Journal of Microbiology (2013) Vol. 51, No. 6, pp. 736–740
DOI 10.1007/s12275-013-3499-5

Aestuariibaculum scopimerae sp. nov., Isolated from the Globular Ghost Crab, *Scopimera globosa*

Jae-Bong Lee[1†], Byung-Chun Kim[2†], Hyangmi Kim[3], Kyung Sook Bae[3], Jae-Hyeong Yang[1], Young-Yull Chun[1], Seong-Joon Park[4], and Doo-Sang Park[3*]

[1]*Dokdo Fisheries Research Center, National Fisheries Research and Development Institute, Pohang 791-119, Republic of Korea*
[2]*The Research Institute of Industrial Science, Hanyang University, Seoul 133-791, Republic of Korea*
[3]*Microbiological Resources Center, KRIBB, Daejeon 305-806, Republic of Korea*
[4]*National Institute of Environmental Research, Inchon 404-708, Republic of Korea*

(Received September 27, 2013 / Accepted November 18, 2013)

A Gram-staining-negative, non-motile, catalase- and oxidase-positive bacterium, designated strain I-15^T, was isolated from a crab of the Yellow Sea, Korea. On the basis of a 16S rRNA gene sequence analysis, strain I-15^T was shown to belong to *Bacteroidetes*, related to the genus *Aestuariibaculum*. Sequence similarity between strain I-15^T and the only type strain of the genus *Aestuariibaculum*, *Aestuariibaculum suncheonense* SC17^T, was 96.7%. Strain I-15^T grew at 0.5–6.0% (w/v) NaCl, at 10–42°C and at pH 4.5–8.0. It could hydrolyze starch and Tweens 80. Menaquinone-6 was the only respiratory quinone, and summed features 3 ($C_{16:1}$ *ω7c*/$C_{16:1}$ *ω6c*) (16.4%), iso-$C_{15:0}$ (15.6%), and iso-$C_{15:1}$ G (12.6%) were the major cellular fatty acids. The major polar lipids were phosphatidylethanolamine, two unidentified aminolipids and two unidentified lipids. The DNA G+C content was 39.0 mol%. Polyphasic data allowed genotypic and phenotypic distinction of strain I-15^T from the only validly published *Aestuariibaculum* species. Therefore, the organism is considered a novel species of the genus *Aestuariibaculum*, for which the name *Aestuariibaculum scopimerae* sp. nov. is proposed. The type strain is I-15^T (=KCTC 32459^T =JCM 19486^T).

Keywords: *Aestuariibaculum scopimerae*, polyphasic analysis

Introduction

The genus *Aestuariibaculum* (family *Flavobacteriaceae*, phylum *Bacteroidetes*) was proposed by Jeong *et al.* (2013) for Gram-staining-negative, oxidase- and catalase-positive, non-motile, chemoheterotrophic marine bacteria. The genus currently contains only one species, *Aestuariibaculum suncheonense* SC17^T, which was isolated from a tidal flat (Jeong *et al.*, 2013).

In a study of culturable bacteria from animals living at the sea-shore of the Yellow Sea, Korea, a bacterial strain designated I-15^T was isolated from the gut of a globular ghost crab, *Scopimera globosa* De Haan, 1835. Strain I-15^T was determined to be closely related with the genus *Aestuariibaculum* on the basis of a 16S rRNA gene sequence analysis. In this study, a polyphasic approach was applied to identify the exact taxonomic position of strain I-15^T.

Materials and Methods

Bacterial strains

Strain I-15^T was isolated from the gut of a globular ghost crab collected at the sea-shore of the Yellow Sea, Korea (35°36′18.02″N, 126°16′56.23″E), following inoculation of diluted samples on marine agar 2216 (MA; BD, USA) and incubation at 25°C. The isolate was routinely cultured aerobically on MA at 25°C for 3 days, and stored at -70°C as a suspension in an aqueous glycerol solution (20%, w/v) for long-term preservation. *Aestuariibaculum suncheonense* SC17^T was distributed from KACC and used as a reference strain. For physiological tests, the isolate and the reference strain were cultured on MA or in marine broth 2216 (MB; BD) at 25°C and pH 6.5–7.0. *Escherichia coli* KCTC 2441^T was used as a reference for G+C mol%.

