

Sialyltransferases and their use in producing sialylated oligosaccharides

- 5 The present invention relates to sialyltransferases, their use in producing sialylated oligosaccharides, and to the use of said sialylated oligosaccharides in providing nutritional formulations.

Background

- 10 More than 150 structurally distinct human milk oligosaccharides (HMOs) have been identified to date. Although HMOs represent only a minor amount of total human milk nutrients, their highly beneficial effect on the development of breast fed infants became evident over the past decades.

- Up to 20 % of the total HMO content in human milk is acidic. Thus, these HMO
15 molecules possess at least one sialic acid moiety. While only 3 % of the sialic acid contained in human milk is available in free form, 23 % and 74 % are bound to (glyco-)proteins and oligosaccharides, respectively. The most common member of the sialic acid family is *N*-acetylneuraminic acid (Neu5Ac). As part of an oligomeric saccharide, *N*-acetylneuraminic acid often accounts for the saccharide's biological
20 activity.

- Sialylated HMOs (SHMOs) were observed to support the resistance to pathogens as well as gut. Interestingly, recent studies further demonstrated the protective effect of long-chained SHMO against necrotizing enterocolitis, which is one of the most common and lethal diseases in preterm infants. In addition, SHMOs are
25 believed to support an infant's brain development and its cognitive capabilities.

Although extensive variations in the HMO profile between different donors hamper an absolute quantification of acidic oligosaccharides, especially affecting the

structural isomers of sialyllacto-*N*-tetraose, the most abundant acidic HMOs are 3'-sialyllactose (3'-SL), 6'-sialyllactose (6'-SL), sialyllacto-*N*-tetraose a (LST-a), sialyllacto-*N*-tetraose b (LST-b), sialyllacto-*N*-tetraose c (LST-c) and disialyllacto-*N*-tetraose (DSLNT).

- 5 Regarding the structural complexity of the sialylated HMOs (Fig. 1), their chemical or (chemo-)enzymatic syntheses are challenging and associated with extensive difficulties, e.g. control of stereochemistry, formation of specific linkages, availability of feedstocks. Finally, despite the assembly of such synthesizing processes was successful for some SHMO, their dearness and unsatisfying yields limits the cost-efficient production of sialylated HMOs for commercial purposes.
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Generally speaking, metabolic engineering of microorganisms to produce HMOs represents the most promising approach for producing HMOs in an industrial scale, and was already developed for 2'-fucosyllactose, 3-fucosyllactose and 3'-sialyllactose.

- 15 Nevertheless, engineering biosynthetic pathways for the production of HMOs is often limited by the specificity and activity of the glycosyltransferases which are involved in the biosynthesis of the desired HMO, e.g. fucosyl-, galactosyl-, *N*-acetylglucosaminyl- or sialyltransferases, especially within a heterologous expression system such as a recombinant bacterial cell.
- 20 Unfortunately, genes encoding human sialyltransferases are barely expressed in prokaryotic microorganisms. Thus, these genes and enzymes are inapplicable in biotechnological processes using genetically engineered bacterial strains such as *Escherichia coli* for producing sialylated HMOs.

- Several sialyltransferases (SiaTs) from bacterial species have been identified and characterized so far, e. g. from *Neisseria*, *Campylobacter*, *Pasteurella*, *Helicobacter* and *Photobacterium*, as well as from mammals and viruses. Sialyltransferases have been generally classified into six glycosyltransferase (GT) families, based on protein sequence similarities. Therein, all eukaryotic and viral sialyltransferases are grouped into the GT family 29, whereas bacterial SiaTs are comprised in the groups
- 25 GT4, GT38, GT42, GT52 or GT80. Besides, sialyltransferases and polysialyltrans-
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ferases can be distinguished due to the linkages that they form, e. g. into α -2,3-, α -2,6- and α -2,8-sialyltransferases. All these sialyltransferases transfer the sialic acid residue from cytidine 5'-monophosphate sialic acid (e. g. CMP-Neu5Ac) to a variety of acceptor molecules, usually galactose- (Gal), *N*-acetylgalactosamine-
5 (GalNAc) or *N*-acetylglucosamine (GlcNAc) moieties or another sialic acid (Sia) moieties.

Several bacterial sialyltransferases were well characterized in the past and are already proven to be suitable for the production of 3'-SL or 6'-SL. Hardly any knowledge could have been achieved about sialyltransferases enabling the
10 synthesis of sialylated penta- or hexasaccharides such as LST-a, LST-b or DSLNT, thereby limiting the establishment of a production process for any one of these SHMOs. As a consequence, the unavailability of highly pure amounts of these desired oligosaccharides impedes an extensive scientific evaluation of their health beneficial properties.

15 Therefore, there is a need for a cost-efficient process for producing one or more SHMOs, especially tetrasaccharides, pentasaccharides and hexasaccharides possessing one or two sialic acid residues, in high amounts and high purity.

Summary

20 The object is solved, inter alia, by the identification and characterization of new sialyltransferases and their use in the production of sialylated human milk oligosaccharides by means of whole cell fermentation or biocatalysis.

According to a first aspect, provided is a method for producing sialylated oligo-
saccharides, wherein a genetically engineered cell is used for producing said
25 sialylated oligosaccharide. Said genetically engineered cell comprises at least one heterologous sialyltransferase which is capable of transferring a sialic acid residue from a donor substrate to an acceptor molecule, wherein said acceptor molecule is selected from the group consisting of lactose, LNT-II and human milk oligosaccharides.

According to a second aspect, provided is a genetically engineered cell for use in a method for producing sialylated oligosaccharides, wherein said genetically engineered cell has been genetically engineered to express a heterologous sialyltransferase which is capable of transferring a sialic acid residue from a donor
5 substrate to an acceptor molecule, wherein said acceptor molecule is selected from the group consisting of lactose, LNT-II and human milk oligosaccharides.

According to a third aspect, provided is a recombinant nucleic acid molecule for expressing a heterologous sialyltransferase when propagated in a cell, wherein said sialyltransferase is capable of transferring a sialic acid residue from a donor
10 substrate to an acceptor molecule, wherein said acceptor molecule is selected from the group consisting of lactose, LNT-II and human milk oligosaccharides.

According to a fourth aspect, provided are sialyltransferases being capable of transferring a sialic acid residue from a donor substrate to an acceptor molecule, wherein said acceptor molecule is selected from the group consisting of lactose,
15 LNT-II and human milk oligosaccharides.

According to a fifth aspect, provided is the use of a sialyltransferase being capable of transferring a sialic acid residue from a donor substrate to an acceptor molecule, wherein said acceptor molecule is selected from the group consisting of lactose, LNT-II and human milk oligosaccharides, for producing sialylated oligosaccharides.

20 According to a sixth aspect, provided is a method for producing sialylated oligosaccharides by *in vitro* biocatalysis, wherein a sialyltransferase is used, said sialyltransferase being capable of transferring a sialic acid residue from a donor substrate to an acceptor molecule, wherein said acceptor molecule is selected from the group consisting of lactose, LNT-II and human milk oligosaccharides.

25 According to a seventh aspect, provided are sialylated oligosaccharides being produced by a method according to the first aspect or by a method according to the sixth aspect.

According to an eighth aspect, provided is the use of sialylated oligosaccharides according to the seventh aspect for manufacturing a nutritional composition.

According to a ninth aspect, provided is a nutritional composition containing at least one sialylated oligosaccharide according to the seventh aspect.

According to a tenth aspect, provided is an infant formula containing at least one sialylated human milk oligosaccharide.

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Brief description of the drawings

FIG. 1 depicts the chemical structures of the most abundant acidic oligosaccharides in human milk: 3'-SL (A), 6'-SL (B), LST-a (C), LST-b (D), LST-c (E), and DSLNT (F).

10 FIG. 2 shows the maps of sialyltransferase gene overexpressing plasmids pDEST14 and pET11a.

FIG. 3 shows the *in vivo* production of sialyllacto-*N*-tetraose-a and sialyllacto-*N*-tetraose-b (marked by arrows) due to the overexpression of suitable sialyltransferases in that the separation of the intracellular fraction of *siaT9*- or *siaT19*-overexpressing *E. coli* BL21(DE3) #2130 cells by thin layer chromatography is depicted.

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Detailed description

In an attempt to identify sialyltransferases which are suitable for use in a process of manufacturing a sialylated HMO, nucleic acid databases and protein databases were searched. One hundred putative sialyltransferases were identified by means of sequence similarities to known glycosyltransferases. Said putative sialyltransferases were assessed for sialyltransferase activity.

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According to the first aspect, provided is a method for producing a sialylated oligosaccharide, the method comprising the steps of

25 a) providing at least one genetically engineered cell which comprises a heterologous sialyltransferase, said heterologous sialyltransferase being

- capable of possessing an α -2,3-sialyltransferase activity and/or an α -2,6-sialyltransferase activity for transferring a sialic residue from a CMP-activated form as donor substrate to an acceptor molecule selected from the group consisting of lactose, LNT-II and human milk oligosaccharides;
- 5 b) cultivating the at least one genetically engineered cell in a fermentation broth and under conditions permissive for the production of said sialylated oligosaccharide; and
- c) recovering said sialylated oligosaccharide.

The method is a method for producing a sialylated oligosaccharide.

- 10 The term "oligosaccharide" as used herein refers to polymers of monosaccharide residues, wherein said polymers comprise at least three monosaccharide residues, but no more than 10 monosaccharide residues, preferably no more than 7 monosaccharide residues. The oligosaccharides are either a linear chain of monosaccharides or are branched. In addition, the monosaccharide residues of the
- 15 oligosaccharides may feature a number of chemical modifications. Accordingly, the oligosaccharides may comprise one or more non-saccharide moieties.

- The term "sialylated oligosaccharide" as used herein refers to oligosaccharides comprising one or more sialic acid residues. In a preferred embodiment, the sialic acid residue is an *N*-acetylneuraminic acid (Neu5Ac) residue. The *N*-acetyl-
- 20 neuraminic acid residue is typically transferred from CMP-Neu5Ac as donor substrate to an acceptor molecule.

- The method for producing a sialylated oligosaccharide comprises the step of providing a genetically engineered cell comprising a heterologous sialyltransferase which is capable of possessing an α -2,3-sialyltransferase activity and/or an
- 25 α -2,6-sialyltransferase activity.

The genetically engineered cell is a prokaryotic cell or a eukaryotic cell. Preferably, the genetically engineered cell is a microbial cell. Appropriate microbial cells include yeast cells, bacterial cells, archaeobacterial cells, algae cells, and fungal cells.

- In an additional and/or alternative embodiment, the microbial cell is a prokaryotic
- 30 cell, preferably a bacterial cell, more preferably a bacterial cell selected from the

group consisting of *Bacillus*, *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Bifidobacterium*, *Sporolactobacillus* spp., *Micromonospora* spp., *Micrococcus* spp., *Rhodococcus* spp., and *Pseudomonas*. Suitable bacterial species are *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus coagulans*, *Bacillus thermophilus*, *Bacillus*
 5 *laterosporus*, *Bacillus megaterium*, *Bacillus mycoides*, *Bacillus pumilus*, *Bacillus lentus*, *Bacillus cereus*, *Bacillus circulans*, *Bifidobacterium longum*, *Bifidobacterium infantis*, *Bifidobacterium bifidum*, *Citrobacter freundii*, *Clostridium cellulolyticum*, *Clostridium ljungdahlii*, *Clostridium autoethanogenum*, *Clostridium acetobutylicum*, *Corynebacterium glutamicum*, *Enterococcus faecium*, *Enterococcus thermophiles*,
 10 *Escherichia coli*, *Erwinia herbicola* (*Pantoea agglomerans*), *Lactobacillus acidophilus*, *Lactobacillus salivarius*, *Lactobacillus plantarum*, *Lactobacillus helveticus*, *Lactobacillus delbrueckii*, *Lactobacillus rhamnosus*, *Lactobacillus bulgaricus*, *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus casei*, *Lactobacillus reuteri*, *Lactobacillus jensenii*, *Lactococcus lactis*, *Pantoea citrea*, *Pectobacterium*
 15 *carotovorum*, *Propionibacterium freudenreichii*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Streptococcus thermophiles* and *Xanthomonas campestris*.

In an alternative embodiment, the eukaryotic cell is a yeast cell, an insect cell, a plant cell or a mammalian cell. The yeast cell is preferably selected from the group
 20 consisting of *Saccharomyces* sp., in particular *Saccharomyces cerevisiae*, *Saccharomycopsis* sp., *Pichia* sp., in particular *Pichia pastoris*, *Hansenula* sp., *Kluyveromyces* sp., *Yarrowia* sp., *Rhodotorula* sp., and *Schizosaccharomyces* sp.

The genetically engineered cell has been genetically engineered to comprise a heterologous sialyltransferase.

25 The term "genetically engineered" as used herein refers to the modification of the cell's genetic make-up using molecular biological methods. The modification of the cell's genetic make-up may include the transfer of genes within and/or across species boundaries, inserting, deleting, replacing and/or modifying nucleotides, triplets, genes, open reading frames, promoters, enhancers, terminators and other
 30 nucleotide sequences mediating and/or controlling gene expression. The modification of the cell's genetic make-up aims to generate a genetically modified organism possessing particular, desired properties. Genetically engineered cells

can contain one or more genes that are not present in the native (not genetically engineered) form of the cell. Techniques for introducing exogenous nucleic acid molecules and/or inserting exogenous nucleic acid molecules (recombinant, heterologous) into a cell's hereditary information for inserting, deleting or altering the nucleotide sequence of a cell's genetic information are known to the skilled artisan. Genetically engineered cells can contain one or more genes that are present in the native form of the cell, wherein said genes are modified and re-introduced into the cell by artificial means. The term "genetically engineered" also encompasses cells that contain a nucleic acid molecule being endogenous to the cell, and that has been modified without removing the nucleic acid molecule from the cell. Such modifications include those obtained by gene replacement, site-specific mutations, and related techniques.

