Description of *Klebsiella spallanzanii* sp. nov. and of *Klebsiella pasteurii* sp. nov.

Cristina Merla[1,2], Carla Rodrigues[3], Virginie Passet[3], Marta Corbella[1], Harry A. Thorpe[4], Teemu V.S. Kallonen[5,6], Zhiyong Zong [7], Piero Marone[1], Claudio Bandi[8], Davide Sassera[9], Jukka Corander[5,6,10], Edward J. Feil[4], and Sylvain Brisse[3,#]

[1] Fondazione IRCCS Policlinico San Matteo, Unità Operativa Complessa Microbiologia e Virologia, Pavia, Italy.

[2] Scuola di Specializzazione in Microbiologia e Virologia, Università degli Studi di Pavia, Pavia, Italy

[3] Institut Pasteur, Biodiversity and Epidemiology of Bacterial Pathogens, Paris, France.

[4] The Milner Centre for Evolution, Department of Biology and Biochemistry, University of Bath, Bath, United Kingdom.

[5] Infection Genomics, Wellcome Sanger Institute, Cambridge, United Kingdom.

[6] Department of Biostatistics, University of Oslo, Oslo, Norway.

[7] Center of Infectious Diseases, West China Hospital, Sichuan University, Chengdu, PR China.

[8] Department of Biosciences, University of Milan, Milan, Italy; Pediatric Clinical Research Center "Romeo ed Enrica Invernizzi", University of Milan, Milan, Italy.

[9] Dipartimento di Biologia e Biotecnologie "L. Spallanzani", Università di Pavia, Pavia, Italy.

[10] Helsinki Institute for Information Technology HIIT, Department of Mathematics and Statistics, University of Helsinki, Finland

 # Corresponding author:

Sylvain Brisse

Institut Pasteur,

Biodiversity and Epidemiology of Bacterial Pathogens,

28 rue du Docteur Roux, F-75724 Paris, France.

e-mail: sylvain.brisse@pasteur.fr; Phone +33 1 45 68 83 34

Running title: Klebsiella spallanzanii and Klebsiella pasteurii

Abstract

Klebsiella oxytoca causes opportunistic human infections and post-antibiotic haemorrhagic diarrhoea. This *Enterobacteriaceae* species is genetically heterogeneous and is currently subdivided into seven phylogroups (Ko1 to Ko4, Ko6 to Ko8). Here we investigated the taxonomic status of phylogroups Ko3 and Ko4. Genomic sequence-based phylogenetic analyses demonstrate that Ko3 and Ko4 formed well-defined sequence clusters related to, but distinct from, *Klebsiella michiganensis* (Ko1), *Klebsiella oxytoca* (Ko2), *K. huaxiensis* (Ko8) and *K. grimontii* (Ko6). The average nucleotide identity of Ko3 and Ko4 were 90.7% with *K. huaxiensis* and 95.5% with *K. grimontii*, respectively. In addition, three strains of *K. huaxiensis*, a species so far described based on a single strain from a urinary tract infection patient in China, were isolated from cattle and human faeces. Biochemical and MALDI-ToF mass spectrometry analysis allowed differentiating Ko3, Ko4 and Ko8 from the other *K. oxytoca* species. Based on these results, we propose the names *Klebsiella spallanzanii* for the Ko3 phylogroup, with SPARK_775_C1^{T} (CIP 111695^{T}, DSM 109531^{T}) as type strain, and *Klebsiella pasteurii* for Ko4, with SPARK_836_C1^{T} (CIP 111696^{T}, DSM 109530^{T}) as type strain. Strains of *K. spallanzanii* were isolated from human urine, cow faeces and farm surfaces, while strains of *K. pasteurii* were found in faecal carriage from humans, cows and turtles.

Keywords: *Klebsiella oxytoca* complex; phylogeny; taxonomy; genome sequencing; *bla*$_{OXY}$; MALDI-ToF mass spectrometry

Abbreviations: ANI, average nucleotide identity; HCCA, a-cyano-4-hydroxycinnamic acid; isDDH, *in silico* DNA-DNA hybridization; SCAI, Simmons citrate agar with inositol; MALDI-ToF MS: Matrix-assisted laser desorption/ionization time of flight mass spectrometry

Accession numbers

The nucleotide sequences generated in this study were deposited in ENA and are available through the INSDC databases under accession numbers MN091365 (SB6411^{T} = SPARK775C1^{T}), MN091366 (SB6412^{T} = SPARK836C1^{T}) and MN104661 to MN104677 (16S rRNA), MN076606 to MN076643 (*gyrA* and *rpoB*), and MN030558 to MN030567 (bla_{OXY}). Complete genomic sequences were submitted to European Nucleotide Archive under the BioProject number PRJEB15325.