Phylogenetic and genomic analyses

The genomic DNA from strain I-15^T and KCTC 2441^T was extracted according to Sambrook and Russell (2001). The 16S rRNA gene of strain I-15^T was amplified by PCR using the universal primers 27F and 1492R (Lane, 1991). The sequence of the amplified 16S rRNA gene was analyzed as described by Kim *et al.* (2012a). The closely related taxa were determined by database searches, and their sequences were retrieved from the EzTaxon-e (http://eztaxon-e.ezbiocloud.net/) server (Kim *et al.*, 2012b) and GenBank (http://www.ncbi.nlm.nih.gov/blast/). The sequences were multiply aligned using Clustal_X (Thompson *et al.*, 1997).

An alignment of 16S rRNA gene sequences was used for tree construction. Neighbor-joining (Saitou and Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum parsimony (Fitch, 1971) phylogenetic trees were constructed using MEGA5 software (Tamura *et al.*, 2011). Phylogenetic distances were calculated using Kimura's two-parameter method (Kimura, 1980). A bootstrap analysis using 1000

[†]These authors contributed equally to this work.
*For correspondence. E-mail: dspark@kribb.re.kr; Tel.: +82-42-860-4656; Fax: +82-42-860-4677

replicates was performed to assess the confidence limits of phylogenetic trees (Felsenstein, 1985).

G+C mol% of strain I-15^T was calculated according to a method reported by Mesbah *et al.* (1989). The genomic DNA was hydrolyzed and dephosphorylated with nuclease P1 and alkaline phosphatase, respectively, and the mixture of nucleosides was analyzed by HPLC (Shimadzu, Japan) equipped with a reversed-phase column (YMC-Pack ODS-A).

Morphological, physiological, and biochemical characterization

Phenotypic characteristic tests were performed with cells cultured for 3 days at 25°C on MA. Gram type was determined by using a Gram stain kit (BD). Oxidase activity was determined by color change after addition of oxidase reagent (bioMérieux), and catalase activity was determined by bubble production in a 3% (v/v) hydrogen peroxide solution. Cell morphology was observed by light (Eclipse 80i; Nikon, Japan), transmission electron (TEM; CM20, Philips, Netherlands) and scanning electron (SEM; S4300N; Hitachi, Japan) microscopy. Gliding motility was observed with microscopy according to the hanging drop method (Bernardet *et al.*, 2002). The presence of flexirubin-type pigments was assessed according to a previously described method using 20% KOH (Fautz and Reichenbach, 1980). The initial pH range for growth was determined in MB. For different pH values, the pH of MB was adjusted to pH 4.0–10.0 (in 0.5 pH unit increments) using buffers (0.1 M sodium citrate-citric acid, 0.2 M sodium phosphate, and 0.1 M sodium carbonate-bicarbonate), and the medium was sterilized by filtration. The growth temperature range was determined on MA after cultivation at 4, 10, 15, 20, 25, 30, 37, 42, and 45°C. The NaCl concentration for growth was determined in MB prepared according to the composition of the BD medium without NaCl and subsequently supplemented with 0–7% (w/v) NaCl (in 0.5% increments). Growth in liquid media was measured by OD_{600}. Anaerobic growth was determined on a MA plate in a GasPak EZ Anaerobe Pouch System (BD). Hydrolysis of different substrates was assessed after incubation at 25°C for 7 days. Hydrolysis of casein, carboxymethyl cellulose (CMC), and starch was assessed on MA containing 3% (w/v) skim milk (BD), 0.5% (w/v) CMC (Sigma), and 0.2% (w/v) soluble starch (BD), respectively. Hydrolysis of CMC and starch was confirmed by the presence of a clear halo zone around the colonies after staining with 1% Congo red solution or Lugol's iodine solution (Teather and Wood, 1982), respectively. Hydrolysis of DNA was tested on DNase agar (BD). Hydrolysis of Tweens 20, 40, 60, and 80 was assessed by the formation of an opaque halo (Sierra, 1957). Other physiological characteristics of strain I-15^T were tested using API 20 NE and API ZYM strips (bioMérieux, France) and Biolog GN microplates (Biolog, USA) according to the manufacturers' instructions. After inoculation, API 20 NE strips and Biolog GN microplates were incubated at 25°C for 2 days. API ZYM strips were incubated for 4 h at 25°C for strain I-15^T.