The genetically engineered cell comprises a heterologous sialyltransferase.

The term "sialyltransferase" as used herein refers to polypeptides being capable of possessing sialyltransferase activity. "Sialyltransferase activity" refers to the transfer of a sialic acid residue, preferably of an *N*-acetylneuraminic acid (Neu5Ac) residue, from a donor substrate to an acceptor molecule. The term "sialyltransferase" comprises functional fragments of the sialyltransferases described herein, functional variants of the sialyltransferases described herein, and functional fragments of the functional variants. "Functional" in this regard means that the fragments and/or variants possess sialyltransferase activity. Functional fragments of a sialyltransferase encompass truncated versions of a sialyltransferase as encoded by it naturally occurring gene, which truncated version is capable of possessing sialyltransferase activity. Examples of truncated versions are sialyltransferases which do not comprise a so-called leader sequence which typically directs the polypeptide to a specific subcellular localization. Typically, such leader sequences are removed from the polypeptide during its subcellular transport, and are also absent in the naturally occurring mature sialyltransferase.

The heterologous sialyltransferase is capable of transferring a sialic acid residue from a donor substrate to an acceptor molecule. The term "capable of" with respect to the heterologous sialyltransferase refers to the sialyltransferase activity of the heterologous sialyltransferase and the provision that suitable reaction conditions are

required for the heterologous sialyltransferase to possess its enzymatic activity. In the absence of suitable reaction conditions, the heterologous sialyltransferase does not possess its enzymatic activity, but retains its enzymatic activity and possesses its enzymatic activity when suitable reaction conditions are restored. Suitable

5 reaction conditions include the presence of a suitable donor substrate, the presence of suitable acceptor molecules, the presence of essential cofactors such as – for example – monovalent or divalent ions, a pH value in an appropriate range, a suitable temperature and the like. It is not necessary that the optimum values for each and every factor effecting the enzymatic reaction of the heterologous

10 sialyltransferase is met, but the reaction conditions have to be such that the heterologous sialyltransferase performs its enzymatic activity. Accordingly, the term “capable of” excludes any conditions upon which the enzymatic activity of the heterologous sialyltransferase has been irreversibly impaired, and also excluded exposure of the heterologous sialyltransferase to any such condition. Instead,

15 “capable of” means that the sialyltransferase is enzymatically active, i.e. possesses its sialyltransferase activity, if permissive reactions conditions (where all requirements being necessary for the sialyltransferase to perform its enzymatic activity) are provided to the sialyltransferase.

Sialyltransferases can be distinguished on the type of sugar linkage they form. As

20 used herein, the terms “ α -2,3-sialyltransferase” and “ α -2,3-sialyltransferase activity” refer to polypeptides and their enzymatic activity which add a sialic acid residue with an alpha-2,3 linkage to galactose or a galactose residue of the acceptor molecule. Likewise, the terms “ α -2,6-sialyltransferase” and “ α -2,6-sialyltransferase activity” refer to polypeptides and their enzymatic activity which add a sialic acid residue with

25 an alpha-2,6 linkage to galactose, *N*-acetylgalactosamine and/or *N*-acetylglucosamine, a galactose residue or a *N*-acetylgalactosamine residue and/or a *N*-acetylglucosamine residue of an acceptor molecule. Likewise, the terms “ α -2,8-sialyltransferase” and “ α -2,8-sialyltransferase activity” refer to polypeptides and their enzymatic activity which add a sialic acid residue with an alpha-2,8 linkage to

30 galactose, *N*-acetylgalactosamine and/or *N*-acetylglucosamine, a galactose residue or a *N*-acetylgalactosamine residue and/or a *N*-acetylglucosamine residue of an acceptor molecule.

The term "heterologous" as used herein refers to a polypeptide, amino acid sequence, nucleic acid molecule or nucleotide sequence that is foreign to a cell or organism, i.e. to a polypeptide, amino acid sequence, nucleic acid molecule or nucleotide sequence that does not naturally occurs in said cell or organism. A

5 "heterologous sequence" or a "heterologous nucleic acid" or "heterologous polypeptide", as used herein, is one that originates from a source foreign to the particular host cell (e.g. from a different species), or, if from the same source, is modified from its original form. Thus, a heterologous nucleic acid operably linked to a promoter is from a source different from that from which the promoter was

10 derived, or, if from the same source, is modified from its original form. The heterologous sequence may be stably introduced, e.g. by transfection, transformation, conjugation or transduction, into the genome of the host microbial host cell, thus representing a genetically modified host cell. Techniques may be applied which will depend on the host cell the sequence is to be introduced. Various

15 techniques are known to a person skilled in the art and are, e.g., disclosed in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989). Accordingly, a "heterologous polypeptide" is a polypeptide that does not naturally occur in the cell, and a "heterologous sialyltransferase" is a sialyltransferase that does not naturally

20 occur in the cell.

The heterologous sialyltransferase is capable of transferring a sialic acid residue, e.g. a *N*-acetylneuraminic acid (Neu5Ac) residue, from a donor substrate, e.g. CMP-Neu5Ac, to an acceptor molecule. The acceptor molecule is lactose, lacto-*N*-triose II (LNT-II) or an oligosaccharide selected from the group consisting of human milk

25 oligosaccharides.

In an additional and/or alternative embodiment, the acceptor molecule is a human milk oligosaccharide selected from the group consisting of trisaccharides, tetrasaccharides and pentasaccharides.

In an additional and/or alternative embodiment, the acceptor molecule is a human

30 milk oligosaccharide selected from the group consisting of lacto-*N*-tetraose, lacto-*N*-neotetraose, LST-a and LST-b.

In an embodiment, the heterologous sialyltransferase is selected from the group consisting of

- I. polypeptides comprising or consisting of an amino acid sequence as represented by any one of SEQ ID NOs: 1 to 33;
- 5 II. polypeptides comprising or consisting of an amino acid sequence having a sequence similarity of at least 80% to any one of the amino acid sequences as represented by any one of SEQ ID NOs: 1 to 33; and
- III. fragments of any one of the polypeptides of I. and II.

In an additional and/or alternative embodiment, the genetically engineered cell has
10 been transformed to contain a nucleic acid molecule which comprises a nucleotide sequence encoding the heterologous sialyltransferase. Preferably, the nucleotide sequence is selected from the group consisting of

- i. nucleotide sequences encoding a polypeptide as represented by any one of SEQ ID NOs: 1 to 33;
- 15 ii. nucleotide sequences as represented by any one of SEQ ID NOs: 34 to 66;
- iii. nucleotide sequences having at least 80% sequence similarity to one of the nucleotide sequences to one of the nucleotide sequences encoding a polypeptide as represented by any one of SEQ ID NOs: 1 to 33;
- iv. nucleotide sequences having a sequence similarity of at least 80 % to any one
20 of the nucleotide sequences represented by SEQ ID NOs: 34 to 66;
- v. nucleotide sequences which are complementary to any one of the nucleotide sequences of i., ii., iii. and iv; and
- vi. fragments of any one of the nucleotide sequences of i., ii., iii., iv. and v.

The expression "any one of SEQ ID NOs: 1 to 33" refers to any one of the group
25 consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13. SEQ ID NO: 14. SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, and SEQ ID NO: 33. The same principle
30 applies to the expression "any one of SEQ ID NOs: 34 to 66". Generally speaking,

the expression "any one of SEQ ID NOs: X to Z", wherein "X" and "Z" represent a natural number, refers to all sequences (nucleotide sequences or amino acid sequences) represented by any one of the "SEQ ID NOs" comprising an identification number from X to Z.

- 5 In addition, the genetically engineered cell has been genetically engineered to express the nucleotide sequence encoding the heterologous sialyltransferase. To this end, the nucleotide sequence encoding the heterologous sialyltransferase is operably linked to at least one expression control effecting transcription and/or translation of said nucleotide sequence encoding the heterologous sialyltransferase
10 in the genetically engineered cell.

The term "operably linked" as used herein, refers to a functional linkage between the nucleotide sequence encoding the heterologous sialyltransferase and a second nucleotide sequence, the nucleic acid expression control sequence (such as promoter, operator, enhancer, regulator, array of transcription factor binding sites,
15 transcriptional terminator, ribosome binding site), wherein the expression control sequence affects transcription and/or translation of the nucleic acid corresponding to the nucleotide sequence encoding the heterologous sialyltransferase. Accordingly, the term "promoter" designates DNA sequences which usually "precede" a gene in a DNA polymer and provide a site for initiation of the
20 transcription into mRNA. "Regulator" DNA sequences, also usually "upstream" of (i.e., preceding) a gene in a given DNA polymer, bind proteins that determine the frequency (or rate) of transcriptional initiation. Collectively referred to as "promoter/regulator" or "control" DNA sequence, these sequences which precede a selected gene (or series of genes) in a functional DNA polymer cooperate to
25 determine whether the transcription (and eventual expression) of a gene will occur. DNA sequences which "follow" a gene in a DNA polymer and provide a signal for termination of the transcription into mRNA are referred to as transcription "terminator" sequences.

In an embodiment, the genetically engineered cell comprises a heterologous
30 sialyltransferase being capable of possessing α -2,3-sialyltransferase activity, and the human milk oligosaccharide is LNT. The thus produced sialylated oligosaccharide is LST-a.

In an additional and/or alternative embodiment, the heterologous sialyltransferase being capable of possessing α -2,3-sialyltransferase activity is selected from the group consisting of

- I. polypeptides comprising or consisting of an amino acid sequence as
5 represented by any one of SEQ ID NOs: 1 to 27;
 - II. polypeptides comprising or consisting of an amino acid sequence having a similarity of at least 80% to any of the amino acid sequences as represented by any one of SEQ ID NOs: 1 to 27; and
 - III. fragments of any one of the polypeptides of I. and II.
- 10 In an additional and/or alternative embodiment, the genetically engineered cell comprises a recombinant or synthetic nucleic acid molecule which comprises at least one nucleotide sequence encoding said heterologous sialyltransferase being capable of possessing α -2,3-sialyltransferase activity, wherein said at least one nucleotide sequence is selected from the group consisting of
- 15 i. nucleotide sequences encoding a polypeptide as represented by any one of SEQ ID NOs: 1 to 27;
 - ii. nucleotide sequences as represented by any one of SEQ ID NOs: 34 to 60;
 - iii. nucleotide sequences having at least 80% sequence similarity to one of the nucleotide sequences encoding a polypeptide as represented by any one of
20 SEQ ID NOs: 1 to 27;
 - iv. nucleotide sequences having a sequence similarity of at least 80 % to any one of the nucleotide sequences represented by SEQ ID NOs: 34 to 60;
 - v. nucleotide sequences which are complementary to any one of the nucleotide sequences of i., ii., iii. and iv; and
 - 25 vi. fragments of any one of the nucleotide sequences of i., ii., iii., iv. and v.

- In an additional and/or alternative embodiment, the heterologous sialyltransferase being capable of possessing α -2,3-sialyltransferase activity has a relative efficacy of at least 100-fold, at least 200-fold, at least 300-fold, at least 1000-fold, at least 10,000-fold, as compared to the relative efficacy of SiaT16 as represented by SEQ
- 30 ID NO: 27 by means of quantitative analysis of LNT sialylation using LC-MS/MS following the method as described in example 5.

In another embodiment, the heterologous sialyltransferase is capable of possessing α -2,6-sialyltransferase activity, and the human milk oligosaccharide is LNT. The thus produced sialylated oligosaccharide is LST-b.

- In an additional embodiment, the heterologous sialyltransferase being capable of
- 5 possessing α -2,6-sialyltransferase activity is selected from the group consisting of
- I. polypeptides comprising or consisting of an amino acid sequence as represented by any one of SEQ ID NOs: 28 to 33;
 - II. polypeptides comprising or consisting of an amino acid sequence having a similarity of at least 80% to any of the amino acid sequences as represented by
 - 10 any one of SEQ ID NOs: 28 to 33; and
 - III. fragments of any one of the polypeptides of I. and II.

- In an additional and/or alternative embodiment, the genetically engineered cell comprises a recombinant or synthetic nucleic acid molecule which comprises at
- 15 least one nucleotide sequence encoding said heterologous sialyltransferase being capable of possessing α -2,6-sialyltransferase activity, wherein said at least one nucleotide sequence is selected from the group consisting of
- i. nucleotide sequences encoding a polypeptide as represented by any one of SEQ ID NOs: 28 to 33;
 - 20 ii. nucleotide sequences as represented by any one of SEQ ID NOs: 61 to 66;
 - iii. nucleotide sequences having at least 80% sequence similarity to one of the nucleotide sequences encoding a polypeptide as represented by any one of SEQ ID NOs: 28 to 33;
 - iv. nucleotide sequences having a sequence similarity of at least 80 % to any one
 - 25 of the nucleotide sequences represented by SEQ ID NOs: 61 to 66;
 - v. nucleotide sequences which are complementary to any one of the nucleotide sequences of i., ii., iii. and iv; and
 - vi. fragments of any one of the nucleotide sequences of i., ii., iii., iv. and v.

- In an additional and/or alternative embodiment, the heterologous sialyltransferase
- 30 being capable of possessing α -2,3-sialyltransferase activity has a relative efficacy of at least 100-fold, more preferably of at least 200-fold, most preferably of at least 300-fold, as compared to the relative efficacy of SiaT5 as represented by SEQ ID

NO: 33 by means of quantitative analysis of LNT sialylation using LC-MS/MS following the method as described in example 5.

