Novel Fructosyltransferases

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(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 posses 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, wich 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→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 fructo-oligosaccharides, 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 β(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 β(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→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 β(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 β(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 prefarably 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 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 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

[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 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. 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 characteristics. Carboxymethylation is achieved by reaction of the fructans with mono-chloroacetic acid or its alkali metal salts and results in anionic polymers suitable for various purposes including crystallisation inhibitors, and metal complexants. Amino-alkylation 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 (*ftfA*) 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 fructosyltranferase 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 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 isloated with a combination of standard and inverse PCR techniques. Briefly, *Lactobacillus reuteri* DNA was cut with restriction enzyme *Xho*I and ligated. PCR with the primers 7ftf and 8ftfi, using the ligation product as a template, yielded a 290 bp PCR product which 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 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 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 primers ftfA1 and ftfA2i. Both primers contained suitable restriction enzyme recognition sites (a *Nco*I site at the 5'end of ftfA1 and a *Bgl*II 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 *Nco*I and *Bgl*II restriction sites. The PCR product with blunt ends was ligated directly into pCRbluntII-Topo. Using the *Nco*I and *Bgl*II 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 fructosyltranferase activities were determined at 37°C in reaction buffer (25 mM sodium acetate, pH 5.4, 1 mM $CaCl_2$, 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. ^{1}H-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 chromotography 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 Briljant 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)
ftfAC1	1176	CTG-ATA-ATA-ATG-GAA-ATG-TAT-CAC (SEQ ID No. 12)
ftfAC2i	1243	CAT-GAT-CAT-AAG-TTT-GGT-AGT-AAT-AG (SEQ ID No. 13)
ftfac1	1176	GTG-ATA-CAT-TTC-CAT-TAT-TAT-CAG (SEQ ID No. 14)
ftfAC2	1243	CTA-TTA-CTA-CCA-AAC-TTA-TGA-TCA-TG (SEQ ID No. 15)
ftfA1		CCA-TGG-CCA-TGG-TAG-AAC-GCA-AGG-AAC-ATA-AAA-AAA-TG (SEQ ID No. 16)

ftfA2i		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 *ftfA* 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).

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 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 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 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 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 (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.

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 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 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

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/cm^2 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 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 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*.

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 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 proceded 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 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 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

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 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 good adhering properties) were cultured in MRS broth supplemented with 5 µl/ml of methyl-1,2-[^{3}H]-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 10^9 cfu/ml. Prior to the adhesion assay, the cell suspensions in PBS 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 10^9 cfu/ml.

Caco-2 cells were cultured as follows. Subcultures of Caco-2 cells (ATCC, code 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 75cm^2 tissue culture flasks containing culture medium and cultured in a humidified incubator at 37°C in air containing 5% CO_2. 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.

Lactobacillus strain	Extra incubation	Polysaccharide produced	Group
reuteri 121	PBS sucrose, 37°C for 1 hr	glucan and fructan	As
reuteri 35-5	PBS sucrose, 37°C for 1 hr	glucan	Bs
reuteri K24	PBS sucrose, 37°C for 1 hr	none	Cs
reuteri 121	PBS on ice	none	D
reuteri DSM20016*	PBS on ice	none	E
rhamnosus 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 tranport 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 ± 1 cm^2 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% CO_2 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 µl of a bacterial suspension (2 x 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 exellent 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

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 *Nhe*I restriction sites (at positions 1154 and 2592) used for inverse PCR are underlined. The primers used and their exact posotions 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. Goups 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 β(2-1) linked D-fructosyl units and/or a levan having β(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 taaaaaaatg tataaaagcg gtaaaaattg ggcagtcgtt acactctcga ctgctgcgct
 1         m  y k s  g k n  w a v  v t l  s t a  a

121 ggtatttggt gcaacaactg taaatgcatc cgcggacaca 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 gttaaaagtg 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 aaatactaat acagccaaaa atgatgacac gcaaaaagct gcaccagcta acgaatcttc
118 e  n t n  t a k  n d d  t q k  a p a  n e s