Introduction

The genus *Klebsiella*, a member of the *Enterobacteriaceae* family, includes Gram-negative, non-motile (except *K. aerogenes*) and non-spore-forming capsulated bacteria. Bacteria belonging to the genus *Klebsiella* are found in water, soil and plants, and as commensals in the gut of animals including humans (1,2,3). In humans, *Klebsiella* species are frequently associated with hospital-acquired infections and are increasingly multidrug-resistant (4). *Klebsiella oxytoca* is the second most common *Klebsiella* species causing disease in humans, after *K. pneumoniae* (5). *K. oxytoca* carries a chromosomally encoded β-lactamase gene (*bla*$_{\mathrm{OXY}}$) that confers resistance to amino- and carboxypenicillins (6). This gene was shown to have diversified in parallel to housekeeping genes, and variants were classified into seven groups (*bla*$_{\mathrm{OXY-1}}$ to *bla*$_{\mathrm{OXY-7}}$) (7, 8, 9, 10). *K. oxytoca* phylogenetic lineages were named Ko1, Ko2, Ko3, Ko4, Ko6 and Ko7 reflecting which *bla*$_{\mathrm{OXY}}$ variant they carry; note that Ko5 was not defined, as isolates carrying *bla*$_{\mathrm{OXY-5}}$ represent a sublineage of Ko1 (9). Taxonomic work has shown that *K. oxytoca* (*sensu lato, i.e.*, as commonly identified in clinical microbiology laboratories) is in fact a complex of species, with *K. oxytoca* (*sensu stricto*) corresponding to phylogroup Ko2, *K. michiganensis* to Ko1 (11) and *K. grimontii* to Ko6 (12). The closely related *K. huaxiensis* (13) represents yet another phylogroup, which we here denominate as Ko8 and which carries *bla*$_{\mathrm{OXY-8}}$. Phylogroups Ko3, Ko4, Ko7 and *K. huaxiensis* were so far described only based on a single strain (9, 13), which has limited our ability to define their genotypic and phenotypic characteristics. While analysing a large number of *Klebsiella* strains from multiple human, animal and environmental sources in and around the Northern Italian town of Pavia, we identified 3 Ko3, 13 Ko4 and 3 *K. huaxiensis* strains. The aim of this work was to define the taxonomic status of *K. oxytoca* phylogroups Ko3 and Ko4 and provide identification biomarkers for all members of the *K. oxytoca* species complex.

Material and methods

Bacterial strains. Novel strains (3 Ko3, 13 Ko4 and 3 Ko8) were isolated through enrichment in Luria-Bertani broth supplemented with 10 µg/mL of amoxicillin, followed by isolation on Simmons citrate agar with 1% inositol (SCAI) medium (14) and re-isolation on MacConkey agar. Additional strains, including type and reference strains of each *K. oxytoca* phylogroup and the type strain of *K. pneumoniae* (15) were included in the study (**Table 1**). Strain SG271 (internal strain bank identifier, SB3356) and SG266 (SB3355) were included as reference strains for the phylogroups Ko3 and Ko4, respectively (9).

Genome sequencing and analyses. Colonies from the novel strains grown on MacConkey agar were collected and resuspended in distilled water for DNA purification, which was performed using QIAsymphony automated instrument with the kit QIAsymphony DSP Virus/Pathogen following the manufacturer's recommendation. DNA was stored at -20°C until sequencing on an Illumina HiSeq X Ten platform with a 2 x 150 nt paired-end protocol. Reads were assembled using SPAdes v3.11 and the assemblies were annotated using Prokka v1.12 (16). JSpeciesWS (17) was used to calculate the average nucleotide identity (ANI) using the BLAST algorithm (ANIb), whereas *in silico* DNA-DNA hybridization (isDDH) was performed through GGDC tool (http://ggdc.dsmz.de; formula 2) (18). Sequences of *gyrA* and *rpoB* genes were obtained from genome assemblies using BLASTN, while 16S rRNA gene sequences were obtained using Barrnap (https://github.com/tseemann/barrnap). The chromosomal *bla*$_{OXY}$ sequences were also extracted, and the new amino-acid sequence variants were submitted to the Institut Pasteur MLST nomenclature database (https://bigsdb.pasteur.fr/klebsiella) for variant number attribution, and to NCBI for accession number attribution. 16S rRNA, g*yrA*, *rpoB* and *bla*OXY beta-lactamase gene sequences were aligned using Muscle (19), concatenated (in the case of *rpoB* and *gyrB*) and phylogenetic relationships were assessed using MEGA v7.0 (20). Genetic distances were inferred using the neighbor-joining method with the Jukes-Cantor correction

(21) in the case of nucleotide sequences or maximum-likelihood with Jones-Taylor-Thornton (JTT) (22) model in the case of the beta-lactamase protein sequences. The genome-based phylogenetic analysis was performed on the concatenation of 3,814 core genes defined using Roary v3.12 (23) with a blastP identity cut-off of 80% and presence in more than 90% of the isolates. *K. pneumoniae* ATCC 13883^T (GCA_000742135.1) was used as outgroup. An approximate maximum-likelihood phylogenetic tree was inferred using FastTree v2.1 (24).

Biochemical and proteomic analyses. A representative subset of strains (n=30, 7 Ko1, 5 Ko2, 4 Ko3, 5 Ko4, 6 Ko6, 3 Ko8) of phylogroups of the *K. oxytoca* complex was subjected to API20E (BioMérieux) and to phenotype microarray characterization using plates PM1 and PM2 (Biolog, Hayward, CA, USA) in aerobic conditions as previously described by Blin and colleagues (25). The same subset of strains was also used to perform a MALDI-ToF mass spectrometry (MS) analysis following the protocol described by Rodrigues *et al.* (26). Briefly, cell extracts were spotted onto an MBT Biotarget 96 target plate, air dried and overlaid with 1 µL of a saturated α-cyano-4-hydroxycinnamic acid (HCCA). Mass spectra were acquired on a Microflex LT mass spectrometer (Bruker Daltonics, Bremen, Germany) using the default parameters, preprocessed (applying smoothing and baseline subtraction) with FlexAnalysis software, and then imported and analyzed in a dedicated BioNumerics v7.6 (Applied-Maths, Belgium) database.

Results

The genome-based phylogenetic analysis based on the concatenation of 3,814 core genes (**Figure 1**) showed six distinct and highly supported branches. The thirteen Ko4 strains were clustered with Ko4 reference strain SG266 (SB3355) and this group was related to, but clearly distinct from, *K. grimontii* (Ko6). The three Ko3 strains (SPARK_350_C1, SPARK_775_C1 and SPARK_1442_C1) formed a well-defined cluster with Ko3 reference strain SG271 (SB3356, **Figure 1**), whereas the remaining three strains (SPARK_1445_C2, SPARK_1448_C1, SPARK_1495_C1) clustered with *K. huaxiensis*, which formed a distinct phylogroup that we here name Ko8. We therefore identified novel strains of these three phylogroups, which were each previously recognized based on a single strain. Furthermore, genome-based phylogeny revealed that Ko4 shares a common ancestor with *K. grimontii*, *K. michiganensis* and *K. oxytoca*, whereas Ko3 and *K. huaxiensis* share a common ancestor distinct from the Ko1/Ko4/Ko6 one (**Figure 1**).

