulitermes speratus* KMT001 (Cho *et al.*, 2010). This bacterial strain was deposited at the Korean Collection for Type Culture, under the number KCTC 18449P; at the Korean Culture Collection of Microorganisms, under the number KCCM 76545; and at the National Institute of Technology and Evaluation in Japan, under the number NBRC 112444. *E. miricola* BM10 was cultured in 1% tryptone, 0.5% yeast extract, and 1% sodium carboxymethyl cellulose (TYE-CMC medium) at 26°C for 2 days. For plate cultures, 1.5% agar was added to TYE-CMC medium.

2.2. Analysis of bacterial physiology

The testing of *E. miricola* BM10 bacterial cell physiology was performed by the Korean Culture Center of Microorganisms (Seoul, Korea), using the API® 20NE kit (bioMérieux, Inc., Durham, NC, USA). Cell growth, as based on doubling-time, was measured in tryptic soy broth (TSB, Difco™, Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

2.3. Fatty acid analysis

For fatty acid composition analysis, the four *Elizabethkingia* strains were grown on tryptic soy agar (TSA, Difco™, Becton, Dickinson and Company) at 28°C for 2 days. The fatty acid composition of the four strains was analyzed, according to the Miller method, by the Korean Culture Center of Microorganisms (Miller, 1982). Fatty acid composition was analyzed using the Agilent 6890 Gas Chromatograph (Agilent Technologies, Santa Clara, CA, USA) and HP-1 capillary column (Agilent Technologies). The results were analyzed Sherlock™ Microbial ID System software version 6.2 (MIDI, Inc., Newark, DE, USA). Peaks were identified by retention time, and the relative peak area was calculated by comparison with standard calibration solutions.

2.4. Analysis of genetic information

For the chromosomal DNA analysis, *E. miricola* BM10 was cultivated on TYE-CMC agar plates at 26℃ for 2 days. The colonies on the plates were sampled for 16S rRNA sequence analysis and whole-genome sequencing.

For the initial identification of *E. miricola* BM10, the 16S rRNA gene sequence was analyzed using two primers: 27F (5′-AGAGTTTGATCNTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Kämpfer *et al.*, 2011). The complete genome sequence of *E. miricola* BM10 was analyzed and deposited in the GenBank of the National Center for Biotechnology Information (NCBI) under the accession number CP011059 (Lee *et al.*, 2015).The average nucleotide identity (ANI) of the whole genome sequence of *E. miricola* BM10 was calculated using the ANI calculator (http://enve-omics.ce.gatech.edu/ani/) with 70% minimum identity in alignment options by pairwise comparison with the reference genome sequences of three other type strains: *E. anophelis* R26 (GenBank assembly accession: GCA_000331815.1), *E. meningoseptica* ATCC 13253 (GenBank assembly accession: GCA_000367325.1), and *E. miricola* GTC 862 (GenBank assembly accession: GCA_000769445.1) (Rodriguez-R and Konstantinidis, 2016). Phylogenetic analysis was performed using MEGA 4 software (http://www.megasoftware.net/mega4/mega.html) using three methods: neighbor-joining method, maximum-parsimony method, and unweighted pair-group method with arithmetic mean with the bootstrap phylogeny test with 1000 replications. The 16S rRNA gene sequences were trimmed and used for the phylogenetic analysis.

3. RESULTS and DISCUSSION

The termite species *R. speratus* KMT001 occurs on Bukhan Mountain in Seoul, Korea (Cho *et al.*, 2010). Symbiotic bacteria from the hindgut of *R. speratus* KMT001 were isolated and *E. miricola* BM10 (named *Elizabethkingia* sp. BM10 by Cho *et al*. (2010)) was one of 16 bacterial species isolated (Cho *et al.*, 2010). The bacteria were Gram-negative, non-motile, and non-spore-forming rods (0.5 × 1 μm). Good growth was observed on TSA, TYE-CMC, and Luria-Bertani agar at 26°C, while very slow growth was observed at 4°C. The organism's doubling-time in the exponential growth phase was 1.12 h at 26°C in TSB medium. Colonies were white-yellow, circular, and semi-translucent, and had entirely shiny edges.

