

University of Groningen

Novel Fructosyltransferases

van Geel-Schutten, Gerritdina Hendrika ; Rahaoui, Hakim; Dijkhuizen, Lubbert; Van Hijum, Sacha Adrianus Fokke Taco

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version

Publisher's PDF, also known as Version of record

Publication date:

2001

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

van Geel-Schutten, G. H., Rahaoui, H., Dijkhuizen, L., & Van Hijum, S. A. F. T. (2001). Novel Fructosyltransferases. (Patent No. *WO0190319*).

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
29 November 2001 (29.11.2001)

PCT

(10) International Publication Number
WO 01/90319 A2

- (51) International Patent Classification⁷: C12N 9/00
- (74) Agent: **JORRITSMA, Ruurd**; Nederlandsch Octrooibureau, Scheveningseweg 82, P.O. Box 29720, NL-2502 LS The Hague (NL).
- (21) International Application Number: PCT/NL01/00392
- (22) International Filing Date: 23 May 2001 (23.05.2001)
- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
00201872.9 25 May 2000 (25.05.2000) EP
01200049.3 9 January 2001 (09.01.2001) EP
- (71) Applicant (*for all designated States except US*): **NED-ERLANDSE ORGANISATIE VOOR TOEGEPAST-WETENSCHAPPELIJK ONDERZOEK** [NL/NL]; Schoemakerstraat 97, NL-2628 VK Delft (NL).
- (72) Inventors; and
- (75) Inventors/Applicants (*for US only*): **VAN GEEL-SCHUTTEN, Gerritdina, Hendrika** [NL/NL]; Bosstraat 78, NL-3971 XG Driebergen (NL). **RAHAOUI, Hakim** [NL/NL]; Sextant 4 b, NL-3813 VS Amersfoort (NL). **DIJKHUIZEN, Lubbert** [NL/NL]; Ter Borch 28, NL-9742 RB Zuidlaren (NL). **VAN HIJUM, Sacha, Adrianus, Fokke, Taco** [NL/NL]; Bedumerstraat 137, NL-9716 BH Groningen (NL).
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.



WO 01/90319 A2

(54) Title: NOVEL FRUCTOSYLTRANSFERASES

(57) Abstract: The present invention describes two novel proteins having fructosyltransferase activity. Both enzymes are derived from lactobacilli, which are food-grade microorganisms with the Generally Recognized As Safe (GRAS) status. One of these proteins produces an inulin and fructo-oligosaccharides, while the other produces a levan. According to the invention lactobacilli capable of producing an inulin and/or a levan and/or fructo-oligosaccharides using one or both of the fructosyltransferases can be used as a probiotic or a symbiotic.

Novel fructosyltransferases

[0001] The present invention is in the field of enzymatic production of biomolecules. The invention is particularly concerned with two novel fructosyltransferases derived from lactobacilli and with a process for recombinant production of the enzymes and for the production of useful levans, inulins and fructo-oligosaccharides from sucrose.

Background of the invention

[0002] Lactic acid bacteria (LAB) play an important role in the fermentative production of food and feed. Traditionally, these bacteria have been used for the production of for instance wine, beer, bread, cheese and yoghurt, and for the preservation of food and feed, e.g. olives, pickles, sausages, sauerkraut and silage. Because of these traditional applications, lactic acid bacteria are food-grade micro-organisms that possess the Generally Recognised As Safe (GRAS) status. Due to the different products which are formed during fermentation with lactic acid bacteria, these bacteria contribute positively to the taste, smell and preservation of the final product. The group of lactic acid bacteria encloses several genera such as *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, etc.

[0003] In recent years also the health promoting properties of lactic acid bacteria have received much attention. They produce an abundant variety of exopolysaccharides (EPS's). These polysaccharides are thought to contribute to human health by acting as prebiotic substrates, nutraceuticals, cholesterol lowering agents or immunomodulants.

[0004] To date high molecular weight polysaccharides produced by plants (such as cellulose, starch and pectin), seaweeds (such as alginate and carrageenan) and bacteria (such as alginate, gellan and xanthan) are used in several industrial applications as viscosifying, stabilising, emulsifying, gelling or water binding agents. Although all these polysaccharides are used as food additives, they originate from organisms not having the GRAS status. Thus they are less desirable than the exopolysaccharides of microorganisms, such as lactic acid bacteria, which have the GRAS status.

[0005] The exopolysaccharides produced by LAB can be divided in two groups, heteropolysaccharides and homopolysaccharides; these are synthesized by totally different mechanisms. The former consist of repeating units in which residues of different types of sugars are present and the latter consist of one type of monosaccharide. The synthesis of heteropolysaccharides by lactic acid bacteria, including lactobacilli, has

been studied extensively in recent years. Considerably less information is available on the synthesis of homopolysaccharides from lactobacilli, although some studies have been performed. Homopolysaccharides with fructose as the constituent sugar can be divided into two groups, inulins and levans. Inulins consist of 2,1-linked β -fructofuranoside residues, whereas levans consist of 2,6-linked β -fructofuranoside residues. Both can be linear or branched. The size of bacterial levans can vary from 20 kDa up to several MDa. There is limited information on the synthesis of levans. In most detail this synthesis has been studied in *Zymomonas mobilis* and in *Bacillus* species. Within lactic acid bacteria, fructosyltransferases have only been studied in streptococci. So far no fructosyltransferases have been reported in lactobacilli.

[0006] In a recent report the *Lactobacillus reuteri* strain LB 121 was found to produce both a glucan and a fructan when grown on sucrose, but only a fructan when grown on raffinose (van Geel-Schutten, G.H. *et al.*, Appl. Microbiol. Biotechnol. (1998) 50, 697-703). In another report the glucan and fructan were characterised by their molecular weights (of 3,500 and 150 kDa respectively) and the glucan was reported to be highly branched with a unique structure consisting of a terminal, 4-substituted, 6-substituted, and 4,6-disubstituted α -glucose in a molar ratio 1.1 : 2.7 : 1.5 : 1.0 (van Geel-Schutten, G.H. *et al.*, Appl. Environ. Microbiol. (1999) 65, 3008-3014). The fructan was identified as a linear (2 \rightarrow 6)- β -D-fructofuranan (also called a levan). This was the first example of fructan synthesis by a *Lactobacillus* species.

Summary of the invention

[0007] Two novel genes encoding enzymes having fructosyltransferase activity have now been found in *Lactobacillus reuteri*, and their amino acid sequences have been determined. These are the first two enzymes identified in a *Lactobacillus* species capable of producing a fructan. One of the enzymes is an inulosucrase which produces a high molecular weight ($>10^7$ Da) fructan containing β (2-1) linked fructosyl units and fructooligosaccharides, while the other is a levansucrase which produces a fructan containing β (2-6) linked fructosyl units. The invention thus pertains to the enzymes, to DNA encoding them, to recombinant cells containing such DNA and to their use in producing carbohydrates, as defined in the appending claims.

Description of the invention

[0008] It was found according to the invention that one of the novel fructosyltransferases (FTFA; an inulosucrase) produces a high molecular weight inulin with $\beta(2-1)$ linked fructosyl units and fructo-oligosaccharides. The fructo-oligosaccharides synthesis was also observed in certain *Lactobacillus* strains, in particular in certain strains of *Lactobacillus reuteri*. However, the inulin has not been found in *Lactobacillus reuteri* culture supernatants, but only in extracts of *E. coli* cells expressing the above-mentioned fructosyltransferase. This inulosucrase consists of either 798 amino acids (2394 nucleotides) or 789 amino acids (2367 nucleotides) depending on the potential start codon used. The molecular weight (MW) deduced of the amino acid sequence of the latter form is 86 kDa and its isoelectric point is 4.51, at pH 7.

[0009] The amino acid sequence of the inulosucrase is shown in SEQ ID No. 1 (figure 1, amino acid residues 1-789). As mentioned above, the nucleotide sequence contains two putative start codons leading to either a 2394 (see SEQ ID No. 3) or 2367 (see SEQ ID No. 2) nucleotide form of the inulosucrase. Both putative start codons are preceded by a putative ribosome binding site, GGGG (located 12 base pairs upstream its start codon) or AGGA (located 14 base pairs upstream its start codon), respectively (see figure 1 and SEQ ID No. 4).

[0010] The present invention covers a protein having inulosucrase activity with an amino acid identity of at least 65%, preferably at least 75%, and more preferably at least 85%, compared to the amino acid sequence of SEQ ID No. 1. The invention also covers a part of a protein with at least 15 contiguous amino acids which are identical to the corresponding part of the amino acid sequence of SEQ ID No. 1.

[0011] Fructosyltransferases have been found in several bacteria such as *Zymomonas mobilis*, *Erwinia amylovora*, *Acetobacter amylovora*, *Bacillus polymyxa*, *Bacillus amyloliquefaciens*, *Bacillus stearothermophilus*, and *Bacillus subtilis*. In lactic acid bacteria this type of enzyme previously has only been found in some streptococci. Most bacterial fructosyltransferases have a molecular mass of 50-100 kDa (with the exception of the fructosyltransferase found in *Streptococcus salivarius* which has a molecular mass of 140 kDa). Amino acid sequence alignment revealed that the novel inulosucrase of lactobacilli has high homology with fructosyltransferases originating from Gram positive bacteria, in particular with *Streptococcus* enzymes. The highest homology (figure 2) was found with the SacB enzyme of *Streptococcus mutans* Ingbritt A (62% identity within 539 amino acids).

[0012] Certain putative functions based on the alignment and site-directed mutagenesis studies can be ascribed to several amino acids of the novel inulosucrase. Asp-263, Glu-330, Asp-415, Glu-431, Asp-511, Glu-514, Arg-532 and/or Asp-551 of the amino acid sequence of SEQ ID No. 1 are identified as putative catalytic residues. Noteworthy, a hydrophobicity plot according to Kyte and Doolittle (1982) J. Mol. Biol. 157, 105-132 suggests that the novel inulosucrase contains a putative signal sequence according to the Von Heijne rule. The putative signal peptidase site is located between Gly at position 21 and Ala at position 22. Furthermore, it is striking that the C-terminal amino acid sequence of the novel inulosucrase contains a putative cell wall anchor amino acid signal LPXTG (SEQ ID No. 5) and a 20-fold repeat of the motif PXX (see figure 1), where P is proline and X is any other amino acid. In 15 out of 20 repeats, however, the motif is PXT. This motif has so far not been reported in proteins of prokaryotic and eukaryotic origin.

[0013] A nucleotide sequence encoding any of the above mentioned proteins, mutants, variants or parts thereof is also a subject of the invention. Furthermore, the nucleic acid sequences corresponding to expression-regulating regions (promoters, enhancers, terminators) of at least 30 contiguous nucleic acids contained in the nucleic acid sequence (1)-(67) or (2438)-(2592) of SEQ ID No. 4 (see also figure 1) can be used for homologous or heterologous expression of genes. Such expression-regulating sequences are operationally linked to a polypeptide-encoding nucleic acid sequence such as the genes of the fructosyltransferase according to the invention. A nucleic acid construct comprising the nucleotide sequence operationally linked to an expression-regulating nucleic acid sequence is also covered by the invention.

[0014] A recombinant host cell, such as a mammalian (with the exception of human), plant, animal, fungal or bacterial cell, containing one or more copies of the nucleic acid construct mentioned above is an additional subject of the invention. The inulosucrase gene (starting at nucleotide 41) has been cloned in an *E. coli* expression vector under the control of an *ara* promoter in *E. coli* Top10. *E. coli* Top10 cells expressing the recombinant inulosucrase hydrolysed sucrose and synthesized fructan material. SDS-PAGE of arabinose induced *E. coli* Top10 cell extracts suggested that the recombinant inulosucrase has a molecular weight of 80-100 kDa, which is in the range of other known fructosyltransferases and in line with the molecular weight of 86 kDa deduced of the amino acid sequence depicted in figure 1.

[0015] The invention further covers an inulosucrase according to the invention which, in the presence of sucrose, produces a inulin having $\beta(2-1)$ -linked D-fructosyl units and fructo-oligosaccharides. Two different types of fructans, inulins and levans, exist in nature. Surprisingly, the novel inulosucrase expressed in *E. coli* Top10 cell synthesizes a high molecular weight ($>10^7$ Da) inulin and fructo-oligosaccharides, while in *Lactobacillus reuteri* culture supernatants, in addition to the fructo-oligosaccharides, a levan and not an inulin is found. This discrepancy can have several explanations: the inulosucrase gene may be silent in *Lactobacillus reuteri*, or may not be expressed in *Lactobacillus reuteri* under the conditions tested, or the inulosucrase may only synthesize fructo-oligosaccharides in its natural host, or the inulin polymer may be degraded shortly after synthesis, or may not be secreted and remains cell-associated, or the inulosucrase may have different activities in *Lactobacillus reuteri* and *E. coli* Top10 cells.

[0016] It was furthermore found according to the invention that certain lactobacilli, in particular *Lactobacillus reuteri*, possess another fructosyltransferase, a levansucrase (FTFB), in addition to the inulosucrase described above. The N-terminal amino acid sequence of the fructosyltransferase purified from *Lactobacillus reuteri* supernatant was found to be QVESNNYNGVAEVNTERQANGQI (SEQ ID No. 6). Furthermore, three internal sequences were identified, namely (M)(A)HLDVWDSWPVQDP(V) (SEQ ID No. 7), NAGSIFGT(K) (SEQ ID No. 8), V(E)(E)VYSPKVSTLMASDEVE (SEQ ID No. 9). The N-terminal amino acid sequence could not be identified in the deduced inulosucrase sequence. Also the amino acid sequences of the three internal peptide fragments of the purified fructosyltransferase were not present in the putative inulosucrase sequence. Evidently, the inulosucrase gene does not encode the purified fructosyltransferase synthesizing the levan. The complete amino acid sequence of the levansucrase is shown in SEQ ID No. 11 and the nucleotide sequence is shown in SEQ ID No. 10. The levansucrase comprises a putative membrane anchor (see amino acids 761-765 in SEQ ID No. 11) and a putative membrane spanning domain (see amino acids 766-787 in SEQ ID No. 11). The fructan produced by the levansucrase was identified in the *Lactobacillus reuteri* culture supernatant as a linear (2 \rightarrow 6)- β -D-fructofuranan with a molecular weight of 150 kDa. The purified enzyme also produces this fructan.