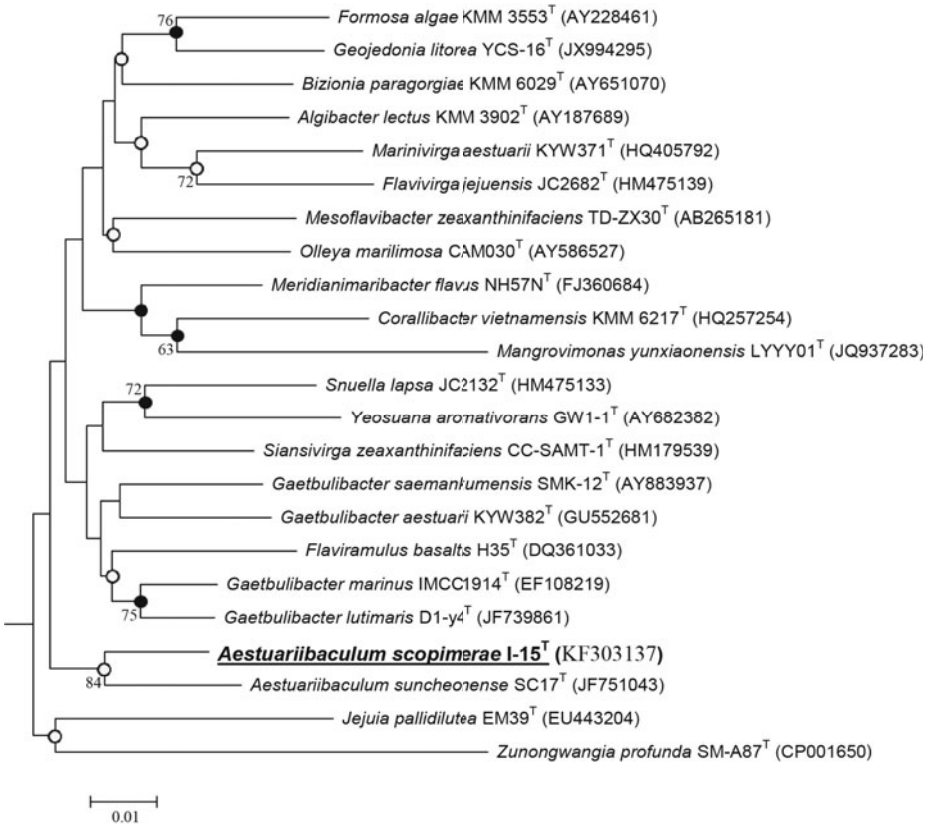

Fig. 1. Phylogenetic tree showing the relationships of strain I-15^T, *Aestuariibaculum suncheonense* SC17^T and representatives of the family *Flavobacteriaceae*. The tree was based on an alignment of 1,189 bases 16S rRNA gene sequences and constructed by using the neighbor-joining method. Black circles indicate that the corresponding branches are also recovered in both the maximum-likelihood and maximum parsimony trees. White circles indicate that the corresponding branches are recovered in only the maximum-likelihood or the maximum parsimony tree. Bootstrap values (expressed as percentages of 1,000 replications) greater than 70% are shown at the nodes. GenBank accession numbers of the 16S rRNA gene sequences are given in parentheses. Bar, 0.01 substitutions per nucleotide position.

