

HMO production

Technical field

5 The present invention relates to the field of recombinant production of biological molecules in host cells. More particularly it relates to a method for recombinant production of human milk oligosaccharides (HMO) using genetically modified cell expressing a protein of the major facilitator superfamily (MFS).

10 Background of the invention

 Human milk oligosaccharides (HMOs) constitute the third largest solid component in human milk and are highly resistant to enzymatic hydrolysis. As a consequence, a substantial fraction of HMOs remains largely undigested and unabsorbed, which enables their passage through to the colon. In the colon, HMOs
15 may serve as substrates to shape the gut ecosystem by selectively stimulating the growth of specific saccharolytic bacteria. This selectivity is viewed as beneficial for both infants and adults since strains of *Bifidobacterium* species are believed to have a positive effect on gut health (Chichlowski M. *et al.*, (2012) J. Pediatr. Gastroenterol. Nutr. 5:251-258; Elison E. *et al.*, (2016) Brit J. Nutr, 116: 1356–1368).

20 Besides their prebiotic properties, HMOs have been linked to additional positive effects, which expands their field of application (Kunz C. *et al.*, (2014) Food Oligosaccharides: Production, Analysis and Bioactivity, 1st Edition, p 5-20, Eds. Moreno J. and Luz Sanz M., John Wiley & Sons, Ltd).

 The obvious health benefits of HMOs have enabled their approval for use in
25 foods, such as infant formulas and foods, and for consumer health products. Biotechnological production of HMOs is a valuable cost-efficient and large-scale way of HMO manufacturing. It relies on genetically engineered bacteria constructed so as to express the glycosyltransferases needed for synthesis of the desired oligosaccharides and takes advantage of the bacteria's innate pool of nucleotide
30 sugars as HMO precursors. Recent developments in biotechnological production of HMOs have made it possible to overcome certain inherent limitations of bacterial expression systems. For example, HMO-producing bacterial cells may be genetically modified to increase the limited intracellular pool of nucleotide sugars in the bacteria (WO2012112777), to improve activity of enzymes involved in the HMO production

(WO2016040531), or to facilitate the secretion of synthesized HMOs into the extracellular media (WO2010142305, WO2017042382). Further, expression of genes of interest in recombinant cells may be regulated by using particular promoters or other gene expression regulators, like e.g. what has recently been
5 described in WO2019123324.

The approach described in WO2010142305 and WO2017042382 has an advantage in that it allows to reduce the metabolic burden inflicted on the producing cell by high levels of recombinant gene expression, e.g. using methods of WO2012112777, WO2016040531 or WO2019123324. This approach attracts
10 growing attention in recombinant HMO-producing cells engineering, e.g. recently there have been described several new sugar transporter genes encoding proteins and fermentation procedures that can facilitate efflux of a recombinantly produced 2'-fucosyllactose (2'-FL), the most abundant HMO of human milk (WO2018077892, US201900323053, US201900323052). However, at present, there is no algorithm
15 that is able to pinpoint the right transporter protein capable of efflux of different recombinantly produced HMO structures among numerous bacterial proteins with predicted transporter function in multiple protein databases, e.g. UniProt, since the structures/factors defining substrate specificity of sugar transporters are still not well-studied and remain to be highly unpredictable.

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Summary of the invention

Identification of new efficient sugar efflux transporter proteins having specificity for different recombinantly produced HMOs and development of recombinant cells expressing said protein are advantageous for high scale industrial
25 HMO manufacturing.

This invention provides recombinant cells capable of producing a human milk oligosaccharide (HMO), wherein the cells are expressing a heterologous gene encoding a putative MFS (major facilitator superfamily) transporter protein, originating from the bacterium *Rosenbergiella nectarea*. More specifically, the
30 invention relates to a genetically modified cell optimized for the production of an oligosaccharide, in particular an HMO, comprising a recombinant nucleic acid encoding a protein having at least 80 % sequence similarity to the amino acid sequence of SEQ ID NO: 1. The amino acid sequence identified herein as SEQ ID NO: 1 is the amino acid sequence that is 100 % identical to the amino acid sequence
35 having the GenBank accession ID WP_092672081.1

(https://www.ncbi.nlm.nih.gov/protein/WP_092672081.1). The MFS transporter protein having the amino acid sequence of SEQ ID NO: 1 is identified herein as "Nec protein" or "Nec transporter" or "Nec", interchangeably; a nucleic acid sequence encoding nec protein is identified herein as "Nec coding nucleic acid/DNA" or "*nec* gene" or "*nec*".