481 tgaagctaaa aatgaaccag ctgtaaacgt taatgattct tcagctgcaa aaaatgatga
138 s  e a k  n e p  a v n  v n d  s s 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 cattaaataa
178 v  k k a  g i d  p n s  l t d  d q i  k a l  n
```

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 tgaaatcaaa 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 caacttgtca tcgcaatgat gggaattcca aaccaaaatg 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 tattttatac 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

Fig 1 (3)

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

1381 ttatcaaggc gaggaccaaa 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 atcggtatcc 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 gttatactca 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

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618 g k g m d s t w a p s f l l q i n p d n

Fig 1 (4)

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658 e n l d m i g d l d s a a l p g e r d k

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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 aaacaccaaa
698 d p e t p t t p e t p e t p n t p k t p

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718 k t p e n p g t p q t p n t p n t p e i

2281 tttaactcca gaaacgccta agcaacctga aacccaaact aataatcgtt tgccacaaac
738 p l t p e t p k q p e t q t n n r l p q

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758 t g n n a n k a m i g l g m g t l l s m

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

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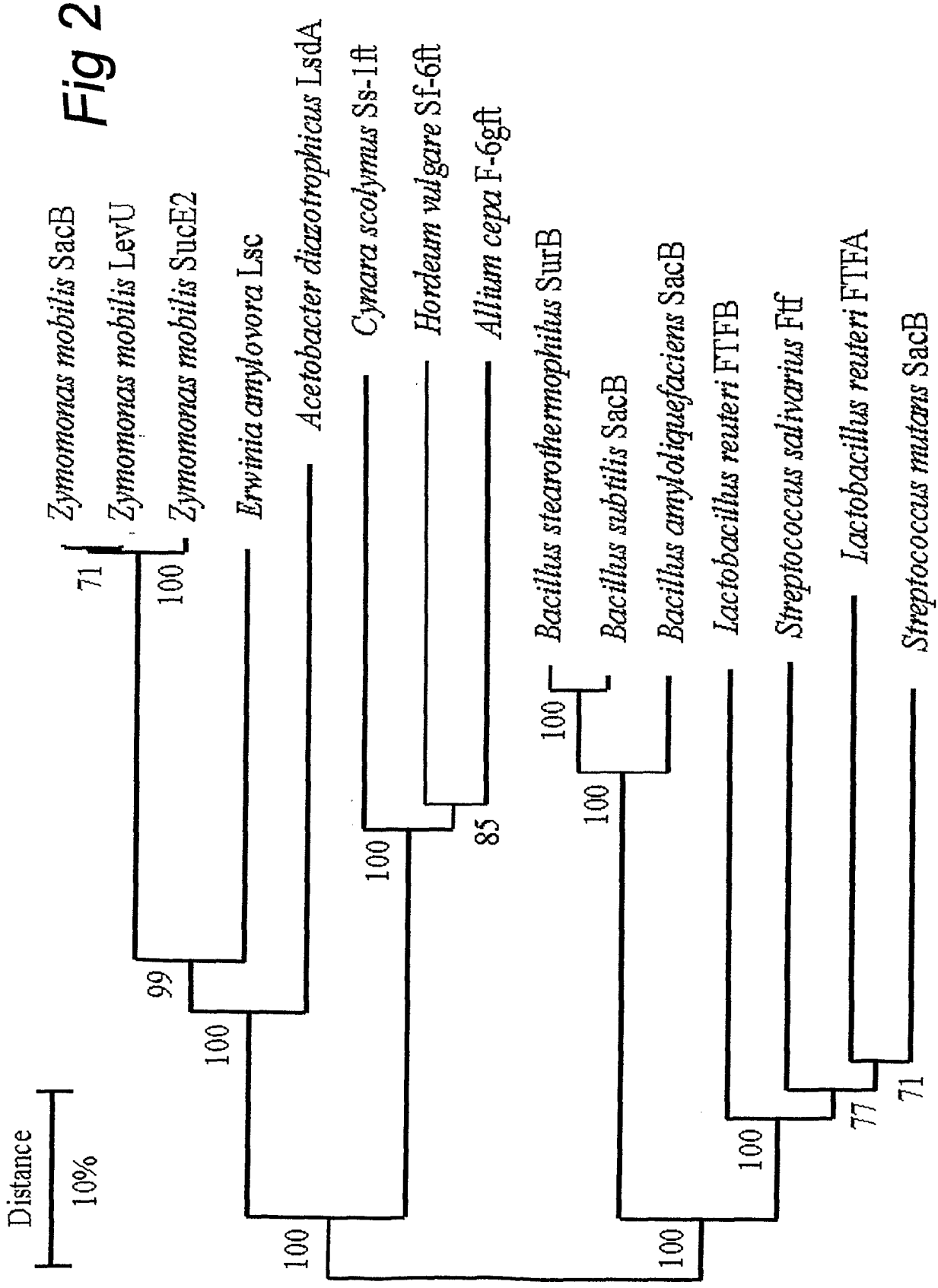
NheI

Fig 2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Hordeum vulgare Sf-6ft
Allium cepa F-6gft
Bacillus stearothermophilus SurB
Bacillus subtilis SacB
Bacillus amyloliquefaciens SacB
Lactobacillus reuteri FTFB
Streptococcus salivarius Ftf
Lactobacillus reuteri FTFA
Streptococcus mutans SacB
71
100
99
100
100
100
85
100
100
100
100
77
71
Distance
10%

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
B. amyloliquefaciens SacB  80   GLDVWDSWPLQNAD 93
B. subtilis SacB           82   GLDVWDSWPLQNAD 95
S. mutans SacB             243  DLDVWDSWPVQDAK 256
S. salivarius Ftf          282  EIDVWDSWPVQDAK 295
                                :*********:*:*.

6ftfi
B. amyloliquefaciens SacB  156  QTQEWSGSATFTSDGK 171
B. subtilis SacB           158  QTQEWSGSATFTSDGK 173
S. mutans SacB             312  LTQEWSGSATVNEDGS 327
S. salivarius Ftf          351  DDQQWSGSATVNSDGS 366