In an additional and/or alternative embodiment, the at least one genetically engineered cell possesses an increased production of one or more nucleotide-activated sugars selected from the group consisting of CMP-*N*-acetylneuraminic acid, UDP-*N*-acetylglucosamine, UDP-galactose and GDP-fucose. Preferably, the at least one genetically engineered cell has been further genetically engineered to possess an increased production of one or more of said nucleotide-activated sugars. The production of the at least one of said nucleotide activated sugars is increased in the further genetically engineered cell as compared to the production of the same nucleotide-activated sugar(s) in the progenitor cell of the further genetically engineered cell prior to being further genetically engineered to possess an increased production of at least one of said nucleotide-activated sugars.

In an additional and/or alternative embodiment, the at least one cell has been further genetically engineered to overexpress one or more genes encoding for a polypeptide being capable of possessing an enzymatic activity selected from the group consisting of L-glutamine:D-fructose-6-phosphate aminotransferase, *N*-acetylglucosamine-1-phosphate uridylyltransferase, glucosamine-1-phosphate acetyltransferase, phosphoglucosamine mutase, glucosamine-6-phosphate-*N*-acetyltransferase, *N*-acetylglucosamine-2-epimerase, UDP-*N*-acetylglucosamine-2-epimerase, sialic acid synthase, phosphoenolpyruvate synthase, CMP-sialic acid synthetase, UDP-galactose-4-epimerase, galactose-1-phosphate uridylyltransferase, phosphoglucomutase, glucose-1-phosphate uridylyltransferase, phosphomannomutase, mannose-1-phosphate guanosyltransferase, GDP-mannose-4,6-dehydratase, GDP-L-fucose synthase and fucose kinase/L-fucose-1-phosphate-guanyltransferase. Said overexpression of the one or more genes is an overexpression as compared to the progenitor cell of the further genetically engineered cell prior to being further genetically engineered to possess overexpression of said one or more genes.

Overexpression of one or more of said genes increases the amount of the corresponding enzyme(s) in the genetically engineered cell, and hence increases

the corresponding enzymatic activity in the cell to enhance intracellular production of at least one of said nucleotide-activated sugars.

In an additional and/or alternative embodiment, the at least one genetically engineered cell lacks or possesses a decreased activity of one or more enzymatic activities selected from the group consisting of β -galactosidase activity, gluco-
5 samine-6-phosphate deaminase, *N*-acetylglucosamine-6-phosphate deacetylase, *N*-acetylmannosamine kinase, *N*-acetylmannosamine-6-phosphate epimerase and *N*-acetylneuraminic acid aldolase as compared to the cell prior to be genetically engineered.

10 In an additional and/or alternative embodiment, one or more of the genes encoding a β -galactosidase, a glucosamine-6-phosphate deaminase, a *N*-acetylglucosamine-6-phosphate deacetylase, a *N*-acetylmannosamine kinase, a *N*-acetylmanno-
samine-6-phosphate epimerase and a *N*-acetylneuraminic acid aldolase has/have been deleted from the genome of the genetically engineered cell or the expression
15 of one or more of the genes encoding a β -galactosidase, a glucosamine-6-phosphate deaminase, a *N*-acetylglucosamine-6-phosphate deacetylase, a *N*-acetylmannosamine kinase, a *N*-acetylmannosamine-6-phosphate epimerase and a *N*-acetylneuraminic acid aldolase has/have been inactivated or at least decrease
in the genetically engineered cell by further genetically engineering of cell. The
20 expression of said genes is decreased in the further genetically engineered cell as compared to the progenitor cell of the further genetically engineered cell prior to being further genetically engineered to possess a decreased expression of said genes.

In an additional and/or alternative embodiment, the at least one genetically
25 engineered cell comprises at least one selected from the group consisting of a functional lactose permease, a functional fucose permease and a functional sialic acid transporter (importer), preferably comprises and expresses at least one nucleotide sequence encoding one selected from the group consisting of a
functional lactose permease, a functional fucose permease and a functional sialic
30 acid transporter (importer).

In an additional and/or alternative embodiment, the genetically engineered cell possesses activity of at least one glycosyltransferase selected from the group consisting of a β -1,3-*N*-acetylglucosaminyltransferase, a β -1,3-galactosyltransferase, a β -1,4-galactosyltransferase, a α -2,3-sialyltransferase and a α -2,6-sialyltransferase.

In an additional and/or alternative embodiment, the at least one genetically engineered cell is cultivated in a fermentation broth and under conditions permissive for the production of the sialylated oligosaccharide.

The fermentation broth contains at least one carbon source for the genetically engineered cells. The at least one carbon source is preferably selected from the group consisting of glucose, fructose, sucrose, glycerol, and combinations thereof.

In an additional and/or alternative embodiment, the fermentation broth contains at least one selected from the group consisting of *N*-acetylglucosamine, galactose and sialic acid.

In an additional and/or alternative embodiment, wherein the at least one genetically engineered cell is cultivated in the absence of and/or without addition of one or more selected from the group consisting of *N*-acetylglucosamine, galactose and sialic acid, the at least one genetically engineered cell is cultivated in the presence of lactose, lacto-*N*-triose II (LNT-II) or at least one HMO, preferably an HMO selected from the group consisting of trisaccharides, tetrasaccharides and pentasaccharides, more preferably an HMO selected from the group consisting, LNT and LN_nT.

The method comprises the optional step of recovering the sialylated oligosaccharide that has been produced by the at least one genetically engineered cell during its cultivation in the fermentation broth. The sialylated oligosaccharide can be recovered from the fermentation broth after the genetically engineered cell have been removed, for example by centrifugation, and/or can be recovered from the cells, for example in that the cells are harvested from the fermentation broth by centrifugation, and are subjected to a cell lysis step. Subsequently, the sialylated oligosaccharides can be further purified from the fermentation broth and/or cell

lysates by suitable techniques known to the skilled artisan. Suitable techniques include microfiltration, ultrafiltration, diafiltration, simulated moving bed type chromatography, electrodialysis, reverse osmosis, gel filtration, anion exchange chromatography, cation exchange chromatography, and the like.

- 5 According to a second aspect, provided is a genetically engineered cell for use in a method for producing sialylated oligosaccharides. Said genetically engineered cell and preferred embodiments of said genetically engineered cell has/have been described herein before in connection with the method. Hence, the genetically engineered cell comprises a heterologous sialyltransferase, said heterologous
- 10 sialyltransferase being capable of possessing an α -2,3-sialyltransferase activity and/or an α -2,6-sialyltransferase activity for transferring a sialic acid residue, e.g. *N*-acetylneuraminic acid (Neu5Ac) residue from a nucleotide-activated form as donor substrate, e.g. CMP-Neu5Ac, to an acceptor molecule, wherein the acceptor molecule is selected from the group consisting of lactose, lacto-*N*-triose II and
- 15 human milk oligosaccharides.

- According to the third aspect, provided are recombinant nucleic acid molecules for expressing a sialyltransferase when propagated in a cell, said sialyltransferase being a heterologous sialyltransferase when expressed in the cell. The recombinant nucleic acid molecule(s) comprise(s) a nucleotide sequence encoding a sialyltrans-
- 20 ferase which is capable of transferring a sialic acid residue, e.g. a *N*-acetylneuraminic acid residue, from a donor substrate to an acceptor molecule, wherein said acceptor molecule is selected from the group consisting of lactose, lacto-*N*-triose II and human milk oligosaccharides.

- Preferred embodiments of the nucleotide sequences encoding a sialyltransferase which is capable of transferring a sialic acid residue from a donor substrate to an acceptor molecule, wherein said acceptor molecule is selected from the group consisting of lactose, lacto-*N*-triose II and human milk oligosaccharides, such as preferred nucleotide sequences, are disclosed herein before in connection with the method of producing sialylated oligosaccharides. For example, the sialyltransferase
- 25 is capable of transferring a *N*-acetylneuraminic acid residue from CMP-Neu5Ac to lactose, lacto-*N*-triose II or a human milk oligosaccharide.
- 30

The nucleotide sequence encoding the sialyltransferase is operably linked to at least one expression control sequence. Thus, in an additional and/or alternative embodiment, the recombinant nucleic acid molecule comprises at least one expression control sequence mediating transcription and/or translation of the nucleotide sequence encoding the sialyltransferase when said recombinant nucleic acid molecule is propagated in the cell.

According to the fourth aspect, provided are sialyltransferases being capable of possessing an α -2,3-sialyltransferase activity and/or an α -2,6-sialyltransferase activity transferring a sialic acid residue, e.g. a *N*-acetylneuraminic acid residue from a donor substrate, e.g. CMP-Neu5Ac, to an acceptor molecule, wherein said acceptor molecule is lactose, lacto-*N*-triose II or a human milk oligosaccharide.

In an embodiment, the acceptor molecule is selected from the group consisting of trisaccharides, tetrasaccharides and pentasaccharides. In an additional and/or alternative embodiment, the acceptor molecule is selected from the group consisting of LST-a and LST-b.

In an additional and/or alternative embodiment, the sialyltransferase is selected from the group consisting of

- I. polypeptides comprising or consisting of an amino acid sequence as represented by any one of SEQ ID NOs: 1 to 33;
- II. polypeptides comprising or consisting of an amino acid sequence having a sequence similarity of at least 80% to any one of the amino acid sequences as represented by any one of SEQ ID NOs: 1 to 33; and
- III. fragments of any one of the polypeptides of I. and II.

According to the fifth aspect, provided is the use of the sialyltransferases described herein before and being capable of transferring a sialic acid residue from a donor substrate, e.g. a *N*-acetylneuraminic acid residue from CMP Neu5AC, to an acceptor molecule, wherein said acceptor molecule is lactose, lacto-*N*-triose II or a human milk oligosaccharide, for producing sialylated oligosaccharides.

Said sialyltransferases are capable of transferring a sialic acid residue to an acceptor molecule, said acceptor molecule being a human milk oligosaccharide, thereby producing a sialylated oligosaccharide.

The human milk oligosaccharide may be a neutral oligosaccharide or an acidic oligosaccharide, i.e. a human milk oligosaccharide comprising at least one sialic acid residue.

The sialylated oligosaccharide produced by using the sialyltransferases as described herein before, may be a human milk oligosaccharide or may be a sialylated oligosaccharide not found in naturally occurring human milk.

According to the sixth aspect, provided is a method for producing sialylated oligosaccharides by *in vitro* biocatalysis, wherein a sialyltransferase is used, said sialyltransferase being capable of transferring a sialic acid residue from a donor substrate, e.g. a *N*-acetylneuraminic acid residue from CMP-Neu5Ac, to an acceptor molecule, wherein said acceptor molecule is a human milk oligosaccharide.

The method comprises the steps of:

- providing – in a reaction mixture - a sialyltransferase being capable of transferring a sialic acid residue, preferably *N*-acetylneuraminic acid, from a donor substrate to an acceptor molecule, a donor substrate, and an acceptor molecule;
- allowing the sialyltransferase to transfer the sialic acid residue from the donor substrate to the acceptor molecule to obtain a sialylated oligosaccharide; and
- recovering the sialylated oligosaccharide from the reaction mixture.

According to a seventh aspect, provided are sialylated oligosaccharides being produced by a method according to the first aspect or by a method according to the sixth aspect.

In an embodiment, the sialylated oligosaccharide is a human milk oligosaccharide, preferably a tetrasaccharide, a pentasaccharide or a hexasaccharide, more preferably a sialylated oligosaccharide selected from the group consisting of LST-a, LST-b and DSLNT.

According to the eight aspect, provided is the use of a sialylated oligosaccharide being produced by a whole cell fermentation approach or an in vitro biocatalysis as described herein before for manufacturing a nutritional composition. Said nutritional composition contains at least one sialylated oligosaccharide which has been
5 produced by a method as disclosed herein before.

Thus, according to the ninth aspect, provided is a nutritional composition containing at least one sialylated oligosaccharide which has been produced by a method as disclosed herein before. Preferably, the at least one sialylated oligosaccharide is 3'-sialyllactose, 6'-sialyllactose, LST-a, LST-b, LST-c or DSLNT.

10 In an additional and/or alternative embodiment, the nutritional composition further contains at least one neutral HMO, preferably 2'-FL.

In an additional and/or alternative embodiment, the nutritional composition contains 3-SL, 6-SL and 2'-FL.

15 In an additional embodiment, the nutritional composition is selected from the group consisting of medicinal formulations, infant formula and dietary supplements.

The nutritional composition may be present in liquid form or in solid form including, but not limited to, powders, granules, flakes and pellets.

According to the tenth aspect, provided is an infant formula containing at least one sialylated HMO. Said sialylated HMO is a HMO selected from the group of sialylated
20 oligosaccharides that have been produced by a method as described herein before.

In an embodiment, the at least one sialylated HMO that is contained in the infant formula is selected from the group consisting of 3-SL, 6-SL, LST-a, LST-b, LST-c and DSLNT.

25 In an additional and/or alternative embodiment, the infant formula contains the at least one sialylated HMO and one or more neutral HMOs.

In an additional and/or alternative embodiment, the infant formula contains 3-SL, 6-SL and 2'-FL.

The present invention will be described with respect to particular embodiments and with reference to drawings, but the invention is not limited thereto but only by the claims. Furthermore, the terms first, second and the like in the description and in the claims, are used for distinguishing between similar elements and not necessarily
5 for describing a sequence, either temporally, spatially, in ranking or in any other manner. It is to be understood that the terms so used are interchangeable under appropriate circumstances and that the embodiments of the invention described herein are capable of operation in other sequences than described or illustrated herein.

