Klebsiella singaporenensis sp. nov., a novel isomaltulose-producing bacterium

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Cells of strain LX3T, isolated from soil, were Gram-negative, facultatively anaerobic, non-motile, capsulated and non-endospore-forming straight rods, able to grow at 10 °C, unable to produce gas from lactose at 45 °C and unable to produce indole. The isolate converted sucrose to isomaltulose and did not produce detectable glucose by-products. The G+C content of the DNA was 56.4 mol%. Furthermore, comparison of 16S rRNA and rpoB gene sequences showed that the isolate clearly belongs to the genus Klebsiella. The closest phylogenetic relative was Klebsiella pneumoniae, there being 99.3 and 97.5% similarity in 16S rRNA and rpoB gene sequences, respectively. DNA–DNA hybridization analysis demonstrated a very low level of relatedness to other members of the genus Klebsiella, indicating that the isolated strain and other species in the genus Klebsiella were not related at the species level. The isolate could be differentiated from other previously described members of the genus Klebsiella on the basis of phenotypic differences and 16S rRNA and rpoB gene sequence divergence, together with DNA–DNA reassociation data. Therefore, it is proposed that strain LX3T (=DSM 16265T = JCM 12419T) should be classified as the type strain of a novel species of genus Klebsiella, Klebsiella singaporenensis sp. nov.

Isomaltulose (6-O-α-D-glucopyranosyl-D-fructofuranose), a functional isomer of sucrose, is a non-cariogenic reducing disaccharide found naturally in small quantities in honey. It has physical and organoleptic properties very similar to those of sucrose, except that its sweetness is about 42% of that of sucrose. Isomaltulose is decomposed only slightly by Streptococcus mutans, and by dental plaque suspensions. Much less acid and insoluble glucan are produced from isomaltulose compared with that from sucrose in human dental plaque (Takazoe et al., 1982; Topitsoglou et al., 1984). Since isomaltulose was first reported by Stodola et al. (1956) as a by-product during dextran production from sucrose, microbial transformation of sucrose to isomaltulose has been reported for several bacteria such as ‘Protaminobacter rubrum’ (Weidenhagen & Lorenz, 1957), Erwinia rhapontici (Cheetham et al., 1982), Serratia plymuthica (McAllister et al., 1990), Klebsiella planticola (Tsuyuki et al., 1992) and ‘Pseudomonas mesoacidophila’ (Miyata et al., 1992). However, these isomaltulose-producing strains not only transform sucrose to isomaltulose and trehalulose, but also produce about 2–7% glucose as by-products (Tsuyuki et al., 1992), which is a considerable industrial problem because elaborate purification is necessary to remove them. In our efforts to screen for efficient isomaltulose-producing bacterial strains, one isolate, designated LX3T, was obtained from soil samples. This strain produced isomaltulose efficiently but glucose was not detectable.

The latest edition of Bergey’s Manual of Systematic Bacteriology (Orskov, 1984) classified the genus Klebsiella into four species, Klebsiella pneumoniae, Klebsiella oxytoca, Klebsiella terrigena and K. planticola, with K. pneumoniae comprising three subspecies, K. pneumoniae subsp. pneumoniae, K. pneumoniae subsp. ozaenae and K. pneumoniae subsp. rhinoscleromatis. Klebsiella ornithinolytica was subsequently described for ornithine-positive strains of K. oxytoca (Sakazaki et al., 1989). Klebsiella trevisanii has been incorporated into K. planticola as a later heterotypic synonym, and Klebsiella mobilis is a later homotypic synonym of Enterobacter aerogenes. Calymmatobacterium granulomatis was reclassified as Klebsiella granulomatis on the basis of phylogenetic data (Carter et al., 1999). The genus Klebsiella is polyphyletic and has not been subjected to extensive 16S rRNA gene sequence analysis until recently. After phylogenetic analysis of 16S rRNA and rpoB gene

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and rpoB nucleotide sequences of strain LX3T are respectively AF250286 and AF250286.
sequences, Drancourt et al. (2001) confirmed that the genus *Klebsiella* is heterogeneous and composed of species that form three clusters: cluster I, containing *K. granulomatis* and the three subspecies of *K. pneumoniae*, cluster II, containing *K. ornithinolytica*, *K. planticola* and *K. terrigena*; and cluster III, containing *K. oxytoca*. The genus *Klebsiella* was therefore divided into two genera, *Klebsiella* and *Raoultella*, and an unnamed group.