E. miricola BM10 could not reduce nitrates to nitrites. It produced indole from tryptophan. Its glucose acidification properties were negative. The bacterium could produce β-glucosidase (esculin hydrolysis), protease (gelatin hydrolysis), β-galactosidase, and cytochrome oxidase, but did not produce arginine dihydrolase and urease. It could assimilate glucose, mannose, N-acetyl-glucosamine, and citrate, but not arabinose, mannitol, maltose, gluconate, caprate, adipate, malate, or phenyl acetate. The biochemical test results are summarized and any differences among the four species are shown in Table 1.

The fatty acid content of *E. miricola* BM10 was compared with that of the three type-strains in the genus *Elizabethkingia*: *E. anophelis* R26, *E. meningoseptica* ATCC 13253, and *E. miricola* GTC 862 (Table 2). The major fatty acids found in *E. miricola* BM10 were iso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH, and summed feature 3 (iso-$C_{16:1}$ ω7*c*/$C_{16:1}$ω6*c*). The iso-$C_{17:0}$ 3-OH content in *E. miricola* BM10 was higher compared with that of the other three *Elizabethkingia* strains, but the iso-$C_{16:0}$ 3-OH and summed feature 3 content in *E. miricola* BM10 was lower compared with that of the other strains.

E. miricola BM10 had two 16S rRNA sequences, and their homology with the 16S rRNA sequences of type strains of the *Elizabethkingia* genus in the NCBI database was analyzed. The homology of the two 16S

Table 1. Characteristics differentiating the *E. miricola* BM10 strain from the other three type-strains of the genus *Elizabethkingia*

Strain	*E. miricola* BM10	*E. anophelis* R26	*E. meningoseptica* ATCC 13253	*E. miricola* GTC 862
Urease	Negative	Negative	Negative	Positive
Mannitol assimilation	Negative	Positive	Positive	Positive
Maltose assimilation	Negative	Positive	Positive	Positive
Adipate assimilation	Negative	Positive	Negative	Negative
Citrate assimilation	Positive	Positive	Negative	Positive

Table 2. Cellular fatty acids of *E. miricola* BM10 and comparison with the three type-strains of *Elizabethkingia* spp. Fatty acids are listed using standard abbreviations (number of carbon atoms:number of double bonds). Fatty acids which had < 1% content of total fatty acids in all four strains are not shown

Fatty acid	*E. miricola* BM10	*E. anophelis* R26	*E. meningoseptica* ATCC 13253	*E. miricola* GTC 862
iso-$C_{13:0}$	1.8 ± 0.1	Tr	1.8 ± 0.0	1.1 ± 0.0
ECL 13.566	ND	ND	ND	ND
iso-$C_{15:0}$	42.6 ± 0.9	45.7 ± 2.2	40.8 ± 0.5	43.5 ± 0.1
iso-$C_{15:0}$3-OH	4.2 ± 0.1	4.6 ± 0.1	4.2 ± 0.0	4.6 ± 0.0
anteiso-$C_{15:0}$	2.6 ± 0.2	2.3 ± 0.2	2.9 ± 0.2	1.8 ± 0.0
$C_{16:0}$	1.3 ± 0.5	0.8 ± 0.1	1.4 ± 0.0	Tr
$C_{16:0}$3-OH	2.7 ± 0.1	2.0 ± 0.2	2.5 ± 0.0	2.5 ± 0.0
iso-$C_{16:0}$3-OH	Tr	1.1 ± 0.3	2.1 ± 0.2	1.8 ± 0.0
ECL 16.580	ND	ND	ND	ND
iso-$C_{17:0}$3-OH	18.2±0.2	16.0 ± 1.6	15.6 ± 1.2	15.6 ± 0.2
iso-$C_{17:1}1\omega 9c$	ND	ND	ND	ND
summed feature 4*	ND	ND	ND	ND
iso-$C_{14:0}$	1.3±0.0	ND	1.9 ± 0.1	1.6 ± 0.1
iso-$C_{16:0}$	Tr	Tr	1.5 ± 0.1	1.0 ± 0.1
summed feature 3†	16.2 ± 0.7	19.4 ± 1.0	19.2 ± 0.5	19.4 ± 1.0
summed feature 9‡	4.1 ± 0.1	3.8 ± 0.1	3.4 ± 0.2	3.4 ± 0.2