10 It is to be noticed that the term "comprising", used in the claims, should not be interpreted as being restricted to the means listed thereafter; it does not exclude other elements or steps. It is thus to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not preclude the presence or addition of one or more other features, integers, steps or
15 components, or groups thereof. Thus, the scope of the expression "a device comprising means A and B" should not be limited to devices consisting only of components A and B. It means that with respect to the present invention, the only relevant components of the device are A and B.

Reference throughout this specification to "one embodiment" or "an embodiment"
20 means that a particular feature, structure or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, appearances of the phrases "in one embodiment" or "in an embodiment" in various places throughout this specification are not necessarily all referring to the same embodiment, but may. Furthermore, the particular features,
25 structures or characteristics may be combined in any suitable manner, as would be apparent to one of ordinary skill in the art from this disclosure, in one or more embodiments.

Similarly, it should be appreciated that in the description of exemplary embodiments of the invention, various features of the invention are sometimes grouped together
30 in a single embodiment, figure, or description thereof for the purpose of streamlining the disclosure and aiding in the understanding of one or more of the various inventive aspects. This method of disclosure, however, is not to be interpreted as

reflecting an intention that the claimed invention requires more features than are expressly recited in each claim. Rather, as the following claims reflect, inventive aspects lie in less than all features of a single foregoing disclosed embodiment. Thus, the claims following the detailed description are hereby expressly
5 incorporated into this detailed description, with each claim standing on its own as a separate embodiment of this invention.

Furthermore, while some embodiments described herein include some but not other features included in other embodiments, combinations of features of different embodiments are meant to be within the scope of the invention, and form different
10 embodiments, as would be understood by those in the art. For example, in the following claims, any of the claimed embodiments can be used in any combination.

Furthermore, some of the embodiments are described herein as a method or combination of elements of a method that can be implemented by a processor of a computer system or by other means of carrying out the function. Thus, a processor
15 with the necessary instructions for carrying out such a method or element of a method forms a means for carrying out the method or element of a method. Furthermore, an element described herein of an apparatus embodiment is an example of a means for carrying out the function performed by the element for the purpose of carrying out the invention.

20 In the description and drawings provided herein, numerous specific details are set forth. However, it is understood that embodiments of the invention may be practiced without these specific details. In other instances, well-known methods, structures and techniques have not been shown in detail in order not to obscure an understanding of this description.

25 The invention will now be described by a detailed description of several embodiments of the invention. It is clear that other embodiments of the invention can be configured according to the knowledge of persons skilled in the art without departing from the true spirit or technical teaching of the invention, the invention being limited only by the terms of the appended claims.

Examples

Example 1: Development of an *E. coli* Neu5Ac production strain enabling *in vivo* screening of sialyltransferases using lactose as acceptor

Metabolic engineering included mutagenesis and deletions of specific genes,
5 respectively, and genomic integrations of heterologous genes. The genes *lacZ* and *araA* were inactivated by mutagenesis using mismatch-oligonucleotides as described by Ellis *et al.*, (Proc. Natl. Acad. Sci. USA 98: 6742-6746 (2001)).

Genomic deletions were performed according to the method of Datsenko and Wanner (Proc. Natl. Acad. Sci. USA 97:6640-6645 (2000)). To prevent intracellular
10 degradation of *N*-acetylneuraminic acid the genes encoding *N*-acetylglucosamine-6-phosphate deacetylase (*nagA*) and glucosamine-6-phosphate deaminase (*nagB*) as well as the whole *N*-acetylneuraminic acid catabolic gene cluster, encoding *N*-acetylmannosamine kinase (*nanK*), *N*-acetylmannosamine-6-phosphate epimerase (*nanE*), *N*-acetylneuraminic acid aldolase (*nanA*) and the sialic acid permease
15 (*nanT*) were deleted from the genome of the *E. coli* strain BL21 (DE3) strain. Also genes *wzxC-wcaJ* were deleted. *WcaJ* encodes an UDP-glucose:undecaprenyl phosphate glucose-1-phosphate transferase catalysing the first step in colanic acid synthesis (Stevenson *et al.*, J. Bacteriol. 1996, 178:4885-4893). In addition, the genes *fucI* and *fucK*, coding for L-fucose isomerase and L-fuculose kinase,
20 respectively, were removed.

Genomic integration of heterologous genes was performed by transposition. Either the EZ-Tn5TM transposase (Epicentre, USA) was used to integrate linear DNA-fragments or the hyperactive C9-mutant of the mariner transposase *Himar1* (Lampe
et al., Proc. Natl. Acad. Sci. 1999, USA 96:11428-11433) was employed for
25 transposition. To produce EZ-Tn5 transposomes the gene of interest together with a FRT-site flanked antibiotic resistance marker was amplified with primer 1119 and 1120 (all primers used are listed in table 3 below); the resulting PCR-product carried on both sites the 19-bp Mosaic End recognition sites for the EZ-Tn5 transposase. For integration using *Himar1* transposase expression constructs (operons) of

interest were similarly cloned together with a FRT-site flanked antibiotic resistance marker into the pEcomar vector. The pEcomar vector encodes the hyperactive C9-mutant of the mariner transposase *Himar1* under the control of the arabinose inducible promoter P_{araB} . The expression fragment $\langle P_{tet}-lacY-FRT-aadA-FRT \rangle$ (SEQ ID NO: 67) was integrated by using the EZ-Tn5 transposase. After successful

5 integration of the gene for the lactose importer LacY from *E. coli* K12 TG1 (acc. no. ABN72583) the resistance gene was eliminated from streptomycin resistant clones by the FLP recombinase encoded on plasmid pCP20 (Datsenko and Wanner, Proc. Natl. Acad. Sci. 2000, USA 97:6640-6645), generating strain #534. Besides, the

10 *csc*-gene cluster of *E. coli* W (acc. no. CP002185.1) comprising the genes for sucrose permase, fructokinase, sucrose hydrolase, and a transcriptional repressor (genes *cscB*, *cscK*, *cscA*, and *cscR*, respectively), that enable the strain to grow on sucrose as sole carbon source, was inserted in the genome. This *csc*-cluster was integrated into the genome of the *E. coli* BL21(DE3) strain by transposition using

15 plasmid pEcomar-*cscABKR*. To enhance *de novo* synthesis of UDP-*N*-acetylglucosamine, genes encoding L-glutamine:D-fuctose-6-phosphate aminotransferase (*glmS*), phosphoglucosamine mutase from *E. coli* K-12 substr. MG1655 (*glmM*) and *N*-acetylglucosamine-1-phosphate uridylyltransferase/ glucosamine-1-phosphate acetyltransferase (*glmU*) from *E. coli* K-12 substr. MG1655 (acc. no.

20 NP_418185, NP_417643, NP_418186, respectively) were codon-optimized and obtained by gene synthesis. The operon *glmUM* was cloned under the control of the constitutive tetracyclin promoter P_{tet} while *glmS* was cloned under the constitutive P_{T5} promoter. The transposon cassette $\langle P_{tet}-glmUM-P_{T5}-glmS-FRT-dhfr-FRT \rangle$ (SEQ ID NO: 68), flanked by the inverted terminal repeats specifically recognized by

25 the mariner-like element *Himar1* transposase was inserted from pEcomar-*glmUM-glmS*. Altogether, the described genome modifications were leading to the *E. coli* BL21(DE3) strain #942, which represents the chassis for strain development. Tables 1, 2 and 3 comprise all strains, oligonucleotides used for cloning as well as general plasmids used within this study, respectively.

30 Strain #942 was modified for the production of sialic acid by the genomic integration of the expression cassettes $\langle P_{tet}-glmSm-gna1-FRT-aacC1-FRT \rangle$ (SEQ ID NO: 69), $\langle P_{tet}-slr1975-FRT-cat-FRT \rangle$ (SEQ ID NO: 70), $\langle P_{tet}-neuBC-FRT-kan-FRT \rangle$ (SEQ ID NO: 71) and $\langle P_{tet}-ppsA-FRT-aad1-FRT \rangle$ (SEQ ID NO: 72). All genes were codon-

optimized for expression in *E. coli* and prepared synthetically by GenScript cooperation. GlmSm represents a mutagenized version of GlmS, thus, eliminating the feed-back inhibition by glucosamine-6-phosphate. The gene *gna1* encodes a glucosamine-6-phosphate acetyltransferase originating from *Saccharomyces cerevisiae*. Genes were subcloned as an operon behind the constitutive promotor *Ptet* and fused to the FRT-site flanked gentamycin resistance gene using primers glmSm/gna1_1-8. Similarly, the genes *neuB* (acc. no. AF305571), encoding a sialic acid synthase, and *neuC* (acc. no. AF305571), encoding an UDP-*N*-acetylglucosamine-2-epimerase, both originating from *Campylobacter jejuni*, were subcloned as an operon behind the constitutive promotor *Ptet* and fused to the FRT-site flanked kanamycin resistance gene using primers neuBC_1-6. The gene *slr1975* (acc. no. BAL35720), also cloned behind the constitutive promotor *Ptet* and fused to the FRT-site flanked chloramphenicol resistance gene using primers slr_1-4, encodes a *N*-acetylglucosamine 2-epimerase from *Synechocystis* sp. PCC6803. The gene *ppsA* (acc. no. ACT43527) encoding the phosphoenolpyruvate synthase of *E. coli* BL21(DE3) was similarly cloned for constitutive expression and fused to the FRT-site flanked streptomycin resistance gene using primers ppsA_1-4. The genomic integrations finally led to the Neu5Ac producing strain #1363, which was used for the screening of the sialyltransferases 1 to 26.

Strain	Genotype	Ref.
<i>E. coli</i> BL21(DE3)	F ⁻ <i>ompT hsdSB(rB⁻, mB⁻) gal dcm</i> (DE3)	Merck KGaA, Darmstadt, Germany
<i>E. coli</i> BL21(DE3) #287	<i>E. coli</i> BL21(DE3) Δ <i>lacZ</i>	This study
<i>E. coli</i> BL21(DE3) #534	<i>E. coli</i> BL21(DE3) Δ <i>lacZ</i> Δ <i>ara</i> Δ <i>wcaJ</i> Δ <i>fucIK</i> Δ <i>nagAB</i> harbouring genomic integrations of: <i>galETKM</i> , <i>lacy</i>	This study
<i>E. coli</i> BL21(DE3) #942	<i>E. coli</i> BL21(DE3) Δ <i>lacZ</i> Δ <i>ara</i> Δ <i>wcaJ</i> Δ <i>fucIK</i> Δ <i>nagAB</i> Δ <i>nanKETA::zeo</i> harbouring genomic integrations of: <i>galETKM</i> , <i>lacy</i> , <i>cscBKAR</i> , <i>glmUM-glmS-dhfr</i>	This study
<i>E. coli</i> BL21(DE3) #1363	<i>E. coli</i> BL21(DE3) Δ <i>lacZ</i> Δ <i>ara</i> Δ <i>wcaJ</i> Δ <i>fucIK</i> Δ <i>nagAB</i> Δ <i>nanKETA::zeo</i> harbouring genomic integrations of: <i>galETKM</i> , <i>lacy</i> , <i>cscBKAR</i> , <i>glmUM-glmS-dhfr</i> , <i>glmSm-gna1-aacC1</i> , <i>slr1975-cat</i> , <i>neuBC-kan</i> , <i>ppsA-aad1</i>	This study
<i>E. coli</i> BL21(DE3) #1730	<i>E. coli</i> BL21(DE3) Δ <i>lacZ</i> Δ <i>ara</i> Δ <i>wcaJ</i> Δ <i>fucIK</i> Δ <i>nagAB</i> Δ <i>nanKETA::zeo</i> harbouring genomic integrations of: <i>galETKM</i> , <i>lacy</i> , <i>cscBKAR</i> , <i>glmUM-glmS-dhfr</i> , <i>neuA-nanT-kan</i>	This study
<i>E. coli</i> BL21(DE3) #2130	<i>E. coli</i> BL21(DE3) Δ <i>lacZ</i> Δ <i>ara</i> Δ <i>wcaJ</i> Δ <i>fucIK</i> Δ <i>nagAB</i> Δ <i>nanKETA</i> harbouring genomic integrations of: <i>galETKM</i> , <i>lacY</i> , <i>lgtA-galT-kan</i> , <i>glmUM-glmS-dhfr</i> , <i>wbdO-galE-cat</i> , <i>neuA-nanT-aacC1</i>	This study

Table 1: List of bacterial strains used.

