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(71) Applicant *(for all designated States except US)*: **INSTITUTE OF MOLECULAR AGROBIOLOGY** [SG/SG]; 1 Research Link, The National University of Singapore, Singapore 117604 (SG).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **ZHANG, Lian, Hui** [AU/SG]; 360 Pasir Panjang Road, #03-11, Goldcoast Condominum, Singapore 118699 (SG). **LI, Xianzhen** [CN/SG]; 16 West Coast Crescent, #03-11 Tower 1A, Westcove Condominium, Singapore 12844 (SG). **ZHANG, Daohai** [CN/SG]; Blk 709 Clementi West Street 2, #08-281, Singapore 120709 (SG).

(74) Agent: **ELLA CHEONG & G. MIRANDAH**; P.O. Box 0931, Raffles City, Singapore 911732 (SG).

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(54) Title: BACTERIAL ISOLATES OF THE GENUS KLEBSIELLA, AND AN ISOMALTULOSE SYNTHASE GENE ISOLATED THEREFROM

(57) **Abstract:** Two strains of a novel bacterial species, *Klebsiella singaporensis* LX3 and LX21, are claimed. A nucleotide sequence (*kis*) encoding a novel form of isomaltulose synthase, KIS, is also claimed. Also claimed is a method for production of isomaltulose in a plant, which method comprises introducing into a cell of such plant a nucleic acid sequence which encodes an enzyme which converts sucrose into isomaltulose in a manner which allows said cell to express said nucleic acid sequence, as well as a functional cloning method of isolating nucleotide sequence encoding the KIS protein comprising the steps of (a) preparing a gene bank from a donor organism that contains a DNA sequence coding for an isomaltulose biosynthesis activity in a suitable host organism, (b) screening the clones of interest from the gene bank by their enhanced reducing sugar content, and (c) isolating the clones which contain a DNA coding for a protein with isomaltulose biosynthesis activity.

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BACTERIAL ISOLATES OF THE GENUS KLEBSIELLA, AND AN ISOMALTULOSE SYNTHASE GENE ISOLATED THEREFROM

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to the fields of bacterial molecular biology and recombinant DNA technology.

2. Description of the Related Art

Isomaltulose (6-O-α-D-glucopyranosyl-D-fructofuranose) is a reducing sugar with physical properties and taste very similar to sucrose. It is a structural isomer of sucrose found naturally in honey (Sporns et al., 1992). Isomaltulose has been regarded as an alternative to sucrose with several attractive features: (1) it resists metabolism by cariogenic oral streptococci and does not cause dental decay (Minami et al., 1990); (2) its ingestion leads to very small effects on the concentrations of glucose and insulin in blood, indicating potential as a parenteral nutrient acceptable both to diabetics and non-diabetics (Kawai, et al., 1989); (3) unlike sucrose, it is not fermented by most bacteria and yeasts and is more stable in acid solutions -- these properties of isomaltulose facilitate the maintenance

of sweetness and taste in fermented foods and beverages (Takazoe, 1989; Schiweck et al., 1991); (4) it does not possess hydroscopic properties like sucrose or lactose -- isomaltulose-containing foods are more stable than those containing sucrose (Takazoe, 1989); (5) it selectively promotes bifidobacteria growth among human intestinal microflora (Mizutani, T. 1989). Evidence is accumulating that bifidobacteria are helpful for maintenance of human health and slowing the aging process (Mitsuoka, T. 1990). In Japan isomaltulose has been widely used as a sugar substitute in food (Takazoe, 1989). Biochemical conversion of sucrose for the production of isomaltulose has reached a scale of more than 10,000 tons annually worldwide (Kunz, 1993).

Isomaltulose is also a raw material for the synthesis of surfactants and polymers. With an annual production of around 110 million tons, sucrose is the world's most abundantly produced renewable organic compound. However, because of the acid instability of its intersaccharidic bond and its lacking of specific chemical reaction possibilities, the utility of sucrose as a raw material for the chemical industry has been very limited (Kunz, 1993). Isomaltulose, the reducing isomer of sucrose, on the other hand, can be specifically oxidized and derivatised for production of different chemical products. Isomaltulose has been successfully used for the production of polyamides and polyureas (Kunz, 1993).

Several bacterial species are known to be able to convert sucrose into isomaltulose. United States patent No. 4,359,531 describes a process for the production of isomaltulose, consisting of immobilizing

whole cell or solvent extracts of whole or disrupted cells of *Erwinia rhapontici*. About 70-95% of sucrose was converted to isomaltulose products. United States patent No. 4,390,627 describes a method for immobilization of the sucrose mutase enzyme from *Protaminobacter rubrum* for production of isomaltulose. United States patent No. 4,670,387 describes a fermentation process to produce isomaltulose using immobilized cells of *Erwinia rhapontici*, *Protaminobacter rubrum*, *Serratia plymuthica*, *Esevinia carotovora var atroseptica*, *Erwinia dissolvens*, *Serratia merscescens*. Again about 70-95% of sucrose was converted. United States patent No.4,857,461 discloses a continuous process for the enzymatic preparation of isomaltulose by immobilized crude enzyme from *Protaminobacter rubrum*, *Serratia plymuthica*, and *Erwinia carotovora*. United States patents No. 5,229,276 and No. 5,336,617 describe a process for preparing trehalulose and isomaltulose with immobilized cells of *Pseudomonas mesoacidophila* and *Agrobacterium radiobacter*.

A single enzyme, isomaltulose synthase (EC 5.4.99.10), is responsible for converting sucrose to isomaltulose. Although several groups of bacteria can convert sucrose to isomaltulose, yields of isomaltulose in the converted products were variable, ranging from 8% to 86% (Tsuyuki et al., 1992; Nagai et al., 1994; Huang et al., 1998). Moreover, these isomaltulose producing strains not only transform sucrose to isomaltulose and trehalulose but also produce about 2-7% of glucose as by-products (McAllister et al.,

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1990; Tsuyuki et al., 1992; Huang et al., 1998). This is a considerable industrial problem because elaborate purification procedures are necessary to remove these contaminating compounds (Sugitani et al., 1993, U.S. Patent No. 5,229,276). Some strains are even capable of hydrolysis of isomaltulose into glucose and fructose.

More recently, U.S. Patent No. 5,786,140 disclosed isolation of genes of sucrose isomerase from several organisms. By using a DNA probe based on the amino acid sequence deduced from the N-terminus of purified sucrose isomerase, the genes for sucrose isomerase were cloned from *Protaminobacter rubrum*, and *Enterobacter sp*. Partial DNA sequences for sucrose isomerase were further isolated from *E. rhapontici* and *P. mesoacidphila* by utilizing PCR.

BRIEF SUMMARY OF THE INVENTION

Bacterial isolates and enzymes capable of high efficiency production of isomaltulose are of considerable interest for biotechnology applications and for further understanding of enzymatic mechanisms. In the present invention a pure culture of a new bacterial isolate is provided, identified taxonomically as *Klebsiella singaporensis*. This bacterial isolate is capable of high efficiency enzymatic conversion of sucrose to isomaltulose.

The present invention further encompasses a process for the production of isomaltulose using immobilized cells of *K. singaporensis*. In contrast to those disclosed in the prior art, the process is highly efficient, converting more than 99% of sucrose to products which consist of more than 87% of isomaltulose

and less than 1% of glucose. A new gene encoding sucrose isomerase from *K. singaporensis* has been cloned by a functional cloning approach, which shows a high level of similarities to, but differs in several amino acids from, the sucrose isomerase from *Enterobacter sp.*

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is an electron micrograph of the negatively stained cell of strain *Klebsiella singaporensis* grown on sucrose medium.

