

# *Klebsiella michiganensis* sp. nov., A New Bacterium Isolated from a Tooth Brush Holder

Ratul Saha · Christine E. Farrance ·  
Bindhu Verghese · Sunhee Hong · Robert S. Donofrio

Received: 24 August 2012 / Accepted: 20 September 2012 / Published online: 6 October 2012  
© Springer Science+Business Media New York 2012

**Abstract** Isolate W14<sup>T</sup> recovered from a household tooth brush holder was found to be gram-negative, a facultative anaerobic, non-motile, capsulated, and a non-endospore-forming straight rod. Based on phylogenetic analysis with 16S rRNA gene sequence, isolate W14<sup>T</sup> was affiliated to the genus *Klebsiella*. The closest phylogenetic relative was *K. oxytoca* with 99 % similarity in the 16S rRNA gene sequence. The major whole-cell fatty acids were C<sub>16:0</sub> (31.23 %), C<sub>18:1ω6c</sub>/C<sub>18:1ω7c</sub> (21.10 %), and C<sub>16:1ω7c</sub>/C<sub>16:1ω6c</sub> (19.05 %). The sequence similarities of isolate W14<sup>T</sup> based on *rpoB*, *gyrA*, and *gyrB* were 97, 98, and 98 % with *K. oxytoca*, and 97, 93, and 90 % with *K. mobilis* (= *Enterobacter aerogenes*), respectively. The ribotyping pattern showed a 0.46 similarity with *K. oxytoca* ATCC 13182<sup>T</sup> and 0.24 with *K. mobilis* ATCC 13048<sup>T</sup>. The DNA G+C content of isolate W14<sup>T</sup> was 54.6 mol%. The DNA–DNA relatedness was 55.7 % with *K. oxytoca* ATCC 13182<sup>T</sup>. Using the identification technology of MALDI-TOF mass spectrometry, the top matches for this isolate were *K. oxytoca* ATCC 13182<sup>T</sup> (Match Factor Score 1.998) and *K. mobilis* (Score 1.797). On the basis of

phenotypic, biochemical, chemotaxonomic, and molecular studies, isolate W14<sup>T</sup> could be differentiated from other members of the genus *Klebsiella* including *K. mobilis*. Therefore, it is proposed that isolate W14<sup>T</sup> (=ATCC BAA-2403<sup>T</sup>=DSM 25444<sup>T</sup>) should be classified as the type strain of a novel species of the genus *Klebsiella*, *K. michiganensis* sp. nov.

## Introduction

The genus *Klebsiella* belongs to the *Enterobacteriaceae* family and was named by Trevisan in 1885. The genus is heterogeneous and originally composed of three major clusters, cluster I consisting of *K. granulomatis* and *K. pneumoniae* along with its three subspecies (*K. pneumoniae* subsp. *pneumoniae*, *K. pneumoniae* subsp. *rhinoscleromatis*, and *K. pneumoniae* subsp. *ozaenae*) and cluster III consisting of *K. oxytoca*. The organisms originally belonging to cluster II were placed into a new genus, *Raoultella* containing *R. terrigena*, *R. ornithinolytica*, and *R. planticola*, based on biochemical, DNA–DNA hybridization and phylogenetic relationship [7]. It is also important to note that *K. mobilis* (= *E. aerogenes*) is more closely related to the genus *Klebsiella* than *E. cloacae* as evident from the phylogenetic relationship based on *rpoB*, *gyrA*, and *gyrB* gene sequences, shown here.

During a study investigating the microbial hot spots in the residential household, the overall levels of contamination on different surfaces, and the identification of the isolates was determined. One isolate, designated as W14<sup>T</sup>, was recovered from one of the Michigan households located in the town of Ypsilanti during the microbial hot-spot study. A polyphasic approach was implemented for the characterization of isolate W14<sup>T</sup>. Evidence indicated

The GenBank accession numbers for the 16S rRNA gene, *rpoB*, *gyrB*, and the *gyrA* nucleotide sequences for strain W14<sup>T</sup> are JQ070300, JQ269337, JQ284304, and JQ990329.

**Electronic supplementary material** The online version of this article (doi:10.1007/s00284-012-0245-x) contains supplementary material, which is available to authorized users.

R. Saha (✉) · R. S. Donofrio  
Microbiology Division, NSF International, 789 Dixboro Road,  
Ann Arbor, MI 48105, USA  
e-mail: rsaha@nsf.org

C. E. Farrance · B. Verghese · S. Hong  
Accugenix Inc., 223 Lake Drive, Newark, DE 19702, USA

that this isolate belonged to the genus *Klebsiella* within the *K. oxytoca* cluster [7, 12].