5 The present invention shows that use of HMO producing recombinant cells that express Nec protein results in very distinct improvements of the HMO manufacturing process related both to fermentation and purification of the HMOs. The disclosed herein recombinant cells and methods for HMO production provide
10 both higher yields of total produced HMOs, lower by-product formation or by-product-to-product ratio, lower biomass formation per fermentation and facilitated recovery of the HMOs during downstream processing of the fermentation broth.

 Surprisingly, expression of a DNA sequence encoding Nec in different HMO producing cells is found to be associated with accumulation of some particular HMOs
15 in the extracellular media and other HMOs inside of the producing cells, and in an increase in total production of the HMOs. Surprisingly, an increase in the efflux of the produced HMOs is found to be characteristic for HMOs that consist of either tri or tetra units of monosaccharides, i.e. HMOs that are trisaccharides and tetrasaccharides, e. g. 2'-fucosyllactose (2'-FL), 3-fucosyllactose (3-FL), 3-
20 sialyllactose (3'-SL), 6-sialyllactose (6'-SL), lacto-N-triose 2, (LNT-2), lacto-N-neotetraose (LNnT) and lacto-N-tetraose (LNT), but not for larger oligosaccharide structures, like pentasaccharides and hexasaccharides, which accumulate inside of the producing cells. Surprisingly, it is also found that the total production of the major HMO, e.g. 2'-fucosyllactose (2'-FL), 3-fucosyllactose (3-FL), lacto-N-triose, (LNT II)
25 and lacto-N-tetraose (LNT), in the corresponding HMO producing cells expressing nec is also increased, while the by-product production, e.g. di-fucosyllactose (DFL), lacto-N-fucopentaose V (LNFP V) or para-lacto-neo-hexaose-I (pLNH-I), in these cells, correspondingly, is often decreased and said by-product oligosaccharides typically accumulate inside of the production cells. Further, highly unexpectedly,
30 expression of Nec protein in HMO producing cells leads to reduction in formation of the biomass during fermentation and to healthier cell cultures reflected by reduction in the number of dead cells at the end of fermentation, which makes the manufacturing process more efficient as more product is produced per biomass unit.

35 Accordingly, a first aspect of the invention relates to a genetically modified cell capable of producing one or more HMO, wherein said cell comprises a

recombinant nucleic acid encoding a protein of SEQ ID NO: 1 (Figure 6), or a functional homologue thereof which amino acid sequence is at least 80 % identical, preferably at least 85 % identical, more preferably at least 90 % identical to SEQ ID NO: 1.

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A second aspect of the invention relates to a nucleic acid construct comprising a nucleic acid sequence(s) encoding an MFS transporter protein, wherein the nucleic acid sequence encoding the protein has at least 70 % sequence identity to SEQ ID NO: 2, such as at least 80 %, such as at least 85%, such as at least 95 %, such as at least 99%, as well as to a genetically modified cell comprising the nucleic acid construct, which is *Escherichia coli*.

In one aspect, the nucleic acid construct comprises a nucleic acid sequence(s) encoding an MFS transporter protein, wherein the nucleic acid sequence is at least 70% identical to SEQ ID NO: 2.

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A third aspect of the invention relates to a method for the production of one or more oligosaccharides, the method comprising the steps of:

- (i) providing a genetically modified cell capable of producing an HMO, wherein said cell comprises a recombinant nucleic acid encoding a protein of SEQ ID NO: 1, or a functional homologue thereof which amino acid sequence is at least 80 % identical, preferably at least 85 % identical, more preferably at least 90 % identical to SEQ ID NO: 1;
- (ii) culturing the cell according to (i) in a suitable cell culture medium to express said recombinant nucleic acid;
- (iii) harvesting one or more HMOs produced in step (ii).

The invention also relates to the use of a genetically modified cell or a nucleic acid construct comprising a heterologous nucleic acid sequence encoding a Major facilitator superfamily (MFS) protein, said nucleic acid sequence having at least 70 % sequence identity to SEQ ID NO: 2, for the production of one or more Human Milk Oligosaccharides (HMOs).

As mentioned above, during the culturing of genetically modified cells capable of producing one or more HMOs, which cells comprise a nucleic acid encoding Nec transporter protein, it has surprisingly been found that the corresponding one or more HMOs are produced in high yields, while by-product and

biomass formation is reduced. This facilitates recovery of the HMOs during downstream processes, e.g. the overall recovery and purification procedure may comprise less steps and overall time of purification may be shortened.