                                  *:*******...**.

12ftfi
B. amyloliquefaciens SacB  440  KATFGPSFLMN 450
B. subtilis SacB           440  QSTFAPSFLLN 450
S. mutans SacB             609  NSTWAPSFLIQ 619
S. salivarius Ftf          655  KSTWAPSFLIK 665
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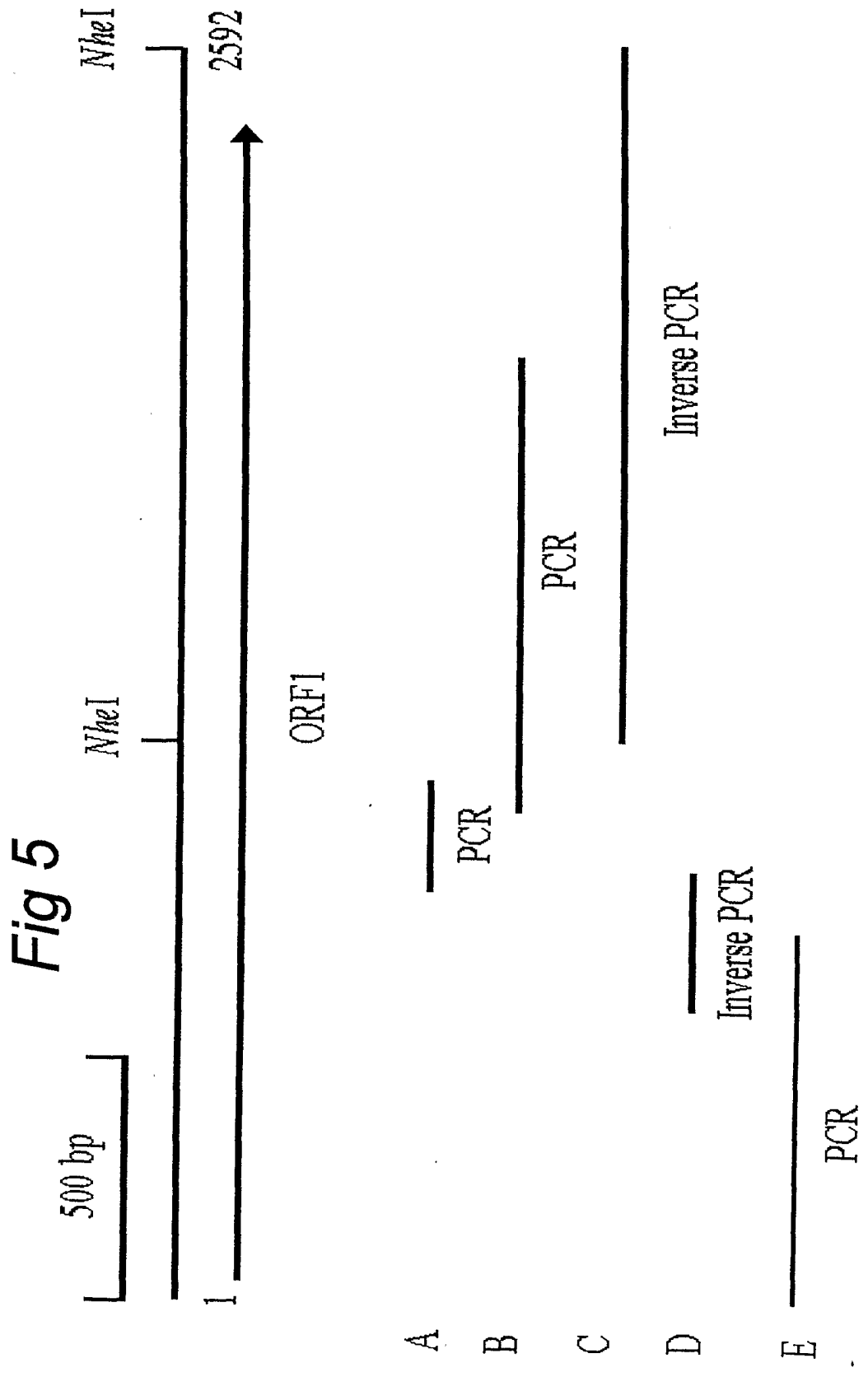
Fig 5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C
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         35                  40                  45

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
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Ala Ile Thr Ser Ser Thr Ser Ser Ala Ala Ser Leu Asn Asn Thr Asp
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Ser Lys Ala Ala Gln Glu Asn Thr Asn Thr Ala Lys Asn Asp Asp Thr
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Gln Lys Ala Ala Pro Ala Asn Glu Ser Ser Glu Ala Lys Asn Glu Pro
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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
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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
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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
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Leu Asn Tyr Gly Gly Asp Asp Ala Phe Asn Ile Lys Ser Leu Phe Arg
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Val Leu Ala Lys Met Thr Asn Gln Gly Asp Trp Ile Trp Asp Asp Ser
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Ser Glu Asn Leu Asp Met Ile Gly Asp Leu Asp Ser Ala Ala Leu Pro
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Gly Glu Arg Asp Lys Pro Val Asp Trp Asp Leu Ile Gly Tyr Gly Leu
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Lys Pro His Asp Pro Ala Thr Pro Asn Asp Pro Glu Thr Pro Thr Thr
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Pro Glu Thr Pro Glu Thr Pro Asn Thr Pro Lys Thr Pro Lys Thr Pro
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Glu Asn Pro Gly Thr Pro Gln Thr Pro Asn Thr Pro Asn Thr Pro Glu
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Ile Pro Leu Thr Pro Glu Thr Pro Lys Gln Pro Glu Thr Gln Thr Asn
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Asn Arg Leu Pro Gln Thr Gly Asn Asn Ala Asn Lys Ala Met Ile Gly
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taaaggaatg gattcaactt gggcaccgag tttcttacta caaattaacc cggataacac 1980
aactactgtt ttagctaaaa tgactaatca aggggattgg atttgggatg attcaagcga 2040
aaatcttgat atgattggtg atttagactc cgctgcttta cctggcgaac gtgataaacc 2100