## Materials and Methods

### Bacterial Strain and Cultivation

Isolate W14<sup>T</sup> was recovered from a tooth brush holder on R2A agar medium at 30 °C. The swab utilized to collect the sample was 3 M<sup>TM</sup> Quick Swab, a rayon-tipped swab containing letheen neutralizing buffer (3 M, Minneapolis, MN, USA). The isolate was grown on Tryptic Soy Agar (TSA) (BD, Franklin Lakes, NJ) at its optimum temperature of 35 °C for further maintenance and biochemical analysis. The reference strains used in the study, *K. oxytoca* ATCC 13182<sup>T</sup>, *K. mobilis* ATCC 13048<sup>T</sup>, *K. pneumoniae* ATCC 13883<sup>T</sup>, and *R. terrigena* ATCC 33257<sup>T</sup> were grown and maintained as per ATCC's instructions.

### Biochemical and Phenotypic Characteristics

Phenotypic characteristics were observed on TSA after 24 h of incubation at 35 °C. Differential-selective media such as MacConkey, XLD, and Levine EMB (BD, Franklin Lakes, NJ) were used to confirm its position in the family *Enterobacteriaceae* and also to determine its growth potential on different media. Gram staining (Four-step Gram Stain Kit, BD, Franklin Lakes, NJ), motility (Motility Agar Stab), microscopic observations (Axioskop 2 Plus Imaging system, Carl Zeiss, Göttingen, Germany), and biochemical analysis were performed according to standard laboratory procedure [1]. The API20E (bioMérieux, Inc. Durham, NC), Biolog (GN2 MicroPlates, Biolog, Inc. Hayward, CA), and Enterotube II (BD, Franklin Lakes, NJ) were used to obtain the identification and biochemical profile according to the manufacturers' instructions.

### Fatty Acid Methyl Ester Analysis (FAME)

A pure culture of isolate W14<sup>T</sup> was used to perform FAME according to the protocols and software provided by Microbial ID (MIDI, Newark, DE) for the Sherlock Microbial Identification System (MIS) using automated gas chromatography. The FAME analysis was performed by growing isolate W14<sup>T</sup> on Tryptic Soy Broth Agar (TSBA) at 28 °C for 24 h and the derived FAME profile was compared against the TSBA66 database.

### MALDI-TOF Analysis

Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectroscopy-based microbial

identification was performed on the autoflex<sup>TM</sup> mass spectrometer according to the manufacturer's recommendations (Bruker Daltonics, Inc., Fremont, CA). Briefly, an ethanol extract method was used to process the sample which was less than 72-h old. Ethanol extraction consisted of resuspending a 1- $\mu$ L loop full of the bacterial sample in 300  $\mu$ L of nuclease free water and adding 900  $\mu$ L of 100 % ethanol. The cell material was then pelleted by centrifugation. The cell pellets were resuspended in formic acid and acetonitrile and 1  $\mu$ L spotted onto the target plate. After samples had dried on the plate, they were overlaid with the  $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA, Protea Biosciences, Inc., Morgantown, WV) matrix and spectra produced for comparison to the reference library for identification of the isolate.

### DNA Base Composition and DNA–DNA Hybridization

The DNA base composition and the DNA–DNA hybridization were performed at DSMZ. Determination of DNA base (G+C) composition was performed by the HPLC method. Cell disruption was performed by using a constant systems TS 0.75 KW instrument (IUL Instruments, Germany). The DNA was purified on hydroxyapatite according to the procedure of Cashion et al. [4]. The DNA was hydrolyzed with P1 nuclease and the nucleotides dephosphorylated with bovine alkaline phosphatase [13]. The resulting deoxyribonucleosides were analyzed by HPLC. For DNA–DNA relatedness, cells of a pure culture of isolate W14<sup>T</sup> and *K. oxytoca* ATCC 13182<sup>T</sup> were disrupted by using constant systems TS 0.75 KW instrument (IUL Instruments, Germany), and the DNA in the crude lysate was purified by chromatography on hydroxyapatite as described by Cashion et al. [4]. The DNA–DNA hybridization test was conducted using the spectroscopic method described by De Ley et al. [6], with modification described by Huss et al. [8] in 2 $\times$  SSC + 5 % formamide at 70 °C using a model Cary100 Bio UV/Vis-spectrophotometer equipped with a Peltier-thermostatted 6  $\times$  6 multi-cell changer and a temperature controller with in situ temperature probe (Varian, Palo Alto, CA). DNA similarity values were determined in duplicate.

### Automated Ribotyping

Automated ribotyping of the isolate W14<sup>T</sup> was performed with *Eco*RI digested samples using a RiboPrinter<sup>®</sup> system (DuPont Qualicon, Wilmington, DE) using the recommended methods from the manufacturer.