In this paper, the results of an examination of the phenotypic characteristics of strain LX3T are described, along with the phylogenetic placement of the strain. The name *Klebsiella singaporensis* sp. nov. is proposed with strain LX3T as the type strain.

Soil samples were suspended in sterilized saline water and spread over sucrose agar plates in serial dilution. All plates were incubated at 30 °C for 24 h. A part of each single colony on the sucrose agar plate was transferred to a new sucrose agar plate as a temporary stock culture. Another part was inoculated into sucrose/peptone/yeast extract (SPY) medium and incubated at 30 °C with shaking overnight. This culture was used to detect strains producing isomaltulose, by the dinitrosalicylate method (Miller, 1959) and TLC. Sucrose agar medium contained (per 1000 ml) 40 g sucrose, 5 g yeast extract, 0.5 g MgSO4·7H2O, 0.7 g KNO3, 1 g KH2PO4, 0.5 g NH4Cl, 1 g NaCl and 20 g agar; the pH was adjusted to 7.0 with NaOH. SPY medium comprised (per 1000 ml) 40 g sucrose, 10 g peptone and 4 g yeast extract (pH 7.0). Basal medium consists of (per 1000 ml) 0.5 g MgSO4·7H2O, 0.7 g KNO3, 1 g KH2PO4, 0.5 g NH4Cl and 1 g NaCl (pH 7.0–7.2).

With this method, single colonies producing isomaltulose from sucrose were isolated from soil samples collected from sugar-cane roots and Clementi Woods in Singapore. The isolate was purified further on the sucrose agar plate by streaking; the purified strain was designed as LX3T.


A 0.5 µl aliquot of the culture supernatant, in which reducing sugar could be detected by the dinitrosalicylate method (Miller, 1959), was spotted onto a TLC plate (silica gel on polyester; Aldrich) and developed. The solvent system was ethylacetate/acetonic acid/water (4:3:0.8 by vol.). The spots were visualized with the diphenylamine/aniline/phosphoric acid reagent (Schwimmer & Bevenue, 1956) at 80 °C for 5 min. The spot colour of isomaltulose is yellowish-green. If the spot that occurred was of the same colour, and in the same position, as that of an isomaltulose standard sample on the silica-gel plate, it demonstrated that the strain could produce isomaltulose.

Unless otherwise indicated, all experiments were conducted in triplicate.

The cultures were grown on sucrose agar for 16 h at 30 °C and bacterial cells were examined with a phase-contrast microscope and a transmission electron microscope. For transmission microscopy, bacterial cells were fixed with 5 % (w/v) glutaraldehyde and 1 % (w/v) osmium tetroxide. Ultrathin sections of the sample embedded in epoxy resin were prepared with an ultramicrotome (Leica Microsystems), stained with uranyl acetate and lead citrate and then examined with a model JEM-1200 EX transmission electron microscope (Japan Electron Optics Laboratory). Cellular morphology was also assessed after Gram staining and was observed by light microscopy; the morphology of fixed specimens was compared with that of living cells. Gram-staining characteristics were determined using the Hucker method, as described by Doetsch (1981).

Oxidase activity was determined by oxidation of 1 % (w/v) tetramethyl-p-phenylenediamine on filter paper, and catalase activity was detected by bubble formation in a 3 % (w/v) hydrogen peroxide solution after incubation in SPY medium for 18–48 h (Smibert & Krieg, 1994). The media used to evaluate utilization of various substrates for growth were prepared by adding 0.2 % (w/v) of each substrate to a basal medium. In tests of the utilization of carbon sources or nitrogen sources, the optical density at 600 nm of a culture after cultivation in each medium was compared with the optical density of a culture grown in basal medium. Tests for gelatin hydrolysis, indole production, hydrogen sulphide production, nitrate reduction, acid-fast staining, the Voges–Prevot reaction, the methyl red reaction and the hydrolysis of starch, casein and agar were performed by using methods described previously (Smibert & Krieg, 1994). Acid production from carbohydrates was determined in basal medium supplemented with various carbohydrates as described by Smibert & Krieg (1994).

Growth at different temperatures between 5 and 60 °C was measured by inoculating a loopful of young culture onto sucrose agar and incubating the inoculated plates at the required temperatures. The growth results were observed at 2 and 10 days.