Figure 2 shows the phylogenetic tree derived from 16S rRNA sequence data analysis, showing the position of strain *Klebsiella singaporensis*. The branching pattern was generated by the neighbor-joining method. The numbers shown next to the nodes indicate percentage bootstrap values from 1000 data sets.

Figure 3 details the phylogenetic position of *Klebsiella singaporensis* among selected bacteria belonging to the family of *Enterobacteriaceae* based on partial *rpoB* gene sequence data analysis. The tree was generated by the neighbor-joining method.

Figure 4 shows isomaltulose production and KIS enzyme activity in *K. singaporensis* cell culture. The first graph, A, details bacterial growth over a 24 hour time period. The second graph, B, details the KIS enzyme activity in the same time period. The last graph, C, shows the isomaltulose detected in the culture medium over the same time period.

Figure 5 maps the cloning strategy for the KIS gene from *Klebsiella singaporensis*. The selected clones are incubated for 18 hrs in the LB broth containing 5%

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sucrose at 37°C and 200 rpm. The reducing sugar in the broth was determined by the DNS method (Miller, 1959). The insert sizes cloned in the pBluescript II SK(+) are provided in brackets.

Figure 6(A) is the nucleotide sequence of the KIS gene of *Klebsiella singaporensis*. The DNA sequences of upstream and terminator regions are shown in initialized fonts, while the DNA sequences in the ORF are indicated by normal fonts. The putative potential ribosome binding site (SD) is underlined. The putative sucrose inducible region in the upstream is indicated by double underlines. A sequence homologous to the helix-loop-helix binding site is boxed. Bold fonts indicate the factor-independent termination sites in the downstream. Figure 6(B) shows the deduced amino acid sequence of KIS protein. The putative sucrose binding site is underlined.

Figure 7 is the homologous sequence associated with the AT-rich sequence of the putative sucrose-inducible promoter in the KIS gene. *Kis* is the gene for isomaltulose synthase from *Klebsiella singarporesis* LX3. Numerals are distance (bp) from the transcription site, except the *kis* where the numbers are the distance (bp) from the translational initiation site ATG.

Figure 8 details a comparison of amino acid sequence in the putative sucrose binding site between *kis* and other enzymes which use sucrose as a substrate. The numbers represent the position from the N termini of the enzymes.

DETAILED DESCRIPTION OF THE INVENTION

Two pure isolates (LX3 and LX21), that can convert sucrose into isomaltulose, were isolated from soil samples of the Republic of Singapore and from the root zone of sugarcane.

According to the description of the genus *Klebsiella* previously published(Orskov, 1984), this genus has the following morphological characteristics: negative reaction in Gram stain and oxidase, facultatively aerobe, rod-shape, non-motion, positive reaction in V.P. test and producing capsulate. These characteristics are consistent with those of the isolates of the present invention, suggesting that strain LX3 and LX21 should belong to the genus *Klebsiella*.

The isolates of the present invention can be distinguished from all of these known species of the genus *Klebsiella* by its phenotypic characteristics, including indole production, growth at 10°C, gas production from lactose, and methyl red test (Table 1). The difference of the isolates from the closely related species *Klebsiella pneumoniae* is that the latter can produce gas from lactose at 44.5 °C but can not grow at 10°C.

Table 1. Comparison of phenotypic characteristics of the two new isolates and related species.

Isolates	LX3	LX21	K. pneumoniae	K. oxytoca	K. ozaenae	K. rhinoschleromatis	S. liquefaciens
Indole production	-	-	-	-	+	-	-
Gas production from Lactose at 44.5°	-	-	+	-	+	+	
Growth at 10°C	+	+	-	+	-	-	+
M.R.	-	-	-	-	+	+	+
V.P.	+	+	+	+	-	-	-
Growth on cellobiose	+	+	+	+	+	+	-
Acid from lactose	+	+	+		(+)	-	-

A phylogenetic analysis indicated that the strain of the present invention clusters with genus *Klebsiella*. The closest relatives are *K. pneumoniae*, *K. rhinoscleromatis*, *K. ozaenae* and *S. liquefaciens*. It has been proposed that strains sharing less than 97% similarity usually do not belong to the same species (Stackebrandt and Goebel, 1994). The similarity of 16s

rRNA sequences between strain LX3 and *Klebsiella pneumoniae* was only 94.8%.

There are many similar phenotypic properties among the classified species of genus *Klebsiella*. To overcome this classification problem, phylogenetic analysis based on rpoB gene sequences was also carried out. It has been found that levels of divergence between the rpoB sequences of different strains in the family *Enterobacteriaceae* were markedly higher than those between their 16s rRNA genes (Mollet et al., 1997). The intraspecies comparison exhibited 98-100% of similarity among enteric strains, whereas a 2-21.9% difference occurred interspecies. As shown in Figure 3, analysis of rpoB gene sequences enabled an estimation of the phylogenetic position of the strains of the present invention. Although the rpoB gene analysis showed the similar phylogenetic position of the isolates to that of 16s rRNA sequence analysis, it is noteworthy that the rpoB gene divergence of the strains of the present invention from the other related strains is higher than that of 16s rRNA sequences. The range of similarities among strains tested is from 88.3% to 94.9%.

According to the physiological and phylogenetic characteristics of strains LX3 and LX21, it is apparent that these strains cannot be assigned to any previously recognized bacterial species. It is therefor proposed that strain LX3 and LX21 should be placed in a new species, *Klebsiella singaporensis sp. nov.* The type strain for this new species is designated LX3^T.

Isomaltulose synthase catalyzes the conversion of sucrose into both isomaltulose and trehalulose

(Cheetham et al., 1982). The *K. singaporensis* strains LX3^T (type strain) and LX21 convert sucrose into isomaltulose (87.3%), a small amount of trehalulose (11.6%) and trace amount of glucose (< 1 %). In comparison to other isomaltulose-producing strains (McAllister et al., 1990; Tsuyuki et al., 1992; and Huang et al., 1998), the isolates of the present invention produced more isomaltulose and less trehalulose from sucrose medium, and especially much less glucose.

The isomaltulose synthase enzyme activity of *K. singaporensis* is significantly higher than that of *Erwinia rhapontici* ATCC 29283 and *Serratia plymuthica* ATCC 15928, two strains known for isomaltulose production (Bucke and Cheetham, 1987, U.S. patent No. 4,670,387; Egerer et al., 1989, U.S. patent No. 4,857,461). Under the same growth conditions, the isomaltulose synthase activity of *K. singaporensis* is 43% and 19% higher, respectively, than that of *Erwinia rhapontici* ATCC 29283 and that of *Serratia plymuthica* ATCC 15928. This explains the very high sucrose conversion rate of *K. singaporensis*. The immobilized cells of *K. singaporensis* provide almost complete conversion of sucrose (99.7%), whereas the conversion rate of other known strains was up to 95% only (Bucke and Cheetham, 1987, U.S. patent No. 4670387). This novel bacterial strain, *K. singaporensis*, could have a significant industry potential for efficient production of isomaltulose.