[0017] Additionally, the invention thus covers a protein having levansucrase activity with an amino acid identity of at least 65%, preferably at least 75%, and more preferably at least 85%, compared to the amino acid sequence of SEQ ID NO. 11. The second novel fructosyltransferase produces a high molecular weight fructan with $\beta(2-6)$ linked

fructosyl units with sucrose or raffinose as substrate. The invention also covers a part of a protein with at least 15 contiguous amino acids which are identical to the corresponding part of the amino acid sequence of SEQ ID No. 11. A nucleotide sequence encoding any of the above-mentioned proteins, mutants, variants or parts thereof is a subject of the invention as well as a nucleic acid construct comprising the nucleotide sequence mentioned above operationally linked to an expression-regulating nucleic acid sequence. A recombinant host cell, such as a mammalian (with the exception of human), plant, animal, fungal or bacterial cell, containing one or more copies of the nucleic acid construct mentioned above is an additional subject of the invention. The invention further covers a protein according to the invention which, in the presence of sucrose, produces a fructan having $\beta(2-6)$ -linked D-fructosyl units.

[0018] The invention also pertains to a process of producing an inulin-type and/or a levan-type of fructan as described above using isolated fructosyltransferases according to the invention and a suitable fructose source such as sucrose or raffinose. The fructosyltransferase enzyme may be isolated by conventional means from the culture of fructosyltransferase-positive lactobacilli, especially a *Lactobacillus reuteri*, or from a recombinant organism containing the fructosyltransferase gene or genes.

[0019] Additionally, the invention concerns a process of producing fructo-oligosaccharides containing the characteristic structure of the fructans described above using an isolated fructosyltransferase according to the invention. There is a growing interest in oligosaccharides derived from homopolysaccharides, for instance for prebiotic purposes. Several fructo- and gluco-oligosaccharides are known to stimulate the growth of bifidobacteria in the human colon. Fructo-oligosaccharides produced by the fructosyltransferase described above are also part of the invention. Another way of producing fructo-oligosaccharides is by hydrolysis of the fructans described above. This hydrolysis can be performed by known hydrolysis methods such as enzymatic hydrolysis with enzymes such as levanase or inulinase or by acid hydrolysis. The fructo-oligosaccharides to be produced according to the invention preferably contain at least 2, more preferably at least 3, up to about 20 anhydrofructose units, optionally in addition to one or more other (glucose, galactose, etc.) units. These fructo-oligosaccharides are useful as prebiotics.

[0020] The invention also concerns chemically modified fructans and fructo-oligosaccharides based on the fructans described above. Chemical modification can be achieved by oxidation, such as hypochlorite oxidation resulting in ring-opened 2,3-

dicarboxy-anhydrofructose units (see e.g. EP-A-427349), periodate oxidation resulting in ring-opened 3,4-dialdehyde-anhydrofructose units (see e.g. WO 95/12619), which can be further oxidised to (partly) carboxylated units (see e.g. WO 00/26257), TEMPO-mediated oxidation resulting in 1- or 6-carboxy-anhydrofructose units (see e.g. WO 5 95/07303). The oxidised fructans have improved water-solubility, altered viscosity and a retarded fermentability and can be used as metal-complexing agents, detergent additives, strengthening additives, bioactive carbohydrates, emulsifiers and water binding agents. They can also be used as starting materials for further derivatisation such as cross-linking and the introduction of hydrophobes. Oxidised fructans coupled to amino compounds 10 such as proteins, or fatty acids can be used as emulsifiers and stabilizers. Partial hydrolysis of fructans according to the invention and modified fructans according to the invention results in fructo-oligosaccharides, which can be used as bioactive carbohydrates or prebiotics. The oxidised fructans of the invention preferably contain 0.05-1.0 carboxyl groups per anhydrofructose unit, e.g. as 6- or 1-carboxyl units

15 [0021] Another type of chemical modification is phosphorylation, as described in O.B. Wurzburg (1986) Modified Starches: properties and uses. CRC Press Inc., Boca Raton, 97-112. One way to achieve this modification is by dry heating fructans with a mixture of monosodium and disodium hydrogen phosphate or with tripolyphosphate. The phosphorylated fructans are suitable as wet-end additives in papermaking, as binders in 20 paper coating compositions, as warp sizing-agents, and as core binders for sand molds for metal casting. A further type of derivatisation of the fructans is acylation, especially acetylation using acetic or propionic anhydride, resulting in products suitable as bleaching assistants and for the use in foils. Acylation with e.g. alkenyl succinic anhydrides or (activated) fatty acids results in surface-active products suitable as e.g. 25 surfactants, emulsifiers, and stabilizers.

[0022] Hydroxyalkylation, carboxymethylation, and aminoalkylation are other methods of chemical derivatisation of the fructans. Hydroxyalkylation is commonly performed by base-catalysed reaction with alkylene oxides, such as ethylene oxide, propylene oxide or epichlorohydrine; the hydroxyalkylated products have improved solubility and viscosity 30 characteristics. Carboxymethylation is achieved by reaction of the fructans with monochloroacetic acid or its alkali metal salts and results in anionic polymers suitable for various purposes including crystallisation inhibitors, and metal complexants. Aminoalkylation can be achieved by reaction of the fructans with alkylene imines, haloalkyl amines or amino-alkylene oxides, or by reaction of epichlorohydrine adducts of the

fructans with suitable amines. These products can be used as cationic polymers in a variety of applications, especially as a wet-end additive in paper making to increase strength, for filler and fines retention, and to improve the drainage rate of paper pulp. Other potential applications include textile sizing and wastewater purification. The above mentioned modifications can be used either separately or in combination depending on the desired product. Furthermore, the degree of chemical modification is variable and depends on the intended use. If necessary 100% modification, *i.e.* modification of all anhydrofructose units can be performed. However, partial modification, *e.g.* from 1 modified anhydrofructose unit per 100 up to higher levels, will often be sufficient in order to obtain the desired effect. The modified fructans have a DP (degree of polymerisation) of at least 100, preferably at least 1000 units.

[0023] Use of a *Lactobacillus* strain capable of producing a levan, inulin or fructo-oligosaccharides or a mixture thereof, as a probiotic, is also covered by the invention. Preferably, the *Lactobacillus* strain is also capable of producing a glucan, especially an 1,4/1,6- α -glucan as referred to above. The efficacy of some *Lactobacillus reuteri* strains as a probiotics has been demonstrated in various animals such as for instance poultry and humans. The administration of some *Lactobacillus reuteri* strains to pigs resulted in significantly lower serum total and LDL-cholesterol levels, while in children *Lactobacillus reuteri* is used as a therapeutic agent against acute diarrhea. For this and other reasons *Lactobacillus reuteri* strains, which were not reported to produce the glucans or fructans described herein, have been supplemented to commercially available probiotic products. The mode of action of *Lactobacillus reuteri* as a probiotic is still unclear. Preliminary studies indicated that gut colonization by *Lactobacillus reuteri* may be of importance. According to the invention, it was found that the mode of action of *Lactobacillus reuteri* as a probiotic may reside partly in the ability to produce polysaccharides. *Lactobacillus* strains, preferably *Lactobacillus reuteri* strains, and more preferably *Lactobacillus reuteri* strain LB 121 and other strains containing one or more fructosyltransferase genes encoding proteins capable of producing inulins, levans and/or fructo-oligosaccharides can thus advantageously be used as a probiotic. They can also, together with these polysaccharides, be used as a symbiotic.

Examples

Example 1: Isolation of DNA from *Lactobacillus reuteri*, nucleotide sequence analysis of the inulosucrase (*ffa*) gene, construction of plasmids for expression of the inulosucrase

gene in *E. coli* Top10, expression of the inulosucrase gene in *E. coli* Top10 and identification of the produced polysaccharides produced by the recombinant enzyme.

General procedures for cloning, DNA manipulations and agarose gel electrophoresis were essentially as described by Sambrook *et al.* (1989) Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York. Restriction endonuclease digestions and ligations with T4 DNA ligase were performed as recommended by the suppliers. DNA was amplified by PCR techniques using ampliTAQ DNA polymerase (Perkin Elmer) or Pwo DNA polymerase. DNA fragments were isolated from agarose gels using the Qiagen extraction kit (Qiagen GMBH), following the instructions of the suppliers. *Lactobacillus reuteri* strain 121 (LMG 18388) was grown at 37°C in MRS medium (DIFCO) or in MRS-s medium (MRS medium containing 100 g/l sucrose instead of 20 g/l glucose). When fructo-oligosaccharides production was investigated phosphate was omitted and ammonium citrate was replaced by ammonium nitrate in the MRS-s medium. *E. coli* strains were grown aerobically at 37°C in LB medium, where appropriate supplemented with 50 µg/ml ampicillin (for selection of recombinant plasmids) or with 0.02% (w/v) arabinose (for induction of the inulosucrase gene).

Total DNA of *Lactobacillus reuteri* was isolated according to Verhasselt *et al.* (1989) FEMS Microbiol. Lett. 59, 135-140 as modified by Nagy *et al.* (1995) J. Bacteriol. 177, 676-687.

The inulosucrase gene was identified by amplification of chromosomal DNA of *Lactobacillus reuteri* with PCR using degenerated primers (5ftf, 6ftfi, and 12ftfi, see table 1) based on conserved amino acid sequences deduced from different bacterial fructosyltransferase genes (SacB of *Bacillus amyloliquefaciens*, SacB of *Bacillus subtilis*, *Streptococcus mutans* fructosyltransferase and *Streptococcus salivarius* fructosyltransferase, see figure 4) and *Lactobacillus reuteri* DNA as template. Using primers 5ftf and 6ftfi, an amplification product with the predicted size of about 234 bp was obtained (figure 5A). This 234 bp fragment was cloned in *E. coli* JM109 using the pCR2.1 vector and sequenced. Transformations were performed by electroporation using the BioRad gene pulser apparatus at 2.5 kV, 25 µF and 200 Ω, following the instructions of the manufacturer. Sequencing was performed according to the method of Sanger *et al.* (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467. Analysis of the obtained sequence data confirmed that part of a fructosyltransferase (*ftf*) gene had been isolated. The 234 bp amplified fragment was used to design primers 7ftf and 8ftfi (see table 1). PCR with the

primers 7ftf and 12ftfi gave a product of the predicted size of 948 bp (see figure 5B); its sequence showed clear similarity with previously characterized fructosyltransferase genes. The 948 bp amplified fragment was used to design the primers ftfAC1(i) and ftfAC2(i) (see table 1) for inverse PCR. Using inverse PCR techniques a 1438 bp
5 fragment of the inulosucrase gene was generated, including the 3' end of the inulosucrase gene (see figure 5C). The remaining 5' fragment of the inulosucrase gene was isolated with a combination of standard and inverse PCR techniques. Briefly, *Lactobacillus reuteri* DNA was cut with restriction enzyme *XhoI* and ligated. PCR with the primers 7ftf and 8ftfi, using the ligation product as a template, yielded a 290 bp PCR product which
10 was cloned into pCR2.1 and sequenced. This revealed that primer 8ftfi had annealed aspecifically as well as specifically yielding the 290 bp product (see figure 5D).

At this time, the N-terminal amino acid sequence of a fructosyltransferase enzyme (FTFB) purified from the *Lactobacillus reuteri* strain 121 was obtained. This sequence consisted of the following 23 amino acids: QVESNNYNGVAEVNTERQANGQI (SEQ
15 ID No. 6). The degenerated primer 19ftf (YNGVAEV) was designed on the basis of a part of this N-terminal peptide sequence and primer 20ftfi was designed on the 290 bp PCR product. PCR with primers 19ftf and 20ftfi gave a 754 bp PCR product (see figure 5E), which was cloned into pCR2.1 and sequenced. Both DNA strands of the entire fructosyltransferase gene were double sequenced. In this way the sequence of a 2.6 kb
20 region of the *Lactobacillus reuteri* DNA, containing the inulosucrase gene and its surroundings were obtained.

The plasmids for expression of the inulosucrase gene in *E. coli* Top10 were constructed as described hereafter. A 2414 bp fragment, containing the inulosucrase gene starting at the first putative start codon at position 41, was generated by PCR, using
25 primers ftfA1 and ftfA2i. Both primers contained suitable restriction enzyme recognition sites (a *NcoI* site at the 5'end of ftfA1 and a *BglII* site at the 3'end of ftfA2i). PCR with *Lactobacillus reuteri* DNA, Pwo DNA polymerase and primers ftfA1 and ftfA2i yielded the complete inulosucrase gene flanked by *NcoI* and *BglII* restriction sites. The PCR product with blunt ends was ligated directly into pCRbluntII-Topo. Using the *NcoI* and
30 *BglII* restriction sites, the putative *ftfA* gene was cloned into the expression vector pBAD, downstream of the inducible arabinose promoter and in frame upstream of the Myc epitope and the His tag. The pBAD vector containing the inulosucrase gene (pSVH101) was transformed to *E. coli* Top10 and used to study inulosucrase expression. Correct construction of plasmid containing the complete inulosucrase gene was confirmed by

restriction enzyme digestion analysis and by sequence analysis, showing an in frame cloning of the inulosucrase gene using the ribosomal binding site provided by the pBAD vector and the first putative start codon (at position 41) of inulosucrase (see figure 1).

Plasmid DNA of *E. coli* was isolated using the alkaline lysis method of Birnboim and Doly (1979) *Nucleic Acids Res.* 7, 1513-1523 or with a Qiagen plasmid kit following the instructions of the supplier. Cells of *E. coli* Top10 with pSVH101 were grown overnight in LB medium containing 0.02% (w/v) arabinose and were harvested by centrifugation. The pellet was washed with 25 mM sodium acetate buffer pH 5.4 and the suspension was centrifuged again. Pelleted cells were resuspended in 25 mM sodium acetate buffer pH 5.4. Cells were broken by sonication. Cell debris and intact cells were removed by centrifugation for 30 min at 4°C at 10,000xg and the resulting cell free extract was used in the enzyme assays.

The fructosyltransferase activities were determined at 37°C in reaction buffer (25 mM sodium acetate, pH 5.4, 1 mM CaCl₂, 100g/l sucrose) by monitoring the release of glucose from sucrose, by detecting fructo-oligosaccharides or by determining the amount of fructan polymer produced using *E. coli* cell free extracts or *Lactobacillus reuteri* culture supernatant as enzyme source. Sucrose, glucose and fructose were determined enzymatically using commercially available kits.

