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

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Cells of strain LX3^T, 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 LX3^T (= DSM 16265^T = JCM 12419^T) should be classified as the type strain of a novel species of genus *Klebsiella*, *Klebsiella singaporensis* 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 % 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 rhapsodica* (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 LX3^T, 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* (Ørskov, 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 LX3^T are respectively AF250285 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 genogroup.

In this paper, the results of an examination of the phenotypic characteristics of strain LX3^T are described, along with the phylogenetic placement of the strain. The name *Klebsiella singaporensis* sp. nov. is proposed with strain LX3^T 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·H₂O, 0.7 g KNO₃, 1 g K₂HPO₄, 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·H₂O, 0.7 g KNO₃, 1 g K₂HPO₄, 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 LX3^T.

The reference strains used in DNA reassociation studies were *K. pneumoniae* subsp. *pneumoniae* ATCC 13883^T, *K. pneumoniae* subsp. *ozaenae* ATCC 11296^T, *K. pneumoniae* subsp. *rhinoscleromatis* ATCC 13884^T, *K. terrigena* ATCC 33257^T, *K. planticola* ATCC 33531^T, *K. trevisanii* ATCC 33558^T, *K. ornithinolytica* ATCC 31898^T, *K. oxytoca* ATCC 13182^T and *K. mobilis* (= *Enterobacter aerogenes*) ATCC 13048^T.

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/acetic 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–Proskauer 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 LX3^T 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'-TGACGAGTGGCG-GACGGGTG-3' (forward primer) and 5'-CCATGGTGTG-ACGGGCGGTGTG-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-joining 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 LX3^T encoding the RNA polymerase β -subunit (*rpoB*) gene, corresponding to positions 1468–2114 of *Escherichia coli rpoB*, was amplified by a PCR using primers designed with reference to the consensus regions of the published sequences for *Escherichia coli* (GenBank accession no. V00340), *Salmonella typhimurium* (X04642), *Pseudomonas putida* (X15849) and *K. pneumoniae* (U77443): 5'-CAGTTCGCGGTTGGCCTG-3' (forward primer) and 5'-CGGTTGGCGTCATCGTGTTC-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 LX3^T 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 LX3^T 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. LX3^T was facultatively anaerobic and capsulated. 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* (Ørskov, 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 LX3^T should belong to the genus *Klebsiella*.

The G+C content of strain LX3^T 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 LX3^T exhibited 16.7–28.2 % relatedness with members of *Klebsiella* cluster I, except for *K. pneumoniae* subsp. *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 LX3^T. With *K. oxytoca* and *K. mobilis* (= *Enterobacter aerogenes*), strain LX3^T 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 (Etchebere *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 LX3^T 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 LX3^T 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

Fig. 1. Phylogenetic tree derived from 16S rRNA gene sequence analysis, showing the position of *K. singaporensis* sp. nov. among related members of the *Enterobacteriaceae*. The tree was generated by the neighbour-joining method. Bootstrap percentages are given at the nodes. *Serratia marcescens* was used as an outgroup. GenBank accession numbers are given in parentheses. Bar, 1% sequence divergence.

generated by the neighbour-joining method is shown in Fig. 1.

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 *Raoultella*, and grouped *K. oxytoca* in cluster III. It was evident from tree topology that strain LX3^T had a close phylogenetic relationship with members of cluster I (i.e. *Klebsiella sensu stricto*). These data demonstrated that strain LX3^T clearly belongs to the genus *Klebsiella* and that the closest relative is *K. pneumoniae* (99.3% similarity). This relationship between the cluster of strain LX3^T, *K. pneumoniae* and *K. granulomatis* was supported by the bootstrap analysis at a confidence level of 99%. Strain LX3^T 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 LX3^T and cluster I shared a higher similarity than did strain LX3^T 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-sorbose as a sole carbon source. Although strain LX3^T and *K. pneumoniae* had high 16S rRNA gene sequence similarity (99.3%), the separation of strain LX3^T

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 LX3^T 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 LX3^T 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 LX3^T 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 2–21.9% interspecies difference occurred (Mollet *et al.*, 1997). Phylogenetic analysis showed that the smallest divergence is 2.5% (*K. pneumoniae*), suggesting that strain LX3^T 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 LX3^T also fell within *Klebsiella* cluster I. The most closely related member was *K. pneumoniae* (97.5% similarity), which was supported by an 81%

Fig. 2. Phylogenetic tree showing the relationship between *K. singaporensis* sp. nov. and related members of the *Enterobacteriaceae* based on partial *rpoB* gene sequences. The tree was generated by the neighbour-joining method. Bootstrap percentages are given at the nodes. *Enterobacter cloacae* was used as an outgroup. GenBank accession numbers are given in parentheses. Bar, 1% sequence divergence.

bootstrap value. Compared with other members of *Klebsiella* cluster I, strain LX3^T showed 96.9–97.3 % *rpoB* gene sequence similarity. Strain LX3^T 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 LX3^T 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 LX3^T 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 LX3^T 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 LX3^T 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 LX3^T and *K. granulomatis* (urease-negative), the latter usually needed growth factors (Carter *et al.*, 1999) while strain LX3^T does not. To distinguish strain LX3^T from *K. mobilis*, the urease test may be decisive; strain LX3^T is urease-positive and *K. mobilis* is urease-negative. The level of DNA–DNA relatedness revealed that strain LX3^T was distinct from other members of genus *Klebsiella*.

Table 1. Differential phenotypic characteristics of strain LX3^T and *Klebsiella* species

Taxa: 1, *K. pneumoniae* subsp. *pneumoniae*; 2, *K. pneumoniae* subsp. *rhinoscleromatis*; 3, *K. pneumoniae* subsp. *ozaenae*; 4, *K. oxytoca*; 5, *K. planticola*; 6, *K. ornithinolytica*; 7, *K. terrigena*; 8, *K. mobilis* (= *Enterobacter aerogenes*). Results: +, positive; –, negative; ND, not determined.

Characteristic	LX3 ^T	1	2	3	4	5*	6	7	8
Indole production	–	–	–	–	+	+	+	–	–
Gas production from lactose at 44.5 °C	–	+	+	+	–	–	–	–	+
Growth at 10 °C	+	–	–	–	+	+	+	+	ND
Methyl red reaction	–	–	+	+	–	ND	ND	+	ND
Voges–Proskauer test	+	+	–	–	+	+	ND	+	+
Utilization of:									
Palatinose	–	+	–	–	+	+	+	+	ND
L-Sorbose	–	–	–	–	+	+	+	+	+
Urease	+	+	–	–	+	+		+	–

*Including *K. trevisanii* isolate.

On the basis of the physiological and phylogenetic characteristics of strain LX3^T, 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 LX3^T should be placed in a novel species, *Klebsiella singaporensis* sp. nov.

Description of *Klebsiella singaporensis* sp. nov.

Klebsiella singaporensis (sin.ga.po.ren'sis. N.L. fem. adj. *singaporensis* 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 viscous when grow 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 LX3^T (= DSM 16265^T = JCM 12419^T).

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