Tr, Trace amount (< 1.0%); ECL, equivalent chain length (i.e., the identity of the fatty acid is unknown); ND, not detected

*feature 4: iso-$C_{15:0}$2-OHand/or$C_{16:1}\omega 7c/t$

†feature 3: iso-$C_{16:1}$ω7c/$C_{16:1}\omega 6c$

‡feature 9: $C_{16:0}$10-methyl

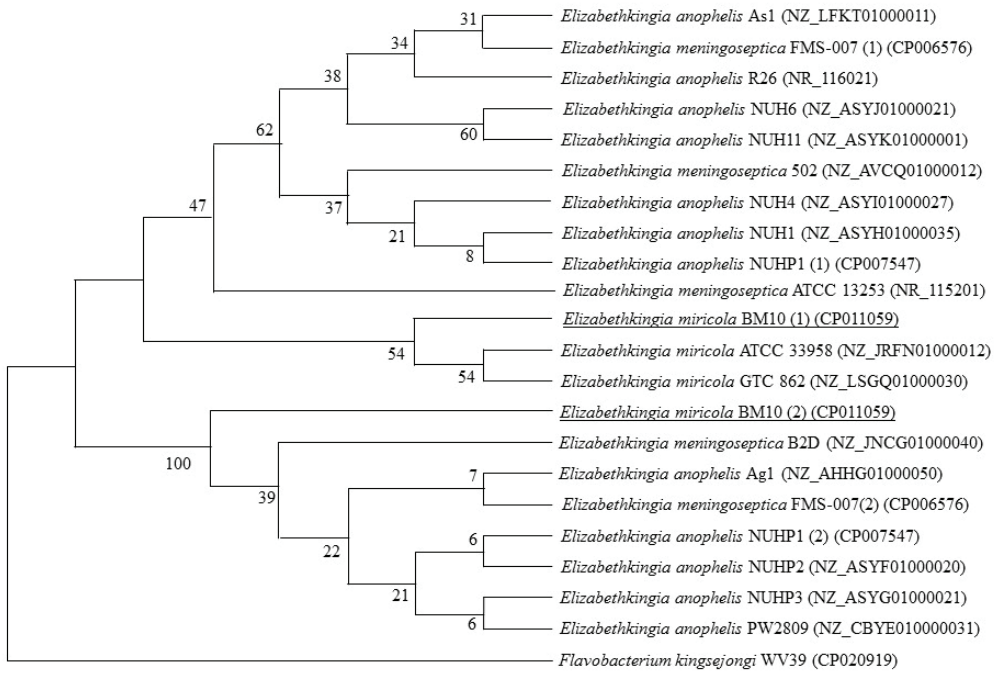

Fig. 1. Phylogenetic analysis of *Elizabethkingia miricola* BM10. A total of 22 sequences of 16S rRNA from 19 strains of *Elizabethkingia* spp., including the other three types-strains, were analyzed to generate the phylogenetic tree. The numbers beside the lines indicate the probability of the same species within the line. This phylogenetic tree was constructed with MEGA version 4 software using the neighbor-joining method and the bootstrap phylogeny test with 1000 replications. The two *E. miricola* BM10 16S rRNA sequences are underlined.

rRNA sequences of *E. miricola* BM10 were 98.3% and 98.7% with *E. anophelis* R26, 98.1% and 98.6% with *E. meningoseptica* ATCC 13253, and 99.4% and 99.4% with *E. miricola* GTC 862.