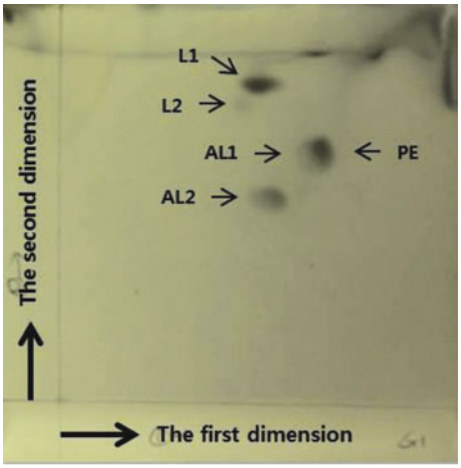

Fig. 2. Thin-layer chromatography of the total polar lipids of strain I-15^T. PE, phosphatidylethanolamine; AL1-2, unidentified aminolipids; and L1-2, unidentified polar lipids.

Chemotaxonomy

Fatty acid methyl esters (FAMEs) were analyzed according to the standard protocol of the Microbial Identification System (MIDI) (Sasser, 2001). Colonies approximately 1 mm in diameter from quadrant sectors on MA after about 3 days of incubation at 25°C were collected; FAMEs were extracted according to Sasser (2001). Fatty acids were separated by gas chromatography (HP 6890 N; Agilent) and identified with the Sherlock software package (MIDI Sherlock system 6.1, TSBA library version 6.0, MIDI).

For isoprenoid quinone and polar lipid analyses, strain I-15^T was incubated on MB at 25°C for 3 days. Isoprenoid quinones of strain I-15^T were extracted from freeze-dried cells according to a method described previously (Komagata and Suzuki, 1987). The extracted quinone content was purified using preparative thin-layer chromatography (TLC; silica gel F254; Merck, Germany) and identified by HPLC (Shimadzu).

Polar lipids were extracted from strain I-15^T and analyzed by two-dimensional TLC (silica gel F254) according to the methods described by Tindall (1990) and Altenburger *et al.* (1996). The total lipids were detected by spraying one TLC plate with 5% ethanolic molybdophosphoric acid. Amino lipids, phospholipids, and glycolipids were specifically detected by spraying other TLC plates with ninhydrin, molybdenum blue and alpha-naphthol, respectively.

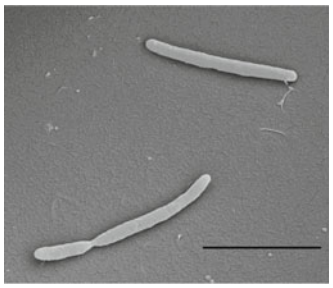

Fig. 3. Scanning electron micrograph of strain I-15^T. Cells were cultured for 3 days at 25°C on MA. Bars, 3 μm.

Results and Discussion

Phylogenetic and genomic analysis

The nearly complete 16S rRNA gene sequence of strain I-15^T, comprising 1449 nucleotides, was determined. This 16S rRNA gene sequence was most closely related to the genus *Aestuariibaculum*. The nearest phylogenetic neighbor of strain I-15^T was *Aestuariibaculum suncheonense* SC17^T with 96.7% sequence similarity. The next closest relatives were members of the genera *Gaetbulibacter*, *Siansivirga*, and *Bizionia*. The 16S rRNA gene sequence of strain I-15^T is deposited in GenBank under accession number KF303137. Strain I-15^T fell within the cluster comprising *Flavobacteriaceae* species in the neighbor-joining, maximum parsimony and maximum-likelihood phylogenetic trees (Fig. 1). The DNA G+C content of strain I-15^T was 39.0 mol%, while that of *A. suncheonense* SC17^T was 46.4 mol% (Jeong *et al.*, 2013).

