Systematic and Applied Microbiology 39 (2016) 1–7

Contents lists available at ScienceDirect

Systematic and Applied Microbiology

journal homepage: www.elsevier.de/syapm

Kosakonia pseudosacchari sp. nov., an endophyte of *Zea mays*☆

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ARTICLE INFO

Article history:
Received 4 April 2015
Received in revised form
15 September 2015
Accepted 18 September 2015

Keywords:
Kosakonia
Pseudosacchari
Taxonomy
MLSA
ANI
Endophyte

ABSTRACT

A beige pigmented bacterial strain (JM-387^T), isolated from field-grown corn root tissue, Tallassee, Alabama, was studied for its taxonomic allocation. A comparison of the 16S rRNA gene sequence with those of the type strains of most closely related species of the family *Enterobacteriaceae* showed highest sequence similarities to the type strain of *Kosakonia sacchari* (99.5%), "*Enterobacter oryzendophyticus*" (98.8%), and *Kosakonia radicincitans* (98.6%).

Construction of phylogenetic trees based on the 16S rRNA gene and partial sequences of four protein-coding genes, *rpoB*, *gyrB*, *infB*, and *atpD* (multilocus sequence analysis, MLSA) showed a distinct clustering of strain JM-387^T with *Kosakonia sacchari*. DNA-DNA hybridizations between JM-387^T and the type strains of most similar *Kosakonia*/"*Enterobacter*" species including *K. sacchari* LMG 26783^T, "*E. oryzendophyticus*" LMG 26432^T, *K. radicincitans* D5/23^T, *K. oryzae* LMG 24251^T, *E. cancerogenus* LMG 2693^T, and *E. cloacae* subsp. *dissolvens* CCUG 25230^T were in the range of 14.4–60.2%. The average nucleotide identity (ANI) of the genome sequence of the new strain to *K. sacchari* SP1^T was 94.47%.

Strain JM-387^T had a typical enterobacterial fatty acid pattern consisting of the major fatty acids $C_{16:0}$, $C_{16:1}$ ω7c/$C_{16:1}$ ω6c/$C_{15:0}$ 2OH, $C_{18:1}$ ω7c/$C_{18:1}$ ω6c with $C_{14:0}$ 3-OH as hydroxylated fatty acid.

Genotypic data and the differentiating biochemical and chemotaxonomic properties showed that strain JM-387^T represents a novel species of the genus *Kosakonia*, for which the name *Kosakonia pseudosacchari* sp. nov. (type strain JM-387^T = CIP 110597^T = DSM 27151^T) is proposed.

On the basis of 16S rRNA gene sequence data, the genus *Enterobacter* is a heterogeneous group and recent attempts have been made to reclassify several "*Enterobacter*" species into novel genera, like *Cronobacter*, *Kosakonia*, *Lelliotia*, and *Pluralibacter* [1,15]. The main criterion for this reclassification was the formation of a distinct cluster of *Enterobacter* species in a phylogenetic tree based on concatenated sequences of four housekeeping genes, the RNA polymerase β subunit (*rpoB*), DNA gyrase (*gyrB*), initiation translation factor 2 (*infB*), and the ATP synthase β subunit (*atpD*) (Multi Locus Sequence analysis, MLSA). Physiological and fatty acid profile data were investigated additionally to confirm the reclassification. Members of the genus *Enterobacter* are detected in various environments, such as infant formula [29,30], clinical specimens [4,13,31] but also in association with plants [6,21]. The majority of the latter are now classified into the genus *Kosakonia* [1,11] and many of them are known to interact and exert beneficial effects on plant growth. Currently five *Enterobacter* species were reclassified to the genus *Kosakonia*: *K. arachidis* (*E. arachidis* [24]), isolated from rhizosphere soil of field-grown groundnut; *K. cowanii* (*E. cowanii* [14]), isolated form clinical specimens; *K. oryzae* (*E. oryzae* [25]), isolated from surface-sterilized roots of the wild rice species *Oryza latifolia* as endophytic diazotrophs; *K. radicincitans* (*E. radicincitans* [21]), plant growth promoting bacteria, isolated from the phyllosphere of winter wheat; and *K. sacchari* (*E. sacchari* [35]) isolated from stem, root, or rhizosphere soil of sugar cane (*Saccharum officinarum* L.) plants.

Here we describe strain JM-387^T, a novel species of the genus *Kosakonia*, which was isolated from field-grown corn root tissue in Tallassee, Alabama, USA.

This beige-pigmented strain was isolated on Tryptic Soy Agar (TSA; Oxoid) and subsequently maintained and subcultivated on nutrient agar (NA, Oxoid) at 30 °C for 48 h. The isolate was

☆ The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, rpoB, gyrB, infB, and atpD gene sequences and the whole genome sequence of strain JM-387^T are KP345901, KP345903, KP345906, KP345910, KP345914, and ERS827601, respectively.