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gactcctgaa aatcctggga cacctcaaac tcctaataca cctaatactc cggaaattcc 2280
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gccttaaatt cagcttaacg gttttttatt ttaaaagttt ttattgtaaa aaagcgaatt 2520
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catctttgtc gg 2592

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<213> Lactobacillus reuteri

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1 5

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1 5 10 15

Arg Gln Ala Asn Gly Gln Ile
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<213> Lactobacillus reuteri

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1 5 10 15

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<213> Lactobacillus reuteri

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Asn Ala Gly Ser Ile Phe Gly Thr Lys
1 5

<210> 9
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Glu Val Glu

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aatgtgccta ggatgcataa tggatgtaaa ttactagatg gcggttttta tacattaacc 180

tcgcaggaga gaaaagaagc aattagtaag gatccatatg cagataaatt tattaggcct 240

tatttaggtg ctaaaaattt cattcatgga actgctaggt actgtatttg gttaaaggac 300

gcaaacccga aagatatcca tcaatcgcca tttatactgg atagaatcaa taaagtagcg 360

gaattcagat cgcagcaaaa aagtaaagat acacaaaaat atgcaaaacg gcccatgcta 420

acaacacgac ttgcctatta tagccacgat gtacatacgg atatgctgat agtacctgca 480

acatcatcgc aacgtagaga atatcttcca attggatatg tttcagaaaa gaatattgtg 540

tcttattcac taatgctaat ccccaatgct agtaatttta atttcggtat tctagaatct 600

aaagttcact atatttggtt aaaaaacttt tgcggtcggt tgaagtccga ttatcgttat 660

tcaaacacta ttatttataa taatttccct 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 atctatttaa gatgtatgaa aaactgacta aaggtgaaag ataactttgt 960

aaaaccaata ttttataaag acagtaaatg ttaatttgat aaaaacatat atttaataaa 1020

caaaagtgat ataatcaagt agttctttgt attacaaaat acatttaata tctctcagca 1080

ttttgcatac tgggagattt tttattgaca aattgtttga aagtgcttat gatgaaaccg 1140

tgtagaaact aattcaattt gataaacgtt agacatttct gaggaggaag tcattttgga 1200

gtacaaagaa cataagaaa atg tat aaa gtc ggc aag aat tgg gcc gtt 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 gtt 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 gtt gct gaa gtt 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

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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
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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