These effects of increased product yields and facilitation of the product recovery makes the present invention superior to the disclosures of the prior art.

Other aspects and advantageous features of the present invention are described in detail and illustrated by non-limiting working examples below.

Brief description of figures

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Figure 1 shows the relative production of 2'-FL (Fig. 1A), the relative distribution of 2'-FL inside and outside the cells (Fig. 1B), the relative ratio of DFL to 2'-FL (Fig. 1C), and the relative optical density (Fig. 1D) of a modified *E. coli* with and without the overexpression of the MFS transporter protein with SEQ ID NO: 1.

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Figure 2 shows the relative distribution of 3-FL inside and outside the cells in modified *E. coli* with and without the overexpression of the MFS transporter protein with SEQ ID NO: 1.

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Figure 3 shows the percentage (%) relative LNT2 concentrations for the strains MP4002 and MP4039 in total, supernatant and pellet samples. Although both strains show optimal expression of the glycosyltransferase gene *lgtA*, only MP4039 expresses the heterologous transporter gene *nec*

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Figure 4 shows the percentage (%) relative LNT and by-product concentrations for the strains MP4473 and MP4537 in total, supernatant and pellet samples. Although both strains show optimal expression of the glycosyltransferase genes *lgtA* and *galTK*, it is only MP4537 that expresses the heterologous transporter gene *nec*

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Figure 5 shows the percentage (%) relative LNFP-I, 2'-FL, LNT and HMO sum concentrations for the strains MP2789 and MP4597 in total samples. Although both strains show optimal expression of the glycosyltransferase genes *lgtA*, *galTK* and *futC*, it is only MP4597 that expresses the heterologous transporter gene *nec*

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Figure 6 presents Nec protein amino acid sequence (SEQ ID NO: 1)

Detailed description

5 In the following, embodiments of the invention will be described in further detail. Each specific variation of the features can be applied to other embodiments of the invention unless specifically stated otherwise.

Generally, all terms used herein are to be interpreted according to their ordinary meaning in the technical field, and applicable to all aspects and embodiments of the invention, unless explicitly defined or stated otherwise. All
10 references to "a/an/the [cell, sequence, gene, transporter, step, etc]" are to be interpreted openly as referring to at least one instance of said cell, sequence, gene, transporter, step, etc., unless explicitly stated otherwise. The steps of any method disclosed herein do not have to be performed in the exact order disclosed, unless explicitly stated.

15 The present invention in general relates to a genetically modified cell for efficient production of oligosaccharides and use of the said genetically modified cell in a method of producing the oligosaccharides. In particular, the present invention relates to a genetically modified cell enabled to synthesise an oligosaccharide, preferably a heterologous oligosaccharide, in particular a human milk
20 oligosaccharide (HMO). Accordingly, a cell of the invention is modified to express a set of recombinant nucleic acids that are necessary for synthesis of one or more HMOs by the cells (which enable the cell to synthesise one or more HMOs), such as genes encoding one or more enzymes with glycosyltransferase activity described below. The oligosaccharide producing recombinant cell of the invention is further
25 modified to comprise a heterologous recombinant nucleic acid sequence, preferably, a DNA sequence, encoding a putative MFS (**m**ajor **f**acilitator **s**uperfamily) transporter protein, originating from the bacterium *Rosenbergiella nectarea*. More specifically, the invention relates to a genetically modified cell optimized for the production of one or more particular oligosaccharides, in particular one or more particular HMOs,
30 comprising a recombinant nucleic acid encoding a protein having at least 80 % sequence similarity, preferably at least 85 %, more preferably at least 90 %, and even more preferably at least 95 % sequence similarity to the amino acid sequence of SEQ ID NO: 1 (Figure 6). The amino acid sequence identified herein as SEQ ID NO: 1 is an amino acid sequence that has 100 % identity with the amino acid
35 sequence having the GenBank accession ID: WP_092672081.1.

Accordingly, a first aspect of the invention relates to a genetically modified cell capable of producing one or more HMOs, wherein said cell comprises a recombinant nucleic acid encoding a protein of SEQ ID NO: 1, or a functional homologue thereof which amino acid sequence is at least 80 % identical, preferably at least 85 % identical, more preferably at least 90 % identical to SEQ ID NO: 1. By the term "functional homolog" in the present context is meant a protein that has an amino acid sequence that is 80 %-99,9% identical to SEQ ID NO: 1 and has a function that is beneficial to achieve at least one advantageous effect of the invention, e.g. an increase the total HMO production by the host cell, facilitate recovery of the produced HMO(s), HMO production efficiency and/or viability of an HMO producing cell.