Fructan production by *Lactobacillus reuteri* was studied with cells grown in MRS-s medium. Product formation was also studied with cell-free extracts of *E. coli* containing the novel inulosucrase incubated in reaction buffer (1 mg protein/10 ml buffer, incubated overnight at 37°C). Fructans were collected by precipitation with ethanol. ¹H-NMR spectroscopy and methylation analysis were performed as described by van Geel-Schutten *et al.* (1999) *Appl. Environ. Microbiol.* 65, 3008-3014. The molecular weights of the fructans were determined by high performance size exclusion chromatography coupled on-line with a multi angle laser light scattering and a differential refractive index detector. Fructo-oligosaccharide synthesis was studied in *Lactobacillus reuteri* culture supernatants and in extracts of *E. coli* cells containing the novel inulosucrase incubated in reaction buffer (1 mg protein/10 ml buffer, incubated overnight at 37°C). Glucose and fructose were determined enzymatically as described above and fructo-oligosaccharides produced were analyzed using a Dionex column. The incubation mixtures were centrifuged for 30 min at 10,000xg and diluted 1:5 in a 100% DMSO solution prior to injection on a Dionex column. A digest of inulin (DP1-20) was used as a standard. Separation of compounds was achieved with anion-exchange

chromatography on a CarboPac Pa1 column (Dionex) coupled to a CarboPac PA1 guard column (Dionex). Using a Dionex GP50 pump the following gradient was generated: % eluent B is 5% (0 min); 35% (10 min); 45% (20 min); 65% (50 min); 100% (54-60 min); 5% (61-65 min). Eluent A was 0.1 M NaOH and eluent B was 0.6 M NaAc in a 0.1 M NaOH solution. Compounds were detected using a Dionex ED40 electrochemical detector with an AU working electrode and a Ag/AgCl reference-electrode with a sensitivity of 300 nC. The pulse program used was: +0.1 Volt (0-0.4 s); +0.7 Volt (0.41-0.60 s); -0.1 Volt (0.61-1.00 s). Data were integrated using a Perkin Elmer Turbochrom data integration system. A different separation of compounds was done on a cation exchange column in the calcium form (Benson BCX4). As mobile phase Ca-EDTA in water (100 ppm) was used. The elution speed was 0.4 ml/min at a column temperature of 85°C. Detection of compounds was done by a refractive index (Jasco 830-RI) at 40°C. Quantification of compounds was achieved by using the software program Turbochrom (Perkin Elmer).

SDS-PAGE was performed according to Laemmli (1970) Nature 227, 680-685 using 7.5% polyacrylamide gels. After electrophoresis gels were stained with Coomassie Brilliant Blue or an activity staining (Periodic Acid Schiff, PAS) was carried out as described by Van Geel-Schutten *et al.* (1999) Appl. Environ. Microbiol. 65, 3008-3014.

Table 1 Nucleotide sequence of primers used in PCR reactions to identify the inulosucrase gene.

Primer name	Location (bp)	Nucleotide sequence (and SEQ ID No)
fftAC1	1176	CTG-ATA-ATA-ATG-GAA-ATG-TAT-CAC (SEQ ID No. 12)
fftAC2i	1243	CAT-GAT-CAT-AAG-TTT-GGT-AGT-AAT-AG (SEQ ID No. 13)
fftac1	1176	GTG-ATA-CAT-TTC-CAT-TAT-TAT-CAG (SEQ ID No. 14)
fftAC2	1243	CTA-TTA-CTA-CCA-AAC-TTA-TGA-TCA-TG (SEQ ID No. 15)
fftA1		CCA-TGG-CCA-TGG-TAG-AAC-GCA-AGG-AAC- ATA-AAA-AAA-TG (SEQ ID No. 16)

ffa2i		AGA-TCT-AGA-TCT-GTT-AAA-TCG-ACG-TTT- GTT-AAT-TTC-TG (SEQ ID No. 17)
5ftf	845	GAY-GTN-TGG-GAY-WSN-TGG-GCC (SEQ ID No. 18)
6ftfi	1052	GTN-GCN-SWN-CCN-SWC-CAY-TSY-TG (SEQ ID No. 19)
7ftf	1009	GAA-TGT-AGG-TCC-AAT-TTT-TGG-C (SEQ ID No. 20)
8ftfi	864	CCT-GTC-CGA-ACA-TCT-TGA-ACT-G (SEQ ID No. 21)
12ftfi	1934	ARR-AAN-SWN-GGN-GCV-MAN-GTN-SW (SEQ ID No. 22)
19ftf	1	TAY-AAY-GGN-GTN-GCN-GAR-GTN-AA (SEQ ID No. 23)
20ftfi	733	CCG-ACC-ATC-TTG-TTT-GAT-TAA-C (SEQ ID No. 24)

Listed from left to right are: primer name (i, inverse primer), location (in bp) in *ffa* and the sequence from 5' to 3' according to IUB group codes (N=any base; M=A or C; R=A or G; W=A or T; S=C or G; Y=C or T; K=G or T; B=not A; D=not C; H=not G; and V=not T).

5

Example 2: Purification and amino acid sequencing of the levansucrase (FTFB).

Protein purification

Samples were taken between each step of the purification process to determine the enzyme activity (by glucose GOD-Perid method) and protein content (by Bradford analysis and acrylamide gel electrophoresis). Collected chromatography fractions were
10 screened for glucose liberating activity (GOD-Perid method) to determine the enzyme activity.

One litre of an overnight culture of LB121 cells grown on MRS medium containing 50 grams per litre maltose was centrifuged for 15 min. at 10,000xg. The
15 supernatant was precipitated with 1.5 litre of a saturated ammonium sulphate solution. The ammonium sulphate solution was added at a rate of 50 ml/min. under continuous stirring. The resulting 60% (w/v) ammonium sulphate solution was centrifuged for 15

min. at 10,000xg. The precipitate was resuspended in 10 ml of a sodium phosphate solution (10 mM, pH 6.0) and dialysed overnight against 10 mM sodium phosphate, pH 6.0.

A hydroxylapatite column was washed with a 10 mM sodium phosphate solution
5 pH 6.0; the dialysed sample was loaded on the column. After eluting the column with 200 mM sodium phosphate, pH 6.0 the eluted fractions were screened for glucose releasing activity and fractions were pooled for phenyl superose (a hydrophobic interactions column) chromatography.

The pooled fractions were diluted 1:1 (v:v) with 25 mM sodium acetate, 2 M ammonium
10 sulphate, pH 5.4 and loaded on a phenyl superose column (washed with 25 mM sodium acetate, 1 M ammonium sulphate, pH 5.4). In a gradient from 25 mM sodium acetate, 1 M ammonium sulphate, pH 5.4 (A) to 25 mM sodium acetate, pH 5.4 (B) fractions were collected from 35% B to 50% B.

Pooled fractions from the phenyl superose column were loaded on a gel filtration
15 (superdex) column and eluted by a 25 mM acetate, 0.1 M sodium chloride, pH 5.4 buffer. The superdex fractions were loaded on a washed (with 25 mM sodium acetate, pH 5.4) Mono Q column and eluted with 25 mM sodium acetate, 1 M sodium chloride, pH 5.4. The fractions containing glucose liberating activity were pooled, dialysed against 25 mM sodium acetate, pH 5.4, and stored at -20 °C.

20 A levansucrase enzyme was purified from LB121 cultures grown on media containing maltose using ammonium sulfate precipitation and several chromatography column steps (table 2). Maltose (glucose-glucose) was chosen because both glucansucrase and levansucrase can not use maltose as substrate. LB121 will grow on media containing maltose but will not produce polysaccharide. From earlier experiments
25 it was clear that even with harsh methods the levansucrase enzyme could not be separated from its product levan. These harsh methods included boiling the levan in a SDS solution and treating the levan with HCl and TFA. No levanase enzyme was commercially available for the enzymatic breakdown of levan. Only a single levansucrase was detected in maltose culture supernatants. In order to prove that the
30 enzyme purified from maltose culture supernatant is the same enzyme which is responsible for the levan production during growth on raffinose, biochemical and biophysical tests were performed.

Table 2: Purification of the *Lactobacillus reuteri* LB 121 levansucrase (FTFB) enzyme.

Step	Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Purification (fold)	Yield (%)
Supernatant	128	64	0.5	1	100
Ammonium sulfate precipitation (65%)	35.2	42	1.2	2.4	65.6
Hydroxyl apatite	1.5	30.6	20.4	40.8	47.8
Phenyl superose	0.27	23	85	170	36
Gel Filtration	0.055	10	182	360	16
MonoQ	0.0255	4	176	352	6

15

Amino acid sequencing of FTFB

A 5% SDS-PAA gel was allowed to “age” overnight in order to reduce the amount of reacting chemical groups in the gel. Reaction of chemicals in the PAA gel (TEMED and ammonium persulphate) with proteins can cause some undesired effects, such as N-terminal blocking of the protein, making it more difficult to determine the protein amino acid composition. 0.1 mM thioglycolic acid (scavenger to reduce the amount of reactive groups in the PAA gel material) was added to the running buffer during electrophoresis.

In order to determine the amino acid sequence of internal peptides of protein bands running in a SDS-PAA gel, protein containing bands were cut out of the PAA gel. After fractionating the protein by digestion with chymotrypsin the N-terminal amino acid sequences of the digested proteins were determined (below).

N-terminal sequencing was performed by Western blotting of the proteins from the PAA gel to an Immobilon PVDF membrane (Millipore/ Waters Inc.) at 0.8 mA/cm² for 1 h. After staining the PVDF membrane with Coomassie Brilliant Blue without adding acetic acid (to reduce N-terminal blocking) and destaining with 50% methanol, the corresponding bands were cut out of the PVDF membrane for N-terminal amino acid sequence determination.

Amino acid sequence determination was performed by automated Edman degradation as described by Koningsberg and Steinman (1977) The proteins (third edition) volume 3, 1-178 (Neurath and Hill, eds.). The automated equipment for Edman degradation was an Applied Biosystems model 477A pulse-liquid sequenator described by Hewick *et al.* (1981), J. Biol. Chem. 15, 7990-7997 connected to a RP-HPLC unit (model 120A, Applied Biosystems) for amino acid identification.

The N-terminal sequence of the purified FTFB was determined and found to be:
(A) Q V E S N N Y N G V A E V N T E R Q A N G Q I (G) (V) (D) (SEQ ID No. 6).

Three internal peptide sequences of the purified FTFB were determined: (M) (A) H L D
V W D S W P V Q D P (V) (SEQ ID No. 7); N A G S I F G T (K) (SEQ ID No. 8); and
5 V (E) (E) V Y S P K V S T L M A S D E V E (SEQ ID No. 9).

The following primers were designed on the basis of the N-terminal and internal
peptide fragments of FTFB. Listed from left to right are: primer name, source peptide
fragment and sequence (from 5' to 3'). FTFB1 + FTFB3i yields approximately a 1400 bp
product in a PCR reaction. FTFB1 forward (N-terminal): AA T/C-TAT-AA T/C-GG
10 T/C-GTT-GC G/A-T/C GA-AGT (SEQ ID No. 25); and FTFB3i reverse (Internal 3):
TAC-CGN-A/T C/G N-CTA-CTT-CAA-CTT (SEQ ID No. 26). The FTFB gene was
partly isolated by PCR with primers FTFB1 and FTFB3i. PCR with these primers yielded
a 1385 bp amplicon, which after sequencing showed high homology to *ftfA* and *SacB*
from *Streptococcus mutans*.

15

Example 3: Oxidation of levans

For TEMPO-mediated oxidation, a levan according to the invention prepared as
described above (dry weight 1 g, 6.15 mmol) was resuspended in 100 ml water. Next,
2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO; 1% by weight compared to the
20 polysaccharide (0.01 g, 0.065 mmol)) was added and resuspended in 20 min. Sodium
bromide (0.75 g, 7.3 mmol) was added and the suspension was cooled down to 0°C. This
reaction also proceeded without bromide. A solution of hypochlorite (6 ml, 15% solution,
12.6 mmol) was adjusted to pH 10.0 with 3M HCl and cooled to 0°C. This solution was
added to the suspension of the polysaccharide and TEMPO. The course of the reaction
25 was followed by monitoring the consumption of sodium hydroxide solution, which is
equivalent to the formation of uronic acid. After 30 min, 60 ml 0.1M NaOH was
consumed. This amount corresponds to the formation of 97% uronic acid. Thereafter, the
solution was poured out in 96% ethanol (comprising 70% of the volume of the solution)
causing the product to precipitate. The white precipitate was centrifuged, resuspended in
30 ethanol/water (70/30 v/v) and centrifuged again. Next, the precipitate was resuspended in
96% ethanol and centrifuged. The obtained product was dried at reduced pressure. The
uronic acid content was determined by means of the uronic acid assay according to
Blumenkrantz and Abdoe-Hansen (Anal. Biochem., 54 (1973), 484). A calibration curve
was generated using polygalacturonic acid (5, 10, 15 and 20 µg). With this calibration

curve the uronic acid content in a sample of 20 µg of the product was determined. The obtained result was a content of 95% uronic acid with a yield of 96%.

Partial oxidation

5 For partial oxidation, a levan according to the invention (dry weight 2 g, 12.3 mmol) was resuspended in 25 ml water. Next, TEMPO (1% by weight compared to the polysaccharide (0.02 g, 0.13 mmol)) was added, resuspended in 20 min and cooled to 0°C. A solution of hypochlorite (1 ml, 15% solution, 2.1 mmol) was adjusted to pH 9.0 with 3M HCl and cooled down to 0°C. This solution was added to the suspension of the
10 polysaccharide and TEMPO. Within 5 min the mixture became a solid gel.

Example 4: Adhesion of *Lactobacillus reuteri* strains to Caco-2 cell lines

The adhesion of *Lactobacillus reuteri* strains to Caco-2 cell lines was determined as described below. Firstly, a bacterial suspension was prepared as follows. *Lactobacillus reuteri* strains LB 121, 35-5 and *L. rhamnosus* LGG (a well known probiotic strain with
15 good adhering properties) were cultured in MRS broth supplemented with 5 µl/ml of methyl-1,2-[³H]-thymidine at 37°C for 18-20 h before the adhesion assays. The cultures were harvested by centrifugation, washed with phosphate buffered saline (PBS) and resuspended in PBS or PBS supplemented with 30 g/l sucrose (see Table 3) to a final density of about 2 x 10⁹ cfu/ml. Prior to the adhesion assay, the cell suspensions in PBS
20 with 30 g/l sucrose were incubated for 1 hour at 37°C, whereas the cell suspensions in PBS were kept on ice for 1 hour. After incubation at 37°C, the suspensions in PBS with sucrose were centrifuged and the cells were washed with and resuspended in PBS to a final density of about 2 x 10⁹ cfu/ml.

Caco-2 cells were cultured as follows. Subcultures of Caco-2 cells (ATCC, code
25 HTB 37, human colon adenocarcinoma), stored as frozen stock cultures in liquid nitrogen were used for the adhesion tests. The Caco-2 cells were grown in culture medium consisting of Dulbecco's modified Eagle medium (DMEM), supplemented with heat-inactivated foetal calf serum (10% v/v), non-essential amino acids (1% v/v), L-glutamine (2mM) and gentamicin (50 µg/ml). About 2,000,000 cells were seeded in 75cm² tissue
30 culture flasks containing culture medium and cultured in a humidified incubator at 37°C in air containing 5% CO₂. Near confluent Caco-2 cell cultures were harvested by trypsinisation and resuspended in culture medium. The number of cells was established using a Bürker-Türk counting chamber.