To determine how previously-used phylogenetic markers (7, 8, 9, 27) would group these novel strains, the sequences of internal portions of the housekeeping genes *gyrA* (383 nt) and *rpoB* (501 nt), as well as the *rrs* (1,454 nt) sequence coding for 16S rRNA, were extracted from genomic sequences and compared to previously characterized sequences of reference and type strains from the *K. oxytoca* complex (**Table 1**). The clustering of Ko4 strains and Ko3 strains was supported by phylogenetic analysis of combined *gyrA* and *rpoB* gene sequences (**Figure 2**), as well as by single gene phylogenies (**Figures S1 and S2**), showing that either gene used alone would allow reliable identification. The phylogeny of the chromosomal OXY beta-lactamase gene (**Figure S3**) was also in concordance with previous phylogenetic analyses. However, phylogroup Ko1 and Ko3 each harbored two different types of *bla*$_{OXY}$, coding for OXY-1/OXY-5 and OXY-3/OXY-9, respectively (**Figure S3**). As previously reported (12, 28, 29), the phylogeny based on the *rrs* gene was not reliable for species or phylogroup identification (type strain sequences were > 97.8% similar), with only a few informative variable sites (**Figure S4**).

Average nucleotide identity (ANI) was estimated between Ko3 and Ko4, and the type strains of species of the *K. oxytoca* complex (**Table 2**). The three Ko3 strains, including SPARK_775_C1^{T}, shared high identity (above 98%) with the Ko3 strain SG271 (SB3356) (data not shown). The ANI values of SPARK_775_C1^{T} (Ko3) strain with *K. huaxiensis* (WCHKl090001^{T}), *K. michiganensis* (W14^{T}), *K grimontii* (06D021^{T}) and *K. oxytoca* (ATCC 13182^{T}) were 90.7%, 88.4%, 88.3% and 87.9%, respectively (**Table 2**). The novel Ko4 strains showed approximately 98% ANI with Ko4 strain SG266 (SB3355). The ANI values of SPARK_836_C1^{T} (Ko4) with *K. grimontii*, *K. michiganensis*, *K. oxytoca* and *K. huaxiensis* were 95.5%, 93.3%, 90.6% and 87.1%, respectively (**Table 2**). Finally, the three Ko8 strains presented ANI values >99% with the type strain of *K. huaxiensis* (WCHKl090001^{T}), showing that they belong to this recently described species. The isDDH relatedness range between the Ko3 and Ko4 type strains and other species was 36.3-44.1% and 34.3-67.8%, respectively. In conclusion, both ANI and isDDH values were below the thresholds proposed (30) for species distinction (~95-96% in the case of ANI, ~70% in the case of isDDH), indicating that Ko3 and Ko4 represent two new species.

The phenotypic characteristics of Ko3 and Ko4 strains were analyzed and compared with those of other *Klebsiella* isolates. We confirmed that all strains were non-motile by microscopy and that all isolates were positive for indole, lactose, mannitol, malonate, lysine decarboxylase and the ONPG test, and reduced nitrate to nitrite, whereas they were all negative for ornithine decarboxylase. Ko8 and Ko3 isolates were negative for Voges–Proskauer test and Ko3 isolates were urease positive (similar to Ko2). To define further the biochemical features of the *K. oxytoca* phylogroups, their carbon source utilization profiles were analysed. Among 190 substrates, several appeared useful for differentiating the phylogroups among themselves and to differentiate Ko3 and Ko4 strains from other groups (**Table 3, Figure S5**). The inability to metabolize L-proline and tricarballylic acid differentiated Ko3 strains from other phylogroups except Ko8, which can be differentiated based on its unique ability to utilize 3-O-methyl-glucose. Ko4 had a weak but unique capacity to utilize glyoxylic acid, and differed from Ko6 (*K. grimontii*) by its inability to metabolize D-melezitose; Ko4 was otherwise similar to Ko6 for many features, consistent with their phylogenetic association.

We also analysed the MALDI-ToF MS peak patterns of the different members of the *K. oxytoca* complex. Based on the MALDI Biotyper Compass database version 4.1.80 (Bruker Daltonics, Bremen, Germany), the thirty strains were identified either as *K. oxytoca* (23 strains, all belonging to Ko1, Ko2, Ko4, and Ko6) or as *Raoultella ornithinolytica* (7 strains, all strains of Ko3 and Ko8). These misidentifications can be explained by the lack of reference spectra of most phylogroups in the reference database. **Figure S6** summarizes the peak positions found in each strain. A total of 31 biomarkers (2383–10152 *m/z*) associated with specific members of the *K. oxytoca* complex were identified (**Table S1, Figure S6**). Consistent with genetic and biochemical findings, we also observed that Ko4 shared most of its spectral peaks with Ko1 and Ko6, presenting only one specific peak (which was variably present) at 3681 *m/z*, whereas Ko3 shared six peaks with only Ko8 and presented two unique peaks at 5178 and 6795 *m/z*. For the remaining phylogroups, specific peaks were observed for Ko2 and Ko8, whereas Ko1 and Ko6 could be identified by specific peak combinations. Based on the current dataset, the specificity and sensitivity of their distribution among phylogroups ranged between 60–100% and 80–100%, respectively (**Table S1**). This finding paves the way to identify isolates of the *K. oxytoca* complex at the species (or phylogroup) level based on MALDI-ToF MS analysis, pending incorporation of reference spectra of the various taxa into reference spectra databases.