### Gene Sequencing

Sequencing of the 16S rDNA region was performed by Accugenix, Inc. using proprietary methods. Briefly, the

DNA was isolated with standard alkaline lysis methods and the extract used in PCR amplification with standard M13-tailed 16S primers, 0005F and 1540R, with the primer numbering based on the *Escherichia coli* 16S rRNA gene [3]. Sequencing reactions were carried out using manufacturer's recommendations and BigDye<sup>®</sup> Terminator v1.1 cycle sequencing kits (Life Technologies, Carlsbad, CA) and run on the ABI 3130XL capillary sequencer (Life Technologies, Carlsbad, CA). Protein-coding genes were also amplified using M13-tailed gene specific primers and subjected to cycle sequencing as above. Consensus sequences for *rpoB* and *gyrB* were generated using information resulting from the amplification with two sets of primers for each gene. The full length sequences were used in the analyses. Primers for *gyrA*: entero\_gyrA6F, 5'-GTAAAACGACGGCCAGTCGACCTTGCGAGAGAAAT-3'; entero\_gyrA631R, 5'-CAGGAAACAGCTATGACGTTCCATCAGCCCTTCAA-3' were used. Primers for *rpoB*: entero\_rpoBb\_F, 5'-AA CAACCCGCTGTCTGAGAT-3'; entero\_rpoBb\_R, 5'-CATGTTTCGACCCATCAAT-3'; entero\_rpoBe\_F, 5'-CAGGCGAACTCCAACCTG-3'; entero\_rpoBe\_R, 5'-CGCCA GTTACCCGAGGTC-3' were used. Primers for *gyrB*: entero\_gyrB\_F, 5'-CCGATCCACCCGAATATCT-3'; entero\_gyrB\_R, 5'-CCCTTCCACCAGGTACAGTT-3'; entero\_gyrBd\_F, 5'-GTAAAACGACGGCCAGTYGCNGGNGG NAARTTYGA-3'; entero\_gyrBd\_R, 5'-CAGGAAACAGCTATGACARRTGNGTNCNC-3' were used. Similarity matches for the sequences of the 16S rDNA, *rpoB*, *gyrA*, and *gyrB* genes were obtained by performing BLAST. The Ribosomal Database Project (RDP) database [5] was also searched for the 16S rDNA similarity matches. Multiple alignment of the sequences were performed using the CLUSTALX [19] software, a distance matrix was created using Kimura's 2-parameter model [10] and neighbor-joining phylogenetic method was applied for the construction of the tree using the software MEGA5 [18] for 16S rRNA, *rpoB*, *gyrB*, and *gyrA* gene sequence. Bootstrapping was performed by using 1,000 replicates.

#### PCR Analysis of “*pehX*” and “*gyrA*” Gene

Kovtunovych et al. [11] developed a specific method to discriminate *K. oxytoca* from other species of the genus *Klebsiella* based on PCR amplification of the polygalacturonase (*pehX*) gene (344 bp) for clinical and ecological monitoring of *K. oxytoca*. In this study, the primer pair developed by Kovtunovych et al. was used to detect if the *pehX* gene is present in the isolate W14<sup>T</sup>. Also, the genus specific primer targeting the *gyrA* gene developed by Brisse and Verhoef [2] was used for the amplification and sequencing of the genus specific region (441 bp) for *Klebsiella* to confirm the position of the isolate W14<sup>T</sup> within this genus.

DNA was isolated using the PrepSEQ Rapid Spin reagent (Life Technologies, Foster City, CA) according to

the manufacturer's instructions. The purity and the yield of the DNA were measured using the NanoDrop 1000 spectrophotometer. The primer pair, forward 5' GAT ACG GAG TAT GCC TTT ACG GTG 3' and reverse 5' TAG CCT TTA TCA AGC GGA TAC TGG 3' was used for the amplification of the *pehX* gene. PCR was performed using Thermo-Start Master Mix (Thermo Scientific, Waltham, MA) and the reaction mixture consisted of 10 µL of master mix, 2.5 µL of PCR enhancer, 0.3 µL (10 µM) of forward and reverse primer, 1 µL (100 ng) of DNA template, and 4.9 µL of water to make the final volume of 20 µL. For the *gyrA* amplification the primer pair, forward 5' CGC GTA CTA TAC GCC ATG AAC GTA 3' and reverse 5' ACC GTT GAT CAC TTC GGT CAG G 3' was used. The reaction mixture consisted of 10 µL of master mix, 2.5 µL of PCR enhancer, 1 µL of BSA, 0.3 µL of forward and reverse primer, 0.5 µL of DNA template, and 5.4 µL of water to make the final volume of 20 µL. The reaction conditions for the PCR were initial denaturation at 95 °C for 15 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min 30 s, and final extension at 72 °C for 10 min. The PCR products were visualized on 2 % agarose gel (E-Gel<sup>®</sup>, Life Technologies, Foster City, CA). A 100-bp DNA ladder was used as molecular weight marker. PCR products were purified using QIAquick PCR purification kit (Qiagen, Valencia, CA) and sequenced both directions with the same primers as used in the PCRs. The obtained sequence of the *gyrA* gene was matched using BLAST for sequence similarity.