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http://dx.doi.org/10.1016/j.syapm.2015.09.004
0723-2020/

studied for its 16S rRNA gene sequence, partial sequences of *rpoB*, *gryB*, *infB*, and *atpD* as applied for the proposal of the genus *Kosakonia* [1], fatty acid methyl ester composition of the whole cell hydrolysates, further biochemical/physiological features, and DNA-DNA similarities to those species most closely related on the basis of 16S rRNA gene sequence similarities. The reference strains for physiological and biochemical tests, fatty acid analysis and DNA-DNA hybridization studies (*K. sacchari* LMG 26783^T, "*E. oryzendophyticus*" LMG 26432^T, *K. radicincitans* D5/23^T, *K. oryzae* LMG 24251^T, *Enterobacter cancerogenus* LMG 2693^T, and *E. cloacae* subsp. *dissolvens* CCUG 25230^T) were grown under the same conditions as the new strain.

The cultural and morphological characteristics of the isolate were determined from cultures grown on NA at 28 °C. The Gram reaction was tested as described by Gerhardt *et al.* [9] and the motility test was done under a light microscope on cells grown for 3 days in nutrient broth (Oxoid) at 30 °C. Oxidase activity was tested using the Oxidase reagent (bioMérieux) according to the instructions of the manufacturer. Catalase activity was tested by gas formation after dropping H_2O_2 on the fresh biomass grown for 48 h on NA (Oxoid). Growth was investigated at different temperatures (8, 10, 30, 37, 45, and 50 °C) on NA. NaCl tolerance was investigated in Tryptic Soy Broth (TSB, Oxoid) supplemented with 1.0–9.0% (w/v) NaCl (increasing in 1.0% steps). pH dependent growth was also tested in TSB adjusted to pH 4.5–12.5 (increasing in 1.0 pH units) by the addition of HCl or NaOH; pH values were controlled and finally adjusted after autoclaving.

The isolate showed a Gram-negative staining reaction and formed visible (diameter about 2 mm) beige colonies within 24 h at 30 °C. Strain JM-387^T was negative for oxidase activity. Cells were motile, non-spore forming rods (approx. 1 μm wide and approx. 2 μm long). Growth occurred well on NA, brain heart infusion agar (BHI agar, Fluke), R2A agar (Roth), and TSA (Oxoid) and on MacConkey agar (Oxoid) at 30 °C but also at 37 °C. No growth was observed after 48 h to 3 days at 4 °C and at 50 °C, but growth occurred between 8 and 45 °C. Strain JM-387^T grew with 1–7% NaCl, weakly in the presence of 8% NaCl, but not in the presence of 9% NaCl. Growth was also obtained in TSB adjusted to pH 4.5–11.5.

For phylogenetic analysis cell lysates of strain JM-387^T and reference strains (if required for MLSA analysis) were generated from a loop of biomass suspended in pure water and treated by three freeze–thaw cycles (−20 °C, 10 min, 1 min at 100 °C). The 16S rRNA gene of strain JM-387^T was PCR amplified and sequenced by the Didesoxy method with primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-ACGGCTACCTTGTTACGACTT-3′) [22]. The obtained 16S rRNA gene sequence of strain JM-387^T is a continuous stretch of 1461 nucleotides comprising positions 12–1478, following the numbering of the *Escherichia coli rrnB* gene [5].

Phylogenetic analyses were performed in ARB release 5.2 [23] using the "All-Species Living Tree" Project (LTP) [34] database LTPs119 (November, 2014). All sequences not included in the database were implemented after alignment with the SILVA Incremental Aligner (SINA; v1.2.9) according to the SILVA seed alignment (http://www.arb-silva.de; [26]). The final alignment used for phylogenetic analysis was controlled manually based on secondary structure information. Pairwise sequence similarities were calculated in ARB using the ARB neighbor-joining tool without the use of an evolutionary substitution model. Phylogenetic trees were constructed with the maximum-likelihood method using RAxML v7.04 [29] with GTR-GAMMA and rapid bootstrap analysis, the neighbor-joining method with the Jukes–Cantor correction [17] and the maximum-parsimony method using Phylip DNAPARS [8]. All phylogenetic trees are based on 100 replications (bootstrap analysis [7]) and 16S rRNA gene sequences between gene termini 107 and 1402 (according to *E. coli* [5]).

Strain JM-387^T showed highest 16S rRNA gene sequence similarities to the type strain of *K. sacchari* (99.5%), "*E. oryzendophyticus*" (98.8%), and *K. radicincitans* (98.6%) followed by *K. arachidis*, *K. oryzae*, and *E. cancerogenus* (all 98.5%). Furthermore, the strain showed a high 16S rRNA gene sequence similarity of between 98 and 98.4% to type strains of the genera *Klebsiella*, *Escherichia*, *Yokenella*, and *Enterobacter*, indicating the high 16S rRNA gene sequence similarities among genera of the *Enterobacteriaceae*. Phylogenetic trees based on 16S rRNA sequences including closest related taxa of the *Enterobacteriaceae* showed the placement of strain JM-387^T within the genus *Kosakonia* with distinct clustering (>70% bootstrap support) to the type strain of *K. sacchari* independent of the applied treeing method (Fig. 1). Additionally, all currently proposed *Kosakonia* species, "*E. oryziphilus*" and "*E. oryzendophyticus*", also clustered together with the species of the genus *Kosakonia*. Although the species of the genus *Kosakonia* clustered together, this cluster was not supported by high bootstrap values. Depending on the treeing method applied, the type species *K. cowanii* either clustered as an outlying species in this cluster or was separated from the other *Kosakonia* species (data not shown). This finding is not in agreement with the distinct clustering of the type strains of *K. cowanii*, *K. arachidis*, *K. oryzae*, and *K. radicincitans* as obtained by Brady *et al.* [1] but does confirm the clustering obtained in previous studies [9].