Morphological, physiological, and biochemical characteristics

Strain I-15^T is Gram-straining-negative, non-motile rods (Fig. 3). The colonies are raised, smooth, circular, with an entire edge and dark yellow in color. The other phenotypic characteristics of strain I-15^T are described in the species description. The differentiating characteristics of strain I-15^T and *A. suncheonense* SC17^T are listed in Table 1.

Chemotaxonomy

The only respiratory quinone in strain I-15^T was menaquinone-6 (MK-6). MK-6 is reported to be the major or only

Table 1. Differentiating properties of strain I-15^T and *Aestuariibaculum suncheonense* SC17^T. Both strains are catalase- and oxidase-positive, obligatory aerobic, not motile by flagella or gliding, have MK-6 as the only respiratory quinone, and do not produce flexirubin-type pigments. In the API ZYM and API 20E strip, both strains are positive for esculin, *β*-galactosidase, alkaline phosphatase, leucine arylamidase, *N*-acetyl-*β*-glucosaminidase, acid phosphatase, and maltose assimilation, but negative for valine arylamidase, crystine arylamidase, trypsin, *α*-chymotrypsin, esterase (C4), lipase (C14), *α*-galactosidase, *β*-galactosidase, *β*-glucuronidase, *α*-glucosidase, *α*-mannosidase, *α*-fucosidase, nitrate reduction, and assimilation of glucose, mannose, mannitol, *N*-acetyl-glucosamine, caprate, adipate, malate, citrate, and phenyl-acetate.
+, Positive; w, weakly positive; -, negative.

Characteristics	I-15^T	*A. suncheonense* SC17^T
Cell size (μm)[b]	0.3–0.7 × 1.4–4	0.4–0.5 × 2–3
Growth at/in:[b]		
Temp. range (°C)	10–42 (25–30)[a]	5–40 (25–30)
NaCl range % (w/v)	0.5–6 (2–3)	1–8 (1–2)
pH range	4.5–8.0 (6.5–7.5)	6.0–8.5 (7.0)
DNAse	-	+
Tween 80 hydrolysis	+	-
Esterase lipase (C8)	w	-
Naphtol-AS-BI-phosphohydrolase	w	+
Glucose assimilation	-	+
Arabinose assimilation	-	+
Gluconate assimilation	w	-
GC contents (mol%)[b]	39	46.4

[a] Optimum conditions are given in parentheses
[b] Data for *A. suncheonense* SC17^T are from Jeong *et al.* (2013).

respiratory quinone in all members of the family *Flavobacteriaceae* (Bernardet, 2011). The major cellular fatty acids of strain I-15^T were summed features 3 ($C_{16:1}$ *ω7c*/$C_{16:1}$ *ω6c*) (16.4%), iso-$C_{15:0}$ (15.6%), and iso-$C_{15:1}$ G (12.6%) (Table 2). Phosphatidylethanolamine, two unidentified aminolipids and two unidentified lipids were observed in strain I-15^T as the major polar lipids (Fig. 2). *A. suncheonense* SC17^T contained phosphatidylethanolamine, two unidentified aminolipids and four unidentified lipids (Jeong *et al.*, 2013).

Taxonomic conclusion

Strain I-15^T shared 96.7% 16S rRNA gene sequence similarity with *A. suncheonense* SC17^T demonstrating that this strain represents a new species in the genus *Aestuariibaculum* (Stackebrandt and Goebel, 1994), and the isolate can be differentiated from *A. suncheonense* SC17^T by the phenotypic characteristics (Table 1). Therefore, on the basis of genotypic and phenotypic properties, strain I-15^T is considered to represent a novel species of the genus *Aestuariibaculum*, for which the name *Aestuariibaculum scopimerae* sp. nov. is proposed.

Description of *Aestuariibaculum scopimerae* sp. nov.

Aestuariibaculum scopimerae (sco.pi.me'rae. N.L. gen. n. *scopimerae* of or belonging to Scopimera, isolated from the gut of *Scopimera globosa*, a globular ghost crab where the type strain was isolated).