Primer	Sequence 5'-3'	SEQ ID NO:
1119	P-CTGTCTCTTATACACATCTCCTGAAATTGGCCA GATGATTAATTCCTAATTTTTGTTG	74
1120	P-CTGTCTCTTATACACATCTCAGCATTACACGTC TTGAGCGATTGTGTAGG	75
slr_1	CCTGACGACGGTGAGCGATCATTTGTATATCTCC TTCTTAAAGTTAAACAAAATTATTC	76
slr_2	AACCCTGCAACTGCCGGTCTCTTAAATAACTAG CATAACCCCTTGGGGCCTCTAAACG	77
slr_3	AACTTTAAGAAGGAGATACAAATGATCGCTCA CCGTCGTCAGGAAGTGGCTCAACAG	78
slr_4	AGGCCCCAAGGGGTTATGCTAGTTATTTTAAGAG ACCGGCAGTTGCAGGGTTTCGGC	79
glmSm/gna1_1	GCCCCAAGGGGTTATGCTAGTTATTTTATTCCAC GGTCACGGATTCGCCAGGTTACGCGGC	80
glmSm/gna1_2	AGCACCAACGATACCGCACATTTGTATATCTCCT TCTTAAAGTTAAACAAAATTATTTCTAG	81
glmSm/gna1_3	GCGAAATCCGTGACCGTGGAATAAAATAACTAGC ATAACCCCTTGGGGCCTCTAAACGGGTC	82
glmSm/gna1_4	CTTTAAGAAGGAGATACAAATGTGCGGTATCG TTGGTGCTATCGCACAGCGTGATG	83
glmSm/gna1_5	CGTGGAATGCAAATTAGAAAATAGAATAACTAG CATAACCCCTTGGGGCCTCTAAACGG	84
glmSm/gna1_6	TATAAATCCATCGGGTAAGCTCATGGTTCTATC TCCTTCGTTATTCCACGGTCACGGATTTTCG	85
glmSm/gna1_7	ATCCGTGACCGTGGAATAACGAAGGAGATAGAA CCATGAGCTTACCCGATGGATTTTATATAAGG	86
glmSm/gna1_8	CCGTTTAGAGGCCCAAGGGGTTATGCTAGTTAT TCTATTTTCTAATTTGCATTTCCACG	87
ppsA_1	CTGGTTAAGCCTGGCTGAAGTGAAGAAATAAAAT AACTAGCATAACCCCTTGGGGCCTC	88
ppsA_2	TAGAGGCCCAAGGGGTTATGCTAGTTATTTAT TTCTTCAGTTCAGCCAGGCTTAACC	89
ppsA_3	TTTGTTTGGCGTCGAGAAGGAGATAGAACCATGT CCAACAATGGCTCGTCACCGCTGGTG	90
ppsA_4	AAGCACCAGCGGTGACGAGCCATTGTTGGACAT GGTCTATCTCCTTCTCGACGCCAAAC	91
neuBC_1	CTACCCAGAAAGTGTTCAAAGATATTAATAAAAT AACTAGCATAACCCCTTGGGGCCTC	92
neuBC_2	TTTAGAGGCCCAAGGGGTTATGCTAGTTATTTT ATTTAATATCTTTGAACACTTTCTGGG	93
neuBC_3	GATGATGATGTTCTGGATTTTGATTTCTTTCAATT GTATATCTCCTTCTTAAAGTTAAAC	94

Primer	Sequence 5'-3'	SEQ ID NO:
neuBC_4	TTTGTTTAACTTTAAGAAGGAGATATACAAATGAA AGAAATCAAAATCCAGAACATCATC	95
neuBC_5	AGCTGTCTTATGAAGATTTTCGCCTAATCGAAGGA GATACAACCATGAAGAAAAATTCTGTTTATCACCG GC	96
neuBC_6	TGCCGGTGATAAACAGAATTTTCTTCATGGTTGT ATCTCCTTCGATTAGGCCGAAATCTTCATAAGACA GC	97
neuA/nanT_1	TAATTTTGTTTAACTTTAAGAAGGAGATATACATG AGCCTGGCCATTATCCCGGCACGTG	98
neuA/nanT_2	TGCCGGGATAATGGCCAGGCTCATGTATATCTC CTTCTTAAAGTTAAACAAAATTATTTT	99
neuA/nanT_3	AAAATAAGAGCTCGAGTCGAAGGAGATAGAACCA TGAGTACTACAACCCAGAATATCCCG	100
neuA/nanT_4	TAGTACTCATGGTTCTATCTCCTTCGACTCGAGC TCTTATTTTTTCCAGATCTGTTCCAC	101
neuA/nanT_5	AAAACGATTTAGTCAAAACCAAAAGTTAATAAATC GATACTAGCATAACCCCTTGGGGCC	102
neuA/nanT_6	AAGGGGTTATGCTAGTATCGATTTATTAACTTTTG GTTTTGACTAAATCGTTTTTG	103
6192 nst-neuA attB1	GGGGACAAGTTTGTACAAAAAGCAGGCTAGAA GGAGGTATACAAATGGGCCTGAAAAAGCCTGC CTGACCG	104
6193 cst-neuA attB1	GGGGACAAGTTTGTACAAAAAGCAGGCTAGAA GGAGATATACAAATGACCCGCACCCGTATGAAAA ACGAACTG	105
6194 siaT-neuA attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTTTATT TTTTCCAGATCTGTTCCACTTTTTTCAG	106
6559 siaT3 for	AAAAAGCAGGCTAGAAGGAGGTATACAAATGGG CAAAAAAGTGATTATTGCGGGCAACGGCCCGAG CC	107
6560 siaT3 rev	AGGCTCATAATTGTACCTCCTTCGAGGTTTAGTT GATGTTTTTGCTGAATTTGCCATACGCTTCGC	108
6561 neuA (siaT3) for	TGGCAAATTCAGCAAAAACATCAACTAAACCTCG AAGGAGGTACAATTATGAGCCTGGCCATTATCCC	109
6562 pDest (siaT3) rev	TGCCCGCAATAATCACTTTTTTGCCCATTTGTATA CCTCCTTCTAGCCTGCTTTTTTGTAACAACTTG	110
6563 siaT4 for	AAAAGCAGGCTAGAAGGAGGTATACAAATGAATA AGAAACCGCTGATTATTGCTGGCAACGGGCC	111
6564 siaT4 rev	AGGCTCATAATTGTACCTCCTTCGAGGTTTATCT CTTCAGGAATGCTTTAATGATTGACTTTAGCGCC	112
6565 neuA (siaT4) for	ATCATTAAAGCATTCTGAAGAGATAAACCTCGA AGGAGGTACAATTATGAGCCTGGCCATTATC	113

Primer	Sequence 5'-3'	SEQ ID NO:
6566 pDest (siaT4) rev	AGCAATAATCAGCGGTTTCTTATTCATTTGTATAC CTCCTTCTAGCCTGCTTTTTTGTACAAACTTGTG	114
6567 siaT5 for	AAAAGCAGGCTAGAAGGAGGTATACAAATGGGG ACCATTAAAAAGCCCTTAATCATAGCAGGAAATG	115
6568 siaT5 rev	AGGCTCATAATTGTACCTCCTTCGAGGTTTATGC AGCTCCCCAACGGAACTAACTTTTAATGTTGGG	116
6569 neuA (siaT5) for	TAGTTTCCGTTGGGGAGCTGCATAAACCTCGAA GGAGGTACAATTATGAGCCTGGCCATTATCCCG	117
6570 pDest (siaT5) rev	ATTAAGGGCTTTTTAATGGTCCCCATTTGTATAC CTCCTTCTAGCCTGCTTTTTTGTACAAACTTGTG	118
6748 siaT6 for	AAAAGCAGGCTAGAAGGAGGTATACAAATGAGT GAAGAAAACACCCAGTCCATTATTAACGAC	119
6749 siaT6 rev	AGGCTCATAATTGTACCTCCTTCGAGGTTTCAGAC AGCAATACAGACACCCGTTTCGCAATTCGGCAG	120
6750 neuA (siaT6) for	AAACGGGTGTCTGTATTGCTGTCTGAACCTCGAA GGAGGTACAATTATGAGCCTGGCCATTATCCCG	121
6751 pDest (siaT6) rev	ATGGACTGGGTGTTTTCTTCACTCATTTGTATAC CTCCTTCTAGCCTGCTTTTTTGTACAAACTTGTG	122
6752 siaT7 for	AAAAGCAGGCTAGAAGGAGGTATACAAATGACCA TTTACCTGGACCCGGCGTCTCTGCCGACCC	123
6753 siaT7rev	AGGCTCATAATTGTACCTCCTTCGAGGTTTACAG TTGTTTCAGAGAATCCCAGAAGATAATTTGGC	124
6754 neuA (siaT7) for	TTCTGGGATTCTCTGAAACAACGTAAACCTCGA AGGAGGTACAATTATGAGCCTGGCCATTATCCC	125
6755 pDest (siaT7) rev	ACGCCGGGTCCAGGTAAATGGTCATTTGTATACC TCCTTCTAGCCTGCTTTTTTGTACAAACTTGTG	126
6483 siaT8 for	AAAGCAGGCTAGAAGGAGGTATACAAATGGGCT GTAATAGCGACTCCAACCACAACAACCTCCGACG	127
6484 siaT8 rev	AGGCTCATAATTGTACCTCCTTCGAGGTTTATTG CAGGTCCGAGATCAGTTTCACATCATTACGG	128
6485 neuA (siaT8) for	TGAAACTGATCTCGGACCTGCAATAAACCTCGAA GGAGGTACAATTATGAGCCTGGCCATTATCCC	129
6486 pDest (siaT8) rev	TGGTTGGAGTCGCTATTACAGCCCATTTGTATAC CTCCTTCTAGCCTGCTTTTTTGTACAAACTTGTG	130
6487 siaT9 for	AAAGCAGGCTAGAAGGAGGTATACAAATGAACAA CGACAACTCCACGACCACCAACAATAACGC	131
6488 siaT9 rev	CTCATAATTGTACCTCCTTCGAGGTTTAAATGTCA GAGATCAGTTTAATATTATCGCGGTTAATCAG	132
6489 neuA (siaT9) for	ATATTAACTGATCTCTGACATTTAAACCTCGAAG GAGGTACAATTATGAGCCTGGCCATTATCCCG	133
6490 pDest (siaT9) rev	TGGTCGTGGAGTTGTCGTTGTTTCAATTTGTATACC TCCTTCTAGCCTGCTTTTTTGTACAAACTTGTG	134
6491 siaT10 for	AAAGCAGGCTAGAAGGAGGTATACAAATGAAAAC GATTACCCTGTATCTGGACCCGGCGTCCCTGCC	135

Primer	Sequence 5'-3'	SEQ ID NO:
6492 siaT10 rev	AGGCTCATAATTGTACCTCCTTCGAGGTTTACAG CTGTTTCAGGCTGTCCCAAAGATCACTTGCG	136
6493 neuA (siaT10) for	TTTGGGACAGCCTGAAACAGCTGTAAACCTCGAA GGAGGTACAATTATGAGCCTGGCCATTATCCCG	137
6494 pDest (siaT10) rev	TCCAGATACAGGGTAATCGTTTTTCATTTGTATAC CTCCTTCTAGCCTGCTTTTTTGTACAAACTTGTG	138
6495 siaT11 for	AAAGCAGGCTAGAAGGAGGTATACAAATGAAAAA GATCCTGACCGTCCTGAGCATCTTTATCCTGAGC	139
6496 siaT11 rev	AGGCTCATAATTGTACCTCCTTCGAGGTTTAGTC CAGCATCGTACCGAAGTCATCCGGTTTGGTGTG	140
6497 neuA (siaT11) for	ATGACTTCGGTACGATGCTGGACTAAACCTCGAA GGAGGTACAATTATGAGCCTGGCCATTATCCCG	141
6498 pDest (siaT11) rev	TGCTCAGGACGGTCAGGATCTTTTTTCATTTGTAT ACCTCCTTCTAGCCTGCTTTTTTGTACAAACTTGT	142
6503 siaT13 for	AAAGCAGGCTAGAAGGAGGTATACAAATGACGA ATCGCAAAATCTATGTCTGCCACACCCTGTACC	143
6504 siaT13 rev	AGGCTCATAATTGTACCTCCTTCGAGGTTTATTT AATGTCTTTCAGATCAACCAGCGTAATTTTCTTGT	144
6505 neuA (siaT13) for	TGGTTGATCTGAAAGACATTAATAAACCTCGAA GGAGGTACAATTATGAGCCTGGCCATTATCCC	145
6506 pDest (siaT13) rev	AGACATAGATTTTTCGATTTCGTCATTTGTATACCT CCTTCTAGCCTGCTTTTTTGTACAAACTTGTG	146
6507 siaT14 for	AAAGCAGGCTAGAAGGAGGTATACAAATGTTCCG TGAAGACAATATGAACCTGATTATCTGCTGTACG	147
6508 siaT14 rev	AGGCTCATAATTGTACCTCCTTCGAGGTTTAGAT GTCGATAACTTTGATACCGAAATCTTTCAGG	148
6509 neuA (siaT14) for	TCGGTATCAAAGTTATCGACATCTAAACCTCGAA GGAGGTACAATTATGAGCCTGGCCATTATCCCG	149
6510 pDest (siaT14) rev	AGGTTCATATTGTCTTCACGGAACATTTGTATAC CTCCTTCTAGCCTGCTTTTTTGTACAAACTTGTG	150
6511 siaT15 for	AAAGCAGGCTAGAAGGAGGTATACAAATGAAAGA AATCGCCATCATCTCCAACCAACGCATGTTCTTC	151
6512 siaT15 rev	AGGCTCATAATTGTACCTCCTTCGAGGTTTAGTC AAAGAAATCCAGCAGTTTCGGATGCACCGCGGT	152
6513 neuA (siaT15) for	TCCGAAACTGCTGGATTTCTTTGACTAAACCTCG AAGGAGGTACAATTATGAGCCTGGCCATTATCCC	153
6514 pDest (siaT15) rev	TTGGAGATGATGGCGATTTCTTTTCATTTGTATAC CTCCTTCTAGCCTGCTTTTTTGTACAAACTTGTG	154
6515 siaT16 for	AAAGCAGGCTAGAAGGAGGTATACAAATGCTGAT TCAACAGAACCTGGAAATCTACCTGGACTACGC	155
6516 siaT16 rev	AGGCTCATAATTGTACCTCCTTCGAGGTTTAATT GTGAATGGTGACATAAACGCCTGATCTTCGTTG	156
6517 neuA (siaT16) for	AGGCGTTTATGTGCACCATTCACAATTAACCTC GAAGGAGGTACAATTATGAGCCTGGCCATTATCC	157