Table 3: Incubation of the different *Lactobacillus* strains prior to the adhesion assays.

<i>Lactobacillus</i> strain	Extra incubation	Polysaccharide produced	Group
<i>reuteri</i> 121	PBS sucrose, 37°C for 1 hr	glucan and fructan	As
<i>reuteri</i> 35-5	PBS sucrose, 37°C for 1 hr	glucan	Bs
<i>reuteri</i> K24	PBS sucrose, 37°C for 1 hr	none	Cs
<i>reuteri</i> 121	PBS on ice	none	D
<i>reuteri</i> DSM20016*	PBS on ice	none	E
<i>rhamnosus</i> GG	PBS on ice	none	F

* Type strain of *L. reuteri*

For the following experiments a Caco-2 monolayer transport system was used. Caco-2 cells cultured in a two-compartment transport system are commonly used to study the intestinal, epithelial permeability. In this system the Caco-2 cell differentiates into polarized columnar cells after reaching confluency. The Caco-2 system has been shown to simulate the passive and active transcellular transport of electrolytes, sugars, amino acids and lipophilic compounds (Hillgren *et al.* 1995, Dulfer *et al.*, 1996, Duizer *et al.*, 1997). Also, a clear correlation between the *in vivo* absorption and the permeability across the monolayers of Caco-2 cells has been reported (Artursson and Karlsson, 1990). For the present transport studies, Caco-2 cells were seeded on semi-permeable filter inserts (12 wells Transwell plates, Costar) at ca. 100,000 cells per filter (growth area \pm 1 cm² containing 2.5 ml culture medium). The cells on the insert were cultured for 17 to 24 days at 37°C in a humidified incubator containing 5% CO₂ in air. During this culture period the cells have been subjected to an enterocyte-like differentiation. Gentamycin was eliminated from the culture medium two days prior to the adhesion assays.

The adhesion assay was performed as follows. PBS was used as exposure medium. 25 μ l of a bacterial suspension (2×10^9 cfu/ml) were added to 0.5 ml medium. The apical side of the Caco-2 monolayers was incubated with the bacterial suspensions for 1 hour at 37°C. After incubation, remaining fluid was removed and the cells were washed three times with 1 ml PBS. Subsequently, the Caco-2 monolayers were digested

overnight with 1 ml 0.1M NaOH, 1% SDS. The lysate was mixed with 10 ml Hionic Fluor scintillation liquid and the radioactivity was measured by liquid scintillation counting using a LKB/Wallac scintillation counter. As a control, the radioactivity of the bacterial suspensions was measured. For each test group, the percentage of bacteria attached to the monolayers was calculated. All adhesion tests were performed in quadruple. In Table 4 the results of the bacterial adhesion test to Caco-2 cellines are given. From the results can be concluded that the glucans and the fructans contribute to the adherence of *Lactobacillus reuteri* to Caco-2 cellines. This could indicate that *Lactobacillus reuteri* strains producing EPS possess improved probiotic characteristics or that *Lactobacillus reuteri* and its polysaccharides could function as an excellent symbiotic.

Table 4: The results of the bacterial adhesion test to Caco-2 cellines.

Group (see Table 1)	% of bacteria bound to the monolayer
As	6.5
Bs	5.7
Cs	1.8
D	2.3
E	0.9
F	1.3

15 **Description of the figures**

Figure 1: SEQ ID No. 1; The deduced amino acid sequence of the novel inulosucrase of *Lactobacillus reuteri* (amino acid 1-789). Furthermore, the designations and orientation (< for 3' to 5' and > for 5' to 3') of the primers and the restriction enzymes used for (inverse) PCR, are shown at the right hand side. Putative start codons (ATG, at positions 41 and 68) and stop codon (TAA, at position 2435) are shown in bold. The positions of the primers used for PCR are shown in bold/underlined. The *NheI* restriction sites (at positions 1154 and 2592) used for inverse PCR are underlined. The primers used and their exact positions in the inulosucrase sequence are shown in table 1. Starting at amino acid 690, the 20 PXX repeats are underlined. At amino acid 755 the LPXTG motif is underlined.

Figure 2: Dendrogram of bacterial and plant fructosyltransferases. The horizontal distances are a measure for the difference at the amino acid sequence level. 10% difference is indicated by the upper bar. Bootstrap values (in percentages) are given at the root of each tree. Fructosyltransferases of Gram positive bacteria are indicated in the lower half of the figure (*B. staerothermophilus* SurB; *B. amyloliquefaciens* SacB; *B. subtilis* SacB; *S. mutans* SacB; *L. reuteri* FtfA (inulosucrase); *S. salivarius* Ftf). Plant fructosyltransferases are indicated in the middle part of the figure (*Cynara scolymus* Ss-1ft; *Allium cepa* F-6gft; *Hordeum vulgare* Sf-6ft). Fructosyltransferases of Gram negative bacteria are shown in the upper part of the figure (*Z. mobilis* LevU; *Z. mobilis* SucE2; *Z. mobilis* SacB; *E. amylovora* Lcs; *A. diazotrophicus* LsdA).

Figure 3: The N-terminal and three internal amino acid sequences of the novel levansucrase of *Lactobacillus reuteri*.

Figure 4: Parts of an alignment of the deduced amino acid sequences of some bacterial fructosyltransferase genes. Sequences in bold indicate the consensus sequences used to construct the degenerated primers 5ftf, 6ftfi and 12 ftfi. (*) indicates a position with a fully conserved amino acid residue. (:) indicates a position with a fully conserved 'strong' group: STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, FYW. (.) indicates a position with a fully conserved 'weaker' group: CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, HFY. Groups are according to the Pam250 residue weight matrix described by Altschul *et al.* (1990) J. Mol. Biol. 215, 403-410.

Figure 5: The strategy used for the isolation of the inulosucrase gene from *Lactobacillus reuteri* 121 chromosomal DNA.

Claims

1. A protein having fructosyltransferase activity, exhibiting at least 65% amino acid identity, as determined by the BLAST algorithm, with the amino acid sequence of SEQ ID No. 1 or 11, or a part thereof having at least 15 contiguous amino acids which are identical to the corresponding part of the amino acid sequence of SEQ ID No. 1 or 11.
2. A protein according to claim 1, exhibiting at least 75%, preferably at least 85%, amino acid identity with the amino acid sequence of SEQ ID No. 1 or 11.
3. A protein according to any one of the preceding claims which, in the presence of sucrose, produces an inulin having $\beta(2-1)$ linked D-fructosyl units and/or a levan having $\beta(2-6)$ linked D-fructosyl units and/or fructo-oligosaccharides.
4. A protein according to any one of the preceding claims which is a recombinant protein.
5. A nucleotide sequence encoding a protein according to any one of the preceding claims.
6. A nucleic acid construct comprising the nucleic acid sequence of claim 5, operationally linked to an expression-regulating nucleic acid sequence.
7. A recombinant host cell containing one or more copies of the nucleic acid construct according to claim 6.
8. A process of producing a fructosyltransferase, comprising culturing a host cell according to claim 7 in a culture medium, and recovering the protein from the culture medium or the cell free extract.
9. A process of producing an oligosaccharide or polysaccharide of interest, using a protein according to any one of claims 1-4, or a host cell according to claim 7.
10. A chemically modified fructan, which is obtained by 3,4-oxidation, 1- or 6-oxidation, phosphorylation, acylation, alkylation, hydroxyalkylation, carboxymethylation, aminoalkylation of one or more anhydrofructose units of a fructan containing a degree of polymerization of at least 100, preferably at least 1000 units.

11. Use of a *Lactobacillus* strain containing a protein according to any one of claims 1-4 and capable of producing an inulin, a levan or fructo-oligosaccharides and optionally a glucan as a probiotic or symbiotic.

Fig 1 (1)

1 tacaatgggg tggcggaggt gaagaaacgg ggttacttct atgctagaac gcaaggaaca 19ftf>
 y n g v a e v k k r g y f y a r t
y n g v a e v n t e r q a n g q i

61 taaaaaatg tataaaagcg gtaaaaattg ggcagtcggt acactctcga ctgctgcgct
 1 m y k s g k n w a v v t l s t a a

121 ggtatttggg gcaacaactg taaatgcatc cgcgacaca aatattgaaa acaatgattc
 18 l v f g a t t v n a s a d t n i e n n d

181 ttctactgta caagttacaa caggtgataa tgatattgct gttaaagtg tgacacttgg
 38 s s t v q v t t g d n d i a v k s v t l

241 tagtggtcaa gttagtgcag ctagtgatac gactattaga acttctgcta atgcaaatag
 58 g s g q v s a a s d t t i r t s a n a n

301 tgcttcttct gccgctaata cacaaaattc taacagtcaa gtagcaagtt ctgctgcaat
 78 s a s s a a n t q n s n s q v a s s a a

361 aacatcatct acaagttccg cagcttcatt aaataacaca gatagtaaag cggctcaaga
 98 i t s s t s s a a s l n n t d s k a a q

421 aaataactaat acagccaaaa atgatgacac gcaaaaagct gcaccagcta acgaatcttc
 118 e n t n t a k n d d t q k a a p a n e s

481 tgaagctaaa aatgaaccag ctgtaaactg taatgattct tcagctgcaa aaaatgatga
 138 s e a k n e p a v n v n d s s a a k n d

541 tcaacaatcc agtaaaaaga atactaccgc taagttaaac aaggatgctg aaaacgttgt
 158 d q q s s k k n t t a k l n k d a e n v

601 aaaaaaggcg ggaattgatc ctaacagttt aactgatgac cagattaaag cattaataa
 178 v k k a g i d p n s l t d d q i k a l n

2/8

Fig 1 (2)

661 gatgaacttc tcgaaagctg caaagtctgg tacacaaatg acttataatg atttccaaaa
 198 k m n f s k a a k s g t q m t y n d f q

721 gattgctgat acgttaatca aacaagatgg tcggtacaca gttccattct ttaaagcaag 20ftfi <
 218 k i a d t l i k q d g r y t v p f f k a

781 tgaatcaaaa aatatgcctg ccgctacaac taaagatgca caaactaata ctattgaacc
 238 s e i k n m p a a t t k d a q t n t i e

841 tttagatgta tgggattcat ggccagttca agatgttcgg acaggacaag ttgctaattg 5ftf >
 258 p l d v w d s w p v q d v r t g q v a n 8ftfi <

901 gaatggctat caacttgctca tcgcaatgat ggggaattcca aaccaaaaatg ataatcatat
 278 w n g y q l v i a m m g i p n q n d n h

961 ctatctctta tataataagt atggtgataa tgaattaagt cattggaaga atgtaggtcc 7ftf >
 298 i y l l y n k y g d n e l s h w k n v g

1021 aatttttggc tataattcta ccgcggtttc acaagaatgg tcaggatcag ctgttttgaa 7ftf >
 318 p i f g y n s t a v s q e w s g s a v l 6ftfi <

1081 cagtgataac tctatccaat tattttatatac aagggtagac acgtctgata acaataccaa
 338 n s d n s i q l f y t r v d t s d n n t

1141 tcatcaaaaa attgctagcg ctactcttta tttaactgat aataatggaa atgtatcact NheI
 358 n h q k i a s a t l y l t d n n g n v s AC1(i)<>

1201 cgctcaggta cgaaatgact atattgtatt tgaaggtgat ggctattact accaaactta AC2(i)<>
 378 l a q v r n d y i v f e g d g y y y q t

1261 tgatcaatgg aaagctacta acaaaggtgc cgataatatt gcaatgcgtg atgctcatgt
 398 y d q w k a t n k g a d n i a m r d a h ,

3/8

Fig 1 (3)

1321 aattgaagat ggtaatggtg atcggtagct tgtttttgaa gcaagtactg gtttgaaaa
 418 v i e d g n g d r y l v f e a s t g l e

1381 ttatcaaggc gaggacaaa tttataactg gttaaattat ggcggagatg acgcatttaa
 438 n y q g e d q i y n w l n y g g d d a f

1441 tatcaagagc ttatttagaa ttctttccaa tgatgatatt aagagtcggg caacttgggc
 458 n i k s l f r i l s n d d i k s r a t w

1501 taatgcagct atcggtagct tcaaactaaa taaggacgaa aagaatccta aggtggcaga
 478 a n a a i g i l k l n k d e k n p k v a

1561 gttataactca ccattaattt ctgcaccaat ggtaagcgat gaaattgagc gaccaaatgt
 498 e l y s p l i s a p m v s d e i e r p n

1621 agttaaatta ggtaataaat attacttatt tgccgctacc cgtttaaact gaggaagtaa
 518 v v k l g n k y y l f a a t r l n r g s

1681 tgatgatgct tggatgaatg ctaattatgc cgttggtgat aatggtgcaa tggtcggata
 538 n d d a w m n a n y a v g d n v a m v g

1741 tgttgctgat agtctaactg gatcttataa gccattaaat gattctggag tagtcttgac
 558 y v a d s l t g s y k p l n d s g v v l

1801 tgcttctggt cctgcaaact ggcggacagc aacttattca tattatgctg tccccgttgc
 578 t a s v p a n w r t a t y s y y a v p v

1861 cggaaaagat gaccaagtat tagttacttc atatatgact aatagaaatg gagtagcggg
 598 a g k d d q v l v t s y m t n r n g v a

1921 taaaggaatg gattcaactt gggcaccgag tttcttacta caaattaacc cggataacac 12ftfi <
 618 g k g m d s t w a p s f l l q i n p d n

Fig 1 (4)

1981 aactactggt ttagctaaaa tgactaatca aggggattgg atttgggatg attcaagcga
 638 t t t v l a k m t n q g d w i w d d s s

2041 aaatcttgat atgattggtg atttagactc cgctgcttta cctggcgaac gtgataaacc
 658 e n l d m i g d l d s a a l p g e r d k

2101 tgttgattgg gacttaattg gttatggatt aaaaccgcat gatcctgcta caccaaatga
 678 p v d w d l i g y g l k p h d p a t p n

2161 tcctgaaacg ccaactacac cagaaacccc tgagacacct aatactccca aaacacccaaa
 698 d p e t p t t p e t p e t p n t p k t p

2221 gactcctgaa aatcctggga cacctcaaac tcctaataca cctaatactc cggaaattcc
 718 k t p e n p g t p q t p n t p n t p e i