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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
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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
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cctcttttct tttgtgacga attgtcaaac caagtgcaac ggtttctcaa aaaacacctc 3838

atatggggtt tcataattta acacttttcg aggacggcgg ttcagctgat gttggcagaa 3898

actgacgtcc ttatctgtat aatcatcaat attagccctt ttaggaaagt attccctaat 3958

tagsccattg gtattttcat tgggtcctct ttcctctggt gaatagggat ctggccaata 4018

gatagctact cctaaacgtc ctcgaatatc attcaagcca agaaattcac gcccatgatc 4078

tggagtcaat gaatggacaa attctttagg aatagaccct aagagatcaa ttaagccctg 4138

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caccctcaa caatccaatt tcacggaggt gagtaatcat gccgagagct aggaatgatt 4558

ggaggaacga acacggtcca tgcggcagtg gctatttgga ttttagccaa agcagcgtta 4618

ctgcttgcaa aagctt 4634

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<213> Lactobacillus reuteri

<400> 11

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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

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     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
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305 310 315 320

Gly Thr Ile Gln Leu Phe Phe Thr Ser Asn Asp Thr Ser Asp Tyr Lys
325 330 335

Leu Asn Asp Gln Arg Leu Ala Thr Ala Thr Leu Asn Leu Asn Val Asp
340 345 350

Asp Asn Gly Val Ser Ile Lys Ser Val Asp Asn Tyr Gln Val Leu Phe
355 360 365

Glu Gly Asp Gly Phe His Tyr Gln Thr Tyr Glu Gln Phe Ala Asn Gly
370 375 380

Lys Asp Arg Glu Asn Asp Asp Tyr Cys Leu Arg Asp Pro His Val Val
385 390 395 400

Gln Leu Glu Asn Gly Asp Arg Tyr Leu Val Phe Glu Ala Asn Thr Gly
405 410 415

Thr Glu Asp Tyr Gln Ser Asp Asp Gln Ile Tyr Asn Trp Ala Asn Tyr
420 425 430

Gly Gly Asp Asp Ala Phe Asn Ile Lys Ser Ser Phe Lys Leu Leu Asn
435 440 445

Asn Lys Lys Asp Arg Glu Leu Ala Gly Leu Ala Asn Gly Ala Leu Gly
450 455 460

Ile Leu Lys Leu Thr Asn Asn Gln Ser Lys Pro Lys Val Glu Glu Val
465 470 475 480

Tyr Ser Pro Leu Val Ser Thr Leu Met Ala Cys Asp Glu Val Xaa Xaa
485 490 495

Lys Leu Gly Asp Lys Tyr Tyr Leu Phe Ser Val Thr Arg Val Ser Arg
500 505 510

Gly Ser Asp Arg Glu Leu Thr Ala Lys Asp Asn Thr Ile Val Gly Asp
515 520 525

Asn Val Ala Met Ile Gly Tyr Val Ser Asp Ser Leu Met Gly Lys Tyr
530 535 540

Lys Pro Leu Asn Asn Ser Gly Val Val Leu Thr Ala Ser Val Pro Ala
545 550 555 560

Asn Trp Arg Thr Ala Thr Tyr Ser Tyr Tyr Ala Val Pro Val Ala Gly