The phylogenetic relationship (Fig. 2) of strain JM-387^T was investigated in more detail by MLSA as applied for the proposal of the genus *Kosakonia* performed by Brady *et al.* [1] using partial nucleotide and amino acid sequences of the four protein-coding genes, *rpoB, gyrB*, *infB*, and *atpD*. PCR amplification and sequencing were performed as described by Brady *et al.* [2]. Manual sequence correction, sequence alignment using ClustalW [33], and phylogenetic analysis were performed in MEGA 5 [32]. Nucleotide sequences of reference strains were obtained from GenBank (http://www.ncbi.nlm.nih.gov/). Accession numbers of all sequences applied in this study are listed in Supplementary Table 1. Nucleotide sequences were aligned with respect to the correct open reading frame using full-length gene sequences of genome sequenced type strains (see Supplementary Table 1) as references. Maximum-likelihood trees of individual and concatenated nucleotide and amino acid sequences were calculated using the General time resolved (GTR) model using a discrete Gamma-distribution (+G) with 5 rate categories and by assuming that a certain fraction of sides are evolutionary invariable (+ I) (for nucleotide sequences) and the Jones-Thornton-Taylor model (JTT [16])+G+I (for amino acid sequences). Best fitting substitution models were determined with the Bayesian Information Criterion (BIC, [28]) using the ML model test implemented in MEGA 5. Partial *rpoB* (635 nt), *gyrB* (739 nt), *infB* (607 nt), and *atpD* (642 nt) nucleotide sequences and partial RpoB (211 aa), GyrB (246 aa), InfB (202 aa), and AtpD (214 aa) amino acid sequences were used for analysis. Maximum-likelihood trees generated from concatenated nucleotide and amino acid sequences showed that all *Kosakonia* species, strain JM-387^T and "*E. oryziphilus*" and "*E. oryzendophyticus*", formed a specific cluster within the *Enterobacteriaceae*, which was supported in both nucleotide- and amino-acid-based analysis by high bootstrap values (both 100%). The monophyletic character of the genus *Kosakonia* was obtained in *rpoB*, *gyrB*, and *infB* nucleotide- and amino-acid-based analysis but not always supported by high bootstrap values, especially not between representatives of *K. cowanii* and other *Kosakonia* species (Supplementary Figures 1–3). In phylogenetic trees based on partial *atpD* and AtpD sequences the genus *Kosakonia* formed two distinct clusters in the phylogenetic analysis based on partial nucleotide sequences and even three clusters in the analysis based on amino acid sequences (Supplementary Figure 4).