Based on the above genomic, phenotypic and proteomic characteristics, we propose Ko3 and Ko4 to be considered as two novel species, which we propose to name *K. spallanzanii* and *K. pasteurii*, respectively.

Description of *Klebsiella spallanzanii* sp. nov.

Klebsiella spallanzanii (spal. lan.za 'ni.i N. L. gen. n. referring to Lazzaro Spallanzani, Italian biologist, important contributor to the experimental study of bodily functions and of animal reproduction. He provided what is considered the first disproval of the theory of the spontaneous generation of microbes).

The description is based on 3 strains. Cells are Gram-negative, non-motile, non-spore-forming, straight, rod-shaped and capsulated. Colonies are smooth, circular, white, dome-shaped and glistening. The general characteristics are as described for the genus *Klebsiella*. Indole-positive, ONPG-positive, lysine decarboxylase positive and ornithine decarboxylase negative. Differentiated from the other species of the *K. oxytoca* complex by the urease-positive (similar to Ko2) and Voges–Proskauer test negative (also negative for Ko8). Distinguished from the other members of *K. oxytoca* complex also by the characteristics listed in **Table 3**. Distinguishable from *K. huaxiensis* by the ability to use D-melezitose and the inability to ferment 3-O-methyl-glucose, and from the remaining *K. oxytoca* members by the inability to use L-proline. *K. spallanzanii* isolates were recovered from human urine and cow faeces.

The type strain is strain SPARK_775_C1^{T} (=SB6411, CIP 111695T, DSM 109531), isolated in 2017 from the urine of a patient in Pavia, Italy. The INSDC (GenBank/ENA/DDBJ) accession numbers of the *gyrA*, *rpoB* and *rrs* (coding for 16S rRNA) genes are MN076620, MN076626 and MN091365, respectively. The genome sequence accession number is: *in process*. The DNA G+C content of the type strain is 53.3%.

Description of *Klebsiella pasteurii* sp. nov.

Klebsiella pasteurii (pas. teu 'ri.i N. L. gen. n. referring to Louis Pasteur, a French microbiologist, who made seminal contributions to microbiology and infectious diseases, vaccination and pasteurization. He contributed decisively to disprove the theory of the spontaneous generation of microbes).

The description is based on 14 strains. Cells are Gram-negative, non-motile, non-spore-forming, straight, rod-shaped and capsulated. Colonies are smooth, circular, white, dome-shaped and glistening. The general characteristics are as described for the genus *Klebsiella*. Indole-positive, urease-negative, ONPG-positive, Voges–Proskauer test positive, lysine decarboxylase positive and ornithine decarboxylase negative. Distinguished from the other members of *K. oxytoca* complex by the characteristics listed in **Table 3**. Distinguishable from *K. grimontii* by the ability to ferment D-melezitose and inability to ferment alpha-keto-glutaric acid, and from the remaining *K. oxytoca* groups by the unique weak ability to ferment glyoxylic acid. *K. pasteurii* isolates were recovered from faeces of cows, turtles and humans.

The type strain is strain SPARK_836_C1^{T} (=SB6412, CIP 111696T, DSM 109530), isolated in 2017 from the faeces of a patient in Pavia, Italy. The INSDC (GenBank/ENA/DDBJ) accession numbers of the *gyrA*, *rpoB* and *rrs* (coding for 16S rRNA) genes are MN076619, MN076625 and MN091366, respectively. The genome sequence accession number is: *in process*. The DNA G+C content of the type strain is 55.4%.

Conflicts of interest

The authors declare that there is no conflict of interest.

Author contributions

Isolation of *Klebsiella* from diverse sources: CM, MC, PM, CB, DS; Microbiological characterization of isolates: CM, CR, VP, MC. Genomic sequencing : CM, HAT, TVSK, DS. Sequence data analysis: CM, CR, HAT, TVSK. MALDI-TOF analysis: CR, VP. Phenotypic microarray analyses: VP, SB. Initial manuscript writing: CM, CR, SB. Manuscript revision: all. Funding acquisition: EJF, SB, JC, CB, DS.

Acknowledgements

We acknowledge Marie-Hélène Nicolas-Chanoine and Alan McNally for providing strains included in this study.

Funding

This work received financial support from the SpARK project "The rates and routes of transmission of multidrug resistant *Klebsiella* clones and genes into the clinic from environmental sources", which has received funding under the 2016 JPI-AMR call "Transmission Dynamics" (MRC reference MR/R00241X/1); and by the French government's Investissement d'Avenir program Laboratoire d'Excellence 'Integrative Biology of Emerging Infectious Diseases' (ANR-10-LABX-62-IBEID). CR was supported financially by the MedVetKlebs project, a component of European Joint Programme One Health EJP, which has received funding from the European Union's Horizon 2020 research and innovation programme under Grant Agreement No 773830. JC was funded by the ERC grant no. 742158 and by the Norwegian Research Council JPIAMR grant no. 144501.