In the phylogenetic tree using the neighbor-joining method (Fig. 1), the first 16S rRNA sequences of *E. miricola* BM10 were grouped with two *E. miricola* strains, including *E. miricola* GTC 862, but were distinctly separate from the two strains of *E. miricola*. The second set of 16S rRNA sequences of *E. miricola* BM10 was separate from the rest of the sequences in its subgroup. An additional two phylogenetic analyses with the maximum-parsimony method and unweighted pair group method with arithmetic mean showed similar results (data not shown).

The whole genome of *E. miricola* BM10 was sequenced (Lee *et al.*, 2015) and compared with three type-strains of the genus *Elizabethkingia*: *E. anophelis* R26, *E. meningoseptica* ATCC 13253, and *E. miricola* GTC 862 in Table 3 (Assembly ID in the NCBI database: ASM167528v1) (Kukutla *et al.*, 2013; Lee *et al.*, 2015; Matyi *et al.*, 2013). The chromosomal size of *E. miricola* BM10 was 4.24 M bases, with 35.7% GC content, and 3,720 protein-coding genes. The chromosomal DNA size and GC content of *E. miricola* BM10 was similar to that of other strains. The *E. miricola* BM10 gene number was 3,873, which fell somewhere between the gene numbers of *E. anophelis* R26 and *E. miricola* GTC 862. The ANI of *E. miricola* BM10, as determined by pairwise comparison with *E.*

Table 3. Summary of whole-genome sequence analysis of *E. miricola* BM10 and the three type-strains of the genus *Elizabethkingia*

Strain	*E. miricola* BM10	*E. anophelis* R26	*E. meningoseptica* ATCC 13253	*E. miricola* GTC 862
Size (Mb)	4.2	4.0	3.8	4.3
GC%	35.7	35.4	35.6	35.8
Protein	3,720	3,687	3,370	4,006
rRNA	15	-	-	6
tRNA	53	39	45	46
Other RNA	1	-	-	6
Genes	3,873	3,726	3,415	4,064
Assembly ID*	ASM95566v1	ASM33181v1	ASM40141v1	ASM167528v1
Reference	(Lee *et al.*, 2015)	(Kukutla *et al.*, 2013)	(Matyi *et al.*, 2013)	-

* From the database of the National Center for Biotechnology Information

anophelis R26, *E. meningoseptica* ATCC 13253, and *E. miricola* GTC 862, was 91.5%, 81.2%, and 94.3%, respectively. The ANI values of <95% suggested that *E. miricola* BM10 may be a new species in the genus *Elizabethkingia* (Kim *et al.*, 2014). *E. miricola* BM10, a new species, is proposed as an intestinal symbiotic microorganism that is important for the low-temperature growth of termites. Studies on the diversity of intestinal symbiotic microbes in termites will be the basis for understanding the ecology of termites and various efforts to reduce the damage of termites (Kim and Chung, 2017; Lee *et al*., 2017). Additionally, the further studies on *E. miricola* BM10 will reveal low temperature gene expression mechanism that produces cellulase only at low temperature, absence of endoglucase in adaptation for symbiosis with termites, and enzymatic characteristics of the low temperature expressing β-glucosidas.

4. CONCLUSION

E. miricola BM10, a symbiotic gut bacteria of the termite *Reticulitermes speratus* KMT001, showed high activity of both cellobiohydrolase and β-glucosidase, but did not show any endo-β-1,4-glucanase activity, which is an enzyme required for initial cellulose degradation (Cho *et al.*, 2010). *E. miricola* BM10 produced considerable β-glucosidase at 10°C (Lee *et al.*, 2018). Based on its fatty acid composition, whole-genome sequence, and bacterial physiology, *E. miricola* BM10 appears to be distinct from the other three type-strains in the genus *Elizabethkingia*. It is therefore proposed that *E. miricola* BM10 is a new species in the genus *Elizabethkingia*.

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