2281 tttactcca gaaacgccta agcaacctga aaccxaaact aataatcggt tgccacaaac
 738 p l t p e t p k q p e t q t n n r l p q

2341 tggaataat gccataaag ccatgattgg cctaggtatg ggaacattgc ttagtatggt
 758 t g n n a n k a m i g l g m g t l l s m

2401 tggcttgca gaaattaaca aacgtcgatt taactaaata ctttaaata aaaccgctaa
 778 f g l a e i n k r r f n -

2461 gccttaaatt cagcttaacg gttttttatt ttaaagttt ttattgtaaa aaagcgaatt

2521 atcattaata ctaatgcaat tgttgtaaga cottacgaca gtagtaacaa tgaatttgcc

2581 catctttgtc gg

NheI

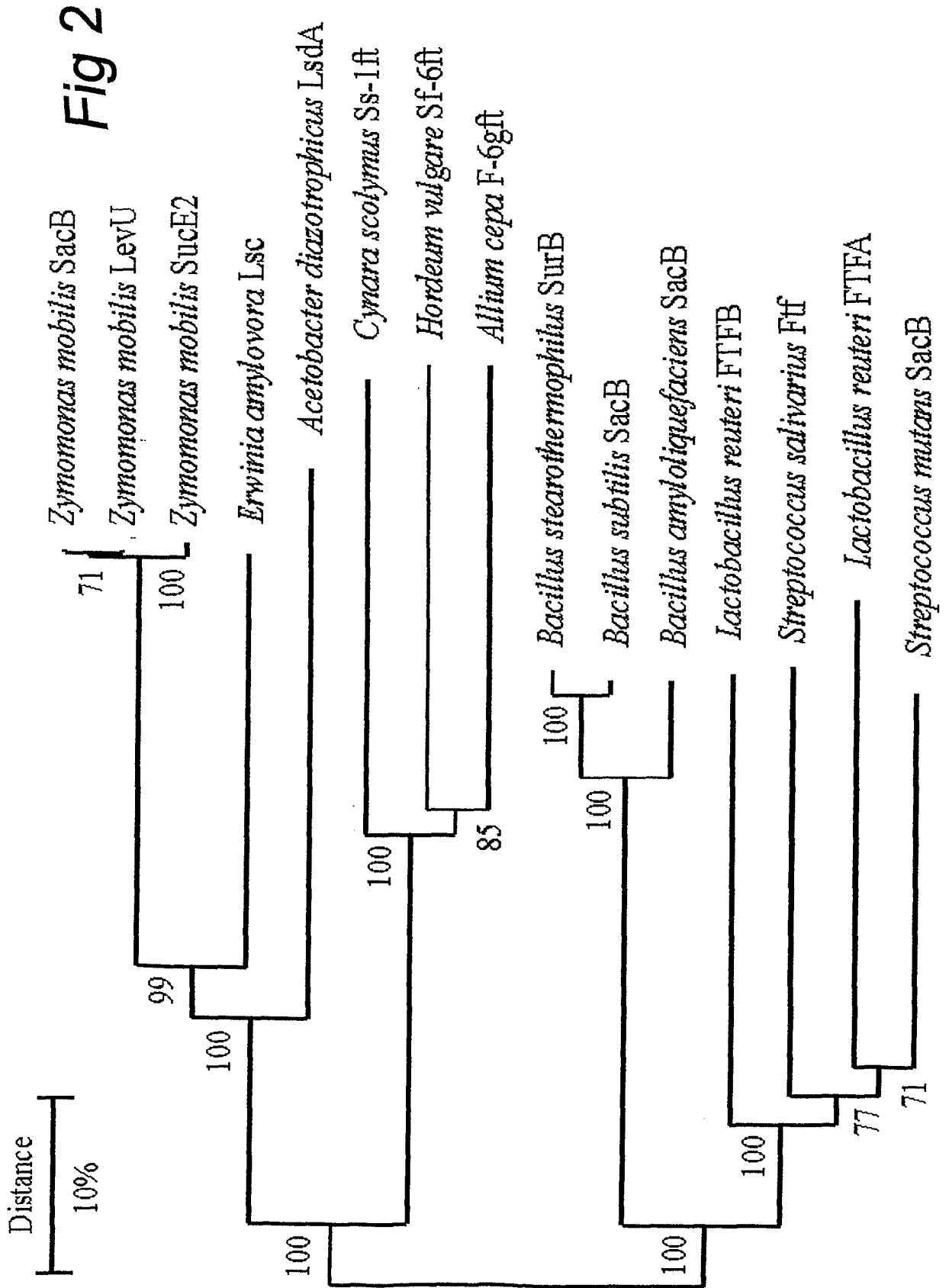


Fig 3

The N-terminal sequence of FTFB (levansucrase):

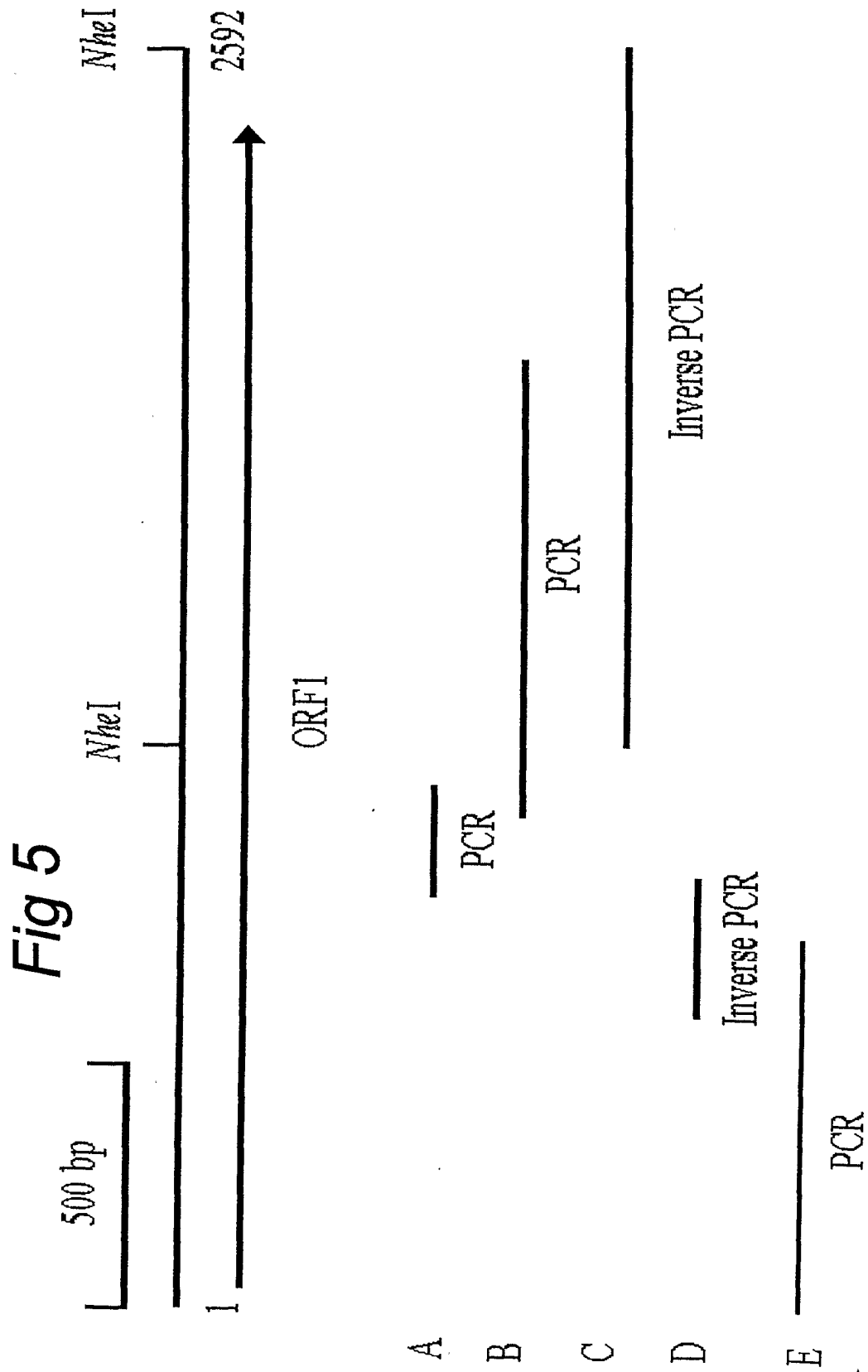
(A) Q V E S N N Y N G V A E V N T E R Q A N G Q I (G) (V) (D).

Internal peptide sequences of FTFB (levansucrase):

- (M) (A) H L D V W D S W P V Q D P (V),
- N A G S I F G T (K),
- V (E) (E) V Y S P K V S T L M A S D E V E.

Fig 4

5ftf				
<i>B. amyloliquefaciens</i> SacB	80	GLDVVWDSWPLQNAD	93	
<i>B. subtilis</i> SacB	82	GLDVVWDSWPLQNAD	95	
<i>S. mutans</i> SacB	243	DLDVVWDSWPVQDAK	256	
<i>S. salivarius</i> Ftf	282	EIDVVWDSWPVQDAK	295	
		:*****:*:*		
6ftfi				
<i>B. amyloliquefaciens</i> SacB	156	QTQEWSGSATFTSDGK	171	
<i>B. subtilis</i> SacB	158	QTQEWSGSATFTSDGK	173	
<i>S. mutans</i> SacB	312	LTQEWSGSATVNEEDGS	327	
<i>S. salivarius</i> Ftf	351	DDQQWSGSATVNSDGS	366	
		*:*****:***.		
12ftfi				
<i>B. amyloliquefaciens</i> SacB	440	KATFGPSEFLMN	450	
<i>B. subtilis</i> SacB	440	QSTFAPSEFLLN	450	
<i>S. mutans</i> SacB	609	NSTWAPSEFLIQ	619	
<i>S. salivarius</i> Ftf	655	KSTWAPSEFLIK	665	
		:*:.*****:*		



SEQUENCE LISTING

<110> TNO

<120> Novel fructosyltransferases

<130> Novel fructosyltransferases

<140>

<141>

<150> 00201872.9

<151> 2000-05-25

<150> 01200049.3

<151> 2001-01-09

<160> 26

<170> PatentIn Ver. 2.1

<210> 1

<211> 789

<212> PRT

<213> Lactobacillus reuteri

<220>

<221> ACT_SITE

<222> (263)

<223> Putative catalytic amino acid residue

<220>

<221> ACT_SITE

<222> (330)

<223> Putative catalytic amino acid residue

<220>

<221> ACT_SITE

<222> (415)

<223> Putative catalytic amino acid residue

<220>

<221> ACT_SITE

<222> (431)

<223> Putative catalytic amino acid residue

<220>

<221> ACT_SITE

<222> (511)
 <223> Putative catalytic amino acid residue

<220>
 <221> ACT_SITE
 <222> (514)
 <223> Putative catalytic amino acid residue

<220>
 <221> ACT_SITE
 <222> (532)
 <223> Putative catalytic amino acid residue

<220>
 <221> ACT_SITE
 <222> (551)
 <223> Putative catalytic amino acid residue

<220>
 <221> SIGNAL
 <222> (1)..(21)
 <223> Putative signal sequence

<220>
 <221> DOMAIN
 <222> (755)..(759)
 <223> Putative cell wall anchor amino acid signal

<220>
 <221> REPEAT
 <222> (690)..(749)
 <223> PXX repeat (20 -fold)

<400> 1
 Met Tyr Lys Ser Gly Lys Asn Trp Ala Val Val Thr Leu Ser Thr Ala
 1 5 10 15
 Ala Leu Val Phe Gly Ala Thr Thr Val Asn Ala Ser Ala Asp Thr Asn
 20 25 30
 Ile Glu Asn Asn Asp Ser Ser Thr Val Gln Val Thr Thr Gly Asp Asn
 35 40 45
 Asp Ile Ala Val Lys Ser Val Thr Leu Gly Ser Gly Gln Val Ser Ala
 50 55 60
 Ala Ser Asp Thr Thr Ile Arg Thr Ser Ala Asn Ala Asn Ser Ala Ser
 65 70 75 80