References

1. Brisse S, Grimont F, Grimont PAD. “The genus Klebsiella,”. In Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E, editors. The Prokaryotes-A Handbook on the Biology of Bacteria. New York, NY: Springer (2006). p. 159-197.
2. Caltagirone M, Nucleo E, Spalla M, Zara F, Novazzi F, Marchetti VM, et al. Occurrence of Extended Spectrum β-Lactamases, KPC-Type, and MCR-1.2-Producing Enterobacteriaceae from Wells, River Water, and Wastewater Treatment Plants in Oltrepò Pavese Area, Northern Italy. Front microbiol (2017) 8:2232. 10.3389/fmicb.2017.02232.
3. Schmitz RA, Klopprogge K, Grabbe R. Regulation of nitrogen fixation in Klebsiella pneumoniae and Azotobacter vinelandii: NifL, transducing two environmental signals to the nif transcriptional activator NifA. J Mol Microbiol Biotechnol (2002) 4(3):235-42.
4. Paczosa MK, Mecsas J. Klebsiella pneumoniae: Going on the Offense with a Strong Defense. Microbiol Mol Biol Rev (2018) 80(3):629-661. 10.1128/MMBR.00078-15.
5. Broberg CA, Palacios M, Miller VL. Klebsiella: a long way to go towards understanding this enigmatic jet-setter. F1000 Prime Rep (2014) 6:64. 10.12703/P6-64.
6. Fournier B, Roy PH. Variability of Chromosomally Encoded b-Lactamases from Klebsiella oxytoca. Antimicrob Agents Chemother (1997) 41(8):1641–1648.
7. Granier SA, Plaisance L, Leflon-Guibout V, Lagier E, Morand S, Goldstein FW, et al. Recognition of two genetic groups in the Klebsiella oxytoca taxon on the basis of chromosomal b-lactamase and housekeeping gene sequences as well as ERIC-1 R PCR typing. Int J Syst Evol Microbiol (2003) 53:661–668.10.1099/ijs.0.02408-0.
8. Granier SA, Leflon-Guibout V, Goldstein FW, Nicolas-Chanoine MH. New Klebsiella oxytoca beta-lactamase genes bla(OXY-3) and bla(OXY-4) and a third genetic group of

K oxytoca based on bla (OXY-3). Antimicrob Agents Chemother (2003) 47:2922–2928. 10.1128/AAC.47.9.2922-2928.2003.

9. Fevre C, Jbel M, Passet V, Weill FX, Grimont PA, Brisse S. Six groups of the OXY b-lactamase evolved over millions of years in Klebsiella oxytoca. Antimicrob Agents Chemother (2005) 49:3453–3462. 10.1128/AAC.49.8.3453-3462.2005.
10. Izdebski R, Fiett J, Urbanowicz P, Baraniak A, Derde LP, Bonten MJ, et al. Phylogenetic lineages, clones and β-lactamases in an international collection of Klebsiella oxytoca isolates non susceptible to expanded-spectrum cephalosporins. J Antimicrob Chemother (2015) 70(12):3230-7. 10.1093/jac/dkv273.
11. Saha R, Farrance CE, Verghese B, Hong S, Donofrio RS. Klebsiella michiganensis sp. nov., a new bacterium isolated from a toothbrush holder. Curr Microbiol (2013) 66:72–78. 10.1007/s00284-012-0245-x.
12. Passet V, Brisse S. Description of Klebsiella grimontii sp. nov. Int J Syst Evol Microbiol (2018) 68:377–381. 10.1099/ijsem.0.00251
13. Hu Y, Wei L, FengY, Xie Y, Zong Z. Klebsiella huaxiensis sp. nov., recovered from human urine. Int J Syst Evol Microbiol (2019) 69(2):333-336. 10.1099/ijsem.0.003102.
14. Van Kregten E, Westerdaal NA, Willers JM. New, simple medium for selective recovery of Klebsiella pneumoniae and Klebsiella oxytoca from human feces. J Clin Microbiol (1984) 20(5):936-41.
15. Brisse S, Passet V, Grimont PA. Description of Klebsiella quasipneumoniae sp. nov., isolated from human infections, with two subspecies, Klebsiella quasipneumoniae subsp. Quasipneumoniae subsp. nov. and Klebsiella quasipneumoniae subsp. similipneumoniae subsp. nov., and demonstration that Klebsiella singaporensis is a junior heterotypic synonym of Klebsiella variicola. Int J Syst Evol Microbiol (2014) 64:3146–3152. 10.1099/ijs.0.062737-0.

16. Seemann T. Prokka: rapid prokaryotic genome annotation. Bioinformatics (2014) 30(14):2068-9. 10.1093/bioinformatics/btu153.
17. Richter M, Rosselló-Móra R, Glöckner FO, Peplies J. JSpeciesWS: a web server for prokaryotic species circumscription based on pairwise genome comparison. Bioinformatics (2016) 32(6):929-31. 10.1093/bioinformatics/btv681.
18. Meier-Kolthoff JP, Auch AF, Klenk HP, Göker M. Genome sequence-based species delimitation with confidence intervals and improved distance functions. BMC Bioinformatics (2013) 14:60.
19. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput Nucleic Acids Res (2004) 32(5):1792-1797. 10.1093/nar/gkh340.
20. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol (2016) 33(7):1870-4. 10.1093/molbev/msw054.
21. Jukes TH, Cantor CR. “Evolution of protein molecules”. In: Munro HN, editor. Mammalian Protein Metabolism, New York, NY: Academic Press (1969) p. 21-132.
22. Jones DT, Taylor WR, Thornton JM. The rapid generation of mutation data matrices from protein sequences. Comput Appl Biosci (1992) 8:275–282.
23. Page AJ, Cummins CA, Hunt M, Wong VK, Reuter S, Holden MTG, et al. Roary: Rapid large-scale prokaryote pan genome analysis. Bioinformatics (2015). 31(22):3691–3693. 10.1093/bioinformatics/btv421.
24. Price MN, Dehal PS, Arkin AP. FastTree 2 - Approximately Maximum-Likelihood Trees for Large Alignments. PLoS ONE (2010) 5(3):e9490. 10.1371/journal.pone.0009490.
25. Blin C, Passet V, Touchon M, Rocha EPC, Brisse S. Metabolic diversity of the emerging pathogenic lineages of Klebsiella pneumoniae. Environ Microbiol (2017) 19(5):1881-1898. 10.1111/1462-2920.13689.