Interestingly, the isomaltulose synthase (KIS) in strain *K. singaporensis* is not constitutively

expressed. It is significantly induced by fructose containing carbohydrates, including fructose, sucrose, raffinose, and isomaltulose. Among them, sucrose is the most effective inducer, followed by raffinose and fructose. Only low, or no enzyme activity has been detected when other sugars were used as a carbon source. This is different from the previously reported isomaltulose synthase, which is basically constitutively expressed (Huang et al., 1998).

The *kis* gene encoding isomaltulose synthase from *Klebsiella singaporensis* has been cloned and characterized and found to have the nucleic acid sequence shown in Fig. 6A (SEQ ID NO. 1). The present invention encompasses nucleic acid sequences within the scope of the degeneracy of the genetic code that encoded essentially the same amino acid sequence encoded by SEQ ID NO. 1 (SEQ ID NO. 2). The gene encodes a peptide of 598 amino acids with a calculated molecular mass of 69.94 kDa. Sequence comparison indicates that it is highly similar to but different from the sucrose isomerase cloned from *Enterobacter sp.* strain SZ62. There are 3 amino acid differences at the C-terminal region.

A functional cloning method for isolating the DNA encoding the KIS protein can be stated as follows: (a)prepare a gene bank from a donor organism that contains a DNA sequence coding for an isomaltulose biosynthesis activity in a suitable host organism, (b)screen the clones of interest from the gene bank by their enhanced reducing sugar content, and (c)isolate the clones which contain a DNA coding for a protein with isomaltulose biosynthesis activity. In a

preferred embodiment, *E. coli* is used as the host organism for the gene bank of step (a), most preferably an *E. coli* strain that does not produce significant amounts of reducing sugar with the time frame needed to perform the steps (a) - (c), above.

A putative promoter sequence in the upstream of ORF of the *kis* gene has been identified. The promoter activity has been confirmed in *E. coli*, where it directs inducible expression of the *kis* gene in the presence of sucrose or fructose. The putative AT-rich sucrose responsive region ($^{-54}$TTTTCTTTAATAACAATT^{-37}) [SEQ ID. 3] is found at -10 region by comparison with other sucrose-responsive regions (Yokoyama et al., 1994).

The isomaltulose synthase belongs to the family of glucosyltransferases. Comparison of the deduced amino acid sequence of the *kis* gene suggests the existence of a potential sucrose-binding site, ^{321}F-D-L-I-R-L-D-R-D-S-N-E^{332} [SEQ ID NO. 4]. It has been established that aspartic acid residues are important for the binding of the glucosyl group of sucrose and its derivatives. Changing Asp to Asn in the sucrose-binding site in glucosyltransferases resulted in reduced or completely inhibited enzyme activity, indicating the role of carboxyl groups in the catalytic site (Mooser et al., 1991; Kato et al., 1992; Monchois et al., 1996, 1997). It seems likely that, by comparison, the Asp-327 in the KIS enzyme is responsible for the catalytic binding of sucrose in isomaltulose biosynthesis. The role of a second highly conserved amino acid, *i.e.* Arg-325, in the substrate binding and catalysis, has not yet been established.

The isomaltulose synthase of the present invention can be expressed in any number of cell types, such as bacterial, plant, mammalian, insect or fungal cells, and the present invention encompasses prokaryotic and eukaryotic cells transformed to express KIS. In one preferred embodiment, the *kis* gene is transferred into a bacterial cell, such as *E. coli*, by conventional methods, and the KIS enzyme expressed therein. Such a transformed bacterial cell line can be used to produce isomaltulose in fermentation culture. In another preferred embodiment, the *kis* gene is transferred into a plant cell. Such a transformed plant cell could be used to produce isomaltulose in culture, or regenerated by conventional methods into a transgenic plant expressing the *kis* gene in its tissues, leading to the production of isomaltulose through the conversion of sucrose. Such a plant would be a rich source of isomaltulose. Preferred plants for use in this embodiment are sugarcane, maize, watermelon and sugarbeet.

Cell transformation can be accomplished using conventional means, such as transformation by an appropriate expression vector that is propagated in the host cell (such as a plasmid), by mechanical means (e.g., particle bombardment, microinjection), or by other means known in the art, such a liposome fusion or electroporation. A preferred embodiment for the transformation of plants and plant cells is an *Agrobacterium tumefaciens* Ti plasmid vector, of which there are numerous examples in the literature (*e.g.*, U.S. Patent Nos. 4,940,838, 4,762,785 and 5,068,193). Transformation with a Ti plasmid vector can be achieved

via *Agrobacterium*-mediated transformation, or by any other means that effects the transferral of Ti plasmid into the target cell. In a preferred embodiment, the *kis* gene is incorporated into an expression vector that propagates in a prokaryotic or eukaryotic cell, and the present invention encompasses transformed prokaryotic and eukaryotic cells harboring such vectors. An appropriate expression vector would comprise the *kis* coding sequence and a promotor active in the host cell that causes expression of the *kis* coding sequence, as well as sequences necessary for the maintenance and/or propagation of the vector in the host cell. Additionally, the vector may comprise sequences that lead to the stable insertion of the *kis* gene into the host cell genome, for example the Ti plasmid border regions (*c.f.* U.S. Patent Nos. 4,762,785 and 5,068,193), or any region of homology that can lead to cross-over or insertion events with the host genome.

In another embodiment, the *kis* gene is fused to a signal peptide sequence. Signal peptides effect the targeting of the expressed peptide to a particular cellular local, such as the vacuole (Bednarek and Raikhel, 1991; Matsuoka and Nakamura, 1991), the chloroplast (Van den Broeck et al., 1985), the endoplasmic reticulum (Borisjuk et al., 1999), or the cell membrane (Okamoto et al., 1991). In a preferred embodiment, the *kis* gene is fused to a vacuole-targeting signal peptide sequence. In still another embodiment, the *kis* gene is linked to a membrane attachment domain coding sequence, which would be

important for enzyme stability (Hsu et al., 1993; Okamoto et al., 1991).

EXAMPLE 1

Isolation, Purification and Characterization of Isomaltulose Producing Bacteria *Klebsiella singaporensis sp. nov.*

Soil samples collected at Woods Park of Clementi, Singapore, or from the root zone of sugarcane, and the tissue of various over-ripe tropical fruits and sugarcane were suspended in sterilized saline water and spread over sucrose agar plates in different dilutions. The sucrose agar medium used for the screening of bacteria producing isomaltulose, contained (per 1000 ml): 40 g sucrose, 5 g yeast extract, 0.5 g $MgSO_4$ H_2O, 0.7 g KNO_3, 1 g K_2HPO_4, 0.5 g NH_4Cl, 1 g NaCl, 20 g agar, adjusted pH to 7.0 with 1 N NaOH.

Isolation and purification of isomaltulose-producing bacteria: All plates were incubated at 30°C for 24 h. A portion of each single colony on the sucrose agar plate was transferred to a new sucrose agar plate for further purification, and another portion was inoculated into SPY medium overnight. SPY medium was composed of (per 1000 ml) 40 g sucrose, 10 g peptone and 4 g yeast extract (pH 7.0).

Bacterial isolates from different samples were purified into homogeneous single-colony cultures by repeating streaking on the SPY plates. The purified bacterial isolates were screened for production of reducing sugars using sucrose as a sole carbon source. The bacterial isolates capable of producing reducing sugars in high yield were further tested for

isomaltulose and glucose production. Two bacterial isolates (LX3 and LX21) capable of efficient production of isomaltulose with trace amounts of glucose by-product were selected for further study. These two isolates were originally isolated from the soil sample close to the root of sugarcane and from soil samples at Woods Park of Clementi of Singapore, respectively.