## Results and Discussion

### Morphology

Well-isolated colonies of W14<sup>T</sup> appeared within 24 h of incubation at 35 °C on both R2A agar and TSA media. The colonies were white, circular with entire margin and convex elevation (supplementary file). They had a glistening texture due to capsule production (negative stain). The size of a single colony of an 18–24 h culture was about 0.25–0.3 cm in diameter. The isolate was capable of growing over a temperature range of 10–45 °C with an optimum of 35 °C.

### Biochemical Characteristics

The isolate was also capable of growing on MacConkey Agar (bright pink colonies indicating lactose fermentation), XLD (yellow colonies, turned the complete plate from pink to yellow indicating coliform), and Levine EMB (dark colonies with green metallic sheen indicating lactose

fermentation with strong acid production as seen with some *Enterobacter* species). The cells were gram-negative straight rods (0.5–0.8  $\mu\text{m}$   $\times$  1.0–2.0  $\mu\text{m}$ ) and non-motile. The NaCl and pH tolerance were up to 6 % and 10.0, respectively. Biochemical tests were performed on isolate W14<sup>T</sup> to confirm its position in the genus *Klebsiella* and to specifically differentiate it from *K. oxytoca* and *K. mobilis*. The isolate W14<sup>T</sup> was oxidase, arginine, ornithine, phenylalanine and H<sub>2</sub>S negative, glucose fermenter, and citrate positive indicating that it belongs to the genus *Klebsiella*. The key physiological and biochemical characteristics are listed in Table 1. Biolog failed to identify the isolate and the closest match was *K. oxytoca* with a similarity and distribution values of 0.279 and 8.97, respectively. The API20E results indicated that the isolate W14<sup>T</sup> could be *K. oxytoca* with a probability value of 97.7 %.

#### FAME Analysis

The major fatty acids found in isolate W14<sup>T</sup> were C<sub>16:0</sub> (31.23 %), sum feature 8, C<sub>18:1 $\omega$ 6c</sub>/C<sub>18:1 $\omega$ 7c</sub> (21.10 %), and sum feature 3, C<sub>16:1 $\omega$ 7c</sub>/C<sub>16:1 $\omega$ 6c</sub> (19.05 %). The comparative fatty acid profile of isolate W14<sup>T</sup> with *K. oxytoca* and *K. mobilis* is shown in the supplementary file.

#### MALDI-TOF Analysis

Upon analysis of the spectrum produced from the MALDI-TOF system, the top match in the reference databases was *K. oxytoca* ATCC 13182<sup>T</sup> (Score 1.998) followed by three additional strains of *K. oxytoca*, then *K. mobilis*, and *R. ornithinolytica* (Score 1.788). These data indicate that the protein spectrum from W14<sup>T</sup> shares similarity to organisms in the *Enterobacteriaceae* group and is close to *K. oxytoca*.

**Table 1** Phenotypic profiles of isolate W14<sup>T</sup> and other related bacteria

Characteristics	Isolate W14 <sup>T</sup>	<i>K. oxytoca</i> ATCC 13182 <sup>T</sup>	<i>K. mobilis</i> ATCC 13048 <sup>T</sup>	<i>K. pneumoniae</i> ATCC 13883 <sup>T</sup>	<i>R. terrigena</i> ATCC 33257 <sup>T</sup>
Indole production	+	+	–	–	–
Growth at 10 °C	+	+	ND	–	+
Growth at 44.5 °C	+	+	–	+	–
Gas from lactose at 44.5 °C	–	–	–	+	–
Voges–Proskauer	+	+	+	+	+
Urease production	–	+	–	+	+
Ornithine decarboxylation	–	–	+	–	–
Motility	–	–	+	–	–
Histamine	–	–	+	–	+
D-melezitose	+	+	–	–	+
Putrescine	–	+	–	–	+
Degradation of pectate	–	+	–	–	–

Result: +, positive; –, negative; ND not determined

**Table 2** Comparative ribotyping patterns of isolate W14<sup>T</sup> and other related bacteria, using the restriction enzyme *EcoRI*

Bacterial ID	Similarity Index	RiboPrint™ pattern				
		1 kbp	5	10	15	50
<i>K. mobilis</i> (ATCC 13048 <sup>T</sup> )	0.24					
Isolate W14 (ATCC BAA-2403 <sup>T</sup> )	1.00					
<i>K. oxytoca</i> (ATCC 13182 <sup>T</sup> )	0.46					

#### DNA Base Composition and DNA–DNA Hybridization

The DNA G+C content of isolate W14<sup>T</sup> was 54.6 mol%, which falls within the range of the genus *Klebsiella*. DNA–DNA hybridization result indicated that the isolate W14<sup>T</sup> does not belong to the species *K. oxytoca* ATCC 13182<sup>T</sup> (55.7  $\pm$  6.2 %) when the recommendations by the *ad hoc* committee [20] for the definition of bacterial species are considered. These recommendations include a threshold value of 70 % DNA–DNA similarity, a level clearly above the observed level of the isolate.