26. Rodrigues C, Passet V, Rakotondrasoa A, Brisse S. Identification of Klebsiella pneumoniae, Klebsiella quasipneumoniae, Klebsiella variicola and Related Phylogroups by MALDI-TOF Mass Spectrometry. Front Microbiol (2018) 9:3000. 10.3389/fmicb.2018.03000.

27. Brisse S, Verhoef J. Phylogenetic diversity of Klebsiella pneumoniae and Klebsiella oxytoca clinical isolates revealed by randomly amplified polymorphic DNA, gyrA and parC genes sequencing and automated ribotyping. Int J Syst Evol Microbiol (2001) 51:915-24. 10.1099/00207713-51-3-915.

28. Naum M, Brown EW, Mason-Gamer RJ. Is 16S rDNA a reliable phylogenetic marker to characterize relationships below the family level in the Enterobacteriaceae? J Mol Evol (2008) 66(6):630-42. 10.1007/s00239-008-9115-3.

29. Boye K, Hansen DS. Sequencing of 16S rDNA of Klebsiella: taxonomic relations within the genus and to other Enterobacteriaceae. Int J Med Microbiol (2003) 292(7-8):495-503. 10.1078/1438-4221-00228.

30. Rossello-Mora R, Amann R. Past and future species definitions for Bacteria and Archaea. Syst Appl Microbiol (2015) 38:209-216. 10.1016/j.syapm.2015.02.001.

Figure legends

Figure 1. Maximum likelihood phylogenetic tree inferred based on the concatenated nucleotide sequence alignments of 3,814 core genes.

The tree was rooted using *K. pneumoniae* DSM 30104^T (=ATCC 13883^T). Taxonomic groups are indicated in front of the branches. Branch lengths represent the number of nucleotide substitutions per site (scale, 0.01 substitution per site). Bootstrap values are indicated at major nodes. Strain labels are given as Strain Bank ID (*e.g.*, SB73) followed by original strain name, followed by the phylogroup. A 'T' after the strain name indicates that the strain is the type strain of its taxon.

Figure 2. Phylogenetic relationships (neighbor-joining method, Jukes-Cantor correction) based on the concatenated sequences of *gyrA* and *rpoB* genes.

The tree was rooted using *K. pneumoniae* DSM 30104^T (=ATCC 13883^T). Taxonomic groups are indicated in front of the branches. Bootstrap proportions obtained after 1000 replicates are indicated at the nodes. Branch lengths represent the number of nucleotide substitutions per site (scale, 0.01 substitution per site). Strain labels are given as Strain Bank ID (e.g., SB73) followed by original strain name, followed by phylogroup. A 'T' after the strain name indicates that the strain is the type strain of its taxon.

Table 1. Strains included in the study, with provenance and genomic information

Taxonomic designation	PhG[a]	Strain bank (SB) ID[b]	Strain Name	Isolation year	Host	Source	Country	City	Accession no.	Intrinsic Beta-lactamase[c] (Accession no.)
Klebsiella michiganensis	Ko1	**SB4934**	W14 T (=CIP 110787 T)	2010	n.a.	Tooth brush holder	USA	Michigan	GCA_901556995	**OXY_1-7** (MN030558)
K. michiganensis	Ko1	**SB9**	16A079	1997	Human	Blood	Spain	Sevilla	GCA_901553745	OXY_1-2 (AY077484)
K. michiganensis	Ko1	**SB2908**	10A188	1997	Human	Blood	Italy	Genoa	GCA_901563895	OXY_5-1 (AJ871868)
K. oxytoca	Ko2	**SB175**	ATCC 13182 T	NA	NA	NA	NA	NA	GCA_900977765	OXY_2-2 (AF473577)
K. spallanzanii	Ko3	**SB6408**	SPARK_350_C1	2017	n.a.	Boot	Italy	Pavia	ERS3550822	**OXY_3-2** (MN030559)
K. spallanzanii	Ko3	**SB6411**	SPARK_775_C1 T (=CIP 111695T)	2017	Human	Urine	Italy	Pavia	ERS3550824	**OXY_3-3** (MN030560)
K. spallanzanii	Ko3	**SB6419**	SPARK_1442_C2	2018	Cow	Faeces	Italy	Valle Salimbene	ERS2601707	**OXY_9-1** (MN030564)
K. spallanzanii	Ko3	**SB3356**	SG271	2000	Human	Peritoneal fluid	France	Paris	GCA_901563875	OXY_3-1 (AF491278)
K. pasteurii	Ko4	**SB3355**	SG266	2000	Human	Wound	France	Paris	GCA_901563825	OXY_4-1 (AY077481)
K. pasteurii	Ko4	**SB6407**	SPARK_327_C1	2017	Cow	Faeces	Italy	Pavia	ERS3550826	OXY_4-1 (AY077481)
K. pasteurii	Ko4	**SB6410**	SPARK_613_C1	2017	Turtle	Faeces	Italy	Sant'Alessio con Vialone	ERS2600949	OXY_4-1 (AY077481)
K. pasteurii	Ko4	**SB6412**	SPARK_836_C1 T (=CIP111696T)	2017	Human	Faeces	Italy	Pavia	ERS3550825	**OXY_4-2** (MN030561)
K. pasteurii	Ko4	**SB6424**	SPARK_1489_C1	2018	n.a.	Soil	Italy	San Genesio	ERS2601773	OXY_4-1 (AY077481)
K. pasteurii	Ko4	**SB6409**	SPARK_534_C3	2017	Turtle	Faeces	Italy	Sant'Alessio con Vialone	ERS3550823	OXY_4-1 (AY077481)