Preparation of crude isomaltulose synthase and enzyme assay: After incubation overnight in SPY medium, the cells and supernatant were collected respectively by centrifugation at 14000 rpm for 10 min. The supernatant was kept at 4 °C for the detection of reducing sugars by the DNS method (Miller, 1959), and isomaltulose by thin-layer chromatography (Schwimmer and Bevenue, 1956), assays described *infra*. The cell-pellet was washed three times with deionized water and suspended in original volume of 0.1M citrate-phosphate buffer (pH 6.6). The resuspended bacterial cells were divided into two aliquots. One aliquot was used directly for enzyme assay as a whole cell fraction. The cells of the other aliquot were broken by sonification, the crude protein extract and the cell-debris fractions were separated by centrifugation at 14000 rpm. The cell-debris fraction was washed three times and resuspended in the same buffer for enzyme assay.

The enzyme activity was determined by incubating a mixture of 450 µl of 4% sucrose in 0.1 M citrate-phosphate buffer (pH 6.6) and 50 µl of enzyme preparation or bacteria suspension at 37°C for 15 min. The reducing sugars formed were determined by the DNS method. One unit of isomaltulose synthase activity is

defined as the amount of enzyme that can convert sucrose into one µmol of reducing sugar in a minute at the conditions specified above.

For determination of the enzyme activity in *E. coli* host cells, the same conditions were applied except that the bacteria were grown in LB medium supplemented with different concentrations of sucrose as indicated, bacterial suspension culture was doubled to 100 µl and sucrose solution was reduced correspondingly to 400 µl and the reaction time was increased to 30 minutes.

Detection of bacteria introducing isomaltulose by thin-layer chromatography: The 0.5 µl of supernatant of culture, in which reducing sugars can be detected by the DNS method, was spotted on TLC plates (Silica Gel on polyester for general-purpose, Aldrich) and developed. The solvent system was ethylacetate-acetic acid-water (4:3:0.8 by volume). The spots were visualized with the diphenylamine-aniline-phosphoric acid reagent (Schwimmer and Bevenue, 1956) at 80°C for 5 min. The presence of isomaltulose is indicated by a typical green-yellow color spot, the isomaltulose from Sigma was used as a standard control.

After sonication no isomaltulose synthase activity was detected in the supernatant fraction and the activity was only found in the cell-pellet fraction, which was not changed by prolonged sonification. The isomaltulose synthase activity of cell-pellet fraction (19.2 U/ml) is almost identical to that of whole cell fraction (20.1 U/ml). The results suggest that the isomaltulose synthase is a cell wall-bound enzyme.

Morphology: The cultures were grown on sucrose agar for 24 h at 30°C and examined with a phase-contrast microscope and transmission electron microscope (TEM). For transmission microscopy, a centrifuged cell pellet was fixed with 5% (wt/vol) glutaraldehyde and 1% (wt/vol) osmium tetroxide. Ultrathin sections of the sample embedded in epoxy resin were prepared with an ultramicrotome, stained with uranyl acetate and lead citrate, and examined with a model JEM-1200 EX transmission electron microscope. Cellular morphology after Gram staining was also checked by light microscopy, and the morphology of fixed specimens was compared to that of living cells.

Strains LX3 and LX21 are Gram-negative, non-motile, straight rods with round ends, appearing singly and sometime in pairs. The cells are of 0.6-0.8 μm in diameter and 0.9-2.0 μm in length. Endospores have not been observed. The size and shape of cells grown on sucrose medium was monitored and is illustrated in Figure 1. Colonies of these two isolates were circular, smooth, pulvinate, entire, opaque, white and viscous when tested with needle.

Physiological and Biochemical Characterization: Basal medium used for physiological and biochemical characterization of bacterial isolates in taxonomic work consisted of (per 1000 ml) 0.5 g $MgSO_4$ H_2O, 0.7 g KNO_3, 1 g K_2HPO_4, 0.5 g NH_4Cl, 1 g NaCl, and pH 7.0-7.2, carbon source was added as specified. The liquid cultures of bacterial isolates were incubated at 30°C with reciprocal shaking.

Gram staining characteristics were determined using the Hucker method as described (Doetsch, 1981).

Oxidase activity was determined by oxidation of 1% (wt/vol) tetramethyl-p-phenylene-diamine on filter paper, and catalase activity was detected by bubble formation in a 3% (wt/vol) hydrogen peroxide solution after incubation in SPY medium for 18-48 h (Smibert and Krieg, 1981). The media used to evaluate utilization of various substrates for growth were prepared by adding 0.2% (wt/vol) of each substrate to the basal medium. Gelatin hydrolysis (method 1), indole production (method 2), hydrogen sulfide production (method 2), nitrate reduction and hydrolysis of starch, casein and agar were tested by using the methods previously described (Smibert and Krieg, 1981). Acid production from carbohydrates was determined in basal medium supplemented with various carbohydrates as described (Smibert and Krieg, 1981).

Growth of bacterial isolates from 5°C to 60°C was determined by inoculation of fresh cultures of isolates onto sucrose agar plates and incubation at different temperatures respectively for 10 days.

The physiological and biochemical properties are shown in Table 2. The two strains show catalase and urease activities but do not have oxidase, nitrate reductase and lipase activities. Both strains can not hydrolyze gelatine, starch and cellulose. Strain LX3 cannot hydrolyze casein, while LX21 can degrade casein. The Voges-Proskauer test is positive but Methyl Red reaction is negative. LX3 and LX21 were facultative, anaerobic and capsulated. They both can use citrate and glucose as a sole carbon source and produce acid and gas from all the tested carbon sources, including glucose, sucrose, lactose, trehalulose, maltose,

fructose, mannitol, glycerol, inositol, mannose, galactose and sorbitol. The nitrogen sources such as peptone, tryptone, yeast extract, beef extract, casein hydrolysate, KNO_3, $(NH_4)_2SO_4$ can support the growth of the isolate, whereas urea can not. There are no special growth factor requirements of the isolates.

Table 2. Characteristics of bacterial isolates LX3 and LX21

Isolates	LX3	LX21
Straight Rod	+	+
Round-end	+	+
Diameter	0.6-0.8	0.6-0.8
Length	0.9-2.0	0.9-2.0
Occurrence		
Single	+	+
Pair	Sometime	Sometime
Capsules and slime layer	+	+
Gram	-	-
Cyst and Microcyst	-	-
Coccoid body	-	-
Endospore	-	-
Motility	-	-
Facultative anaerobic	+	+
Catalase	+	+
Oxidase		
Urease	+	+
Lecithinase	-	-
Lipase	-	-
Citrate utilization	+	+
Hydrolysis of		
Gelatine	-	-
Starch	-	-
Cellulose	-	-
Casein	-	-

Glucose as carbon source	+	+
Catabolism	Fermentation	Fermentation
H_2S production from		
Cysteine	+	+
Thiosulfate	-	-
TSI	-	-
M.R.	-	-
V.P.	+	+
Nitrate reduction	-	-
Indole production	-	-
Gas production	+	+
Acid	+	+

These characteristics allow grouping of strains LX3 and LX21 to the genus *Klebsiella*. The closest members are *K. oxytoca* and *K. pneumoniae*. But strains LX3 and LX21 differ from *K. oxytoca* in indole production and differ from *K. pneumoniae* in their abilities to grow at 10 °C and to produce gas from lactose at 44.5°C (Table 1).