DNA was extracted from cells grown at 30 °C overnight in SPY medium, and purified by using the methods described (Wilson, 1990). The G+C content of DNA was determined by the thermal denaturation method described by Marmur & Doty (1962). Levels of DNA relatedness were determined (hybridization at 64 °C) by using the non-radioactive detection system developed by Ziemke et al. (1998).

A 16S rRNA gene DNA fragment from strain LX3T that corresponds to position 95–1395 of *Escherichia coli* 16S
rRNA was amplified by a PCR, using purified DNA and a primer combination consisting of 5′-TGACGAGTGGCGGAGCGGGTTG-3′ (forward primer) and 5′-CCATGGTGTTGACGGCCGGTGG-3′ (reverse primer). The amplification products were purified with a QIAquick PCR purification kit (Qiagen) and were sequenced using a dRhodamine terminator cycle sequencing kit (PE Applied Biosystems) and a model 2400 Perkin Elmer GeneAmp PCR System (PE Applied Biosystems). Sequences were determined with a Perkin Elmer ABI PRISM 377 DNA sequencer. The closest known relatives of the new isolate was determined by performing sequence database searches; the sequences of closely related strains were retrieved from the GenBank and Ribosomal Database Project (Cole et al., 2003) libraries. These sequences were aligned using the CLUSTAL X program (Thompson et al., 1997) and the alignment was corrected manually. Distance matrices were calculated with the DNADIST program of the PHYLIP package (Felsenstein, 1995) and a phylogenetic unrooted tree was constructed with the neighbour-jointing method using the NEIGHBOR program contained in the PHYLIP package (Felsenstein, 1995). The statistical significance of the groups obtained was assessed by bootstrapping (100 replicates) using the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE in the PHYLIP package (Felsenstein, 1995).

The genomic DNA of strain LX3T encoding the RNA polymerase β-subunit (rpoB) gene, corresponding to positions 1468–2114 of Pseudomonas putida (X04642), Salmonella typhimurium (X04642), Pseudomonas putida (X15849) and K. pneumoniae (U77443): 5′-CAGTTCCGGCTGGCTT-3′ (forward primer) and 5′-CCGGTGGCCTACGTTGG-3′ (reverse primer). The PCR products were purified and sequenced as described above. The phylogenetic analysis of rpoB was carried out using the same procedure and program as that for 16S rRNA.

Colonies on sucrose agar medium were circular, smooth, pulvinate, entire, opaque, white and viscous when tested with a needle. Strain LX3T comprised Gram-negative, non-motile, straight rods with round ends, arranged singly and sometimes in pairs. The mean cell dimensions on sucrose agar medium were 0.6–0.8 μm (diameter) and 0.9–2.0 μm (length). Endospores were never observed.

Strain LX3T exhibited catalase and urease activities, but not oxidase, nitrate reductase or lipase activities. It failed to hydrolyse gelatin, starch, cellulose or casein. The Voges–Proskauer test was positive but the methyl red reaction was negative. LX3T was facultatively anaerobic and capsule.s. The isolate grew when citrate or glucose was used as a sole carbon source. It produced acid and gas from the carbon sources tested, including glucose, sucrose, lactose, trehalulose, maltose, fructose, mannitol, glycerol, inositol, mannose, galactose and sorbitol. It could not utilize palatinose or L-sorbose as sole carbon sources. Nitrogen sources such as peptone, tryptone, yeast extract, beef extract, casein hydrolysate, KNO3 and (NH4)2SO4 supported growth of the isolate, whereas urea did not. The isolate had no special growth-factor requirements. H2S was produced from cysteine but not from triple-sugar-iron agar. The test for indole production was positive. The isolate could grow at 10°C but could not produce gas from lactose at 45°C.

According to the previous description of the genus Klebsiella (Örskov, 1984), this genus has the following morphological characteristics: a negative reaction in Gram-stain and oxidase tests, is facultatively anaerobic, rod-shaped and non-motile, gives a positive reaction in the Voges–Proskauer test, and produces capsules. These characteristics were consistent with those of our isolate, suggesting that strain LX3T should belong to the genus Klebsiella.