The MFS transporter protein having the amino acid sequence of SEQ ID NO: 1 is identified herein as "Nec protein" or "Nec transporter" or "Nec", interchangeably; a nucleic acid sequence encoding Nec protein is identified here in as "Nec coding nucleic acid /DNA" or "*nec* gene" or "*nec*".

By the term "Major Facilitator Superfamily (MFS)" is meant a large and exceptionally diverse family of the secondary active transporter class, which is responsible for transporting a range of different substrates, including sugars, drugs, hydrophobic molecules, peptides, organic ions, etc. The specificity of sugar transporter proteins is highly unpredictable and the identification of novel transporter protein with specificity towards for example oligosaccharides requires unburden laboratory experimentation (for more details see review by Reddy V.S. *et al.*, (2012), FEBS J. 279(11): 2022–2035). The term "MFS transporter" means in the present context protein that facilitates transport of an oligosaccharide, preferably, an HMO, through the cell membrane, preferably transport of an HMO/oligosaccharide synthesized by the host cell from the cell cytosol to the cell medium, preferably an HMO/oligosaccharide comprising three or four sugar units, e.g. 2'-FL, 3-FL, LNT-2, LNT, LNnT, 3'-SL or 6'-SL. Additionally, or alternatively, the MFS transporter, may also facilitate efflux of molecules that are not considered HMO or oligosaccharides according to the present invention, such as lactose, glucose, cell metabolites or toxins.

The term "sequence identity of [a certain] %" in the context of two or more nucleic acid or amino acid sequences means that the two or more sequences have nucleotides or amino acid residues in common in the given percent when compared and aligned for maximum correspondence over a comparison window or designated sequences of nucleic acids or amino acids (i.e. the sequences have at least 90

percent (%) identity). Percent identity of nucleic acid or amino acid sequences can be measured using a BLAST 2.0 sequence comparison algorithm with default parameters, or by manual alignment and visual inspection (see e.g. <http://www.ncbi.nlm.nih.gov/BLAST/>). This definition also applies to the complement
5 of a test sequence and to sequences that have deletions and/or additions, as well as those that have substitutions. An example of an algorithm that is suitable for determining percent identity, sequence similarity and for alignment is the BLAST 2.2.20+ algorithm, which is described in Altschul *et al. Nucl. Acids Res.* **25**, 3389 (1997). BLAST 2.2.20+ is used to determine percent sequence identity for the
10 nucleic acids and proteins of the invention. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). Examples of sequence alignment algorithms are CLUSTAL Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>), EMBOSS Needle (http://www.ebi.ac.uk/Tools/psa/emboss_needle/), MAFFT
15 (<http://mafft.cbrc.jp/alignment/server/>) or MUSCLE (<http://www.ebi.ac.uk/Tools/msa/muscle/>).

In the context of the invention, the term "oligosaccharide" means a saccharide polymer containing a number of monosaccharide units. In some
20 embodiments, preferred oligosaccharides are saccharide polymers consisting of three or four monosaccharide units, i.e. trisaccharides or tetrasaccharides. Preferable oligosaccharides of the invention are human milk oligosaccharides (HMOs).