The distinct physiological, biochemical properties, and as well as DNA sequence differences examined in Example 3, indicate that isolates should be classified as a new bacterial species within the genus *Klebsiella, i.e. Klebsiella singaporensis*. The type strain is LX3^T.

Description of *Klebsiella singaporensis sp. nov.*:

Klebsiella singaporensis (sin.ga.po.ren'.sis M.L. gen. n. singaporensis of Singapore; pertaining to Singapore, a name of a country in Asia).

Gram-negative facultatively anaerobic straight rod with round ends. Non-motile and capsulated. Not endospore forming. Cells range from 0.6-0.8 μm in

diameter to 0.9-2.0 μm in length and occurs singly and sometimes in pairs. Colonies are circular, smooth, pulvinate, entire, opaque, white and viscous when grown on sucrose agar plates. Catalase and urease are produced, oxidase, and lipase are not produced. Voges-Proskauer test is positive and Methyl Red reaction is negative. Nitrate is not reduced. Does not ferment gelatin, starch, cellulose and casein. There are no special growth factor requirements. Citrate and glucose can be used as a sole carbon source. Lack of production of H_2S. Acids and gas are produced from glucose, sucrose, lactose, trehalulose, maltose, fructose, mannitol, glycerol, inositol, mannose, galactose and sorbitol. Optimum temperature 30°C and optimum pH 7.0. The G + C content of DNA is 56.5 mol %. The type strain is $LX3^T$.

EXAMPLE 2

Immobilization of *K. singarporesis* $LX3^T$ in alginate and production of isomaltulose using immobilized cells

Strain $LX3^T$ was grown in SPY medium at 30°C for 16 h with shaking (250 rpm). Per 5 ml of bacterial culture was mixed with 100 ml of alginatic acid (2%, medium viscosity) and dropped into 0.65% (w/v) $CaCl_2$ solution with a syringe. The solution was stirred constantly and the immobilized cells were hardened gradually in the 0.65% (w/v) calcium chloride solution at 4°C for 4 h. The immobilized cells were then incubated in SPY medium containing 0.2% (w/v) $CaCl_2$ overnight at 30°C with shaking for increasing the bacterial cell density in the alginate particles.

The immobilized cells (100 ml) were packed in 200 ml column reactors. Sucrose solutions at different concentrations were passed through the columns with different retention times. Eluates were collected and the yield of total reducing sugar as well as isomaltulose under different conditions was determined as described above.

Production of isomaltulose by immobilized cells of *K. singaporensis*: The fresh cultures of *K. singaporensis* was immobilized in alginate gel. Table 3 shows the isomaltulose production efficiency of the immobilized cells, which were packed in 200-ml column reactors. Best sucrose conversion rate (99.7%) was achieved when 10% sucrose solution passed through the column reactor in a rate of 6 ml/min. Under these conditions, the yield of isomaltulose was 522.8 mg/min or 31 kg/h. Increments of sucrose concentration up to 20% further increased the yield of isomaltulose, but sucrose conversion rate was decreased. Higher sucrose concentration did not result in better yield.

Table 3. Production of isomaltulose by immobilized *S. singarporensis**

Sucrose concentration	Retention time (ml/min)	Conversion rate (%)	Yield (mg/min)**
10	2	ND	ND
	4	99.7	348.5
	6	99.7	522.8
20	2	98.2	343.3
	4	96.4	674.0
	6	72.1	756.2

30	1	71.8	188.3
	2	65.4	342.9
	3	32.7	357.2
40	1	67.2	234.9
	2	65.1	455.2
	3	34.3	359.7

* The data are means from two replicates per treatment.

** Yield of isomaltulose was converted according to the detected proportion of isomaltulose in total reducing sugar 87.4%).

Determination of products ratio: Washed bacterial cells of strain LX3 were incubated with 4% sucrose in 0.1 M citrate-phosphate buffer (pH 6.6) for 10 min at 37°C. The reaction was stopped immediately by heating in boiling water bath for 5 min. The supernatant of the reaction mixture was spotted onto a TLC plate and developed as described. The TLC plate fractions containing isomaltulose and trehalulose were cut and extracted with distilled water respectively. The sugar concentration was determined by the DNS method and the ratio of these two sugars was calculated.

For determination of glucose content in the reaction mixture, the concentrations of glucose and total reducing sugar of the above supernatant were analyzed respectively using the Glucose (HK) Assay Kit (Kit GAHK-20, Sigma) and the DNS method. The ratio of

isomaltulose, trehalulose and glucose was determined with the above quantification data.

Enzyme and isomaltulose production: Strain LX3^T was cultured aerobically in the basal medium supplemented with different sugars at the same initial concentration (1.5%, w/v) as sole carbon sources at 30°C, and the samples were removed at intervals for determination of isomaltulose synthase activity, cell mass and isomaltulose production. As shown in Table 4, the best enzyme activities were detected when sucrose, raffinose or fructose was used as sole carbon source, which is 15.12, 13.62 or 11.08 U/ml respectively. Enzyme activity lower than 1.7 U/ml was obtained when maltose, mannitol, mannose, lactose, cellobiose or melibiose was used as the carbon source. Isomaltulose was also able to induce the production of isomaltulose synthase, but the activity was only 30% of that in the presence of sucrose. Almost all the tested monosaccharides including glucose, xylose, arabinose and galactose could not induce isomaltulose synthase activity, with the exception of fructose.

Table 4. Effects of carbon source on isomaltulose synthase production by *K. singaporensis* LX3. The basal medium is comprised of 0.1% peptone, 0.2% yeast extract, 0.05% $MgSO_4$, 0.2% NaCl.

Carbon source (1.5%, w/v)	Growth (OD600)	KIS activity (U ml^{-1})

Sucrose	1.89	15.1
Glucose	1.94	0.0
Fructose	1.82	11.1
Raffinose	1.96	13.6
Isomaltulose	1.92	4.5
Melibiose	1.88	1.6
Maltose	1.77	1.5
Mannose	1.84	0.7
Lactose	1.82	0.0
Xylose	1.97	0.0
Arabinose	1.73	0.0
Galactose	1.71	0.0
Mannitol	1.77	1.7
Cellobiose	1.83	0.0

Strain LX3^T did not grow well when inorganic chemicals such as KNO_3, urea or $(NH_4)_2SO_4$ was used respectively, as a sole nitrogen source, and no isomaltulose synthase activity has been detected in the cultures (Table 5). All organic nitrogen sources tested support good growth of the strain LX3^T, but with varied isomaltulose synthase activities. Among them, yeast extract and tryptone are most effective in promoting induction of maximum isomaltulose synthase activities (Table 5).

Table 5. Effect of nitrogen source on isomaltulose synthase production by *K. singaporensis* LX3. The basal medium is comprised of 1.5% sucrose, 0.02% $MgSO_4$, 0.5% NaCl, 0.03% K_2HPO_4. The data are means from two replicates per treatment.