#### Ribotyping

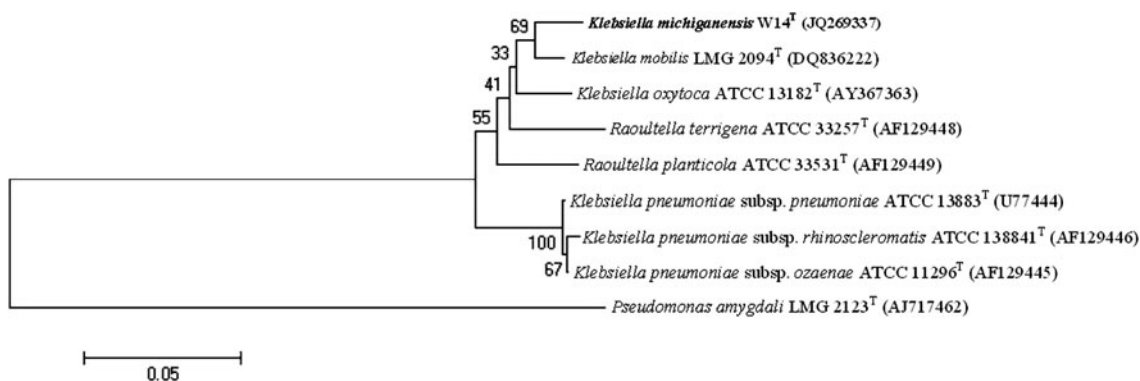
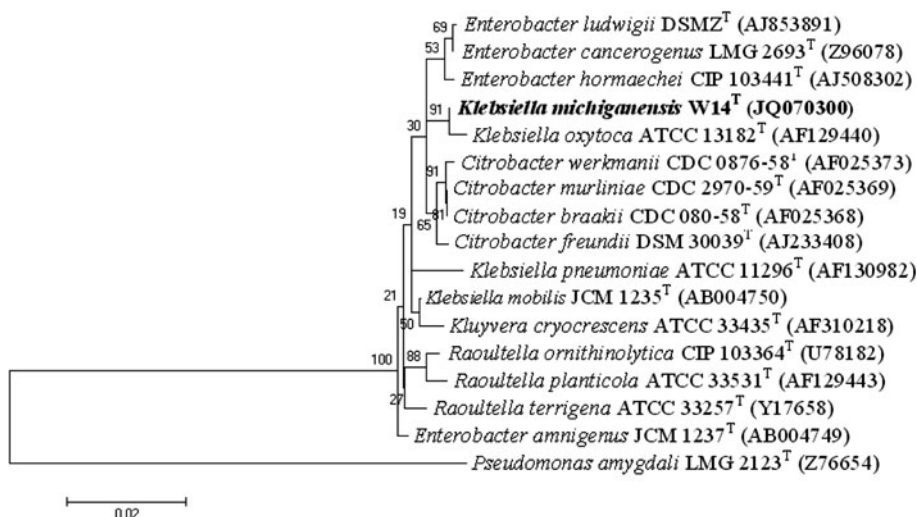
The ribotyping pattern for W14<sup>T</sup> was visibly distinct from *K. oxytoca* ATCC 13182<sup>T</sup> and *K. mobilis* ATCC 13048<sup>T</sup> when cut with *EcoRI* (Table 2). The RiboPrinter system uses a statistical match at or above a similarity index of 0.85 to assign a species level match [16]. The observation that W14<sup>T</sup> held similarity values <0.46 indicated that it was only distantly related to *K. oxytoca* and *K. mobilis*.