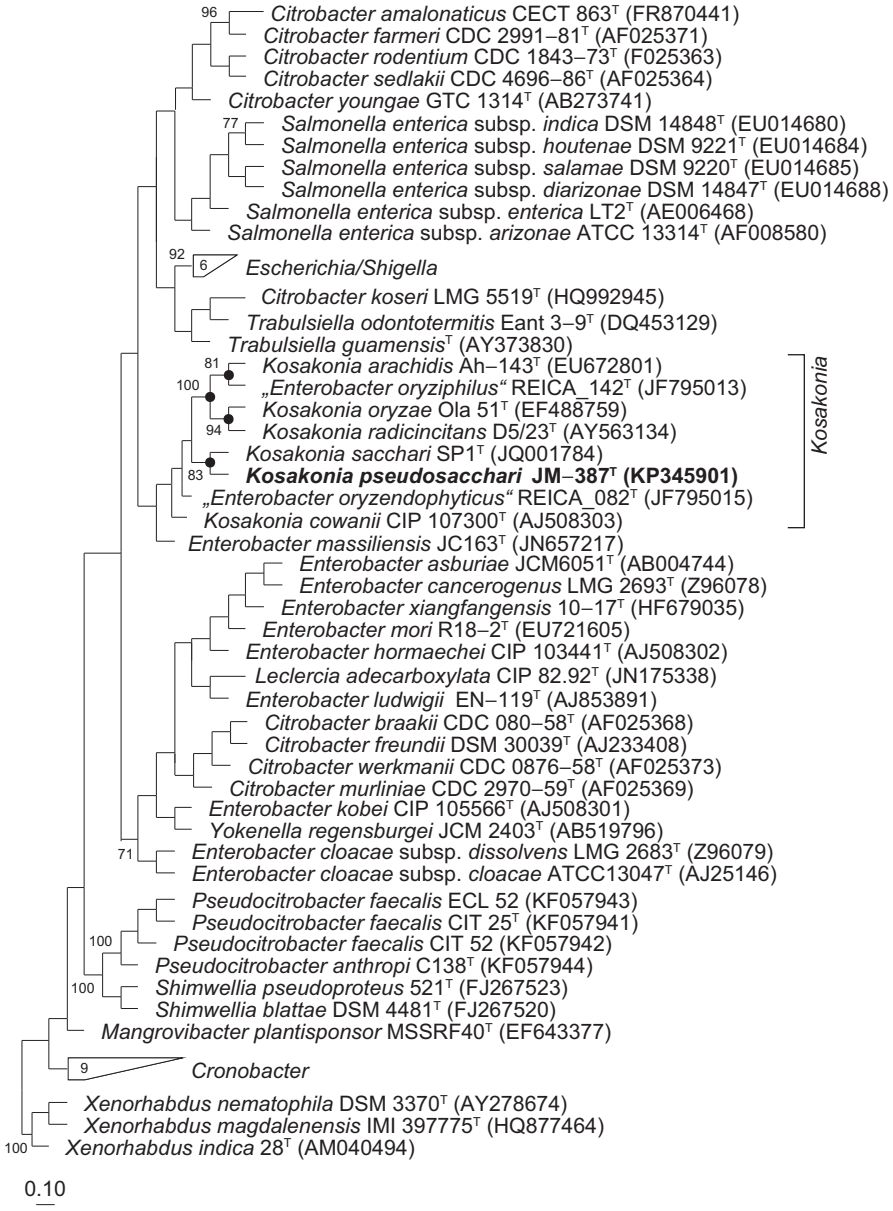

Fig. 1. Phylogenetic placement of strain JM-387^T within the genus *Kosakonia* based on nearly full-length 16S rRNA gene sequences. The maximum-parsimony tree was generated in ARB in the LTP database of November, 2014, and based on 16S rRNA gene sequences between gene termini 102–1402 and 100 replications (bootstrap analysis). Bootstrap values >70% are shown at the nodes. Nodes in the *Kosakonia* genus cluster marked with circles were also present with high bootstrap support (>70%) in the parallel constructed maximum-likelihood and neighbor-joining trees. *Xenorhabdus* type strain sequences were used as outgroup. Bar: 0.1 substitutions per nucleotide position.

Strain JM-387^T clustered among the four *K. sacchari* strains investigated by Brady *et al.* [1], forming a distinct cluster with those within the genus *Kosakonia*. This clustering of strain JM-387^T with the different strains assigned to the genus *K. sacchari* was obtained in all four individual gene-based phylogenetic trees (Supplementary Figures 1–4). At the level of amino acid sequences, strain JM-387^T and the four other *K. sacchari* strains shared 99.9–100% amino acid sequence identity (concatenated sequences) among each other (Supplementary Table 2). Higher sequence variability was obtained at the level of nucleotide sequences. Two of the *K. sacchari* strains, LMG 26785 and LMG 26786, always clustered closer (with slight variations in the single-gene-based trees) to strain JM-387^T than to the type strain of *K. sacchari.* Sequence similarities of the concatenated nucleotide sequences of strain JM-387^T to the two strains were 99.0% (concatenated nucleotide sequences) but only 97.7% and 97.5% to the type strain of *K. sacchari* and the other *K. sacchari* strain LMG 26786, respectively (Supplementary Table 2).

DNA-DNA hybridization (DDH) experiments were performed between strain JM-387^T and the type strains of the five most closely related species (at least those with >98.2% 16S rRNA gene sequence similarities), according to the method of Ziemke *et al.* [36] (except that for nick translation 2 μg of DNA were labeled during 3 h of incubation at 15 °C).

DDHs between strain JM-387^T and the type strains of the most similar *Kosakonia/"Enterobacter"* species resulted in values of 38.8% (39.7% reciprocal) to *K. sacchari* LMG 26783^T, 50.4% (25.9% reciprocal) to "*E. oryzendophyticus*" LMG 26432^T, 25.8% (51.5% reciprocal) to *K. radicincitans* D5/23^T, 45.0% (13.0% reciprocal) to *K. oryzae* LMG 24251^T, 36.0% (60.2% reciprocal) to *E. cancerogenus* LMG 2693^T, and 32.1% (14.4% reciprocal) to *E. cloacae* subsp. *dissolvens* CCUG 25230^T.