The G+C content of strain LX3T was 56.4 ± 0.7 mol%, which was comparable to that of the genus Klebsiella (53–58 mol%). The DNA–DNA relatedness between the newly isolated strain and Klebsiella species ranged from 3-4 to 28-2 %. Strain LX3T exhibited 16–7–28-2 % relatedness with members of Klebsiella cluster I, except for K. pneumoniasubsp. rhinoscleromatis, for which the value was lower (3-4 %). Klebsiella cluster II containing K. planticola, K. trevisanii, K. ornithinolytica and K. terrigena exhibited values of 12-3-25-5 % for relatedness to isolate LX3T. With K. oxytoca and K. mobilis (= Enterobacter aerogenes), strain LX3T showed 15-5-20-5 % DNA–DNA relatedness. Although the values of DNA–DNA relatedness were below the threshold value of 28 % recommended for genus delineation in the Enterobacteriaceae, these values were in the range of those previously reported in the analysis of the genus Klebsiella. Values as low as 13–38 % for K. ornithinolytica to K. planticola and 10–34 % to K. terrigena (Sakazaki et al., 1989) and 7–29 % relatedness of K. planticola and K. pneumoniae (Bagley et al., 1981) have been cited in previous phylogenetic analyses of the genus Klebsiella, and numerous bacterial species in other genera also exhibited much lower DNA–DNA relatedness (Etchebehere et al., 1998; Yoon et al., 2001). Likewise, the low DNA–DNA relatedness with the members of the genus Klebsiella clearly supported the classification of the isolate in a novel species of the genus Klebsiella.

The 16S rRNA gene sequence of strain LX3T was determined using a total of eight primers for double-strand sequencing; a 1282-base sequence was obtained. Comparison with 16S rRNA gene sequences available in databases revealed that strain LX3T was closely related to species belonging to the genus Klebsiella and was peripherally related to some species of the genera Enterobacter and Citrobacter. The most closely related species was K. pneumoniae and the levels of sequence similarity among strains tested ranged from 97.2 to 99.3 %. The dataset used for the construction of the phylogenetic tree contained 1281 unambiguous nucleotides between positions 95 and 1395 (Escherichia coli position numbers). A dendrogram
was obvious that strain LX3T and cluster I shared a higher similarity to the other three species in the genera sequence similarity to 97 % 16S rRNA sequence similarities ranged from 97 to 2134 %.

Recently Drancourt et al. (2001) divided the genus Klebsiella into three clusters, which grouped K. granulomatis and the three subspecies of K. pneumoniae into cluster I as Klebsiella sensu stricto, grouped K. planticola, K. terrigena and K. ornithinolytica into cluster II, given the new genus name Raoultella, and grouped K. oxytoca into cluster III. It was evident from tree topology that strain LX3T had a close phylogenetic relationship with members of cluster I (i.e. Klebsiella sensu stricto). These data demonstrated that strain LX3T clearly belongs to the genus Klebsiella and that the closest relative is K. pneumoniae (99-3 % similarity). This relationship between the cluster of strain LX3T, K. pneumoniae and K. granulomatis was supported by the bootstrap analysis at a confidence level of 99 %. Strain LX3T exhibited 98-6 %–98-8 % 16S rRNA gene sequence similarity to the other three species in Klebsiella cluster I, 97-6 %–97-7 % similarity to Klebsiella cluster II and 97-2 % sequence similarity to K. oxytoca. With other related species in the genera Enterobacter or Citrobacter, however, the 16S rRNA sequence similarities ranged from 97-2 to 97-9 %. It was obvious that strain LX3T and cluster I shared a higher similarity than did strain LX3T and cluster II or III. These data were in accordance with the proposal of 98 % 16S rRNA gene sequence similarity as a cut-off value for delineating different genera (Drancourt et al., 2001). Also, such a differentiation at genus level was further supported by biochemical characteristics, as only cluster II and cluster III could utilize L-sorbosine as a sole carbon source. Though strain LX3T and K. pneumoniae had high 16S rRNA gene sequence similarity (99-3 %), the separation of strain LX3T from the subspecies of K. pneumoniae was supported by the low values for DNA–DNA reassociation.