K. pasteurii	Ko4	**SB6413**	SPARK_1058_C2	2018	Human	Faeces	Italy	Pavia	ERS2601251	OXY_4-1 (AY077481)
K. pasteurii	Ko4	**SB6414**	SPARK_1260_C1	2018	Cow	Faeces	Italy	Magherno	ERS2601488	**OXY_4-3** (MN030562)
K. pasteurii	Ko4	**SB6415**	SPARK_1268_C1	2018	Cow	Milk	Italy	Magherno	ERS2601499	**OXY_4-3** (MN030562)
K. pasteurii	Ko4	**SB6416**	SPARK_1269_C1	2018	Cow	Milk	Italy	Magherno	ERS2601500	OXY_4-1 (AY077481)
K. pasteurii	Ko4	**SB6417**	SPARK_1286_C1	2018	Human	Faeces	Italy	Pavia	ERS2601525	**OXY_4-4** (MN030563)
K. pasteurii	Ko4	**SB6420**	SPARK_1445_C1	2018	Cow	Faeces	Italy	Valle Salimbene	ERS2601710	OXY_4-1 (AY077481)
K. pasteurii	Ko4	**SB6423**	SPARK_1448_C2	2018	Cow	Faeces	Italy	Valle Salimbene	ERS2601714	**OXY_4-5** (MN030567)
K. pasteurii	Ko4	**-**	SPARK_1531_C1	2018	n.a.	Water	Italy	Lardirago	ERS2601825	OXY_4-1 (AY077481)
K. grimontii	Ko6	**SB73**	06D021 T	1997	Human	Wound	France	Lille	GCA_900200035	OXY_6-1 (AJ871873)
K. huaxiensis	Ko8	**SB6421**	SPARK_1445_C2	2018	Cow	Faeces	Italy	Valle Salimbene	ERS2601711	**OXY_8-2** (MN030565)
K. huaxiensis	Ko8	**SB6422**	SPARK_1448_C1	2018	Cow	Faeces	Italy	Valle Salimbene	ERS2601714	**OXY_8-3** (MN030566)
K. huaxiensis	Ko8	**SB6425**	SPARK_1495_C1	2018	Human	Faeces	Italy	Pavia	ERS2601786	OXY_8-1 (WP_112215366)
K. huaxiensis	Ko8	**SB6550**	WCHKl090001 T	2017	Human	Urine	China	Chengdu	GCA_003261575	OXY_8-1 (WP_112215366)

NA, information not available; n.a. not applicable; T, Type strain.

[a]PhG, *K. oxytoca* phylogroup; [b]Internal strain collection number of the Biodiversity and Epidemiology of Bacterial Pathogens unit, Institut Pasteur.[c] bold characters represent the new OXY beta-lactamases submitted to the nomenclature database at https://bigsdb.pasteur.fr/klebsiella/klebsiella.html.

Table 2. Average nucleotide identity (ANI) values obtained among the type strains of members of the *Klebsiella oxytoca* complex.

			Average nucleotide identity of test genome against query genomes					
Query genome[a]	**Size** (nucleotides)	**DNA G+C content** (mol%)	**Ko1**	**Ko2**	**Ko3**	**Ko4**	**Ko6**	**Ko8**
Ko1	6 193 009	56.0	*	91.65	88.53	93.09	93.23	87.47
Ko2	5 672 774	55.1	91.92	*	88.22	90.81	91.06	87.05
Ko3	6 186 380	53.3	88.4	87.9	*	87.99	88.32	90.7
Ko4	6 006 767	55.3	93.29	90.61	88.12	*	95.52	87.11
Ko6	6 168 876	55.4	93.27	90.9	88.5	95.56	*	87.45
Ko8	6 206 993	53.3	87.07	86.64	90.58	86.78	87.07	*

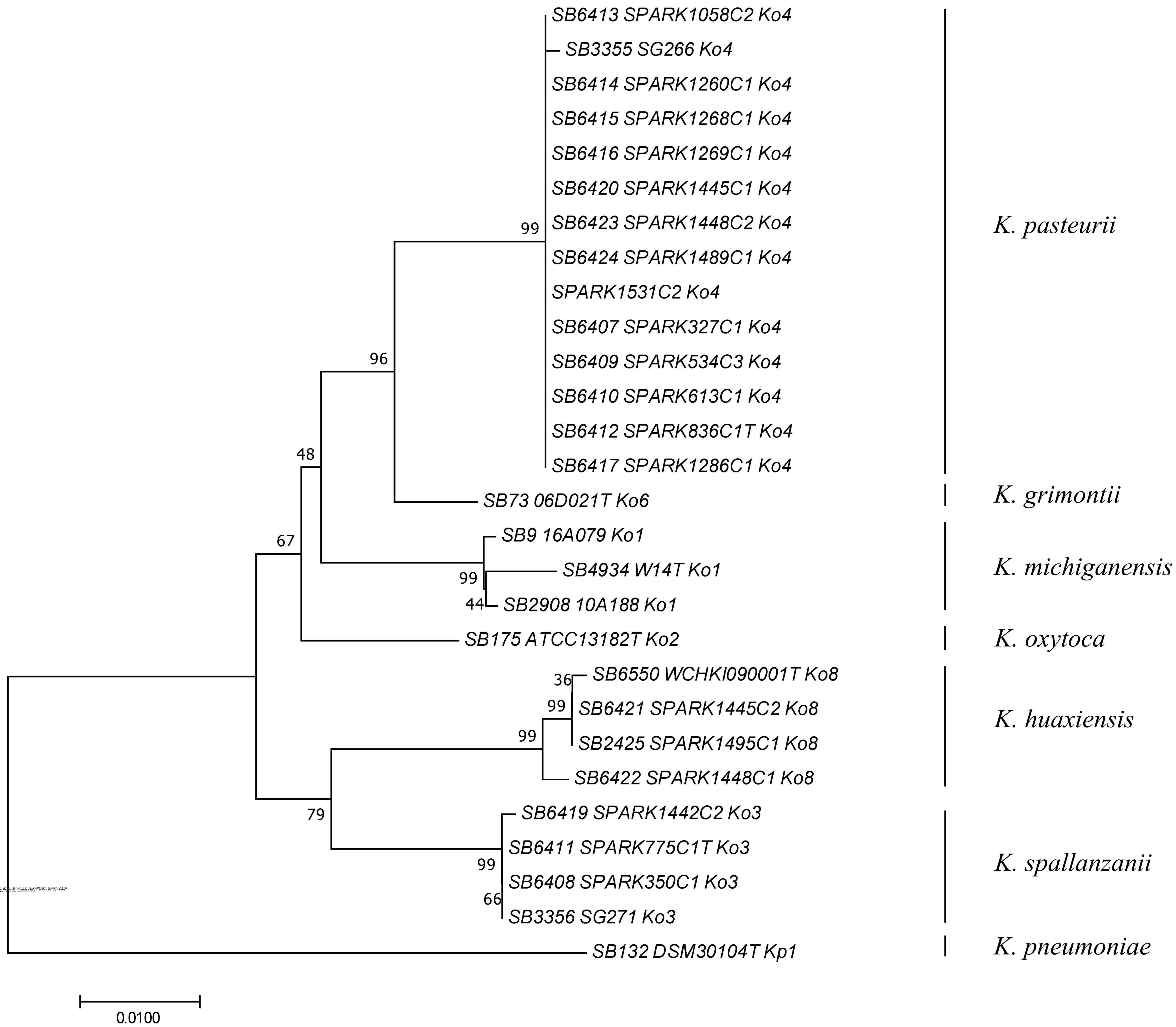
[a]Ko1, *K. michiganensis* W14^T; Ko2, *K. oxytoca* ATCC13182^T; Ko3, *K. spallanzanii* SPARK_775_C1^T; Ko4, *K. pasteurii* SPARK_836_C1^T; Ko6, *K. grimontii* 06D021^T; Ko8, *K. huaxiensis* WCHKl090001^T.