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

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<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 agtttggtag taatag 26

<210> 14
<211> 24
<212> DNA
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<220>
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<400> 14
gtgatacatt tccattatta tcag 24

<210> 15
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<212> DNA
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<220>
<223> Description of Artificial Sequence:primer

<400> 15
ctattactac caaacttatg atcatg 26

<210> 16
<211> 38

<212> DNA
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<220>
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ccatggccat ggtagaacgc aaggaacata aaaaaatg 38

<210> 17
<211> 38
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<400> 17
agatctagat ctgttaaatc gacgtttgtt aatttctg 38

<210> 18
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<212> DNA
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<400> 18
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<210> 19
<211> 23
<212> DNA
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<220>
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<400> 19
gtngcnswnc cnswccayts ytg 23

<210> 20
<211> 22

<212> DNA
<213> Artificial Sequence

<220>
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<400> 20
gaatgtaggt ccaatttttg gc 22

<210> 21
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<212> DNA
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<220>
<223> Description of Artificial Sequence:primer

<400> 21
cctgtccgaa catcttgaac tg 22

<210> 22
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<212> DNA
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<400> 22
arraanswng gngcvmangt nsw 23

<210> 23
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<212> DNA
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<400> 23
tayaayggng tngcngargt naa 23

<210> 24
<211> 22

<212> DNA
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<220>
<223> Description of Artificial Sequence:primer

<400> 24
ccgaccatct tgtttgatta ac 22

<210> 25
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<220>
<223> Description of Artificial Sequence:primer

<400> 25
aaytataayg gygttgcryg aagt 24

<210> 26
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<212> DNA
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<220>
<223> Description of Artificial Sequence:primer

<400> 26
taccgnwsnc tacttcaact t 21