Nitrogen source (0.5%, w/v)	Growth (OD_{600})	Relative KIS enzyme activity (%)
Peptone	1.97	59.2
Tryptone	2.13	85.9

Yeast extract	2.33	100
Beef extract	2.23	54.4
Casein hydrolysate	2.34	53.9
KNO3	0.60	0
Urea	0.33	0
$(NH_4)_2SO_4$	0.56	0

Figure 4 shows a typical profile of isomaltulose and isomaltulose synthase productions by strain LX3^T. Isomaltulose production is rapid and reaches the maximum level after 7 h culture when the concentration of sugar in the medium is 10%. From 7 to 22 h, the concentration of isomaltulose in the culture is maintained basically in the same level, indicating that strain LX3^T is unable to metabolize isomaltulose. It is noted that at 7 h, both bacterial growth and isomaltulose activity in the culture did not reached the maximum level, whereas isomaltulose production basically stopped. One possibility is that when sucrose concentration in the medium reaches a low level, the remaining sucrose is actively pumped into cells to support bacterial growth.

Comparison of isomaltulose synthase enzyme activities of several bacterial strains: Under the same growth conditions (SPY medium), the relative isomaltulose synthase activities of *K. singaporensis*, *Erwinia rhapontici* ATCC 29283 and *Serratia plymuthica* ATCC 15928 were determined to be 100%, 57%, and 81% respectively.

EXAMPLE 3

Manipulation, Cloning, Location and Sequence Analysis of the *kis* Gene

DNA manipulation: Purification of total genomic DNA, digestion of DNA with restriction endonucleases, DNA ligation, agarose gel electrophoresis and transformation of *E. coli* DH5α were carried out by standard procedures (Sambrook et al., 1989) or as recommended by the reagent manufacturers. Plasmid preparation was performed with the kits from Qiagen according to the manufacturer's instructions. DNA restriction fragments were isolated from the agarose gel by using a QIAEX II Gel Extraction Kit (Qiagen).

The bacterial strains and plasmids are listed in Table 6. *E. coli* DH5α was used as the cloning host strain. Plasmid vectors pLAFR3 and pBluescript II SK (+) were used for constructing the genomic DNA library, and for subcloning, respectively. *E.coli* cosmid clones and subclones where grown aerobically at 37°C in Luria-Bertani-sucrose medium (per liter contains Bacto Tryptone 10 g, yeast extract 5 g, NaCl 10 g, and sucrose 50 g, pH 7.0). When necessary, tetracycline (12 mg/liter) or ampicillin (100 mg/liter) was added to the medium.

Table 6. Bacterial strains and plasmids used in this study

Strain	Genotype or phenotype	Reference/source
Bacteria		
K. singaporensis LX3^T	Soil isolate, isomaltulose producer	This study
K. singaporensis LX21	Soil isolate, isomaltulose producer	This study
Erw. rhapontici ATCC 29282	isomaltulose producer	ATCC

S. plymuthica ATCC 15928	isomaltulose producer	ATCC
E.coli DH 5α	*recA1 endA1 hsdR17supE4 gyrA96 relA1 Δ(lac ZYA-argF)U169(80DLACzΔM15)*	Sambrook et al. 1989
Plasmids		
pPLAFR3	Cloning vector, Tc^r	Lab collection
pBluescript II sk(+)	Cloning vector, Amp^r	Stratagene
pPLASI164	PLAFR3 plasmid with ~6.1 kb BamHI/BamHI fragment	this study
pSIBB	~6.1 kb BamHI/BamHI fragment in pBluescript II SK(+)	
pSIBP	~4.8 kb BamHI/PstI fragment in pBluescript II SK(+)	this study
pSICB	~3.6 kb ClaI/BamHI fragment in pBluescript II SK(+)	this study
pSIBX	~3.5 kb BamHI/XhoI fragment in pBluescript II SK(+)	this study
pSIEE	~2.5 kb EcoRV/EcoRV fragment in pBluescript II SK(+)	this study

G+C content of DNA: The guanine-plus-cytosine (G+C) content of the genomic DNA was determined by the thermal denaturation method as described (Marmur & Doty, 1962). The G + C contents of strains LX3 and LX21 were determined to be 56.5 mol% which is comparable to that of the genus *Klebsiella*.

16S rRNA gene sequence: The 16S rRNA gene DNA fragments of the isolates $LX3^T$ (T = type strain) and LX21 that correspond to positions 95 to 1395 of *Escherichia coli* 16S rRNA was amplified by PCR using purified genomic DNA and a primer pair consisting of 5'TGACGAGTGGCGGACGGGTG-3 (forward primer) [SEQ ID NO. 5] and 5'CCATGGTGTGACGGGCGGTGTG-3' (reverse primer) [SEQ ID NO. 6]. The amplification products were purified with a QIAquick PCR purification kit (Qiagen, Germany)

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 from both strands with a Perkin Elmer ABI PRISM 377 DNA sequencer. The primers used for sequence analysis were designed and shown in Table 7. Sequencing was repeated at least two times. The closest known relatives of the new isolate were determined by performing sequence database searches and the sequences of closely related strains were retrieved from GenBank and Ribosomal Database Project (RDP II) libraries. Sequences were aligned with the PILEUP program (Devereux et al., 1984) and the alignment was corrected manually. Distance matrices were produced with the DNADIST program of the PHYLIP package (Felsenstein, 1989) and a phylogenetic unrooted tree was constructed using the NEIGHBOR program contained in the PHYLIP package (V3.5c) (Felsenstein, 1989). The statistical signification of the groups obtained was assessed by bootstrapping (1000 replicates) using the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE (Felsenstein, 1989).

Table 7. Oligonucleotide primers used for PCR amplification and sequencing of 16s rRNA gene from strains LX3 and LX21

Primer	Identification	Sequence (5'-3')	Orientation
1	16sf95	TGACGAGTGGCGGACGGGTG [SEQ. ID NO. 7]	Forward
2	16sr394	CCATGGTGTGACGGGCGGTGTG [SEQ ID NO. 8]	Reverse
3	16sf342	TACGGGAGGCAGCAGTGGGGAATA [SEQ ID NO. 9]	Forward
4	16sr616	ATGCAGTTCCCAGGTTGAGC [SEQ ID NO. 10]	Reverse
5	16sf605	GAAATCCCCGGGCTCAACCTG [SEQ ID NO. 11]	Forward

6	16SR798	ACATCGTTTACGGCGTGGACTACC [SEQ ID NO. 12]	Reverse
7	16sf893	GGCCGCAAGGTTAAAACTCAAATG [SEQ ID NO. 13]	Forward
8	16sr119	CCGGACCGCTGGCAACAAAG [SEQ ID NO. 14]	Reverse

The 16S rRNA gene sequence of strains LX3 and LX21 was determined. Comparison with 16S rRNA sequences available in databases revealed that strains LX3 and LX21 are closely related to the species belonging to the genus *Klebsiella*, and peripherally related to species of the genera *Serratia* and *Enterobacter*. The sequences of the two isolates showed high similarities with that of *Klebsiella pneumoniae* and the levels of sequence similarities among these strains ranged from 91.0 to 94.8%. The data set used for the construction of the phylogenetic tree contained 1282 nucleotides of each sequence as a result of elimination of gaps and ambiguous nucleotides from the sequences between positions 95 and 1395 (corresponding to that of *Escherichia coli*). The phylogenetic tree constructed by the neighbor-joining method is shown in Fig. 2. Strains LX3 and LX21 are closely associated with *Klebsiella pneumoniae*. The clustering of strain LX3 and LX21 with *Klebsiella pneumoniae* was supported by the results of the bootstrap analyses at a confidence level of 100%. But the two strains are different from *K. pneumoniae*; the divergence value of the 16S rRNA gene sequence of strain LX3 with that of *K. pneumoniae* is > 5% (Table 8).