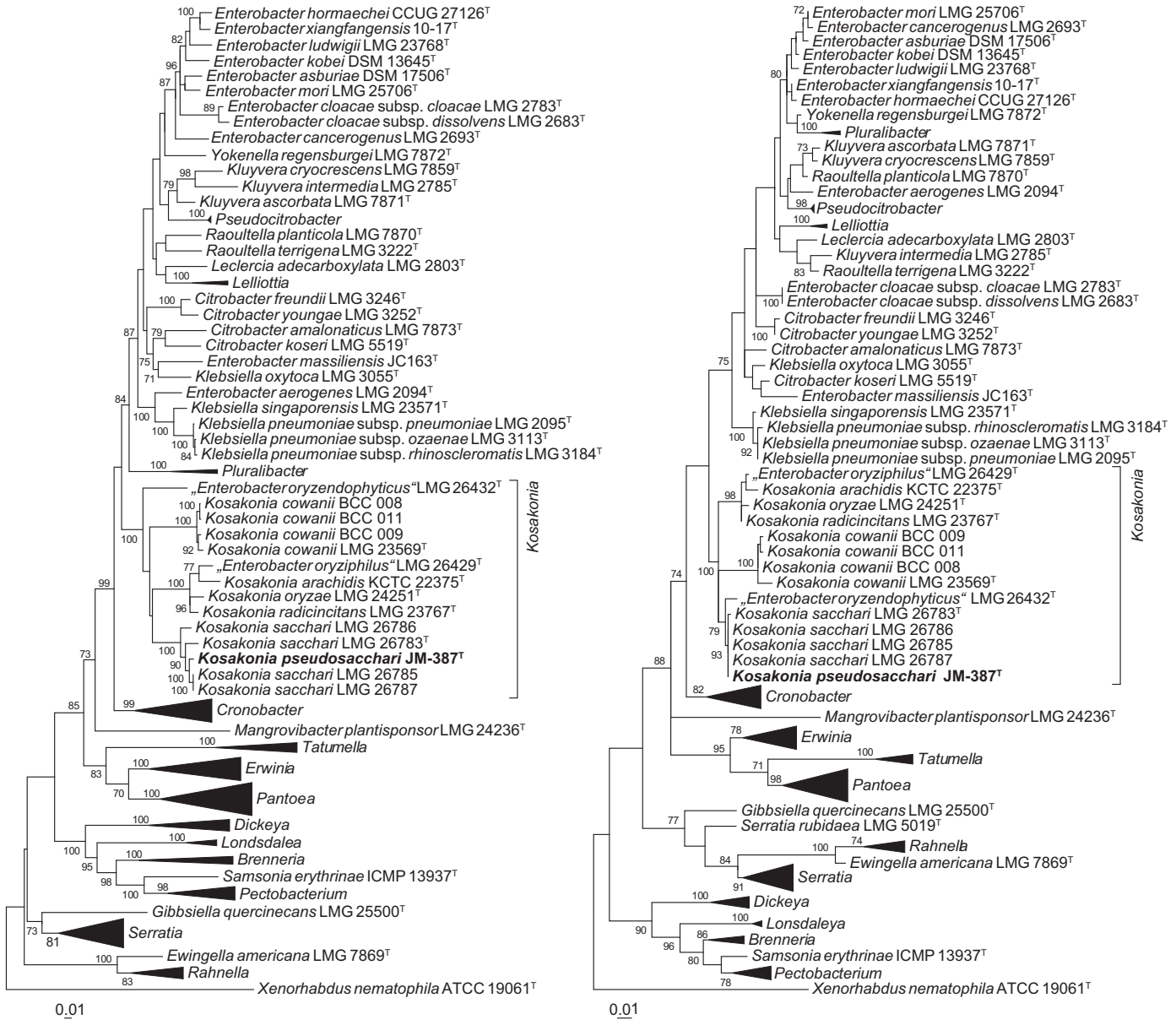

Fig. 2. Phylogenetic placement of strain JM-387^T within the genus *Kosakonia* based on concatenated partial *rpoB*, *gyrB*, *infB*, and *atpD* nucleotide and amino acid sequences (MLSA analysis). Maximum-likelihood trees were generated in MEGA5 using the GTR + G + I model (nucleotide sequences considering all codon positions) and the JTT + G + I model (amino acid sequences) for tree construction. The trees were based on 2623 nucleotide and 867 amino acid sequence positions. Bootstrap values >70% (generated by 100 replications) are presented at the nodes. Clusters contain type strain sequences of species belonging to one genus. *Xenorhabdus nematophilus* ATCC 19061^T was used as outgroup. Bar: 0.01 substitutions per nucleotide or amino acid position.

For genome-based analyses, genomic DNA of strain JM-387^T was isolated by the PureLink® Genomic DNA isolation kit (Invitrogen) and DNA sequencing libraries were prepared using the Nextera XT kit (Illumina, San Diego) following the vendor's instructions. The individually tagged library was sequenced as a part of a flow cell as 2 × 250 base paired-end reads using the Illumina MiSeq platform (Illumina, San Diego). The sequence was assembled after quality control with SPAdes [3]. The ANI was calculated by Jspecies [27]. The ANI between the genome sequences of strain JM-387^T and that of *K. sacchari* SP1^T (Acc. No. CP007215) was determined to be 94.47%, thereby confirming that strain JM-387^T indeed represents a novel species. The G + C content of the genome of strain JM-387^T is 53.55 mol%. This is similar to the G + C content given for the genus *Kosakonia* [1] as well as to the G + C content of the genome of *K. sacchari* SP1^T (53.7 mol%; Acc. No. CP007215).