#### Gene Sequence Analysis

The almost complete 16S rRNA sequence (1,395 bp) analysis and the phylogenetic tree placed isolate W14<sup>T</sup> in the



**Fig. 1** Phylogenetic tree based on 16S rRNA gene sequences indicating the relationship between isolate W14<sup>T</sup> with other species of the family *Enterobacteriaceae*, constructed using CLUSTALX and MEGA5. *Pseudomonas amygdali* LMG 2123<sup>T</sup> was selected as the out group. Confidence limit for the tree was assessed using bootstrap with 1,000 replicates. The evolutionary distances were computed using the Kimura 2-parameter method. Bar 2 % sequence variation

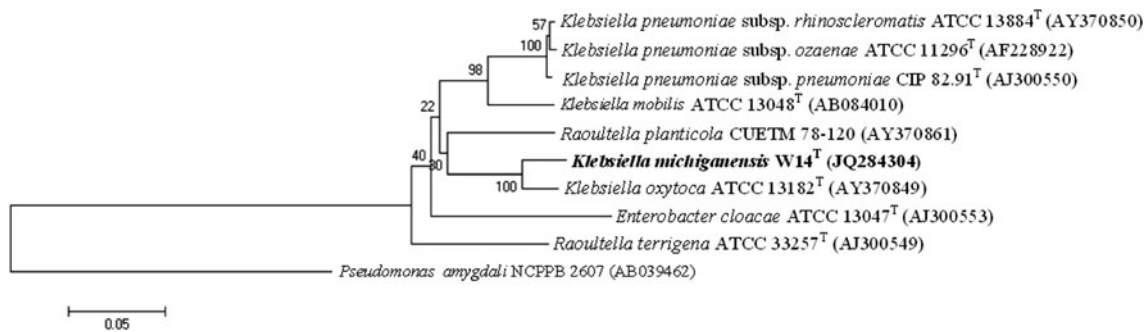


**Fig. 2** Phylogenetic tree based on *rpoB* gene sequences indicating the relationship between isolate W14<sup>T</sup> with other species of the family *Enterobacteriaceae*, constructed using CLUSTALX and MEGA5. *Pseudomonas amygdali* LMG 2123<sup>T</sup> was selected as the out group. Confidence limit for the tree was assessed using bootstrap with 1,000 replicates. The evolutionary distances were computed using the Kimura 2-parameter method. Bar 5 % sequence variation

Confidence limit for the tree was assessed using bootstrap with 1,000 replicates. The evolutionary distances were computed using the Kimura 2-parameter method. Bar 5 % sequence variation

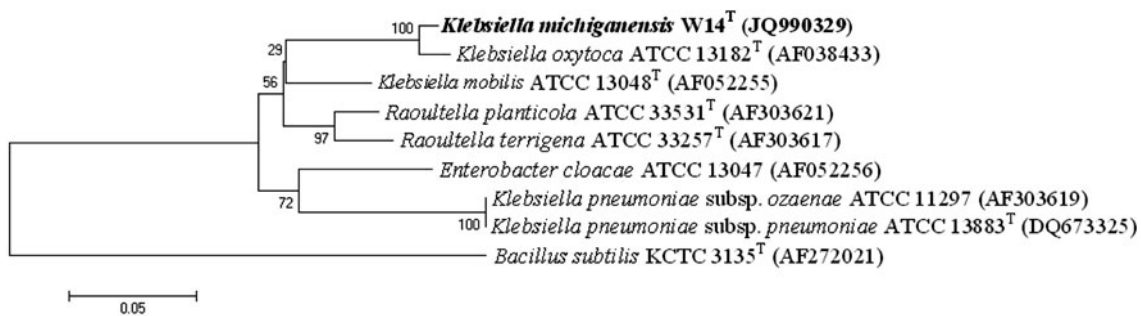
cluster III of the genus *Klebsiella*. The most closely related species was *K. oxytoca* (99 %) and the range of sequence similarity among the tested strains was 96–98 %. A dendrogram is presented in Fig. 1. The 16S rRNA sequence data indicated that isolate W14<sup>T</sup> belongs to the genus *Klebsiella* but did not permit clear differentiation between other closely related bacteria. Previous studies have shown that 16S rRNA sequence does not provide clear differentiation between the species belonging to the genus *Klebsiella* (*K. variicola* and *K. pneumoniae* share 99.3 % 16S rRNA gene sequence similarity) [15]. Therefore, three other protein-coding housekeeping genes, *gyrA*, *gyrB*, and *rpoB*, were sequenced. The genes encoding *gyrA* and *gyrB* (encoding the  $\alpha$ - and  $\beta$ -subunits of DNA gyrase, a type II DNA topoisomerase) and *rpoB* ( $\beta$ -subunit of the bacterial RNA polymerase) are

reported to be better molecular chronometers for the study of phylogenetic relationships in bacteria [14, 21]. It was reported [9, 17] that, due to its high levels of divergence, the *rpoB* gene sequence serves as a useful tool for identification among members of the *Enterobacteriaceae* family. The *rpoB* gene (834 bp) for isolate W14<sup>T</sup> showed 97 % similarity with both the type strains of *K. oxytoca* and *K. mobilis* (Fig. 2). The *rpoB* sequence similarity between the isolate W14<sup>T</sup> and *K. oxytoca* was observed to be lower than the sequence similarity reported by Li et al. [12] for the comparison between *K. singaporensis* and *K. pneumoniae* (97.5 %). The similarity was 2 % lower than the 16S rRNA sequence similarity with *K. oxytoca* and 1 % lower than that seen with *K. mobilis* indicating higher resolution compared to the ribosomal sequence. The *gyrB* gene sequence (978 bp)



**Fig. 3** Phylogenetic tree based on *gyrB* gene sequences indicating the relationship between isolate W14<sup>T</sup> with other species of the family *Enterobacteriaceae*, constructed using CLUSTALX and MEGA5. *Pseudomonas amygdali* NCPPB 2607 was selected as the out group.