The term "human milk oligosaccharide" or "HMO" in the present context means a complex carbohydrate found in human breast milk (for reference, see
25 Urashima *et al.: Milk Oligosaccharides*. Nova Science Publisher (2011); or Chen, *Adv. Carbohydr. Chem. Biochem.* 72, 113 (2015)). The HMOs have a core structure comprising a lactose unit at the reducing end that can be elongated by one or more beta-N-acetyl-lactosaminyl and/or one or more beta-lacto-N-biosyl units, and this core structure can be substituted by an alpha-L-fucopyranosyl and/or an alpha-N-acetyl-neuraminyl (sialyl) moiety. In this regard, the non-acidic (or neutral) HMOs are
30 devoid of a sialyl residue, and the acidic HMOs have at least one sialyl residue in their structure. The non-acidic (or neutral) HMOs can be fucosylated or non-fucosylated. Examples of such neutral non-fucosylated HMOs include lacto-N-triose 2 (LNT-2) lacto-N-tetraose (LNT), lacto-N-neotetraose (LNnT), lacto-N-neohexaose (LNnH), para-lacto-N-neohexaose (pLNnH), para-lacto-N-hexaose (pLNH) and lacto-
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N-hexaose (LNH). Examples of neutral fucosylated HMOs include 2'-fucosyllactose (2'-FL), lacto-N-fucopentaose I (LNFP-I), lacto-N-difucohexaose I (LNDFH-I), 3-fucosyllactose (3-FL), difucosyllactose (DFL), lacto-N-fucopentaose II (LNFP-II), lacto-N-fucopentaose III (LNFP-III), lacto-N-difucohexaose III (LNDFH-III), fucosyl-
 5 lacto-N-hexaose II (FLNH-II), lacto-N-fucopentaose V (LNFP-V), lacto-N-difucohexaose II (LNDFH-II), fucosyl-lacto-N-hexaose I (FLNH-I), fucosyl-para-lacto-N-hexaose I (FpLNH-I), fucosyl-para-lacto-N-neohexaose II (F-pLNnH II) and fucosyl-lacto-N-neohexaose (FLNnH). Examples of acidic HMOs include 3'-sialyllactose (3'-SL), 6'-sialyllactose (6'-SL), 3-fucosyl-3'-sialyllactose (FSL), 3'-O-sialyllacto-N-tetraose a (LST a), fucosyl-LST a (FLST a), 6'-O-sialyllacto-N-tetraose b
 10 (LST b), fucosyl-LST b (FLST b), 6'-O-sialyllacto-N-neotetraose (LST c), fucosyl-LST c (FLST c), 3'-O-sialyllacto-N-neotetraose (LST d), fucosyl-LST d (FLST d), sialyl-lacto-N-hexaose (SLNH), sialyl-lacto-N-neohexaose I (SLNH-I), sialyl-lacto-N-neohexaose II (SLNH-II) and disialyl-lacto-N-tetraose (DSLNT). In the context of the
 15 present invention lactose is not regarded as an HMO species.

In some embodiments of the invention, tri-HMOs and tetra-HMOs may be a preferred, e.g. trisaccharides 2'-FL, 3-FL, LNT-2, 3'-SL, 6'-SL, and tetrasaccharides DFL, LNT, LNnT, FSL.

To be able to synthesize one or more HMOs, the recombinant cell of the
 20 invention comprises at least one recombinant nucleic acid which encodes a functional enzyme with glycosyltransferase activity. The galactosyltransferase gene may be integrated into the genome (by chromosomal integration) of the host cell, or alternatively, it may be comprised in a plasmid DNA and expressed as plasmid-borne. If two or more glycosyltransferases are needed for the production of an HMO,
 25 e.g. LNT or LNnT, two or more recombinant nucleic acids encoding different enzymes with glycosyltransferase activity may be integrated in the genome and/or expressed from a plasmid, e.g. a beta-1,3-N-acetylglucosaminyltransferase (a first recombinant nucleic acid encoding a first glycosyltransferase) in combination with a beta-1,3-galactosyltransferase (a second recombinant nucleic acid encoding a
 30 second glycosyltransferase) for the production of LNT, where the first and second recombinant nucleic acid can independently from each other be integrated chromosomally or on a plasmid. In one preferred embodiment, both the first and second recombinant nucleic acids are stably integrated into the chromosome of the production cell; in another embodiment at least one of the first and second
 35 glycosyltransferase is plasmid-borne. A protein/enzyme with glycosyltransferase

- activity (glycosyltransferase) may be selected in different embodiments from enzymes having the activity of alpha-1,2-fucosyltransferase, alpha-1,3-fucosyltransferase, alpha-1,3/4-fucosyltransferase, alpha-1,4-fucosyltransferase, alpha-2,3-sialyltransferase, alpha-2,6-sialyltransferase, beta-1,3-N-acetylglucosaminyltransferase, beta-1,6-N-acetylglucosaminyltransferase, beta-1,3-galactosyltransferase and beta-1,4-galactosyltransferase. For example, the production of 2'-FL requires that the modified cell expresses an active alpha-1,2-fucosyltransferase enzyme; for the production of 3-FL the modified cell needs expression of an active alpha-1,3-fucosyltransferase enzyme; for the production of
- 10 LNT the modified cell need to express at least two glycosyltransferases, a beta-1,3-N-acetylglucosaminyltransferase and a beta-1,3-galactosyltransferase; for the production of 6'-SL the modified cell has to express an active alpha-2,6-sialyltransferase enzyme and a pathway for CMP-sialic acid synthesis; for the production of 3'-SL the modified cell has to express an active alpha-2,3-
- 15 sialyltransferase enzyme and a pathway for CMP-sialic acid synthesis. Some non-limiting embodiments of proteins having glycosyltransferase activity, which can be encoded by the recombinant genes comprised by the production cell, can be selected from non-limiting examples of Table 1.