Ser Ala Ala Asn Thr Gln Asn Ser Asn Ser Gln Val Ala Ser Ser Ala
 85 90 95

Ala Ile Thr Ser Ser Thr Ser Ser Ala Ala Ser Leu Asn Asn Thr Asp
 100 105 110

Ser Lys Ala Ala Gln Glu Asn Thr Asn Thr Ala Lys Asn Asp Asp Thr
 115 120 125

Gln Lys Ala Ala Pro Ala Asn Glu Ser Ser Glu Ala Lys Asn Glu Pro
 130 135 140

Ala Val Asn Val Asn Asp Ser Ser Ala Ala Lys Asn Asp Asp Gln Gln
 145 150 155 160

Ser Ser Lys Lys Asn Thr Thr Ala Lys Leu Asn Lys Asp Ala Glu Asn
 165 170 175

Val Val Lys Lys Ala Gly Ile Asp Pro Asn Ser Leu Thr Asp Asp Gln
 180 185 190

Ile Lys Ala Leu Asn Lys Met Asn Phe Ser Lys Ala Ala Lys Ser Gly
 195 200 205

Thr Gln Met Thr Tyr Asn Asp Phe Gln Lys Ile Ala Asp Thr Leu Ile
 210 215 220

Lys Gln Asp Gly Arg Tyr Thr Val Pro Phe Phe Lys Ala Ser Glu Ile
 225 230 235 240

Lys Asn Met Pro Ala Ala Thr Thr Lys Asp Ala Gln Thr Asn Thr Ile
 245 250 255

Glu Pro Leu Asp Val Trp Asp Ser Trp Pro Val Gln Asp Val Arg Thr
 260 265 270

Gly Gln Val Ala Asn Trp Asn Gly Tyr Gln Leu Val Ile Ala Met Met
 275 280 285

Gly Ile Pro Asn Gln Asn Asp Asn His Ile Tyr Leu Leu Tyr Asn Lys
 290 295 300

Tyr Gly Asp Asn Glu Leu Ser His Trp Lys Asn Val Gly Pro Ile Phe
 305 310 315 320

Gly Tyr Asn Ser Thr Ala Val Ser Gln Glu Trp Ser Gly Ser Ala Val
 325 330 335

Leu Asn Ser Asp Asn Ser Ile Gln Leu Phe Tyr Thr Arg Val Asp Thr
 340 345 350

Ser Asp Asn Asn Thr Asn His Gln Lys Ile Ala Ser Ala Thr Leu Tyr
 355 360 365

Leu Thr Asp Asn Asn Gly Asn Val Ser Leu Ala Gln Val Arg Asn Asp
 370 375 380

Tyr Ile Val Phe Glu Gly Asp Gly Tyr Tyr Tyr Gln Thr Tyr Asp Gln
 385 390 395 400

Trp Lys Ala Thr Asn Lys Gly Ala Asp Asn Ile Ala Met Arg Asp Ala
 405 410 415

His Val Ile Glu Asp Gly Asn Gly Asp Arg Tyr Leu Val Phe Glu Ala
 420 425 430

Ser Thr Gly Leu Glu Asn Tyr Gln Gly Glu Asp Gln Ile Tyr Asn Trp
 435 440 445

Leu Asn Tyr Gly Gly Asp Asp Ala Phe Asn Ile Lys Ser Leu Phe Arg
 450 455 460

Ile Leu Ser Asn Asp Asp Ile Lys Ser Arg Ala Thr Trp Ala Asn Ala
 465 470 475 480

Ala Ile Gly Ile Leu Lys Leu Asn Lys Asp Glu Lys Asn Pro Lys Val
 485 490 495

Ala Glu Leu Tyr Ser Pro Leu Ile Ser Ala Pro Met Val Ser Asp Glu
 500 505 510

Ile Glu Arg Pro Asn Val Val Lys Leu Gly Asn Lys Tyr Tyr Leu Phe
 515 520 525

Ala Ala Thr Arg Leu Asn Arg Gly Ser Asn Asp Asp Ala Trp Met Asn
 530 535 540

Ala Asn Tyr Ala Val Gly Asp Asn Val Ala Met Val Gly Tyr Val Ala
 545 550 555 560

Asp Ser Leu Thr Gly Ser Tyr Lys Pro Leu Asn Asp Ser Gly Val Val
 565 570 575

Leu Thr Ala Ser Val Pro Ala Asn Trp Arg Thr Ala Thr Tyr Ser Tyr
 580 585 590

Tyr Ala Val Pro Val Ala Gly Lys Asp Asp Gln Val Leu Val Thr Ser
 595 600 605

Tyr Met Thr Asn Arg Asn Gly Val Ala Gly Lys Gly Met Asp Ser Thr
 610 615 620

Trp Ala Pro Ser Phe Leu Leu Gln Ile Asn Pro Asp Asn Thr Thr Thr
 625 630 635 640

Val Leu Ala Lys Met Thr Asn Gln Gly Asp Trp Ile Trp Asp Asp Ser
 645 650 655

Ser Glu Asn Leu Asp Met Ile Gly Asp Leu Asp Ser Ala Ala Leu Pro
 660 665 670

Gly Glu Arg Asp Lys Pro Val Asp Trp Asp Leu Ile Gly Tyr Gly Leu
 675 680 685

Lys Pro His Asp Pro Ala Thr Pro Asn Asp Pro Glu Thr Pro Thr Thr
 690 695 700

Pro Glu Thr Pro Glu Thr Pro Asn Thr Pro Lys Thr Pro Lys Thr Pro
 705 710 715 720

Glu Asn Pro Gly Thr Pro Gln Thr Pro Asn Thr Pro Asn Thr Pro Glu
 725 730 735

Ile Pro Leu Thr Pro Glu Thr Pro Lys Gln Pro Glu Thr Gln Thr Asn
 740 745 750

Asn Arg Leu Pro Gln Thr Gly Asn Asn Ala Asn Lys Ala Met Ile Gly
 755 760 765

Leu Gly Met Gly Thr Leu Leu Ser Met Phe Gly Leu Ala Glu Ile Asn
 770 775 780

Lys Arg Arg Phe Asn
 785

<210> 2

<211> 2367

<212> DNA

<213> Lactobacillus reuteri

<400> 2

```

atgtataaaa gcggtataaaa ttgggcagtc gttacactct cgactgctgc gctggtatth 60
ggtgcaacaa ctgtaaattgc atccgctggac acaaatattg aaaacaatga ttctttctact 120
gtacaagtta caacaggtga taatgatatt gctgttaaaa gtgtgacact tggtagtggt 180
caagttagtg cagctagtga tacgactatt agaacttctg ctaatgcaaa tagtgcttct 240
tctgccgcta atacacaaaa ttctaacagt caagtagcaa gttctgctgc aataacatca 300
tctacaagtt ccgcagcttc attaaataac acagatagta aagcggctca agaaaatact 360
aatacagcca aaaatgatga cacgcaaaaa gctgcaccag ctaacgaatc ttctgaagct 420
aaaaatgaac cagctgtaaa cgttaatgat tcttcagctg caaaaaatga tgatcaacaa 480
tccagtaaaa agaatactac cgctaagtta aacaaggatg ctgaaaacgt tgtaaaaaag 540
gcggaattg atcctaacag ttaactgat gaccagatta aagcattaaa taagatgaac 600
ttctcgaaag ctgcaaagtc tggtagacaa atgacttata atgatttcca aaagattgct 660
gatacgttaa tcaaacaaga tggtagctac acagttccat tctttaaagc aagtgaatc 720
aaaaatatgc ctgccgctac aactaaagat gcacaaacta atactattga acctttagat 780
gtatgggatt catggccagt tcaagatgtt cggacaggac aagttgctaa ttggaatggc 840
tatcaacttg tcatcgcaat gatgggaatt ccaaaccaaa atgataatca tatctatctc 900
ttatataata agtatggtga taatgaatta agtcattgga agaatttagg tccaattttt 960
ggctataatt ctaccgctgt ttcaagaaga tggtagctac cagctgtttt gaacagtgat 1020
aactctatcc aattatttta tacaagggtg gacacgtctg ataacaatac caatcatcaa 1080
aaaattgcta gcgctactct ttatttaact gataataatg gaaatgtatc actcgcctcag 1140
gtacgaaatg actatattgt atttgaaggat gatggctatt actaccaaac ttatgatcaa 1200
tggaagctc ctaacaaagg tgccgataat attgcaatgc gtgatgctca tgtaattgaa 1260
gatggtaatg gtgatcggta ccttgttttt gaagcaagta ctggttttga aaattatcaa 1320
ggcgaggacc aaatttataa ctggttaaat tatggcggag atgacgcatt taatatcaag 1380
agcttattta gaattctttc caatgatgat attaagagtc gggcaacttg ggctaattgca 1440
gctatcggta tcctcaaaact aaataaggac gaaaagaatc ctaagggtggc agagttatac 1500
tcaccattaa tttctgcacc aatggtaagc gatgaaattg agcgaccaa tgtagttaa 1560
ttaggtaata aatattactt atttgcctc acccgtttta atcgaggaag taatgatgat 1620
gcttggatga atgctaatta tgccgttggg gataatgttg caatggtcgg atatggtgct 1680
gatagtctaa ctggatctta taagcatta aatgattctg gagtagtctt gactgcttct 1740
gttctcgtca actggcggac agcaacttat tcatattatg ctgtccccgt tgccggaaaa 1800
gatgaccaag tattagttac ttcatatatg actaatagaa atggagtagc gggtaaagga 1860
atggattcaa cttgggcacc gagtttctta ctacaaatta acccggataa cacaactact 1920
gttttagcta aaatgactaa tcaaggggat tggatttggg atgattcaag cgaaaatctt 1980
gatatgattg gtgatttaga ctccgctgct ttacctggcg aacgtgataa acctgttgat 2040
tgggacttaa ttggttatgg attaaaaccg catgatcctg ctacaccaa tgatcctgaa 2100
acgccaacta caccagaaac ccctgagaca cctaatactc ccaaacacc aaagactcct 2160
gaaaatcctg ggacacctca aactcctaata acacctaata ctccggaaat tcctttaact 2220
ccagaaacgc ctaagcaacc tgaaaccaa actaataatc gtttgccaca aactggaaat 2280
aatgccaata aagccatgat tggcctaggt atgggaacat tgcttagtat gtttggctt 2340
gcagaaatta acaaacgtcg atttaac 2367

```

<210> 3

<211> 2394

<212> DNA

<213> *Lactobacillus reuteri*

<400> 3

```

atgctagaac gcaaggaaca taaaaaatg tataaaagcg gtaaaaattg ggcagtcggt 60
acactctoga ctgctgcgct ggtatttggt gcaacaactg taaatgcatc cgcggacaca 120
aatattgaaa acaatgattc ttctactgta caagttacaa caggtgataa tgatattgct 180
gttaaaagtg tgacacttgg tagtggtcaa gttagtgcaag ctagtgatac gactattaga 240
acttctgcta atgcaaatag tgcttcttct gccgctaata cacaaaattc taacagtcaa 300
gtagcaagtt ctgctgcaat aacatcatct acaagttccg cagcttcatt aaataacaca 360
gatagtaaag cggctcaaga aaataactaat acagccaaaa atgatgacac gcaaaaagct 420
gcaccagcta acgaatcttc tgaagctaaa aatgaaccag ctgtaaactg taatgattct 480
tcagctgcaa aaaatgatga tcaacaatcc agtaaaaaga atactaccgc taagttaaac 540
aaggatgctg aaaacgttgt aaaaaaggcg ggaattgatc ctaacagttt aactgatgac 600
cagattaaag cattaaataa gatgaacttc tcgaaagctg caaagtctgg tacacaaatg 660
acttataatg atttccaaaa gattgctgat acgttaatca aacaagatgg tcggtacaca 720
gttccattct ttaaagcaag tgaatcaaaa aatatgcctg ccgctacaac taaagatgca 780
caactaata ctattgaacc tttagatgta tgggattcat ggccagttca agatgttcgg 840
acaggacaag ttgctaattg gaatggctat caacttgta tcgcaatgat gggaaattcca 900
aaccaaaatg ataatcatat ctatctotta tataataagt atggtgataa tgaattaagt 960
cattggaaga atgtaggtcc aatttttggc tataattcta ccgcggtttc acaagaatgg 1020
tcaggatcag ctgttttgaa cagtgataac tctatccaat tattttatac aagggtagac 1080
acgtctgata acaataccaa tcatcaaaaa attgctagcg ctactcttta ttttaactgat 1140
aataatggaa atgtatcact cgctcaggta cgaaatgact atattgtatt tgaaggtgat 1200
ggctattact accaaactta tgatcaatgg aaagctacta acaaaggtgc cgataatatt 1260
gcaatgcgtg atgctcatgt aattgaagat ggtaatggtg atcgggtacct tgtttttgaa 1320
gcaagtactg gtttgaaaa ttatcaaggc gaggaccaa tttataactg gttaaattat 1380
ggcggagatg acgcatttaa tatcaagagc ttatttagaa ttctttccaa tgatgatatt 1440
aagagtcggg caacttgggc taatgcagct atcggtatcc tcaaactaaa taaggacgaa 1500
aagaatccta aggtggcaga gttatactca ccattaattt ctgcaccaat ggtaagcgat 1560
gaaattgagc gaccaaatgt agttaaatta ggtaataaat attacttatt tgccgctacc 1620
cgtttaaatc gaggaagtaa tgatgatgct tggatgaatg ctaattatgc cgttggtgat 1680
aatgttgcaa tggtcggata tgttgctgat agtctaactg gatcttataa gccattaat 1740
gattctggag tagtcttgac tgcttctggt cctgcaaaact ggcggacagc aacttattca 1800
tattatgctg tccccgttgc cggaaaagat gaccaagtat tagttacttc atatatgact 1860
aatagaaatg gagtagcggg taaaggaatg gattcaactt gggcaccgag tttcttacta 1920
caaattaacc cggataacac aactactggt ttagctaaaa tgactaatca aggggattgg 1980
at ttgggatg attcaagcga aaatcttgat atgattggtg atttagactc cgctgcttta 2040
cctggcgaac gtgataaacc tgttgattgg gacttaattg gttatggatt aaaaccgcat 2100
gatcctgcta caccaaatga tcctgaaacg ccaactacac cagaaacccc tgagacacct 2160
aatactccca aaacaccaa gactcctgaa aatcctggga cacctcaaac tcctaataca 2220
cctaatactc cggaaattcc ttttaactcca gaaacgccta agcaacctga aaccxaaact 2280
aataatcggt tgccacaaac tggaaataat gccataaaag ccatgattgg cctaggtatg 2340
ggaacattgc ttagtatggt tggcttgca gaaattaaca aacgtcgatt taac 2394

```

<210> 4

<211> 2592

<212> DNA

<213> *Lactobacillus reuteri*

<220>

<221> RBS
 <222> (29)..(32)

<220>
 <221> RBS
 <222> (54)..(57)

<220>
 <221> misc_signal
 <222> (1)..(67)
 <223> Putative expression-regulating region

<220>
 <221> misc_signal
 <222> (2438)..(2592)
 <223> Putative expression-regulating region