Table 8. DNA sequence similarities of 16s rRNA and rpoB genes between *Klebsiella singaporensis* sp. nov. and other representatives of the family Enterobacteriaceae

	16s rRNA		rpoB		
Strains	GenBank No.	Similarity of 16s rRNA to K. singaporensis (%)	GenBank No.	Similarity of rpoB to K. singaporensis (%)	
Klebsiella singaporensis		100		100	
Klebsiella pneumoniae	Y17656	94.8	U77444	95.0	
Klebsiella rhinoscleromatis	Y17657	94.7			
Klebsiella terrigena	Y17658	93.3			
Klebsiella planticola	Y17659	92.8			
Klebsiella ozaenae	Y17654	94.5			
Klebsiella ornithinolytica	U78182	93.1	U77440	93.9	
Klebsiella oxytoca	Y17655	92.4	U77442	93.6	
Serratia liquefaciens	AB004752	94.7			
Serratia macescens	M59160	92.2	U77449	88.3	
Serratia rubidaea	AB004751	91.5			
Salmonella typhimurium	U90316	91.2	X04642	93.4	
Salmonella sofia	X80677	91.5	U77452	93.2	
Salmonella waycross	U92194	91.0			
Citrobacter werkmanii	AF025373	92.6			
Citrobacter farmeri	AF025371	93.1			
Citrobacter amalonaticus	AF025370	93.1			
Citrobacter rodentium	AF025363	91.8			
Enterobacter cloacae	Y17665	92.9	U77435	94.9	
Enterobacter cancerogenus	Z96078	93.1			
Enterobacter aerogenes	AB004750	93.5			
Enterobacter intermedius	AB004747	92.6			
Enterobacter asburiae	AB004744	93.6			
Enterobacter					

	nimipressuralis	Z96077	92.4		
	Erwinia wasabiae	U80199			
	Erwinia cypripedii	U80201	91.5		
	Escherichia coli	Z80725	90.8	V00340	94.9
5	*Salmonella typhi*			AF008577	92.8
	Citrobacter freundii			U77434	95.5
	Salmonella arizonae			U77447	92.0
	Salmonella paratyphi			U77450	93.0
	Salmonella shomron			U77451	92.4
10	*Yersinia enterocolitica*			U77453	85.5

Partial rpoB gene DNA of strains LX3 and LX21, which represented the most variable part of the gene, were sequenced to determine the phylogenetic position 15 of these two strains among the members of the *Enterobacteriaceae* family. The sequences for strain LX3 and LX21 were compared to the rpoB sequences of family *Enterobacteriaceae* available in database and the similarity matrix was obtained. As shown in Figure 3, 20 the phylogenetic position is closely related to the species belonging to the genus *Klebsiella*. The closest member is *K. pneumoniae*, but with a divergence value of 5% (Table 8).

Amplification of rpoB and sequencing: The genomic 25 DNA of strain LX3 and LX21 coding RNA polymerase beta-subunit (rpoB) gene, corresponding to positions 1468 to 2114 of *E. coli* rpoB, was amplified by PCR using a primer pair designed with reference to the consensus regions of the published sequences for *E.* 30 *coli* (GenBank V00340), *Salmonella typhimurium* (X04642), *Pseudomonas putide* (X 15849) and *Klebsiella pneumoniae*

(U77443), which is 5'-CAGTTCCGCGTTGGCCTG-3' (Forward) [SEQ ID NO. 15] and 5'-CGGTTGGCGTCATCGTGTTC-3' (Reverse) [SEQ ID NO. 16]. The PCR products were purified and sequenced as described above. The phylogenetic analysis of rpoB was carried out using the same procedure and programs as that of 16S rRNA.

Cloning and Sequencing of the *kis* gene: A genomic DNA library was constructed by partial digestion of genomic DNA from *Klebsiella singaporensis* with BamHI, ligation into the BamHI site of the cosmid vector pLAFR3 and transfection into the *E.coli* DH5α after in vitro packaging using Gigapack II (Stratagene). The transformed *E.coli* DH5α were selected on LB agar plates containing tetracycline and incubated overnight at 37°C. The cosmid colonies were inoculated in the LB medium supplemented with sucrose, and the reducing sugar formed in the broth 24 h later was determined by using the DNS method (Miller, 1959). The presence of isomaltulose in the bacterial cultures was identified by using the thin-layer chromatography. The clones carrying the *kis* gene are selected and the 6.1 kb fragment was purified after digestion with BamHI. The clone pSIEE was sequenced from both strands by the chain-termination method using the ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystem). The primers used for sequencing are listed in Table 9. Sequence analysis and alignment were conducted with the program DNAStar. The BLAST computer program (Altschul et al., 1997) was used for the homology search.

Table 9. Primers for DNA sequencing of *kis* gene.

Primers (Orientation)		Sequence
SI-1	(F)	5'-CCCGCTGGCAATATTTCTGACT3' [SEQ ID NO. 17]
SI-2	(R)	5' -CACGACCACTACCCTTTCTCCTGA-3' [SEQ ID NO. 18]
SI-3	(F)	5' -CCTCGCCTCATTTAAAGACACCAA3' [SEQ ID NO. 19]
SI-4	(R)	5' -TAGGCGCCATATACCAGAGCAG-3' [SEQ ID NO. 20]
SI-5	(F)	5' -CGTGACTATTATTTCTGGCGTGAC-5' [SEQ ID NO. 21]
SI-6	(R)	5' -CGTCGCCCGCTGAGTGAGGTAA-3' [SEQ ID NO. 22]
SI-7	(F)	5' -TAGATAACCATGACAACC-3' [SEQ ID NO. 23]
SI-8	(R)	5' -GCGGTGGCCACATCATACC-3' [SEQ ID NO. 24]
SI-9	(F)	5' -CGCCGTTTATTTATCAAGGTTCAG-3' [SEQ ID NO. 25]
SI-10	(R)	5' -GGAGCATTGCCGTAAAGAT-3' [SEQ ID NO. 26]
SI-11	(F)	5' -CTATACTCTCCCGGCTAATGATGC-3' [SEQ ID NO. 27]
SI-12	(R)	5' -CCACCATGCAGGATATTCACTTT-3' [SEQ ID NO. 28]

The cosmid library of *Klebsiella singaporensis* was screened for isomaltulose biosynthesis activity. Two positive cosmid clones, designed pPLASI164 and pPLASI169, were selected by their ability to produce reducing sugar, which was confirmed to be isomaltulose by TLC analysis. Restriction enzyme analysis of the two clones revealed that they likely contain the same fragment of ~ 6.1 kb released by BamHI digestion. Restriction enzyme mapping of the two clones further verified that the clones pPLASI164 and pPLASI169 are identical. The 6.1 kb fragment contains one PstI site, one ClaI site, one XhoI site and two EcoRV sites as shown in Fig. 5. Deletion analysis narrowed down the

region encoding isomaltulose biosynthesis to a 2.5 kb EcoRV fragment contained in the clone pSIEE.