It was noted that in some taxa, particularly members of the Enterobacteriaceae, variation within the 16S rRNA gene prevented confident species identification. Mollet et al. (1997) found that levels of divergence between rpoB sequences (which represented the most variable part of the gene) of different strains in the family Enterobacteriaceae were markedly higher than those between 16S rRNA genes. Thus rpoB gene analysis is another useful identification tool, at least for the species in the Enterobacteriaceae. The partial rpoB gene of strain LX3T was sequenced to determine its phylogenetic position among the enteric members of the Enterobacteriaceae. The tree topology for rpoB gene sequences was generated as shown in Fig. 2. The rpoB gene sequence of strain LX3T was compared with those of members of the family Enterobacteriaceae available in databases, and a similarity matrix was obtained. In accordance with the inference by Mollet et al. (1997), the highest level of rpoB sequence similarity, between strain LX3T and K. pneumoniae (97-5 %), was about 2 % lower than that in 16S rRNA gene sequence analysis. Accordingly, 98–100 % intraspecies similarity was observed among enteric strains, whereas 2–21 % interspecies difference occurred (Mollet et al., 1997). Phylogenetic analysis showed that the smallest divergence is 2-5 % (K. pneumoniae), suggesting that strain LX3T could be distinguished from other Klebsiella species more confidently. As shown in Fig 2 (which shows a phylogenetic branching pattern similar to that for the 16S rRNA gene), strain LX3T also fell within Klebsiella cluster I. The most closely related member was K. pneumoniae (97-5 % similarity), which was supported by an 81 %
bootstrapped value. Compared with other members of Klebsiella cluster II, strain LX3T showed 96–9–97±3 % rpoB gene sequence similarity. Strain LX3T exhibited low values of sequence similarity (92·4–93·4 %) with cluster II and 93·4 % sequence similarity to cluster III. These data were also in good agreement with the proposal of 94 % as the cut-off value for delineating different genera (Drancourt et al., 2001).

The phenotypic characteristics of strain LX3T were also consistent with its classification in the genus Klebsiella, but there were some notable phenotypic traits that might be very important for distinguishing strain LX3T from other members of Klebsiella: these included indole production, growth at 10 °C, the faecal coliform test and the Voges–Proskauer test (Table 1). The differences between the isolate and the most closely related species, K. pneumoniae, were that the latter produced gas from lactose at 44·5 °C but failed to grow at 10 °C. Among the differential phenotypic characteristics, strain LX3T could be distinguished from Klebsiella cluster II or cluster III including K. terrigena by tests for the utilization of palatinose or L-sorbose as sole carbon sources, strain LX3T being negative and the latter being positive. The problem in evaluating the phylogenetic position of K. granulomatis was the difficulty in culturing the organism (Richens, 1991): although a difference in the urease test occurred between strain LX3T and K. granulomatis (urease-negative), the latter usually needed growth factors (Carter et al., 1999) while strain LX3T does not. To distinguish strain LX3T from K. mobilis, the urease test may be decisive; strain LX3T is urease-positive and K. mobilis is urease-negative. The level of DNA–DNA relatedness revealed that strain LX3T was distinct from other members of genus Klebsiella.

On the basis of the physiological and phylogenetic characteristics of strain LX3T, as well as DNA–DNA relatedness analyses, it was evident that the isomaltulose-producing strain isolated from soil samples cannot be assigned to any previously recognized bacterial species. We propose that strain LX3T should be placed in a novel species, Klebsiella singaporenensis sp. nov.

**Description of Klebsiella singaporenensis sp. nov.**

*Klebsiella singaporenensis* (sin.ga.po.ren’is. N.L. fem. adj. *singaporenensis* of Singapore, the country in which the type strain was isolated).

Gram-negative, facultatively anaerobic, straight rods with rounded ends. Non-motile and capsulated. Not endospore-forming. Cells are 0·6–0·8 µm in diameter and 0·9–2·0 µm in length, occurring singly and sometimes in pairs. Colonies are circular, smooth, pulvinate, entire, opaque, white and viscid when grown on sucrose agar plates. Catalase and urease are produced, but oxidase, lipase and indole are not. Growth occurs at 10 °C but gas is not produced from lactose at 45 °C. The Voges–Proskauer test is positive and the methyl red reaction is negative. Nitrate is not reduced. Gelatin, starch, cellulose and casein are not fermented. There are no special growth-factor requirements. Citrate and glucose can be used as a sole carbon source. Fails to produce H₂S. Acids and gas are produced from glucose, sucrose, lactose, trehalulose, maltose, fructose, mannitol, glycerol, inositol, mannose, galactose and sorbitol. Does not utilize palatinose or L-sorbose as sole carbon sources. The optimum temperature is 30 °C and the optimum pH is 7·0. The G + C content of the DNA is 56·4 mol%.

The type strain is LX3T (= DSM 16265T = JCM 12419T).

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**References**


Table 1. Differential phenotypic characteristics of strain LX3T and Klebsiella species

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*Including K. trevisanii isolate.