<400> 4
 tacaatgggg tggcggaggt gaagaaacgg gggtacttct atgctagaac gcaaggaaca 60
 taaaaaaaaatg tataaaagcg gtaaaaattg ggcagtcggt acactctcga ctgctgcgct 120
 ggtatattggt gcaacaactg taaatgcatc cgcggacaca aatattgaaa acaatgattc 180
 ttctactgta caagttacaa caggtgataa tgatattgct gttaaaagtg tgacacttgg 240
 tagtggtcaa gttagtgcag ctagtgatac gactattaga acttctgcta atgcaaatag 300
 tgcttcttct gccgctaata cacaaaattc taacagtcaa gtagcaagtt ctgctgcaat 360
 aacatcatct acaagttccg cagcttcatt aaataacaca gatagtaaag cggctcaaga 420
 aaataactaat acagccaaaa atgatgacac gcaaaaagct gcaccagcta acgaatcttc 480
 tgaagctaaa aatgaaccag ctgtaaactg taatgattct tcagctgcaa aaaatgatga 540
 tcaacaatcc agtaaaaaga atactaccgc taagttaaac aaggatgctg aaaacgttgt 600
 aaaaaaggcg ggaattgatc ctaacagttt aactgatgac cagattaaag cattaataa 660
 gatgaacttc tcgaaagctg caaagtctgg tacacaaatg acttataatg atttccaaaa 720
 gattgctgat acgttaatca aacaagatgg tcggtacaca gttccattct ttaaagcaag 780
 tgaaatcaaa aatatgcctg ccgctacaac taaagatgca caaactaata ctattgaacc 840
 tttagatgta tgggattcat ggccagttca agatgttcgg acaggacaag ttgctaattg 900
 gaatggctat caacttgtca tcgcaatgat gggaaattcca aacccaaaatg ataatcatat 960
 ctatctctta tataataagt atggtgataa tgaattaaat cattggaaga atgtagggtcc 1020
 aatthtttggc tataattcta ccgcggtttc acaagaatgg tcaggatcag ctgttttgaa 1080
 cagtgataac tctatccaat tattttatac aagggtagac acgtctgata acaataccaa 1140
 tcatcaaaaa attgctagcg ctactcttta ttaactgat aataatggaa atgtatcact 1200
 cgctcaggta cgaaatgact atattgtatt tgaaggtgat ggctattact accaaactta 1260
 tgatcaatgg aaagctacta acaaaggtgc cgataatatt gcaatgcgtg atgctcatgt 1320
 aattgaagat ggtaatggtg atcggtacct tgtttttgaa gcaagtactg gtttgaaaa 1380
 ttatcaaggc gaggaccaa tttataactg gttaaattat ggcggagatg acgcatttaa 1440
 tatcaagagc ttatttagaa ttctttccaa tgatgatatt aagagtcggg caacttgggc 1500
 taatgcagct atcggtatcc tcaactaaa taaggacgaa aagaatccta aggtggcaga 1560
 gttatactca ccattaattt ctgcaccaat ggtaagcgtg gaaattgagc gaccaaaatgt 1620
 agttaaatta ggtaataaat attacttatt tgccgctacc cgtttaaadc gaggaagtaa 1680
 tgatgatgct tggatgaatg ctaattatgc cgttgggtgat aatggtgcaa tggtcggata 1740
 tgttgctgat agtctaactg gatcttataa gccattaat gattctggag tagtcttgac 1800


```

tgcttctggt cctgcaaact ggcggacagc aacttattca tattatgctg tccccgttgc 1860
cggaaaagat gaccaagtat tagttacttc atatatgact aatagaaatg gagtagcggg 1920
taaaggaatg gattcaactt gggcaccgag tttcttacta caaattaacc cggataaacac 1980
aactactggt ttagctaaaa tgactaatca aggggattgg atttgggatg attcaagcga 2040
aaatcttgat atgattggtg atttagactc cgctgcttta cctggcgaac gtgataaacc 2100
tgttgattgg gacttaattg gttatggatt aaaaccgcat gatcctgcta caccaaata 2160
tcctgaaacg ccaactacac cagaaacccc tgagacacct aatactccca aaacacccaa 2220
gactcctgaa aatcctggga cacctcaaac tcctaataca cctaatactc cggaaattcc 2280
tttaactcca gaaacgccta agcaacctga aacccaaact aataatcggt tgccacaaa 2340
tggaataaat gccataaaag ccatgattgg cctaggatg ggaacattgc ttagtatggt 2400
tggtcttgca gaaattaaca aacgtcgatt taactaaata ctttaaaata aaaccgctaa 2460
gccttaaatt cagcttaacg gttttttatt ttaaaagttt ttattgtaaa aaagcgaatt 2520
atcattaata ctaatgcaat tgttgtaaga ccttacgaca gtagtaacaa tgaatttgcc 2580
catctttgtc gg 2592

```

<210> 5

<211> 5

<212> PRT

<213> *Lactobacillus reuteri*

<400> 5

Leu Pro Xaa Thr Gly

1 5

<210> 6

<211> 23

<212> PRT

<213> *Lactobacillus reuteri*

<400> 6

Gln Val Glu Ser Asn Asn Tyr Asn Gly Val Ala Glu Val Asn Thr Glu

1 5 10 15

Arg Gln Ala Asn Gly Gln Ile

20

<210> 7

<211> 16

<212> PRT

<213> *Lactobacillus reuteri*

<400> 7

Met Ala His Leu Asp Val Trp Asp Ser Trp Pro Val Gln Asp Pro Val

1 5 10 15

<210> 8
 <211> 9
 <212> PRT
 <213> Lactobacillus reuteri

<400> 8
 Asn Ala Gly Ser Ile Phe Gly Thr Lys
 1 5

<210> 9
 <211> 19
 <212> PRT
 <213> Lactobacillus reuteri

<400> 9
 Val Glu Glu Val Tyr Ser Pro Lys Val Ser Thr Leu Met Ala Ser Asp
 1 5 10 15

Glu Val Glu

<210> 10
 <211> 4634
 <212> DNA
 <213> Lactobacillus reuteri

<220>
 <221> CDS
 <222> (1220)..(3598)

<220>
 <221> RBS
 <222> (1205)..(1210)

<400> 10
 gttaacaaag acaaaat tttt atataattct tcaaattaa tttcccactg taagaacata 60
 aatgggtacc tgtttgatgg gaataatata tttgtaacta accggccggc acctctttct 120
 aatgtgccta ggatgcataa tggatgtaaa ttactagatg gcggttttta tacattaacc 180

tcgcaggaga gaaaagaagc aattagtaag gatccatagc cagataaatt tattaggcct 240
tatttaggtg ctaaaaattht cattcatgga actgctaggt actgtatttg gttaaaggac 300
gcaaaccgga aagatatcca tcaatcgcca ttataactgg atagaatcaa taaagtagcg 360
gaattcagat cgcagcaaaa aagtaaagat acacaaaaat atgcaaaacg gcccatgcta 420
acaacacgac ttgcctatta tagccacgat gtacatacgg atatgctgat agtacctgca 480
acatcatcgc aacgtagaga atatcttcca attggatagc tttcagaaaa gaatattgtg 540
tcttattcac taatgctaht cccaatgct agtaatttht atttcggtat tctagaatct 600
aaagttcact atatttggtt aaaaaacttht tgcggtcggc tgaagtccga ttatcgttat 660
tcaaacacta ttatttataa taatttcctt tggccgactg ttggtgacaa gccaggamca 720
acaccatctc tgacactcgc tcaaggtata ttaaatactc gcaagctcta tccagacagc 780
tcactggctg atctttatga tccactaaca atgccragtt gaactcgtaa agctcatgaa 840
gccaatgata aagctgttct taaagcatat ggattgagcc ctaaagctac tgagcaagaa 900
atcgtagaac atctatttht gatgtatgaa aaactgacta aagggtgaaag ataactthtgt 960
aaaaccaata ttttataaag acagtaahtg ttaatttgat aaaaacatat atttaataaa 1020
caaaagtgat atahtcaaght agttctthtgt attacaaaht acattthaata tctctcagca 1080
ttthgcatac tgggagatttht tttattgaca aattgthtga aagtgcttat gatgaaaccg 1140
tgtagaaact aattcaatttht gataaacgtht agacattthtct gaggaggaag tcattthtggaa 1200
gtacaaagaa cataagaaa atg tat aaa gtc ggc aag aat tgg gcc gth gct 1252
Met Tyr Lys Val Gly Lys Asn Trp Ala Val Ala
1 5 10
aca ttg gta tca gct tca att tta atg gga ggg gth gta acc gct cat 1300
Thr Leu Val Ser Ala Ser Ile Leu Met Gly Gly Val Val Thr Ala His
15 20 25
gct gat caa gta gaa agt aac aat tac aac ggt gth gct gaa gth aat 1348
Ala Asp Gln Val Glu Ser Asn Asn Tyr Asn Gly Val Ala Glu Val Asn
30 35 40
act gaa cgt caa gct aat ggt caa att ggc gta gat gga aaa att att 1396

Thr	Glu	Arg	Gln	Ala	Asn	Gly	Gln	Ile	Gly	Val	Asp	Gly	Lys	Ile	Ile	
	45					50					55					
agt	gct	aac	agt	aat	aca	acc	agt	ggc	tcg	aca	aat	caa	gaa	tca	tct	1444
Ser	Ala	Asn	Ser	Asn	Thr	Thr	Ser	Gly	Ser	Thr	Asn	Gln	Glu	Ser	Ser	
	60				65					70					75	
gct	act	aac	aat	act	gaa	aat	gct	gtt	gtt	aat	gaa	agc	aaa	aat	act	1492
Ala	Thr	Asn	Asn	Thr	Glu	Asn	Ala	Val	Val	Asn	Glu	Ser	Lys	Asn	Thr	
				80					85					90		
aac	aat	act	gaa	aat	gct	gtt	gtt	aat	gaa	aac	aaa	aat	act	aac	aat	1540
Asn	Asn	Thr	Glu	Asn	Ala	Val	Val	Asn	Glu	Asn	Lys	Asn	Thr	Asn	Asn	
			95					100					105			
act	gaa	aat	gct	gtt	gtt	aat	gaa	aac	aaa	aat	act	aac	aac	aca	gaa	1588
Thr	Glu	Asn	Ala	Val	Val	Asn	Glu	Asn	Lys	Asn	Thr	Asn	Asn	Thr	Glu	
			110					115					120			
aac	gat	aat	agt	caa	tta	aag	tta	act	aat	aat	gaa	caa	cca	tca	gcc	1636
Asn	Asp	Asn	Ser	Gln	Leu	Lys	Leu	Thr	Asn	Asn	Glu	Gln	Pro	Ser	Ala	
	125					130						135				
gct	act	caa	gca	aac	ttg	aag	aag	cta	aat	cct	caa	gct	gct	aag	gct	1684
Ala	Thr	Gln	Ala	Asn	Leu	Lys	Lys	Leu	Asn	Pro	Gln	Ala	Ala	Lys	Ala	
	140				145					150					155	
gtt	caa	aat	gcc	aag	att	gat	gcc	ggt	agt	tta	aca	gat	gat	caa	att	1732
Val	Gln	Asn	Ala	Lys	Ile	Asp	Ala	Gly	Ser	Leu	Thr	Asp	Asp	Gln	Ile	
				160					165					170		
aat	gaa	tta	aat	aag	att	aac	ttc	tct	aag	tct	gct	gaa	aag	ggt	gca	1780
Asn	Glu	Leu	Asn	Lys	Ile	Asn	Phe	Ser	Lys	Ser	Ala	Glu	Lys	Gly	Ala	
				175					180					185		
aaa	ttg	acc	ttt	aag	gac	tta	gag	ggg	att	ggt	aat	gct	att	gtt	aag	1828
Lys	Leu	Thr	Phe	Lys	Asp	Leu	Glu	Gly	Ile	Gly	Asn	Ala	Ile	Val	Lys	
		190						195					200			
caa	gat	cca	caa	tat	gct	att	cct	tat	tct	aat	gct	aag	gaa	atc	aag	1876
Gln	Asp	Pro	Gln	Tyr	Ala	Ile	Pro	Tyr	Ser	Asn	Ala	Lys	Glu	Ile	Lys	
	205					210						215				
aat	atg	cct	gca	aca	tac	act	gta	gat	gcc	caa	aca	ggt	aag	atg	gct	1924
Asn	Met	Pro	Ala	Thr	Tyr	Thr	Val	Asp	Ala	Gln	Thr	Gly	Lys	Met	Ala	
	220				225					230					235	
cat	ctt	gat	gtc	tgg	gac	tct	tgg	cca	gta	caa	gat	cct	gtc	aca	ggt	1972

His	Leu	Asp	Val	Trp	Asp	Ser	Trp	Pro	Val	Gln	Asp	Pro	Val	Thr	Gly	
				240					245					250		
tat	gta	tct	aat	tac	atg	ggt	tat	caa	cta	gtt	att	gct	atg	atg	ggt	2020
Tyr	Val	Ser	Asn	Tyr	Met	Gly	Tyr	Gln	Leu	Val	Ile	Ala	Met	Met	Gly	
			255					260					265			
att	cca	aat	tcg	cca	act	gga	gat	aat	cat	atc	tat	ctt	ctt	tac	aac	2068
Ile	Pro	Asn	Ser	Pro	Thr	Gly	Asp	Asn	His	Ile	Tyr	Leu	Leu	Tyr	Asn	
			270					275					280			
aag	tat	ggt	gat	aat	gac	ttt	tct	cat	tgg	cgc	aat	gca	ggt	tca	atc	2116
Lys	Tyr	Gly	Asp	Asn	Asp	Phe	Ser	His	Trp	Arg	Asn	Ala	Gly	Ser	Ile	
		285					290				295					
ttt	gga	act	aaa	gaa	aca	aat	gtg	ttc	caa	gaa	tgg	tca	ggt	tca	gct	2164
Phe	Gly	Thr	Lys	Glu	Thr	Asn	Val	Phe	Gln	Glu	Trp	Ser	Gly	Ser	Ala	
300					305					310					315	
att	gta	aat	gat	gat	ggt	aca	att	caa	cta	ttt	ttc	acc	tca	aat	gat	2212
Ile	Val	Asn	Asp	Asp	Gly	Thr	Ile	Gln	Leu	Phe	Phe	Thr	Ser	Asn	Asp	
					320				325					330		
acg	tct	gat	tac	aag	ttg	aat	gat	caa	cgc	ctt	gct	acc	gca	aca	tta	2260
Thr	Ser	Asp	Tyr	Lys	Leu	Asn	Asp	Gln	Arg	Leu	Ala	Thr	Ala	Thr	Leu	
			335					340					345			
aac	ctt	aat	gtt	gat	gat	aac	ggt	gtt	tca	atc	aag	agt	gtt	gat	aat	2308
Asn	Leu	Asn	Val	Asp	Asp	Asn	Gly	Val	Ser	Ile	Lys	Ser	Val	Asp	Asn	
			350				355					360				
tat	caa	gtt	ttg	ttt	gaa	ggt	gat	gga	ttt	cac	tac	caa	act	tat	gaa	2356
Tyr	Gln	Val	Leu	Phe	Glu	Gly	Asp	Gly	Phe	His	Tyr	Gln	Thr	Tyr	Glu	
		365				370				375						
caa	ttc	gca	aac	ggc	aaa	gat	cgt	gaa	aat	gat	gat	tac	tgc	tta	cgt	2404
Gln	Phe	Ala	Asn	Gly	Lys	Asp	Arg	Glu	Asn	Asp	Asp	Tyr	Cys	Leu	Arg	
380					385					390					395	
gac	cca	cac	gtt	gtt	caa	tta	gaa	aat	ggt	gat	cgt	tat	ctt	gta	ttc	2452
Asp	Pro	His	Val	Val	Gln	Leu	Glu	Asn	Gly	Asp	Arg	Tyr	Leu	Val	Phe	
				400					405					410		
gaa	gct	aat	act	ggg	aca	gaa	gat	tac	caa	agt	gac	gac	caa	att	tat	2500
Glu	Ala	Asn	Thr	Gly	Thr	Glu	Asp	Tyr	Gln	Ser	Asp	Asp	Gln	Ile	Tyr	
			415					420					425			
aat	tgg	gct	aac	tat	ggt	ggc	gat	gat	gcc	ttc	aat	att	aag	agt	tcc	2548