Cells are Gram-staining-negative, strictly aerobic, non-motile and catalase- and oxidase-positive. Cells are straight or slightly curved rods about 1.4–4.0 μm in length and 0.3–0.7 μm in diameter. Flexirubin-type pigments are not produced. Colonies are dark yellow, circular with entire edges and convex, with a diameter of 1 mm after 3 days incubation on MA. Growth occurs at 10–42°C (optimum, 25–30°C), at pH 4.5–8.0 (optimum, pH 6.5–7.5) and in the presence of 0.5–6% (w/v) NaCl (optimum, 2–3%). Starch and Tweens 80 are hydrolysed, but DNA, casein, CMC and Tweens 20, 40, and 60 are not. In the API 20 NE strip, positive for maltose assimilation, *β*-glucosidase and *β*-galactosidase activities, weakly positive for gluconate assimilation and negative for all other tests. In the API ZYM strip, alkaline phosphatase, leucine arylamidase, acid phospatase and *N*-acetyl-*β*-glucosaminidase activities are present; esterase lipase (C8) and naphtol-AS-BI-phosphohydrolase activities are weakly present; the other enzyme activities are absent. In the Biolog GN microplate, positive for dextrin, glycogen, L-arabinose, D-lactose, maltose, D-trehalose, and succinic acid mono-methyl ester; weakly positive for *α*-cyclodextrin, D-cellobiose, lactulose, acetic acid, *γ*-hydroxybutyric acid, L-glutamic acid, and D-glucose-1-phosphate; negative for other carbohydrate utilization. The only respiratory quinone is MK-6. The major cellular fatty acids (>10%) are summed features 3 ($C_{16:1}$ *ω7c*/$C_{16:1}$ *ω6c*), iso-$C_{15:0}$, iso-$C_{15:1}$ G, and iso-$C_{17:0}$ 3OH. The major polar lipids are phosphatidylethanolamine, two unidentified aminolipids and two unidentified lipids. The DNA G+C content of the type strain is 39.0 mol%. The type strain is I-15^T (=KCTC 32459^T =JCM 19486^T), isolated from the gut of a globular ghost crab collected at a seashore region of the Yellow Sea, Korea.

Table 2. Cellular fatty acid compositions (%) of strain I-15^T and *Aestuariibaculum suncheonense* SC17^T. Fatty acids comprising less than 1% of the total in all strains are not shown. All data from this study. tr, Trace (less than 1%); -, not detected.

Fatty acid	I-15^T	*A. suncheonense* SC17^T
Saturated		
$C_{15:0}$ 2OH	2.0	1.1
$C_{16:0}$	2.6	4.1
$C_{16:0}$ 3OH	2.7	3.3
$C_{17:0}$ 2OH	3.5	2.3
iso $C_{14:0}$	tr	1.1
iso $C_{15:0}$	15.6	22.2
iso $C_{15:0}$ 3OH	7.7	4.7
iso $C_{16:0}$	tr	2.2
iso $C_{16:0}$ 3OH	3.7	4.5
iso $C_{17:0}$ 3OH	11.9	11.1
anteiso $C_{15:0}$	9.6	7.7
Unsaturated		
iso $C_{15:1}$ G	12.6	11.3
iso $C_{16:1}$ H	1.1	1.5
anteiso $C_{15:1}$ A	3.5	1.7
anteiso $C_{17:1}$*ω9c*	1.1	1.0
Summed features[a]		
3	16.4	11.7
9	1.7	1.8

[a] Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 3 consisted of $C_{16:1}$ *ω7c* and/or $C_{16:1}$ *ω6c*. Summed feature 9 consisted of iso-$C_{17:1}$ *ω9c* and/or $C_{16:0}$ 10-methyl.

Acknowledgements

We are grateful to Dr. J.P. Euzéby for his advice on naming this microorganism. This work was supported by a grant from the Dokdo Fisheries Research Center, National Fisheries Research and Development Institute (RP-2013-FR-075) and a grant from the KRIBB Research Initiative Program.