Detailed physiological characterization and biochemical tests were performed to assess the carbon source utilization pattern and hydrolysis of chromogenic substrates as described by Kämpfer *et al.* [20] and Kämpfer [18]. The strains utilized many carbon sources. The biochemical/physiological data are given in Table 1 and in the species description. Biochemical/physiological data of strain JM-387^T were consistent with data given in the *Kosakonia* genus description by Brady *et al.* [1]; but physiological profiles especially substrate utilization patterns further supported the separation from the closest related species by giving some different results (Tables 1 and 2).

The analysis of the cellular fatty acid profiles of whole cell hydrolysates was done as described previously [19] and revealed for JM-387^T a typical enterobacterial pattern consisting of the major fatty acids $C_{16:0}$, $C_{16:1}$ ω7c/$C_{16:1}$ ω6c/$C_{15:0}$ 2OH, $C_{18:1}$ ω7c/$C_{18:1}$ ω6c with $C_{14:0}$ 3–OH as hydroxylated fatty acid and was confirmed with

Table 1
Fatty acid profiles of strains: 1, JM-387^T; 2, *K. sacchari* LMG 26783^T; 3, *K. oryzae* LMG 24251^T; 4, "*E. oryziphilus*" LMG 26429^T; 5, "*E. oryzendophyticus*" LMG 26432^T; 6, *E. radicincitans* D5/23^T; 7, *E. arachidis* Ah-143^T; 8, *E. cowanii*; 9, *E. cloacae* subsp. *cloacae* ATCC 13047^T. Data in brackets for taxa 4–8 from Hardoim *et al.* [12] and Madhaiyan *et al.* [24].

Average	1	2	3	4	5	6	7	8	9
Saturated									
10:0				0.0	0.0	- (0.0/-)	(1.1/-)	(/0.2)	0.0
11:0						(/-)	(/-)	(/-)	
12:0	3.5	4.3		3.5(2.1)	3.7(1.6)	3.9 (1.7/5.2)	(2.5/5.0)	(/3.2)	1.0
13:0		0.7			(0.1)	(0.1/-)	(1.4/0.2)	(/0.6)	0.5
14:0	5.8	4.7	7.3	7.9(4.0)	7.1(2.8)	6.8 (2.8/5.5)	(3.1/7.5)	(/6.3)	3.4
15:0	0.9	4.0		2.3(0.9)	2.1(0.5)	0.6 (3.1/1.4)	(2.3/1.0)	(/-)	6.8
Anteiso 15:0						(/-)	(/-)	(/1.5)	
16:0	28.8	27.3	34.0	31.7(34.4)	29.2(32.6)	32.6 (20.5/28.5)	(22.1/28.3)	(/15.2)	28.6
17:0		1.4		0.9(0.5)	(0.3)	(0.9/0.8)	(2.3/0.6)	(/1.4)	3.0
18:0				(0.3)	(0.4)	(0.4/-)	(2.1/-)	(/-)	0.4
Iso 19:0						1.0			
Unsaturated									
15:1 ω8c						0.1	1.6		0.2
15:1 ω6c						0.0	0.0		0.1
15:1 ω5c				(0.1)	(0.1)	0.1	1.6		0.0
16:1 ω7c/16:1 ω6c/15:0 2OH	19.4	12.1	20.6	10.8(20.7)	22.6(26.4)	11.0 (11.1/22.6)	(14.9/18.5)	(/25.6)	15.0
16:1ω5c				(0.1)	(0.2)	13.2	1.9		0.3
17:1 ω8c				(0.1)	(0.1)	(6.5/-)	(1.9/-)	(/0.4)	0.3
18:1 ω7c/18:1ω6c	22.9	18.1	21.0	12.9(19.2)	24.2(27.6)	19.0 (21.1/15.5)	(15.5/14.3)	(/7.2)	21.1
18:1 ω5c				(0.1)	(0.1)	0.0	0.0		0.0
20:1 ω7c				(0.1)		0.0	2.1		0.0
Hydroxylated									
13:0 3OH/15:1 H						(/-)	(/-)	(/1.9)	
15:0 3OH	0.8			(0.1)	0.0	(0.1/0.2)	(1.8/-)	(/0.4)	0.2
15:0 3OH iso						(/-)	(/-)	(/12.3)	
14:0 3OH/16:1 iso I	8.5	7.8	8.5	7.4(0.8)	8.0(0.8)	7.1 (1.2/9.5)	(2.5/11.5)	(/18.4)	1.2
Cyclopropyl-Branched									
17:0 cyclo	5.9	14.2	8.5	16.0(14.2)	3.1(4.8)	13.5 (14.5/9.5)	(13.4/11.7)	(/4.6)	15.5
19:0 cyclo/19:0 cyclo ω8c		3.9		5.6(0.6)	(0.2)	2.0 (1.1/0.8)	(3.1/0.4)	(/-)	1.5
unknown FA (ECL 10.928)			8.5			8.0			
unknown FA (ECL 13.957)		0.8							
unknown FA (ECL 14.502)	0.8	0.7		1.0(1.7)	(1.2)	1.6 (0.9/-)	(2.6/1.0)	(/-)	0.7
unknown FA (ECL 14.959)	0.6								

the description of the genus *Kosakonia* [1]. Slight, mainly quantitative differences to the other most closely related *Kosakonia* and "*Enterobacter*" species could be detected. The complete fatty acid pattern of strain JM-387^T is shown in Table 2 in comparison with those of the type strains of the most closely related *Kosakonia* and '*Enterobacter*' species.