Asn Trp Ala Asn Tyr Gly Gly Asp Asp Ala Phe Asn Ile Lys Ser Ser
 430 435 440

ttc aag ctt ttg aat aat aag aag gat cgt gaa ttg gct ggt tta gct 2596
 Phe Lys Leu Leu Asn Asn Lys Lys Asp Arg Glu Leu Ala Gly Leu Ala
 445 450 455

aat ggt gca ctt ggt atc tta aag ctc act aac aat caa agt aag cca 2644
 Asn Gly Ala Leu Gly Ile Leu Lys Leu Thr Asn Asn Gln Ser Lys Pro
 460 465 470 475

aag gtt gaa gaa gta tac tca cca ttg gta tct act ttg atg gct tgc 2692
 Lys Val Glu Glu Val Tyr Ser Pro Leu Val Ser Thr Leu Met Ala Cys
 480 485 490

gat gag gta nnn nnn aag ctt ggt gat aag tat tat ctc ttc tcc gta 2740
 Asp Glu Val Xaa Xaa Lys Leu Gly Asp Lys Tyr Tyr Leu Phe Ser Val
 495 500 505

act cgt gta agt cgt ggt tcc gat cgt gaa tta acc gct aag gat aac 2788
 Thr Arg Val Ser Arg Gly Ser Asp Arg Glu Leu Thr Ala Lys Asp Asn
 510 515 520

aca atc gtt ggt gat aac gtt gct atg att ggt tac gtt tcc gat agc 2836
 Thr Ile Val Gly Asp Asn Val Ala Met Ile Gly Tyr Val Ser Asp Ser
 525 530 535

tta atg ggt aag tac aag cca tta aat aac tca ggt gtc gta tta act 2884
 Leu Met Gly Lys Tyr Lys Pro Leu Asn Asn Ser Gly Val Val Leu Thr
 540 545 550 555

gca tca gta cct gca aac tgg cgt act gct act tat tcc tac tat gca 2932
 Ala Ser Val Pro Ala Asn Trp Arg Thr Ala Thr Tyr Ser Tyr Tyr Ala
 560 565 570

gta cct gta gct ggt cat cct gat caa gta tta att act tct tac atg 2980
 Val Pro Val Ala Gly His Pro Asp Gln Val Leu Ile Thr Ser Tyr Met
 575 580 585

agt aac aag gac ttt gct tca ggt gaa gga aac tat gca act tgg gca 3028
 Ser Asn Lys Asp Phe Ala Ser Gly Glu Gly Asn Tyr Ala Thr Trp Ala
 590 595 600

cca agt ttc tta gta caa atc aat cca gat gac acg aca act gta tta 3076
 Pro Ser Phe Leu Val Gln Ile Asn Pro Asp Asp Thr Thr Thr Val Leu
 605 610 615

gca cgt gca act aac caa ggt gac tgg gtg tgg gac gac tct agt cgg 3124

Ala Arg Ala Thr Asn Gln Gly Asp Trp Val Trp Asp Asp Ser Ser Arg
 620 625 630 635

aac gat aat atg ctc ggt gtt ctt aaa gaa ggt gca gct aac agt gcc 3172
 Asn Asp Asn Met Leu Gly Val Leu Lys Glu Gly Ala Ala Asn Ser Ala
 640 645 650

gcc tta cca ggt gaa tgg ggt aag cca gtt gac tgg agt ttg att aac 3220
 Ala Leu Pro Gly Glu Trp Gly Lys Pro Val Asp Trp Ser Leu Ile Asn
 655 660 665

aga agt cct ggc tta ggc tta aag cct cat caa cca gtt caa cca aag 3268
 Arg Ser Pro Gly Leu Gly Leu Lys Pro His Gln Pro Val Gln Pro Lys
 670 675 680

att gat caa cct gat caa caa cct tct ggt caa aac act aag aat gtc 3316
 Ile Asp Gln Pro Asp Gln Gln Pro Ser Gly Gln Asn Thr Lys Asn Val
 685 690 695

aca cca ggt aat ggt gat aag cct gct ggt aag gca act cct gat aac 3364
 Thr Pro Gly Asn Gly Asp Lys Pro Ala Gly Lys Ala Thr Pro Asp Asn
 700 705 710 715

act aat att gat cca agt gca caa cct tct ggt caa aac act aat att 3412
 Thr Asn Ile Asp Pro Ser Ala Gln Pro Ser Gly Gln Asn Thr Asn Ile
 720 725 730

gat cca agt gca caa mct tct ggt caa aac act aag aat gtc aca cca 3460
 Asp Pro Ser Ala Gln Xaa Ser Gly Gln Asn Thr Lys Asn Val Thr Pro
 735 740 745

ggt aat gag aaa caa ggt aag aat acc gat gca aaa caa tta cca caa 3508
 Gly Asn Glu Lys Gln Gly Lys Asn Thr Asp Ala Lys Gln Leu Pro Gln
 750 755 760

aca ggt aat aag tct ggt tta gca gga ctt tac gct ggt tca tta ctt 3556
 Thr Gly Asn Lys Ser Gly Leu Ala Gly Leu Tyr Ala Gly Ser Leu Leu
 765 770 775

gcc ttg ttt gga ttg gca gca att gaa aag cgt cac gct taa 3598
 Ala Leu Phe Gly Leu Ala Ala Ile Glu Lys Arg His Ala
 780 785 790

tagagtaaaa aaacatcctc cactcaagtt acaagtagga taatatgtat tatttctacg 3658

cytagtcaag aggrattact ggacatannn nnnnnnnnnn tccagttacc aagtggaata 3718

tagtattatt ccacgctagt caggaggatt actgacatta ttggctacat ggccggtagt 3778

cctcttttct tttgtgacga attgtcaaac caagtgcaac ggtttctcaa aaaacacctc 3838
 atatgggggt tcataattta aacttttctg aggacggcgg ttcagctgat gttggcagaa 3898
 actgacgtcc ttatctgtat aatcatcaat attagccctt ttaggaaagt attccctaata 3958
 tagscattg gtattttcat tgggtcctct ttcctctggt gaataggat ctggccaata 4018
 gatagctact cctaaacgtc ctcgaatatac attcaagcca agaaattcac gcccatgatc 4078
 tggagtcaat gaatggacaa attctttagg aatagaccct aagagatcaa ttaagccctg 4138
 atatttgaat tcggagaagg ggagttgtcc aacaattgcc gttataatac cagggttaat 4198
 acggccctgg gcctctacgg taatattgta tttttggctc agatcagtga tagaaacca 4258
 cagatttagc ttgccggtgg agtgctgctt gaagtcttca attacttctg taccatgttt 4318
 gattgctaata ctgatgtgtc gttgttggg tgtagtaggc atcataccac ctcctcataa 4378
 aataaggtat aacaggaatt tcttgtacta tatgatcctt ccaatataat aatattaggc 4438
 cgataagaaa tgaccagcta ccatttcttg atgcttagtg aatataatcg gatgatacgt 4498
 caccctcaa caatccaatt tcacggaggt gagtaatcat gccgagagct aggaatgatt 4558
 ggaggaacga acacggtcca tgcggcagtg gctatttga ttttagccaa agcagcgta 4618
 ctgcttgcaa aagctt 4634

<210> 11

<211> 792

<212> PRT

<213> Lactobacillus reuteri

<400> 11

Met Tyr Lys Val Gly Lys Asn Trp Ala Val Ala Thr Leu Val Ser Ala
 1 5 10 15
 Ser Ile Leu Met Gly Gly Val Val Thr Ala His Ala Asp Gln Val Glu
 20 25 30
 Ser Asn Asn Tyr Asn Gly Val Ala Glu Val Asn Thr Glu Arg Gln Ala
 35 40 45
 Asn Gly Gln Ile Gly Val Asp Gly Lys Ile Ile Ser Ala Asn Ser Asn

50	55	60																	
Thr	Thr	Ser	Gly	Ser	Thr	Asn	Gln	Glu	Ser	Ser	Ala	Thr	Asn	Asn	Thr				
65					70					75					80				
Glu	Asn	Ala	Val	Val	Asn	Glu	Ser	Lys	Asn	Thr	Asn	Asn	Thr	Glu	Asn				
				85					90					95					
Ala	Val	Val	Asn	Glu	Asn	Lys	Asn	Thr	Asn	Asn	Thr	Glu	Asn	Ala	Val				
			100					105					110						
Val	Asn	Glu	Asn	Lys	Asn	Thr	Asn	Asn	Thr	Glu	Asn	Asp	Asn	Ser	Gln				
		115						120				125							
Leu	Lys	Leu	Thr	Asn	Asn	Glu	Gln	Pro	Ser	Ala	Ala	Thr	Gln	Ala	Asn				
130						135						140							
Leu	Lys	Lys	Leu	Asn	Pro	Gln	Ala	Ala	Lys	Ala	Val	Gln	Asn	Ala	Lys				
145					150						155				160				
Ile	Asp	Ala	Gly	Ser	Leu	Thr	Asp	Asp	Gln	Ile	Asn	Glu	Leu	Asn	Lys				
				165					170					175					
Ile	Asn	Phe	Ser	Lys	Ser	Ala	Glu	Lys	Gly	Ala	Lys	Leu	Thr	Phe	Lys				
			180						185					190					
Asp	Leu	Glu	Gly	Ile	Gly	Asn	Ala	Ile	Val	Lys	Gln	Asp	Pro	Gln	Tyr				
		195					200							205					
Ala	Ile	Pro	Tyr	Ser	Asn	Ala	Lys	Glu	Ile	Lys	Asn	Met	Pro	Ala	Thr				
			210				215							220					
Tyr	Thr	Val	Asp	Ala	Gln	Thr	Gly	Lys	Met	Ala	His	Leu	Asp	Val	Trp				
225					230							235			240				
Asp	Ser	Trp	Pro	Val	Gln	Asp	Pro	Val	Thr	Gly	Tyr	Val	Ser	Asn	Tyr				
				245							250				255				
Met	Gly	Tyr	Gln	Leu	Val	Ile	Ala	Met	Met	Gly	Ile	Pro	Asn	Ser	Pro				
			260							265					270				
Thr	Gly	Asp	Asn	His	Ile	Tyr	Leu	Leu	Tyr	Asn	Lys	Tyr	Gly	Asp	Asn				
		275							280						285				
Asp	Phe	Ser	His	Trp	Arg	Asn	Ala	Gly	Ser	Ile	Phe	Gly	Thr	Lys	Glu				
	290						295								300				
Thr	Asn	Val	Phe	Gln	Glu	Trp	Ser	Gly	Ser	Ala	Ile	Val	Asn	Asp	Asp				

				565					570					575	
His	Pro	Asp	Gln	Val	Leu	Ile	Thr	Ser	Tyr	Met	Ser	Asn	Lys	Asp	Phe
			580					585					590		
Ala	Ser	Gly	Glu	Gly	Asn	Tyr	Ala	Thr	Trp	Ala	Pro	Ser	Phe	Leu	Val
		595					600					605			
Gln	Ile	Asn	Pro	Asp	Asp	Thr	Thr	Thr	Val	Leu	Ala	Arg	Ala	Thr	Asn
	610					615					620				
Gln	Gly	Asp	Trp	Val	Trp	Asp	Asp	Ser	Ser	Arg	Asn	Asp	Asn	Met	Leu
625					630					635					640
Gly	Val	Leu	Lys	Glu	Gly	Ala	Ala	Asn	Ser	Ala	Ala	Leu	Pro	Gly	Glu
				645				650						655	
Trp	Gly	Lys	Pro	Val	Asp	Trp	Ser	Leu	Ile	Asn	Arg	Ser	Pro	Gly	Leu
			660					665					670		
Gly	Leu	Lys	Pro	His	Gln	Pro	Val	Gln	Pro	Lys	Ile	Asp	Gln	Pro	Asp
		675					680					685			
Gln	Gln	Pro	Ser	Gly	Gln	Asn	Thr	Lys	Asn	Val	Thr	Pro	Gly	Asn	Gly
	690					695					700				
Asp	Lys	Pro	Ala	Gly	Lys	Ala	Thr	Pro	Asp	Asn	Thr	Asn	Ile	Asp	Pro
705					710					715					720
Ser	Ala	Gln	Pro	Ser	Gly	Gln	Asn	Thr	Asn	Ile	Asp	Pro	Ser	Ala	Gln
				725					730					735	
Xaa	Ser	Gly	Gln	Asn	Thr	Lys	Asn	Val	Thr	Pro	Gly	Asn	Glu	Lys	Gln
			740					745					750		
Gly	Lys	Asn	Thr	Asp	Ala	Lys	Gln	Leu	Pro	Gln	Thr	Gly	Asn	Lys	Ser
		755					760					765			
Gly	Leu	Ala	Gly	Leu	Tyr	Ala	Gly	Ser	Leu	Leu	Ala	Leu	Phe	Gly	Leu
	770					775					780				
Ala	Ala	Ile	Glu	Lys	Arg	His	Ala								
785					790										

<210> 12

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer

<400> 12

ctgataataa tggaaatgta tcac 24

<210> 13

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer

<400> 13

catgatcata agtttgtag taatag 26

<210> 14

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer

<400> 14

gtgatacatt tccattatta tcag 24

<210> 15

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer

<400> 15

ctattactac caaacttatg atcatg 26

<210> 16

<211> 38

<212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:primer

<400> 16
 ccatggccat ggtagaacgc aaggaacata aaaaaatg 38

<210> 17
 <211> 38
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:primer

<400> 17
 agatctagat ctgttaaadc gacgtttggt aatttctg 38

<210> 18
 <211> 21
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:primer

<400> 18
 gaygtntggg aywsntgggc c 21

<210> 19
 <211> 23
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:primer

<400> 19
 gtngcnswn cnsbccayts ytg 23

<210> 20
 <211> 22

<212> DNA
 <213> Artificial Sequence

 <220>
 <223> Description of Artificial Sequence:primer

 <400> 20
 gaatgtaggt ccaatttttg gc 22

<210> 21
 <211> 22
 <212> DNA
 <213> Artificial Sequence

 <220>
 <223> Description of Artificial Sequence:primer

 <400> 21
 cctgtccgaa catcttgaac tg 22

<210> 22
 <211> 23
 <212> DNA
 <213> Artificial Sequence

 <220>
 <223> Description of Artificial Sequence:primer

 <400> 22
 arraanswng gngcvmangt nsw 23

<210> 23
 <211> 23
 <212> DNA
 <213> Artificial Sequence

 <220>
 <223> Description of Artificial Sequence:primer

 <400> 23
 tayaayggng tngcngargt naa 23

<210> 24
 <211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer

<400> 24

ccgaccatct tgtttgatta ac

22

<210> 25

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer

<400> 25

aaytataayg gygttgcryg aagt

24

<210> 26

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer

<400> 26

taccgnwsnc tacttcaact t

21