Primer	Sequence 5'-3'	SEQ ID NO:
6518 pDest (siaT16) rev	ATTTCCAGGTTCTGTTGAATCAGCATTGTATAC CTCCTTCTAGCCTGCTTTTTGTACAACTTGTG	158
6519 siaT17 for	AAAGCAGGCTAGAAGGAGGTATACAAATGGGCT GTAACCTCCGATAGCAAACACAATAACAGTGATGG	159
6520 siaT17 rev	AGGCTCATAATTGTACCTCCTTCGAGGTTTATTG CAGGTCACCTAATCAGTTTCACATCATTGCGG	160
6521 neuA (siaT17) for	TGAAACTGATTAGTGACCTGCAATAAACCTCGAA GGAGGTACAATTATGAGCCTGGCCATTATCCCG	161
6522 pDest (siaT17) rev	TGTTTGCTATCGGAGTTACAGCCCATTTGTATAC CTCCTTCTAGCCTGCTTTTTGTACAACTTGTG	162
6523 siaT18 for	AAAGCAGGCTAGAAGGAGGTATACAAATGTGTAA CGATAATCAAAATACGGTCGATGTTGTTGTGAGC	163
6524 siaT18 rev	AGGCTCATAATTGTACCTCCTTCGAGGTTTAATA CTGAGCAATACAAACACCCGAGGAACAATCCGG	164
6525 neuA (siaT18) for	TCGGGTGTTTGTATTGCTCAGTATTAACCTCGA AGGAGGTACAATTATGAGCCTGGCCATTATCCC	165
6526 pDest (siaT18) rev	ACCGTATTTTGATTATCGTTACACATTTGTATACC TCCTTCTAGCCTGCTTTTTGTACAACTTGTG	166
6807 siaT19 for	AAAAGCAGGCTAGAAGGAGGTATACAAATGAAC GATAATCAAAATACGGTGGACGTGGTGGTCTC	167
6808 siaT19 rev	AGGCTCATAATTGTACCTCCTTCGAGGTTTAGCA CCAGAACAGCACATCTTTTCTTTCACAATGCC	168
6809 neuA (siaT19) for	AAAAAGATGTGCTGTTCTGGTGCTAACCTCGAA GGAGGTACAATTATGAGCCTGGCCATTATCCCG	169
6810 pDest (siaT19) rev	TCCACCGTATTTTGATTATCGTTTCAATTTGTATACC TCCTTCTAGCCTGCTTTTTGTACAACTTGTG	170
6811 siaT20 for	AAAAGCAGGCTAGAAGGAGGTATACAAATGCAAA ACGTCATTATCGCTGGTAACGGTCCGAGCCTGC	171
6812 siaT20 rev	AGGCTCATAATTGTACCTCCTTCGAGGTTTATTT CTTTTGTATTCTTCTTCAGTTTTTGTATTTCG	172
6813 neuA (siaT20) for	TGAAGAAAGAATACAAAAAGAAATAAACCTCGAA GGAGGTACAATTATGAGCCTGGCCATTATCCCG	173
6814 pDest (siaT20) rev	TTACCAGCGATAATGACGTTTTGCATTTGTATAC CTCCTTCTAGCCTGCTTTTTGTACAACTTGTG	174
6815 siaT21 for	AAAAGCAGGCTAGAAGGAGGTATACAAATGGATT CTTCGCCGAAAACACCAGCTCTACGCTGG	175
6816 siaT21 rev	AGGCTCATAATTGTACCTCCTTCGAGGTTTATTT GATGTCCGTCGTAAAGCGCACTTTTTCGTCCG	176
6817 neuA (siaT21) for	TGCGCTTTACGACGGACATCAAATAAACCTCGAA GGAGGTACAATTATGAGCCTGGCCATTATCCCG	177
6818 pDest (siaT21) rev	TGGTGTTTTCCGGCGAAGAATCCATTTGTATACC TCCTTCTAGCCTGCTTTTTGTACAACTTGTG	178
6652 siaT22 for	AAAAGCAGGCTAGAAGGAGGTATACAAATGAAGA AAGTCTACTTCTGCCATACGGTCTACCATCTGC	179

Primer	Sequence 5'-3'	SEQ ID NO:
6653 siaT22 rev	AGGCTCATAATTGTACCTCCTTCGAGGTTTAACT ATTTGCTTTCATTTGTTTCAGGGTGATTTTC	180
6654 neuA (siaT22) for	TGAAACAAATGAAAGCAAATAGTTAAACCTCGAA GGAGGTACAATTATGAGCCTGGCCATTATCCCG	181
6655 pDest (siaT22) rev	TATGGCAGAAGTAGACTTTTCTTCATTTGTATACCT CCTTCTAGCCTGCTTTTTTGTACAAACTTGTG	182
6819 siaT24 for	AAAGCAGGCTAGAAGGAGGTATACAAATGCGTAA AATCATCACCTTCTTCAGCCTGTTCTTCTCG	183
6820 siaT24 rev	AGGCTCATAATTGTACCTCCTTCGAGGTTTAAAA GTTAATCGGGTTCGGCATTCTTCAAAGAAAATC	184
6821 neuA (siaT24) for	AAATGCCGAACCCGATTAACCTTTTAAACCTCGAA GGAGGTACAATTATGAGCCTGGCCATTATCCCG	185
6822 pDest (siaT24) rev	TGAAGAAGGTGATGATTTTACGCATTTGTATACC TCCTTCTAGCCTGCTTTTTTGTACAAACTTGTG	186

Table 2: List of oligonucleotides used for PCR

- 5 Example 2: Development of an *E. coli* strain enabling the *in vivo* screening of
sialyltransferases using lactose as an acceptor but requiring
exogenous addition of sialic acid

Escherichia coli BL21(DE3) strain #942 was used to build up a screening strain for the plasmid encoded sialyltransferases 27 to 100. Therefore, to enable the uptake and nucleotide-activation of sialic acid, the genes *nanT* and *neuA*, respectively,
10 were integrated. The *nanT* gene (acc. no. B21_03035), encoding the *E. coli* Neu5Ac major facilitator superfamily transporter, was amplified from genomic DNA of *E. coli* BL21(DE3) and the *neuA* gene, originating from *Campylobacter jejuni* (acc. no. AF305571), was codon-optimized and obtained by synthesis. The genes were cloned as an operon under the control of the constitutive tetracyclin promoter P_{tet}
15 and the resulting expression fragment $\langle P_{tet}\text{-}neuA\text{-}nanT\text{-}lox66\text{-}kan\text{-}lox72 \rangle$ (SEQ ID NO: 73) was integrated by using the EZ-Tn5 transposase, yielding the screening strain #1730.

Plasmid	Relevant genotype	Ref.
pCP20	rep, <i>FLP</i> , <i>bla</i> , <i>cat</i>	Datsenko and Wanner, 2002
pEcomar- <i>cscABKR</i>	<i>cscB</i> , <i>cscK</i> , <i>cscA</i> , and <i>cscR</i> of <i>E. coli</i> W integrated into vector pEcomar; <i>bla</i>	This study
pEcomar- <i>glmUM-glmS</i>	<i>glmU</i> , <i>glmM</i> and <i>glmS</i> of <i>Escherichia coli</i> K12 integrated into vector pEcomar; <i>dhfr</i> , <i>bla</i>	This study
pEcomar- <i>lgtA-galT</i>	<i>lgtA</i> of <i>Neisseria meningitidis</i> and <i>galT</i> of <i>Escherichia coli</i> K12 integrated into vector pEcomar; <i>kan</i> , <i>bla</i>	This study
pEcomar- <i>wbdO-galE</i>	<i>wbdO</i> of <i>Salmonella enterica</i> subsp. <i>salamae</i> serovar Greenside and <i>galE</i> of <i>Escherichia coli</i> K12 integrated into vector pEcomar; <i>cat</i> , <i>bla</i>	This study
pDEST14	<i>bla</i> , <i>cat</i> , <i>ccdB</i>	Thermo Fisher Scientific, Waltham, Massachusetts, USA

Table 3: List of common plasmids used

Example 3: Generation of a plasmid collection coding for sialyltransferases

- 5 Gene sequences of characterized or putative sialyltransferases were received from the literature and public databases. Since sialyltransferases are often described to exhibit higher activity when their signal peptide is deleted, we analyzed the corresponding protein sequences by the on-line prediction tool SignalP (Petersen *et al.*, *Nature Methods*, 2011 Sep 29;8(10):785-6). Genes were synthetically
- 10 synthesized by GenScript cooperation either, as annotated, in a full length form or, when a signal peptide is predicted, as a truncated variant lacking the N-terminal signal peptide (Table 4).

The sialyltransferases 1 to 26 were each subcloned as an operon with *neuA* into pDEST14 by SLIC using gene specific primers (Table 2), yielding plasmids of the

general kind: pDEST14-*siaT-neuA*. The remaining sialyltransferases 27 to 100 were directly subcloned by GenScript cooperation into plasmid pET11a using restriction sites *NdeI* and *BamHI*. Both expression systems allow the IPTG-inducible gene expression (Figure 2). For *in vivo* activity screenings, the plasmids were either transformed to strains #1363 or #1730 whereas *E. coli* BL21(DE3) wild type or a *lacZ*-lacking variant thereof (strain #287) was used for *in vitro* assays.

Plasmid	Origin of the sialyltransferase gene	accession number of the sialyltransferase gene	Cloned as full length (FL) gene or without signal peptide (Δ)
pDEST14-siaT1-neuA	<i>Neisseria meningitidis</i>	U60660	FL (SEQ ID NO: 5)
pDEST14-siaT2-neuA	<i>Campylobacter jejuni</i> strain OH4384	AF130466	FL (SEQ ID NO: 10)
pDEST14-siaT3-neuA	<i>Campylobacter jejuni</i> strain OH4384	AX934425	FL (SEQ ID NO: 12)
pDEST14-siaT4-neuA	<i>Helicobacter acinonychis</i>	NC_008229	FL (SEQ ID NO: 11)
pDEST14-siaT5-neuA	<i>Helicobacter acinonychis</i>	NC_008229	FL (SEQ ID NO: 33)
pDEST14-siaT6-neuA	<i>Photobacterium</i> sp. JT-ISH-224	BAF92026	Δ 17 (SEQ ID NO: 29)
pDEST14-siaT7-neuA	<i>Pasteurella dagmatis</i> strain DSM 22969	AFY98851	FL (SEQ ID NO: 7)
pDEST14-siaT8-neuA	<i>Photobacterium</i> sp. JT-ISH-224	BAF92025	Δ 20 (SEQ ID NO: 3)
pDEST14-siaT9-neuA	<i>Vibrio</i> sp. JT-FAJ-16	BAF91160	Δ 22 (SEQ ID NO: 2)
pDEST14-siaT10-neuA	<i>Pasteurella multocida</i> PM70	AAK02272	Δ 25 (SEQ ID NO: 4)
pDEST14-siaT11-neuA	<i>Photobacterium damsela</i> JT0160	BAA25316	FL (SEQ ID NO: 31)
pDEST14-siaT13-neuA	<i>Streptococcus agalactiae</i>	AB050723	FL (SEQ ID NO: 17)
pDEST14-siaT14-neuA	<i>Haemophilus somnus</i> -2336	ACA31578	FL (SEQ ID NO: 26)
pDEST14-siaT15-neuA	<i>Haemophilus ducreyi</i> 35000HP	AF101047	FL (SEQ ID NO: 14)
pDEST14-siaT16-neuA	<i>Haemophilus ducreyi</i> 35000HP	AAP95068	FL (SEQ ID NO: 27)
pDEST14-siaT17-neuA	<i>Photobacterium phosphoreum</i> JT-ISH-467	BAF63530	Δ 20 (SEQ ID NO: 8)
pDEST14-siaT18-neuA	<i>Photobacterium leiognathi</i> JT-SHIZ-119	AB500947	Δ 15 (SEQ ID NO: 28)
pDEST14-siaT19-neuA	<i>Photobacterium leiognathi</i> JT-SHIZ-145	BAF91416	Δ 15 (SEQ ID NO: 30)
pDEST14-siaT20-neuA	<i>Campylobacter coli</i>	YP_008473374	FL (SEQ ID NO: 1)
pDEST14-siaT21-neuA	<i>Vibrio harveyi</i>	WP_017817635	Δ 24 (SEQ ID NO: 21)

Plasmid	Origin of the sialyltransferase gene	accession number of the sialyltransferase gene	Cloned as full length (FL) gene or without signal peptide (Δ)
pDEST14-siaT22-neuA	<i>Streptococcus entericus</i>	WP_018369230	FL (SEQ ID NO: 13)
pDEST14-siaT24-neuA	<i>Avibacterium paragallinarum</i>	WP_021724759	FL (SEQ ID NO: 9)
pET11a-siaT30	<i>Haemophilus parahaemolyticus</i> HK385	EIJ71207	FL (SEQ ID NO: 19)
pET11a-siaT31	<i>Alistipes</i> sp. CAG:268	CDC95697	Δ 17 (SEQ ID NO: 22)
pET11a-siaT32	<i>Alistipes</i> sp. AL-1	WP_032134786	FL (SEQ ID NO: 15)
pET11a-siaT34	<i>Pasteurella multocida</i> PM70	NC_002663	FL (SEQ ID NO: 6)
pET11a-siaT37	<i>Campylobacter jejuni</i> strain 81-176	AAL09368	FL (SEQ ID NO: 16)
pET11a-siaT39	<i>Alistipes shahii</i> WAL 8301	YP_007816735	Δ 21 (SEQ ID NO: 23)
pET11a-siaT41	<i>Actinobacillus suis</i> ATCC 33415	AIJ32009	FL (SEQ ID NO: 24)
pET11a-siaT42	<i>Actinobacillus capsulatus</i> DSM 19761	WP_018652686	FL (SEQ ID NO: 25)
pET11a-siaT43	<i>Bibersteinia trehalosi</i> USDA-ARS-USMARC-189	AHG84654	FL (SEQ ID NO: 18)
pET11a-siaT51	<i>Photobacterium damsela</i> subsp. <i>damsela</i> CIP 102761	EEZ40509	FL (SEQ ID NO: 32)
pET11a-siaT73	<i>Haemophilus somnus</i> 2336	ACA31170	FL (SEQ ID NO: 20)

Table 4: List of sialyltransferase-encoding plasmids. Sialyltransferases 1 to 11, 13 to 21, and 24 were cloned as an operon with *neuA* (acc. no. AY102622) of *Campylobacter jejuni*. Cloning of sialyltransferases 30 to 32, 34, 37, 39, 41, 42, 51, and 73 occurred via sites *NdeI* and *BamHI*. The sialyltransferase genes were either cloned as full length constructs (FL) or without a predicted signal peptide (Δ). The number behind the Δ indicates the N-terminally amino acids deleted from the corresponding sequence.