The genus *Kosakonia* was proposed based on the distinct clustering of *Kosakonia* species in a MLSA study based on concatenated partial nucleotide sequences of four protein-coding genes [1]. This approach, however, should be critically appraised, as it may not reflect true phylogenetic relationships but rather an averaged relationship in the form of a composite image generated

Table 2
Physiological/biochemical tests of JM-387^T and the most closely related species. 1, JM-387^T; 2, *K. sacchari* LMG 26783^T; 3, *K. oryzae* LMG 24251^T; 4, "*E. oryziphilus*" LMG 26629^T; 5, "*E. oryzendophyticus*" LMG 26432^T; 6, *K. radicincitans* D5/23^T; 7, *E. cloacae* subsp. *cloacae* ATCC 13047^T; 8, *E. cloacae* subsp. *dissolvens*. +, positive; w, weakly positive; -, negative. Data in brackets for taxa 4–8 from Hardoim *et al.* [12]. The percentage of strains giving a positive result is scored as: -, 0–20%; V, 20–80%; +, 80–100%; ND, no data available; cell morphology: R, rods; CR, coccoid rods; SR, straight rods. Data in brackets for taxa 2 from Zhu *et al.* [35] and for 3 from Peng *et al.* [25].

Characteristic[a]	1	2	3	4	5	6	7	8
Voges-Proskauer test (37 °C)[b]	+	+(+)	+(+)	+(+)	+(+)	+(+)	(+)	(+)
Methyl red test	+	+(+)	+(+)	+(+)	+(+)	+(+)	(-)	(-)
Cell morphology	CR	CR	CR	(SR)	(SR)	(R)	(R)	(R)
Ornithine decarboxylase[b]	-	w(+)	+(+)	-(-)	+(+)	-(-)	(+)	(+)
Malonate decarboxylase 48 h)	+	+(+)	+(+)	+(+)	+(+)	+(+)	(-)	(+)
Arginine dihydrolase[b]	+	+(-)	+(+)	+(+)	+(+)	+(+)	(+)	(+)
Esculin hydrolysis	+	+(+)	-(-)	+(+)	+(+)	+(+)	(-)	(+)
Citrate[b]	+	+	+(+)	+(+)	+(+)	+(+)	(+)	(+)
Carbon source utilization[c]								
Sucrose	+	+(+)	+(+)	+(+)	+(+)	+(+)	(+)	(+)
D-melibiose	-	w(+)	+(+)	-(-)	+(V)	-(-)	(+)	(+)
Adonitol	-	-(ND)	+(ND)	+(+)	+(+)	-(-)	(-)	(-)
D-sorbitol	+	+(+)	+(+)	+(+)	+(+)	+(+)	(+)	(+)
L-aspartic acid	-	w(+)	w(+)	+(+)	-(-)	+(+)	(ND)	(ND)
i-inositol	-	-	-	+(+)	+(+)	-(-)	(+)	(+)
D-arabitol	ND	ND(+)	ND(-)	+(+)	+(+)	-(-)	(-)	(-)
D-raffinose	-	-(+)	-(+)	-(-)	+(+)	-(-)	(+)	(+)

[a] For all strains analyzed, the tests were positive for catalase, β-galactosidase (ONPG), and motility, and negative for oxidase, lysine decarboxylase, indole, and H_2S production.
[b] Test results of both the API-20E system and conventional test methods.
[c] The carbon source utilization tests were determined by using Biolog GN2 microplates.

by four different phylogenetic markers. This is especially problematic because single gene-based phylogenetic analyses did not support a clear distinct clustering separating *Kosakonia* species from other *Enterobacteriaceae*. This was obtained for the protein-coding genes and for the 16S rRNA gene-based phylogeny as also indicated in previous studies [9,11]. Differentiation using 16S rRNA gene-based phylogeny is not sufficient to investigate phylogenetic relationships within a genus, and the phylogenetic affiliation should be supplemented with studies based on protein-coding genes. Furthermore, there are no strong phenotypic data that support the distinction of the genera within the *Enterobacteriaceae*. It is promising that the integration of whole genome sequence-based analysis into the polyphasic approach reveals more detail into the phylogenetic relationships of closely related genera of the *Enterobacteriaceae* and could lead to reassessments in previous classification schemes primarily based on the MLSA data [10].