The nucleotide sequence of clone pSIEE was determined from both DNA strands (Fig. 6A). Open reading frame (ORF) search revealed the presence of one ORF of 1797 bp nucleotides. Deletion analysis indicates that it is the coding region of isomaltulose synthase gene *kis*. The putative initiation codon ATG is proceeded at a spacing of 8 bp by a potential ribosome-binding sequence (AAGGA). There is no significant homology to the consensus -35 promoter element (TTGACA). The possible -10 region promoter sequence (TTTAAT), homologous to the consensus promoter sequence (TATAAT) for σ70 factor in *E.coli* is observed 44 bp upstream of the initiation codon. A putative sucrose responsive region ($^{-54}$TTTTCTTTAATAACAATT^{-37}) [SEQ ID NO. 3], an AT-rich sequence, is also located in -10 region which is homologous to the sucrose-responsive region conserved in several sucrose inducible promoters (Fig. 7; Yokoyama et al., 1994). Inducible expression of the *kis* gene has been confirmed in the presence of sucrose and fructose, whereas there is not *kis* gene expression when glucose was used as a sole carbon source. The putative core sequence, "CAACTG", the binding motif of helix-loop-helix transcription factors (Murre et al., 1989; Rocha and Gomes, 1998), is located at position 21bp upstream from the initiation codon site (Fig. 6A). At the downstream, there are three TCTC boxes, one TCTT box and one TGTG box that closely resemble the TCTG consensus sequence of factor-independent termination sites. Inverted repeated

sequences are also found in the downstream of the putative termination codon (TAA). This could create a potential stable hairpin structure, which could block the progression of RNA polymerase (Brendel and Trifonov, 1984).

The cloned *kis* gene from *K. singaporensis* encodes a protein of 598 amino acids with a calculated molecular weight of 69.94 kDa and an isoelectric point at 6.62 (Fig. 6B). The deduced hydrophilic amino acids are 68.4% of all the amino acids. The best homology to the *kis* gene is found in the isomaltulose synthase gene from *Enterobacter sp.* strain SZ62 (Mattes et al., 1998 US patent No. 5786140), which shares 99.3% identical nucleotides in the open reading frame and 99.7% identical amino acids in the predicted peptide sequences.

The differences are in the C-terminal region of the proteins. KIS has one extra amino acid, Ala577, and two substitutions, with Gly577 and Ala593 in the isomaltulose synthase of strain SZ62 being replaced with Ala578 and Val594, respectively. In addition, the KIS protein shares 67% homology to the same enzyme from the *Protaminobacter rubrum* (Mattes, et al., 1998 US patent No. 5786140).

A potential sucrose catalytic binding site, "^{321}F-D-L-I-R-L-D-R-D-S-N-E^{332}" [SEQ ID NO. 4], has been located based on sequence homology comparison with the identified sites of α-glucosyl transferases (Fig. 8). In this motif, two amino acids, arginine and aspartic acid, are highly conserved. The aspartic acid (D) was reported to play a key role in the enzyme activity and its mutation resulted in complete inhibition of the

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sucrose binding property (Mooser et al., 1991; Kato et al., 1992; Monchois et al., 1997).

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CLAIMS:

1. A pure culture of a bacterial strain comprising all identifying characteristics of *Klebsiella* strain LX3.

2. A pure culture of a bacterial strain comprising all identifying characteristics of *Klebsiella* strain LX21.

3. A nucleic acid encoding a *Klebsiella* isomaltulose synthase (KIS), said KIS comprises the amino acid sequence of SEQ ID NO:2 and converts 99% or more of sucrose in a medium to products comprising more than 87% isomaltulose.

4. A *Klebsiella* isomaltulose synthase comprising the amino acid sequence of SEQ ID NO:2, wherein said *Klebsiella* isomaltulose synthase is adapted to convert 99% or more of sucrose in a medium to products comprising more than 87% isomaltulose.

5. The nucleic acid of claim 3 comprising the nucleotide sequence of SEQ ID NO:1.

6. The nucleic acid of claim 3 which comprises nucleotides 214 through 2007 of the nucleotide sequence of SEQ ID NO:1.

7. A nucleic acid comprising the nucleic acid of claim 3 or 6 fused 3' to a nucleic acid encoding a signal peptide.

8. An expression vector which comprises the nucleic acid of claim 3, 5 or 6, wherein the expression vector propagates in a procaryotic or eucaryotic cell.

9. A cell of a procaryote or eukaryote transformed or transfected with the expression vector of claim 8.

10. A method for production of isomaltulose in a plant, comprising

introducing into a cell of such plant a nucleic acid encoding a *Klebsiella* isomaltulose synthase (KIS), said KIS comprises the amino acid sequence of SEQ ID NO:2 and converts 99% or more of sucrose in a medium to products comprising more than 87% isomaltulose

expressing said nucleic acid in said cell to produce KIS, and

converting a carbon source into isomaltulose via KIS.

11. The method of claim 10, wherein the nucleic acid is fused 3' to a nucleic acid encoding a signal peptide.

12. The method of claim 11, wherein the signal peptide is a vacuole targeting signal peptide.

13 The method of claim 10, wherein the nucleic acid is linked to nucleic acid encoding a membrane attachment domain.

14. The method of claim 10, wherein the nucleic acid comprises the nucleotide sequence of SEQ ID NO:1

15. The method of claim 10, wherein the nucleic acid comprises nucleotides 214 through 2007 of the nucleotide sequence of SEQ ID NO:1.

16. The method of claim 15, wherein the nucleic acid is fused 3' to a nucleic acid encoding a signal peptide.

17. The method of claim 15, wherein the nucleic acid is linked to a nucleic acid encoding a membrane attachment domain.

18. The method of claim 10, wherein the plant is selected from the group consisting of sugarcane, maize, watermelon, and sugarbeet.

19. A functional cloning method of isolating the nucleic acid of claim 3 comprising the steps of

(a) preparing a gene bank of clones from a donor organism that contains a nucleic acid encoding a protein with isomaltulose biosynthesis activity in a suitable host organism,

(b) screening the clones of interest from the gene bank by their enhanced reducing sugar content, and

(c) isolating the clones which contain a nucleic acid encoding a protein with isomaltulose biosynthesis activity.

20. A process as claimed in claim 19, wherein *E. coli* is used as the host organism.

21. A process as claimed in claim 19, wherein the steps of preparing a gene bank, screening the clones, and isolating the clones are performed in an *E. coli* strain that does not produce significant amounts of a reducing sugar.

22. A method of converting at least parts of a carbon source into isomaltulose comprising

providing a *Klebsiella* strain comprising an inducible *Klebsiella* isomaltulose synthase gene,

contacting said *Klebsiella* strain with said carbon source,

inducing said *Klebsiella* isomaltulose synthase gene with one or more fructose containing carbohydrates to express said *Klebsiella* isomaltulose synthase,

contacting said Klebsiella isomaltulose synthase with at least parts of said carbon source and converting at least parts of said carbon source into isomaltulose.

23. The method of claim 22, wherein said carbon source that is converted into isomaltulose comprises sucrose, fructose, raffinose, isomaltulose or a combination thereof.

24. The method of claim 22, wherein 99% of sucrose in the carbon source is converted to products comprising more than 87% isomaltulose and less than 1% glucose.

25. The method of claim 22, wherein said *Klebsiella* isomaltulose synthase is induced by sucrose, fructose, raffinose, isomaltulose or combinations thereof.

26. The method of claim 22, wherein said *Klebsiella* strain is a naturally occurring Klebsiella strain which does not metabolize isomaltulose.

27. The method of claim 22, wherein said *Klebsiella* isomaltulose synthase is encoded by SEQ ID NO:1.