Example 4: Identification and characterization of α -2,3- and α -2,6-sialyltransferases using lactose as acceptor substrate

Escherichia coli BL21(DE3) #1363 and #1730 harbouring plasmids encoding for 100 sialyltransferases were grown at 30°C in 100 ml shake flasks filled with 20 ml of mineral salts medium (Samain *et al.*, J. Biotech. 1999, 72:33-47) supplemented with 2 % (wt/vol) glucose, ampicillin 100 $\mu\text{g ml}^{-1}$, kanamycin 15 $\mu\text{g ml}^{-1}$ and zeocin 40 $\mu\text{g ml}^{-1}$. When the cultures reached an OD₆₀₀ of 0.1 to 0.3, gene expression was induced by addition of 0.3 mM IPTG. After an incubation for one hour, 1.5 mM

- lactose was added to #1363 cultures whereas 1.5 mM lactose as well as 1.5 mM sialic acid was added to #1730 cultures. The incubation continued for 72 to 96 hours. Then, cells were harvested by centrifugation and mechanically disrupted in a defined volume using glass beads. Subsequently, samples were applied to thin
- 5 layer chromatography (TLC) on Silica Gel 60 F₂₅₄ (Merck KGaA, Darmstadt, Germany). A mixture of butanol:acetone:acetic acid:H₂O (35/35/7/23 (v/v/v/v)) was used as mobile phase. For detection of the separated substances, the TLC plate was soaked with thymol reagent (0.5 g thymol solved in 95 ml ethanol, 5 ml sulfuric acid added) and heated.
- 10 The results are summarized in Table 5. In the entire screening, thirty two genes were identified to encode for α -2,3-sialyltransferases, thus producing 3'-SL. 19 enzymes synthesized 6'-SL and were depicted as α -2,6-sialyltransferases. An α -2,3- as well as an α -2,6-sialyltransferase activity could only be observed for 3 enzymes. Accordingly, the expression of 46 enzymes did not result in sialyllactose
- 15 formation. The screening appeared highly accurate since the described activities of extensively characterized sialyltransferases, e. g. SiaT1 (Gilbert *et al.*, J Biol Chem. 1996 Nov 8;271(45):28271-6; Gilbert *et al.*, Eur J Biochem. 1997 Oct 1;249(1):187-94), SiaT6 (Tsukamoto *et al.*, J Biochem. 2008 Feb;143(2):187-97) and SiaT11 (Yamamoto *et al.*, J Biochem. 1996 Jul;120(1):104-10), could be confirmed.
- 20 Regarding the product formation, the experimental setting allowed a semi-quantitative comparison of the screened enzymes. But to achieve deeper knowledge about their kinetic properties, the 3 potentially best performing α -2,3- and α -2,6-sialyltransferases were applied to *in vitro* assays.

SiaT #	Relative efficacy of sialyltransferases regarding <i>in-vitro</i> production of sialyllacto- <i>N</i> -tetraoses		Production of sialyllactose <i>in-vivo</i>	SEQ ID NO:	
	LST-a	LST-b		aa	nt
1	2.98 x 10 ³	n.d.	3'-SL	38	5
2	749	n.d.	3'-SL	43	10
3	605	n.d.		45	12
4	654	n.d.		44	11
5	n.d.	1		66	33
6	n.d.	300	6'-SL	62	29
7	2.25 x 10 ³	n.d.		40	7
8	4.00 x 10 ³	n.d.		36	3
9	8.39 x 10 ³	n.d.	3'-SL	35	2
10	3.18 x 10 ³	n.d.	3'-SL, 6'-SL	37	4
11	n.d.	13		64	31
13	24	n.d.	3'-SL	50	17
14	2	n.d.	3'-SL	59	26
15	204	n.d.	3'-SL	47	14
16	1	n.d.	3'-SL	60	27
17	1.24 x 10 ³	n.d.	3'-SL	41	8
18	n.d.	303	6'-SL	61	28
19	n.d.	157	6'-SL	63	30
20	1.00 x 10 ⁴	n.d.	3'-SL	34	1
21	12	n.d.	3'-SL	54	21
22	564	n.d.	3'-SL	46	13
24	835	n.d.	3'-SL	42	9
30	17	n.d.	3'-SL	52	19
31	4	n.d.	3'-SL	55	22
32	64	n.d.	3'-SL, 6'-SL	48	15
34	2.34 x 10 ³	n.d.	3'-SL	39	6
37	40	n.d.	3'-SL	49	16
39	4	n.d.	3'-SL	56	23
41	3	n.d.	3'-SL	57	24
42	3	n.d.	3'-SL	58	25
43	21	n.d.	3'-SL	51	18
51	n.d.	10	6'-SL	65	32
73	15	n.d.	3'-SL	53	20

5 Table 5: Characterization of the product spectrum of sialyltransferases (SiaT). Activities were determined by *in vivo* or *in vitro* experiments using lactose or LNT as glycan substrates, respectively. Identification of sialylated lactose occurred qualitatively via thin layer chromatography. The products of the sialylation of LNT were quantified via LC-MS/MS. Depicted is the relative quantity of LST-a or -b respective to the best performing enzyme. (n.d.: not detectable).

Example 5: Identification and characterization of α -2,3- and α -2,6-sialyltransferases using lacto-*N*-tetraose as acceptor substrate

Escherichia coli BL21(DE3) harbouring plasmids encoding for 100 sialyltransferases were grown at 30°C in 100 ml shake flasks filled with 20 ml of 2YT medium supplemented with ampicillin 100 $\mu\text{g ml}^{-1}$. When the cultures reached an OD₆₀₀ of 0.1 to 0.3, gene expression was induced by addition of 0.3 mM IPTG and the incubation was continued for 12 to 16 hours. Cells were harvested by centrifugation and mechanically disrupted in a defined volume of 50 mM Tris-HCl pH7.5 using glass beads. The protein extract was kept on ice until the assay started. The *in vitro* assay was carried out in a total volume of 25 μl including 50 mM Tris-HCl pH7.5, 5 mM MgCl₂, 10 mM CMP-Neu5Ac and 5 mM LNT. The assay started with the addition of 3 μl protein extract and continued for 16 hours. Product formation was determined by mass spectrometry.

Mass spectrometry analysis was performed by MRM (multiple reaction monitoring) using a LC Triple-Quadrupole MS detection system. Precursor ions are selected and analyzed in quadrupole 1, fragmentation takes place in the collision cell using argon as CID gas, selection of fragment ions is performed in quadrupole 3. Chromatographic separation of lactose, 3'-Sialyllactose and 6'-Sialyllactose after dilution of culture supernatant 1:100 with H₂O (LC/MS Grade), was performed on a XBridge Amide HPLC column (3.5 μm , 2.1 $\times$ 50 mm (Waters, USA) with a XBridge Amide guard cartridge (3.5 μm , 2.1 $\times$ 10 mm) (Waters, USA). Column oven temperature of the HPLC system was 50°C. The mobile phase was composed of acetonitrile:H₂O with 10 mM ammonium acetate. A 1 μl sample was injected into the instrument; the run was performed for 3.60 min with a flow rate of 400 $\mu\text{l/min}$. 3'-Sialyllactose and 6'-Sialyllactose were analyzed by MRM in ESI positive ionization mode. The mass spectrometer was operated at unit resolution. Sialyllactose forms an ion of m/z 656.2 [M+Na]. The precursor ion of Sialyllactose was further fragmented in the collision cell into the fragment ions m/z 612.15, m/z 365.15 and m/z 314.15. Collision energy, Q1 and Q3 Pre Bias were optimized for each analyte individually. Chromatographic separation of lactose, LNT-II, LNT and LST-a or -b after dilution of particle-free biocatalysis-reaction or crude extract, respectively, 1:50

with H₂O (LC/MS Grade), was performed on a XBridge Amide HPLC column (3.5 μ m, 2.1 $\times$ 50 mm (Waters, USA) with a XBridge Amide guard cartridge (3.5 μ m, 2.1 $\times$ 10 mm) (Waters, USA). Column oven was run at 35°C. The mobile phase was composed of acetonitrile:H₂O with 0.1% ammonium hydroxide. A 1 μ l sample was
5 injected into the instrument; the run was performed for 3.50 min with a flow rate of 300 μ l/min. Lactose, LNT-II, LNT as well as LST-a and -b were analyzed by MRM in ESI negative ionization mode. The mass spectrometer was operated at unit resolution. Lactose forms an ion of m/z 341.00 [M-H]. The precursor ion of Lactose was further fragmented in the collision cell into the fragment ions m/z 179.15, m/z
10 161.15 and m/z 101.05. LNT-II forms an ion of m/z 544.20 [M-H]. The precursor ion of LNT-II was further fragmented into the fragment ions m/z 382.10, m/z 161.00 and m/z 112.90. LNT forms an ion of m/z 706.20 [M-H]. The precursor ion of LNT was further fragmented into the fragment ions m/z 382.10, m/z 202.10 and m/z 142.00. LST-a and -b forms an ion of m/z 997.20 [M-H]. The precursor ion of LST-a and -b
15 was further fragmented into the fragment ions m/z 290.15, m/z 202.15 and m/z 142.15. Collision energy, Q1 and Q3 Pre Bias were optimized for each analyte individually. Quantification methods were established using commercially available standards (Carbosynth, Compton, UK).

The results of the *in vitro* screening are summarized in Table 5. Twenty eight genes
20 were identified to produce LST-a whereas only 6 enzymes synthesized LST-b. Accordingly, the expression of 66 enzymes did not result in the formation of either LST-a or LST-b. The assay is regarded to be accurate since the activity of SiaT1, which was already described to sialylate LNT (Gilbert *et al.*, J Biol Chem. 1996 Nov 8;271(45):28271-6; Gilbert *et al.*, Eur J Biochem. 1997 Oct 1;249(1):187-94), could
25 be verified. Irrespectively of the protein overexpression level, sialyltransferases that produced best were selected for determination of K_m and V_{max} .

Example 6: Characterization of the kinetic properties of selected sialyltransferases

To rank the best performing sialyltransferases, their K_m values for donor and acceptor substrates were determined *in vitro*. *Escherichia coli* BL21(DE3) strain #287 was used for overproduction of the enzymes. Cells were incubated in 100 ml 2YT medium in shaking flasks supplemented with 100 $\mu\text{g ml}^{-1}$ ampicillin at 30 °C until an OD_{600} of 0.3 was reached. Then, 0.3 mM IPTG was added and the incubation was continued for 12 to 16 hours. Cells were harvested by centrifugation and mechanically disrupted in a defined volume of 50 mM Tris-HCl pH7.5 using glass beads. The protein extract was kept on ice until the assay started. The *in vitro* assay was carried out in a total volume of 50 μl including 50 mM Tris-HCl pH7.5, 5 mM MgCl_2 and varying concentrations of CMP-Neu5Ac (0.05-30 mM) as well as of lactose or LNT (0.1-50 mM). The assay started with the addition of 35 to 750 μg protein extract. After 1 to 10 minutes of incubation at 30 °C, the assay was inactivated at 95 °C for 5 minutes. Product formation was determined by mass spectrometry. Data were evaluated using the enzyme kinetic module of SigmaPlot v12.5 to calculate K_m and V_{max} .

During screening, the best performing α -2,3-sialyltransferases for LST-a production appeared to be SiaT8, SiaT9 and SiaT20. In contrast, SiaT6, SiaT18 and SiaT19 were observed to sialylate LNT most efficiently among the tested α -2,6-sialyltransferases. Their kinetic parameters for CMP-Neu5Ac and LNT as well as lactose are depicted in Table 6. Solely SiaT20 does not follow a Michaelis-Menten kinetic.

Activity	SiaT#	CMP-Neu5Ac		Lactose		LNT	
		K_m [mM]	V_{max} [μ mol/min]	K_m [mM]	V_{max} [μ mol/min]	K_m [mM]	V_{max} [μ mol/min]
α 2,3	SiaT8	4.0	0.35	4.3	0.37	4.2	0.32
	SiaT9	0.96	0.16	2.9	0.18	2.9	0.15
α 2,6	SiaT6	2	0.16	5.6	0.20	5.5	0.13
	SiaT18	2.8	0.21	7.6	0.21	5.2	0.14
	SiaT19	1.1	0.09	3	0.09	2.9	0.04

Table 6: K_m and V_{max} for selected sialyltransferases using CMP-Neu5Ac as donor substrate and LNT as well as lactose as acceptor substrates.