Confidence limit for the tree was assessed using bootstrap with 1,000 replicates. The evolutionary distances were computed using the Kimura 2-parameter method. Bar 5 % sequence variation



**Fig. 4** Phylogenetic tree based on *gyrA* gene sequences indicating the relationship between W14<sup>T</sup> with other species of the family *Enterobacteriaceae*, constructed using CLUSTALX and MEGA5. *Bacillus subtilis* KCTC 3135<sup>T</sup> was selected as the out group.

Confidence limit for the tree was assessed using bootstrap with 1,000 replicates. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. Bar 5 % sequence variation

of isolate W14<sup>T</sup> showed 98 % similarity with the type strains of *K. oxytoca* and 90 % similarity with *K. mobilis* (Fig. 3). The *gyrA* gene sequence (626 bp) of isolate W14<sup>T</sup> showed 98 % similarity with the type strains of *K. oxytoca* and 93 % similarity with *K. mobilis* (Fig. 4). Phylogenetic analysis of the gene sequences for *rpoB*, *gyrB*, and *gyrA* indicated that isolate W14<sup>T</sup> belongs to the cluster III of the genus *Klebsiella* and is related to *K. oxytoca* (Figs. 2, 3, 4). Based on the gene sequence analysis, the isolate W14<sup>T</sup> also deserves the status of a new species belonging to the genus *Klebsiella*.

#### PCR Analysis of *pehX* and *gyrA* Gene

No PCR product was observed for the amplification of the *pehX* gene for the isolate W14<sup>T</sup> indicating that the polygalacturonase gene is absent in the isolate and it does not belong to the *K. oxytoca* species. The 441-bp amplicon was observed for the *gyrA* gene and the sequence obtained from the PCR product confirmed the position of the isolate in the genus *Klebsiella*.

#### Taxonomic Conclusion

This study was conducted to characterize and identify isolate W14<sup>T</sup> recovered from a tooth brush holder. Based on

16S rRNA, *gyrB*, and *gyrA* gene sequence analyses, the isolate W14<sup>T</sup> was related to *K. oxytoca*. The DNA–DNA hybridization, *rpoB*, FAME, ribotyping, and biochemical analysis data indicated that it deserves a status of a new species belonging to the genus *Klebsiella*. Additionally, the biochemical profile of isolate W14<sup>T</sup> was not only consistent with the characteristics of the genus *Klebsiella* but there were some key differences that might be important for distinguishing this isolate from *K. oxytoca* such as urease production, utilization of putrescine, and pectate degradation (a key test for *K. oxytoca*). Isolate W14<sup>T</sup> was negative for urease production, utilization of putrescine, and pectate degradation while *K. oxytoca* was positive for urease production, utilization of putrescine, and pectate degradation. W14<sup>T</sup> could be easily distinguished from *K. mobilis* based on motility, indole production, and ornithine decarboxylation (a key test for the genus *Enterobacter*). In contrast to *K. mobilis*, isolate W14<sup>T</sup> is non-motile, positive for indole production and negative for ornithine decarboxylation. Therefore, on the basis of physiological, chemotaxonomic, phylogenetic, and DNA–DNA hybridization characteristics of the isolate W14<sup>T</sup>, it cannot be assigned to a previously described bacterial species. We propose that isolate W14<sup>T</sup> is considered as a novel species, *K. michiganensis* sp. nov.

## Description of *K. michiganensis* sp. nov

*Klebsiella michiganensis* (mi.chi.ga.nen.sis. N.L. masc. adj. *michiganensis*, of or belonging to Michigan State, USA, where the type strain was isolated).