Based on the current description of the genus *Kosakonia*, strain JM-387^T can now be assigned to the genus *Kosakonia*. Strain JM-387^T could not be distinguished from *K. sacchari* when using the MLSA approach; however, DDH and genome sequence-based ANI analysis clearly showed the distinction of the strain from the *K. sacchari* type strain. Differences in physiological data further supported the distinction of strain JM-387^T from *K. sacchari.* On the basis of the results of this polyphasic study, it is obvious that strain JM-387^T represents a novel species of the genus *Kosakonia* for, which the name *Kosakonia pseudosacchari* sp. nov. is proposed. Whether or not the other strains characterized by Zhu *et al.* [35], especially LMG 26785 and LMG 26787, belong to the species *K. sacchari* or the new proposed species awaits further studies. However, melting temperature based DDH analysis indicated their assignment to *E. sacchari* [35], MLSA data indicate a closer relationship to the newly proposed species presented herein. Genome sequence-based comparisons permit further unequivocal assessments of their phylogenetic relationship. There is, in addition, a need for the reclassification of the two species "*Enterobacter oryzendophyticus*" and "*Enterobacter oryziphilus*" to the genus *Kosakonia*, because all phylogenetic data clearly indicate their affiliation to the genus *Kosakonia*. However, these species have not been validated so far.

Description of Kosakonia pseudosacchari sp. nov

K. sacchari [pseu.do.sac.caríi, N.L. gen. n., Gr. adj. pseudês false; sac.chaíri. N.L. gen. n. sacchari of Saccharum, isolated from *Saccharum officinarum* L. (sugar cane)].

Cells show a Gram-negative staining. They are non-motile, non-spore forming rods, approx. 1 μm in width and 2 μm in length. Facultatively anaerobic, oxidase-negative, catalase-positive. Good growth occurs after 48 h on NA, BHI agar, TSA, and R2A agar, and on MacConkey agar at 30 and 37 °C. Growth occurs on NA after 24 h to 3 days at 8–45 °C, but not at 4 and 50 °C. Cells grow at 30 °C in the presence of 1.0–7.0% NaCl (weakly at 8%) as additional ingredient of TSB and at pH 4.5–11.5. Colonies on NA are smooth, beige, and circular with entire edges.

Urease-activity, lysine-decarboxylase, ornithine-decarboxylase, indole, and H_2S production are negative, while activity of arginine dihydrolase, malonate, and citrate- alkalinisation (Simmons), Voges-Proskauer and methyl red reactions, and β-galactosidase (ONPG) are positive.

Weak production of acids is found from various sugar and sugar-related compounds, including D-glucose, sucrose, mannose, D-mannitol, salicin, L-arabinose, cellobiose, D-sorbitol, D-maltose, L-rhamnose, D-trehalose, and α-methyl-D-glucoside. No acid is produced from adonitol, D-arabitol, dulcitol, erythritol, *i*-inositol, D-melibiose, raffinose, and D-xylose.

The following compounds are utilized as a sole source of carbon: acetate, *N*-acetylgalactosamine, *N*-acetylglucosamine, L-arabinose, L-arbutine, D-cellobiose, D-galactose, gluconate, D-glucose, D-maltose, D-mannose, D-fructose, D-trehalose, glycerol, D-mannitol, maltitol, L-rhamnose, D-ribose, D-sucrose, salicine, D-xylose, D-sorbitol, fumarate, DL-lactate, pyruvate, citrate, L-alanine, L-aspartate, L-histidine, and L-proline.

The following compounds are not utilized as a sole source of carbon: propionate, α-D-melibiose, adonitol, *i*-inositol, putrescine, *cis*-aconitate, *trans*-aconitate, 4-aminobutyrate, adipate, azelate, glutarate, DL-3-hydroxybutyrate, itaconate, 2-oxoglutarate, suberate, mesaconate, β-alanine, L-ornithine, L-phenylalanine, L-leucine, L-serine, L-tryptophan, 3-hydroxybenzoate, 4-hydroxybenzoate, and phenylacetate.

The chromogenic substrates *p*-nitrophenyl-α-D-glucopyranoside, *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-β-D-xylopyranoside, bis-*p*-nitrophenyl-phosphate, bis-*p*-nitrophenyl-phenyl-phosphonate, bis-*p*-nitrophenyl-phosphoryl-choline, 2-deoxythymidine-2′-*p*-nitrophenyl-phosphate, L-alanine-*p*-nitroanilide, γ-L-glutamate-*p*-nitroanilide, and L-proline-*p*-nitroanilide are hydrolyzed; *p*-nitrophenyl-β-D-glucuronide is not hydrolyzed. Strain JM-387^T clustered within the genus *Kosakonia* by applying the four protein-coding MLSA approach used by Brady *et al.* [1] for the genus proposal.

The major cellular fatty acids are $C_{16:0}$, $C_{16:1}$ ω7c/$C_{16:1}$ ω6c/$C_{15:0}$ 2OH, $C_{18:1}$ ω7c/$C_{18:1}$;ω6c with $C_{14:0}$ 3-OH as hydroxylated fatty acid.

The type strain is JM-387^T (=CIP 110597^T = DSM 27151^T), was isolated as an endophyte from healthy field-grown corn root tissue (*Zea mays*), in Tallassee, Alabama, USA. The DNA G + C content of the type strain is 53.88 mol% (genome sequence based).

Acknowledgement

We thank Gundula Will, Maria Sowinsky, Katja Grebing, and Jan Rodrigues-Fonseca for excellent technical assistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.syapm.2015.09.004.

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