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Example 7: Generation of a lacto-*N*-tetraose-production strain for screening the *in vivo* activity of sialyltransferases using LNT as acceptor substrate

Escherichia coli BL21(DE3) strain #534 was used to construct a lacto-*N*-tetraose (LNT) producing strain. The β -1,3-*N*-acetylglucosaminyltransferase gene *lgtA* from *Neisseria meningitidis* MC58 (acc. no. NP_274923) was codon-optimized for expression in *E. coli* and prepared synthetically by gene synthesis. Together with the gene *galT*, encoding a galactose-1-phosphate uridylyltransferase from *E. coli* K-12 substr. MG1655 (acc. no. NP_415279), that was similarly obtained by gene synthesis, *lgtA* was inserted by transposition (SEQ ID NO: 188) using plasmid pEcomar-*lgtA-galT*. To enhance *de novo* synthesis of UDP-*N*-acetylglucosamine, genes encoding L-glutamine:D-fructose-6-phosphate aminotransferase (*glmS*), phosphoglucosamine mutase from *E. coli* K-12 substr. MG1655 (*glmM*) and *N*-acetylglucosamine-1-phosphate uridylyltransferase/ glucosamine-1-phosphate acetyltransferase (*glmU*) from *E. coli* K-12 substr. MG1655 (acc. no. NP_418185, NP_417643, NP_418186, respectively) were codon-optimized and obtained by gene synthesis. The operon *glmUM* was cloned under the control of constitutive tetracyclin promoter P_{tet} while *glmS* was cloned under the constitutive P_{T5} promoter. The transposon cassette $\langle P_{tet}\text{-}glmUM\text{-}P_{T5}\text{-}glmS\text{-}FRT\text{-}dhfr\text{-}FRT \rangle$, flanked by the inverted terminal repeats specifically recognized by the mariner-like element *Himar1* transposase was inserted from pEcomar-*glmUM-glmS* revealing a lacto-*N*-triose II production strain. Metabolic engineering further included the genomic integration of

the transposon cassettes <P_{tet}-*wbdO*-P_{T5}-*galE*-FRT-*cat*-FRT> (SEQ ID NO: 187), flanked by the inverted terminal repeats specifically recognized by the mariner-like element *Himar1* transposase, which was inserted from pEcomar-*wbdO-galE*. To prevent intracellular degradation of *N*-acetylneuraminic acid the *nanKETA* gene cluster was deleted from the genome of the *E. coli* strain BL21(DE3) strain according to the method of Datsenko and Wanner (Proc. Natl. Acad. Sci. USA 97:6640-6645 (2000)). To provide sufficient donor substrate (CMP-Neu5Ac) for the sialylation of LNT, the uptake mechanism for sialic acid as well as the capability for its nucleotide-activation was implemented into the *E. coli* strain. As described above, the genes *nanT* and *neuA* were cloned as an operon (using primers *neuA/nanT_1-6*) under the control of the constitutive tetracyclin promoter P_{tet} and the resulting expression fragment <P_{tet}-*neuA-nanT-lox66-kan-lox72*> was integrated by using the EZ-Tn5 transposase, finally generating strain #2130.

Example 8: Batch fermentations of *E. coli* BL21(DE3) #2130 expressing different sialyltransferases

Escherichia coli BL21(DE3) #2130 cells harbouring expression plasmids encoding for the sialyltransferases SiaT9 or SiaT19 were grown at 30°C in 100 ml shake flasks filled with 25 ml of mineral salts medium (Samain *et al.*, J. Biotech. 1999, 72:33-47) supplemented with 2 % (wt/vol) glucose, 5g/l NH₄Cl, ampicillin 100 µg ml⁻¹, kanamycin 15 µg ml⁻¹ and gentamycin 5 µg ml⁻¹. When the cultures reached an OD₆₀₀ of 0.5 to 1, 3 mM lactose was added. After 24 hours of incubation sialyltransferase gene expression was induced by addition of 0.3 mM IPTG. Concomitantly, 3 mM of sialic acid was added to the cultures. The incubation continued for 48 hours. Then, cells were harvested by centrifugation and mechanically disrupted in a defined volume using glass beads. Subsequently, thin layer chromatography (TLC) was performed to confirm the intracellular formation of the sialyllacto-*N*-tetraoses-a and -b. As shown in Figure 3, the expression of *siaT9* or *siaT19* in strain #2130 resulted in the formation of LST-a and LST-b, respectively. Results were validated by mass spectrometry.

CLAIMS

1. A method for producing sialylated oligosaccharides, the method comprising the steps of
 - 5 a) providing at least one genetically engineered cell which comprises a heterologous sialyltransferase, said heterologous sialyltransferase being capable of possessing an α -2,3-sialyltransferase activity and/or an α -2,6-sialyltransferase activity for transferring a sialic acid residue from its-nucleotide-activated form as donor substrate to an acceptor molecule,
 - 10 wherein the acceptor molecule is selected from the group consisting of lactose, lacto-*N*-triose II and human milk oligosaccharides;
 - b) cultivating the at least one cell in a fermentation broth and under conditions permissive for the production of said sialylated oligosaccharide; and optionally
 - 15 c) recovering said sialylated oligosaccharide.
2. The method according to claim 1, wherein the sialylated oligosaccharide is selected from the group consisting of 3'-sialyllactose, 6'-sialyllactose, LST-a, LST-b, LST-c and DSLNT.
- 20 3. The method according to any one of claims 1 or 2, wherein the fermentation broth contains at least one carbon source, the at least one carbon source is preferably selected from the group consisting of glucose, fructose, sucrose, glycerol, and combinations thereof.
- 25 4. The method according to any one of claims 1 to 3, wherein the fermentation broth contains at least one selected from the group consisting of *N*-acetylglucosamine, galactose and sialic acid.
- 30 5. The method according to any one of claims 1 to 4, wherein the at least one cell is cultivated in the absence of and/or without addition of one or more selected from the group consisting of *N*-acetylglucosamine, galactose and sialic acid.

6. The method according to any one of claims 1 to 5, wherein the at least one cell is cultivated in the presence of at least one selected from the group consisting of lactose, lacto-*N*-triose II, LNT and LNrt.
- 5 7. A genetically engineered cell for producing sialylated oligosaccharides, wherein the genetically engineered cell comprises a heterologous sialyltransferase, said heterologous sialyltransferase being capable of possessing an α -2,3-sialyltransferase activity and/or an α -2,6-sialyltransferase activity for transferring a sialic acid residue from its nucleotide-activated form
10 as donor substrate to an acceptor molecule, wherein the acceptor molecule is selected from the group consisting of lactose, lacto-*N*-triose II and human milk oligosaccharides.
8. The method according to any one of claims 1 to 6 or the genetically
15 engineered cell according to claim 7, wherein the acceptor molecule is a human milk oligosaccharide selected from the group consisting of trisaccharides, tetrasaccharides and pentasaccharides, preferably selected from the group consisting of 3-fucosyllactose, lacto-*N*-tetraose, lacto-*N*-neotetraose, sialyllacto-*N*-tetraose a, sialyllacto-*N*-tetraose b, lacto-*N*-fucopentaose I and lacto-*N*-fucopentaose II.
20
9. The method according to any one of claims 1 to 6 or 8, or the genetically engineered cell according to claim 7 or 8, wherein the heterologous sialyltransferase, is selected from the group consisting of
25 i. polypeptides comprising or consisting of an amino acid sequence as represented by any one of SEQ ID NOs: 1 to 33;
ii. polypeptides comprising or consisting of an amino acid sequence having a sequence similarity of at least 80 % to any one of the amino acid sequences as represented by any one of SEQ ID NOs: 1 to 33; and
30 iii. fragments of any one of the polypeptides of i. and ii.

10. The method according to any one of claims 1 to 6 or 8 to 9, or the genetically engineered cell according to any one of claims 7 to 9, wherein the genetically engineered cell comprises a nucleic acid molecule comprising a nucleotide sequence which encodes the heterologous sialyltransferase, said nucleotide sequence being selected from the group consisting of
- i. nucleotide sequences encoding a polypeptide as represented by any one of SEQ ID NOs: 1 to 33;
 - ii. nucleotide sequences as represented by any one of SEQ ID NOs: 34 to 66;
 - 10 iii. nucleotide sequences having at least 80% sequence similarity to one of the nucleotide sequences encoding a polypeptide as represented by any one of SEQ ID NOs: 1 to 33;
 - iv. nucleotide sequences having a sequence similarity of at least 80 % to any one of the nucleotide sequences represented by SEQ ID NOs: 34 to 66;
 - 15 v. nucleotide sequences which are complementary to any one of the nucleotide sequences of i., ii., iii. and iv.; and
 - vi. fragments of any one of the nucleotide sequences of i., ii., iii., iv. and v., wherein said nucleotide sequence is operably linked to at least one nucleic acid expression control sequence effecting transcription and/or translation of said nucleotide sequence encoding the heterologous sialyltransferase in the
 - 20 genetically engineered cell.
11. The method according to any one of claims 1 to 6 or 8 to 10, or the genetically engineered cell according to any one of claims 7 to 10, wherein the genetically engineered cell possesses an increased production of one or more nucleotide-activated sugars selected from the group consisting of CMP-*N*-acetylneuraminic acid, UDP-*N*-acetylglucosamine, UDP-galactose and GDP-fucose as compared to cell before being genetically engineered.
12. The method according to any one of claims 1 to 6 or 8 to 11, or the genetically engineered cell according to any one of claims 7 to 11, wherein the genetically engineered cell possesses an overexpression, as compared to the cell prior to be genetically engineered, of one or more genes encoding for a polypeptide

- being capable of possessing an enzymatic activity selected from the group consisting of L-glutamine:D-fructose-6-phosphate aminotransferase, *N*-acetylglucosamine-1-phosphate uridyltransferase, glucosamine-1-phosphate acetyl transferase, phosphoglucosamine mutase, glucosamine-6-phosphate-*N*-acetyltransferase, *N*-acetylglucosamine-2-epimerase, UDP-*N*-acetylglucosamine-2-epimerase, sialic acid synthase, phosphoenolpyruvate synthase, CMP-sialic acid synthetase, UDP-galactose-4-epimerase, galactose-1-phosphate uridyltransferase, phosphoglucomutase, glucose-1-phosphate uridyltransferase, phosphomannomutase, mannose-1-phosphate
- 5 guanosyltransferase, GDP-mannose-4,6-dehydratase, GDP-L-fucose synthase
- 10 and fucosekinase/L-fucose-1-phosphate-guanyltransferase.
13. The method according to any one of claims 1 to 6 or 8 to 12, or the genetically engineered cell according to any one of claims 7 to 12, wherein the cell lacks or
- 15 possesses a decreased activity, as compared to the prior to be genetically engineered, of one or more enzymatic activities selected from the group consisting of β -galactosidase, a glucosamine-6-phosphate deaminase, a *N*-acetylglucosamine-6-phosphate deacetylase, a *N*-acetylmannosamine kinase, a *N*-acetylmannosamine-6-phosphate epimerase and a *N*-acetylneuraminic
- 20 acid aldolase.
14. The method according to any one of claims 1 to 6 or 8 to 13, or the genetically engineered cell according to any one of claims 7 to 13, wherein one or more of the genes encoding a β -galactosidase, a glucosamine-6-phosphate deaminase,
- 25 a *N*-acetylglucosamine-6-phosphate deacetylase, a *N*-acetylmannosamine kinase, a *N*-acetylmannosamine-6-phosphate epimerase and a *N*-acetylneuraminic acid aldolase has been deleted or its expression has been inactivated in the genetically engineered cell.
- 30 15. The method according to any one of claims 1 to 6 or 8 to 14, or the genetically engineered cell according to any one of claims 7 to 14, wherein the at least one cell comprises at least one selected from the group consisting of a functional lactose permease, a functional fucose permease and a functional sialic acid transporter (importer), preferably comprises and expresses at least one

nucleotide sequence encoding one selected from the group consisting of a functional lactose permease, a functional fucose permease and a functional sialic acid transporter (importer).

- 5 16. The method according to any one of claims 1 to 6 or 8 to 15, or the genetically engineered cell according to any one of claims 7 to 15, wherein the cell possesses activity of at least one glycosyltransferase selected from the group consisting of a β -1,3-N-acetylglucosaminyltransferase, a β -1,3-galactosyltransferase, a β -1,4-galactosyltransferase, a α -2,3-sialyltransferase
10 and a α -2,6-sialyltransferase.
17. A method for producing a sialylated oligosaccharide, the method comprising:
a) providing a sialyltransferase in a reaction mixture, the sialyltransferase being capable of capable of possessing an α -2,3-sialyltransferase activity
15 and/or an α -2,6-sialyltransferase activity for transferring a sialic acid residue from its nucleotide-activated form as donor substrate to an acceptor molecule, wherein the acceptor molecule is selected from the group consisting of lactose, lacto-*N*-triose II and human milk oligosaccharides;
20 b) contacting said sialyltransferase with a donor substrate and an acceptor molecule, wherein the acceptor molecule is selected from the group consisting of lactose, lacto-*N*-triose II and human milk oligosaccharides.
18. Use of a sialylated oligosaccharide produced by a method according to any
25 one of claims for manufacturing a nutritional composition, preferably an infant composition.
19. A nutritional composition comprising at least one sialylated oligosaccharide, wherein said at least one sialylated oligosaccharide has been produced by a
30 method according to any one of claims.
20. The nutritional composition according to claim 19, wherein the nutritional composition contains 3'-SL and/or 6'-SL in combination with at least one neutral HMO, said at least one neutral HMO preferably being 2'-FL.