References

Altenburger, P., Kämpfer, P., Makristathis, A., Lubitz, W., and Busse, H.-J. 1996. Classification of bacteria isolated from a medieval wall painting. *J. Biotechnol.* **47**, 39–52.

Bernardet, J.F. 2011. Family I. *Flavobacteriaceae* Reichenbach 1992, pp. 106–111. *In* Whitman, W. (ed.), Bergey's Manual of Systematic Bacteriology, The Williams & Wilkins Co., Baltimore, USA.

Bernardet, J.F., Nakagawa, Y., and Holmes, B. 2002. Proposed minimal standards for describing new taxa of the family *Flavobacteriaceae* and emended description of the family. *Int. J. Syst. Evol. Microbiol.* **52**, 1049–1070.

Fautz, E. and Reichenbach, H. 1980. A simple test for flexirubin-type pigments. *FEMS Microbiol. Lett.* **8**, 87–91.

Felsenstein, J. 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. *J. Mol. Evol.* **17**, 368–376.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.

Fitch, W.M. 1971. Toward defining the course of evolution: minimum change for a specific tree topology. *Syst. Zool.* **20**, 406–416.

Jeong, S.H., Park, M.S., Jin, H.M., Lee, K., Park, W., and Jeon, C.O. 2013. *Aestuariibaculum suncheonense* gen. nov., sp. nov., a marine

bacterium of the family *Flavobacteriaceae* isolated from a tidal flat and emended descriptions of the genera *Gaetbulibacter* and *Tamlana*. *Int. J. Syst. Evol. Microbiol.* **63**, 332–338.

Kim, B.C., Park, D.S., Kim, H., Oh, H.W., Lee, K.H., Shin, K.S., and Bae, K.S. 2012a. *Herbiconiux moechotypicola* sp. nov., a xylanolytic bacterium isolated from the gut of hairy long-horned toad beetles, *Moechotypa diphysis* (Pascoe). *Int. J. Syst. Evol. Microbiol.* **62**, 90–95.

Kim, O.S., Cho, Y.J., Lee, K., Yoon, S.H., Kim, M., Na, H., Park, S.C., Jeon, Y.S., Lee, J.H., Yi, H., and *et al.* 2012b. Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int. J. Syst. Evol. Microbiol.* **62**, 716–721.

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **16**, 111–120.

Komagata, K. and Suzuki, K. 1987. Lipids and cell-wall analysis in bacterial systematics. *Methods Microbiol.* **19**, 161–203.

Lane, D.J. 1991. 16S/23S rRNA sequencing, pp. 115–175. *In* Stackebrandt, E. and Goodfellow, M. (eds.), Nucleic acid techniques in bacterial systematics, Wiley, New York, USA.

Mesbah, M., Premachandran, U., and Whitman, W.B. 1989. Precise measurement of the G+C content of deoxyribonucleic acid by high performance liquid chromatography. *Int. J. Syst. Bacteriol.* **39**, 159–167.

Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**, 406–425.

Sambrook, J. and Russell, D.W. 2001. Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Laboratory, New York, N.Y., USA.

Sasser, M. 2001. Identification of bacteria by gas chromatography of cellular fatty acids, Technical note 101. MIDI Inc., Newark, DE, USA.

Sierra, G. 1957. A simple method for the detection of lipolytic activity of micro-organisms and some observations on the influence of the contact between cells and fatty substrates. *Antonie van Leeuwenhoek.* **23**, 15–22.

Stackebrandt, E. and Goebel, B.M. 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int. J. Syst. Bacteriol.* **44**, 846–849.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28**, 2731–2739.

Teather, R.M. and Wood, P.J. 1982. Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. *Appl. Environ. Microbiol.* **43**, 777–780.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Higgins, D.G. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25**, 4876–4882.

Tindall, B.J. 1990. Lipid composition of *Halobacterium lacusprofundi*. *FEMS Microbiol Lett.* **66**, 199–202.