Isolate W14<sup>T</sup> has small, circular, mucoid, convex, entire, opaque, and white colonies. The cells are gram-negative, facultatively anaerobic, straight rods, non-motile, and capsulated. Cells are 0.5–0.8 µm in diameter and 1.0–2.0 µm in length. The cells are capable of growing at 10–45 °C with an optimum temperature of 35 °C. The optimum pH is 7.0. NaCl tolerance is up to 6 %. It is oxidase, urease, and gelatinase negative, but positive for catalase, indole, adonitol, and lysine. The Voges–Proskauer test is positive and the methyl red reaction is negative. Nitrate reduction test is positive. Citrate can be used as a sole carbon source. It utilizes glucose, mannitol, inositol, lactose, sucrose, arabinose, rhamnose, melibiose, and sorbitol, but fails to produce H<sub>2</sub>S. It is also negative for arginine dihydrolase, ornithine, dulcitol, tryptophan deaminase, phenylalanine, and pectate degradation. The G+C content of the DNA is 54.6 mol%. The type strain is W14<sup>T</sup> (=ATCC BAA-2403<sup>T</sup>=DSM 25444<sup>T</sup>).

**Acknowledgments** The authors would like to extend their sincere thanks to NSF International and Accugenix Inc. for providing funding and facilities for the project and also to Jodie Lee of Bacteriology at American Type Culture Collection (ATCC), Virginia, USA for excellent technical support.

## References

- Barrow GL, Feltham RKA (2004) Cowan and steel's manual for the identification of medical bacteria, 3rd edn. Cambridge University Press, Cambridge
- Brisse S, Verhoef J (2001) Phylogenetic diversity of *Klebsiella pneumoniae* and *Klebsiella oxytoca* clinical isolates revealed by randomly amplified polymorphic DNA, *gyrA* and *parC* gene sequencing and automated ribotyping. *Int J Syst Evol Microbiol* 51:915–924
- Brosius J, Palmer ML, Kennedy PJ et al (1978) Complete nucleotide sequence of a 16S ribosomal gene from *Escherichia coli*. *Proc Natl Acad Sci* 75:4801–4805
- Cashion P, Holder-Franklin MA, McCully J et al (1977) A rapid method for base determination of bacterial DNA. *Anal Biochem* 81:461–466
- Cole JR, Chai B, Marsh TL et al (2003) The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. *Nucleic Acids Res* 31:442–443
- De Ley J, Cattoir H, Reynaerts A (1970) The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* 12:133–142
- Drancourt M, Bollet C, Carta A et al (2001) Phylogenetic analyses of *Klebsiella* species delineate *Klebsiella* and *Raoultella* gen. nov., with description of *Raoultella ornithinolytica* comb. nov., *Raoultella terrigena* comb. nov. and *Raoultella planticola* comb. nov. *Int J Syst Evol Microbiol* 51:925–932
- Huss VAR, Festl H, Schleifer KH (1983) Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. *Syst Appl Microbiol* 4:184–192
- Kämpfer P, Ruppel S, Remus S (2005) *Enterobacter radicincitans* sp. nov., a plant growth promoting species of the family *Enterobacteriaceae*. *Syst Appl Microbiol* 28:213–221
- Kimura M (1980) A simple method for estimating evolutionary rates of base substitution through comparative studies of nucleotide sequences. *J Mol Evol* 16:111–120
- Kovtunovych G, Lytvynenko T, Negrutka V et al (2003) Identification of *Klebsiella oxytoca* using a specific PCR assay targeting the polygalacturonase *pehX* gene. *Res Microbiol* 154: 587–592
- Li X, Zhang D, Chen F et al (2004) *Klebsiella singaporensis* sp. nov., a novel isomaltulose-producing bacterium. *Int J Syst Evol Microbiol* 54:2131–2136
- Mesbah M, Premachandran U, Whitman WB (1989) Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* 39:159–167
- Mollet C, Drancourt M, Raoult D (1997) *rpoB* sequence analysis as a novel basis for bacterial identification. *Mol Microbiol* 26: 1005–1011
- Rosenblueth M, Martinez L, Silva J et al (2004) *Klebsiella variicola*, a novel species with clinical and plant associated isolates. *Syst Appl Microbiol* 27:27–35
- Sistanich D, Sherriff M (2005) The RiboPrinter® characterization system: an overview of automated ribotyping. In: Miller MJ (ed) *Encyclopedia of rapid microbiological methods*, vol 3. DHI Publishing, PDA, Bethesda
- Stephan R, Van Trappen S, Cleenwerck I et al (2007) *Enterobacter turicensis*, sp. nov. and *Enterobacter helveticus*, sp. nov., isolated from fruit powder. *Int J Syst Evol Microbiol* 57:820–826
- Tamura K, Peterson D, Peterson N et al (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28:2731–2739
- Thompson JD, Gibson TJ, Plewniak F et al (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 24:4876–4882
- Wayne LG, Brenner DJ, Colwell RR et al (1987) Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* 37:463–464
- Yamamoto S, Kasai H, Arnold DL et al (2000) Phylogeny of the genus *Pseudomonas*: intrageneric structure reconstructed from the nucleotide sequences of *gyrB* and *rpoD* genes. *Microbiol* 146:2385–2394