Table 3. Differential biochemical characteristics of the taxa under study.

	K. michiganensis (Ko1, n=7)	***K. oxytoca*** (Ko2, n=5)	***K. spallanzanii*** (Ko3, n=4)	***K. pasteurii*** (Ko4, n=5)	***K. grimontii*** (Ko6, n=6)	***K. huaxiensis*** (Ko8, n=3)
Metabolic phenotypes						
L-proline	+	+	-	+	+	-
D,L-a-Glycerol-phosphate	+	+	v	+	v	-
alpha-Keto- Glutaric Acid	-	-	-	-	+	-
Glyoxylic Acid	-	-	-	v	-	-
Tricarballylic acid	+	+	-	+	+	-
Acetyl-b-D-Mannosamine	v	+	v	+	+	+
D-Melezitose	+	+	+	+	-	v
3-O-Methyl-Glucose	-	-	-	-	-	+
g-Amino-Butyric Acid	+	+	-	v	v	-
L-Tartaric Acid	v	v	v	+	+	-

-, less than 20% of positive strains; +, more than 80% of positive strains; v, between 20% and 80% of positive strains.


SB73 06D021T Ko6
SPARK1531C2 Ko4
SB3355 SG266 Ko4
SB6420 SPARK1445C1 Ko4
SB6409 SPARK534C3 Ko4
SB6410 SPARK613C1 Ko4
SB6416 SPARK1269C1 Ko4
SB6423 SPARK1448C2 Ko4
SB6412 SPARK836C1T Ko4
SB6417 SPARK1286C1 Ko4
SB6415 SPARK1268C1 Ko4
SB6424 SPARK1489C1 Ko4
SB6414 SPARK1260C1 Ko4
SB6407 SPARK327C1 Ko4
SB6413 SPARK1058C2 Ko4
SB2908 10A188 Ko1
SB4934 W14T Ko1
SB9 16A079 Ko1
SB175 ATCC13182T Ko2
SB6422 SPARK1448C1 Ko8
SB6421 SPARK1445C2 Ko8
SB6425 SPARK1495C1 Ko8
SB6550 WCHKI090001T Ko8
SB6419 SPARK1442C2 Ko3
SB6408 SPARK350C1 Ko3
SB3356 SG271 Ko3
SB6411 SPARK775C1T Ko3
SB132 DSM30104T Kp1
100
100
100
100
100
100
100
100
K. grimontii
K. pasteurii
K. michiganensis
K. oxytoca
K. huaxiensis
K. spallanzanii
Tree scale: 0.01


SB6413 SPARK1058C2 Ko4
SB3355 SG266 Ko4
SB6414 SPARK1260C1 Ko4
SB6415 SPARK1268C1 Ko4
SB6416 SPARK1269C1 Ko4
SB6420 SPARK1445C1 Ko4
SB6423 SPARK1448C2 Ko4
SB6424 SPARK1489C1 Ko4
SPARK1531C2 Ko4
SB6407 SPARK327C1 Ko4
SB6409 SPARK534C3 Ko4
SB6410 SPARK613C1 Ko4
SB6412 SPARK836C1T Ko4
SB6417 SPARK1286C1 Ko4
K. pasteurii
SB73 06D021T Ko6
K. grimontii
SB9 16A079 Ko1
SB4934 W14T Ko1
SB2908 10A188 Ko1
K. michiganensis
SB175 ATCC13182T Ko2
K. oxytoca
SB6550 WCHKI090001T Ko8
SB6421 SPARK1445C2 Ko8
SB2425 SPARK1495C1 Ko8
SB6422 SPARK1448C1 Ko8
K. huaxiensis
SB6419 SPARK1442C2 Ko3
SB6411 SPARK775C1T Ko3
SB6408 SPARK350C1 Ko3
SB3356 SG271 Ko3
K. spallanzanii
SB132 DSM30104T Kp1
K. pneumoniae
99
96
48
67
99
44
36
99
99
79
99
66
0.0100