Table 1

Gene	Protein Sequence ID (GenBank)	Description	HMO example
<i>lgtA_Nm</i>	WP_002248149.1	β -1,3-N-acetylglucosaminyltransferase	LNT, LNnT, LNFP-I, LNFP-II, LNFP-III, LNFP-V, LNFP-VI, LNDFH-I, LNDFH-II, pLNH, F-pLNH I, pLNnH
<i>lgtA_Nm_MC5</i> ₈	AAF42258.1	β -1,3-N-acetylglucosaminyltransferase	LNT, LNnT, LNFP-I, LNFP-II, LNFP-III, LNFP-V, LNFP-VI, LNDFH-I, LNDFH-II, pLNH, F-pLNH I, pLNnH
<i>lgtA_Hd</i>	AAN05638.1	β -1,3-N-acetylglucosaminyltransferase	LNT, LNnT, LNFP-I, LNFP-II, LNFP-III, LNFP-V, LNFP-VI, LNDFH-I, LNDFH-II, pLNH, F-pLNH I, pLNnH
<i>lgtA_Ng_PID2</i>	AAK70338.1	β -1,3-N-acetylglucosaminyltransferase	LNT, LNnT, LNFP-I, LNFP-II, LNFP-III, LNFP-V, LNFP-VI,

Gene	Protein Sequence ID (GenBank)	Description	HMO example
			LNDFH-I, LNDFH-II, pLNH, F-pLNH I, pLNnH
<i>lgtA_Ng_NCC P11945</i>	<i>ACF31229.1</i>	β -1,3-N-acetylglucosaminyl-transferase	LNT, LNnT, LNFP-I, LNFP-II, LNFP-III, LNFP-V, LNFP-VI, LNDFH-I, LNDFH-II, pLNH, F-pLNH I, pLNnH
<i>lgtA_Past</i>	<i>AAK02595.1</i>	β -1,3-N-acetylglucosaminyl-transferase	LNT, LNnT, LNFP-I, LNFP-II, LNFP-III, LNFP-V, LNFP-VI, LNDFH-I, LNDFH-II, pLNH, F-pLNH I, pLNnH
<i>lgtA_Nc</i>	<i>EEZ72046.1</i>	β -1,3-N-acetylglucosaminyl-transferase	LNT, LNnT, LNFP-I, LNFP-II, LNFP-III, LNFP-V, LNFP-VI, LNDFH-I, LNDFH-II, pLNH, F-pLNH I, pLNnH
<i>lgtA_Nm_8725 5</i>	<i>ELK60643.1</i>	β -1,3-N-acetylglucosaminyl-transferase	LNT, LNnT, LNFP-I, LNFP-II, LNFP-III, LNFP-V, LNFP-VI, LNDFH-I, LNDFH-II, pLNH, F-pLNH I, pLNnH
<i>galT_Hp/HP0826</i>	<i>NP_207619.1</i>	β -1,4-galactosyltransferase	LNnT, LNFP-III, LNFP-VI, pLNH I, F-pLNH I, pLNnH
<i>galT_Nm/lgtB</i>	<i>AAF42257.1</i>	β -1,4-galactosyltransferase	LNnT, LNFP-III, LNFP-VI, pLNH I, F-pLNH I, pLNnH
<i>wbgO</i>	<i>WP_000582563.1</i>	β -1,3-galactosyltransferase	LNT, LNFP-I, LNFP-II, LNFP-V, LNDFH-I, LNDFH-II, pLNH, F-pLNH I
<i>cpsIBJ</i>	<i>AB050723.1</i>	β -1,3-galactosyltransferase	LNT, LNFP-I, LNFP-II, LNFP-V, LNDFH-I, LNDFH-II, pLNH, F-pLNH I
<i>jhp0563</i>	<i>AEZ55696.1</i>	β -1,3-galactosyltransferase	LNT, LNFP-I, LNFP-II, LNFP-V, LNDFH-I, LNDFH-II, pLNH, F-pLNH I
<i>galTK</i>	<i>homologous to BD182026</i>	β -1,3-galactosyltransferase	LNT, LNFP-I, LNFP-II, LNFP-V, LNDFH-I, LNDFH-II, pLNH, F-pLNH I

Gene	Protein Sequence ID (GenBank)	Description	HMO example
<i>futC</i>	WP_080473865.1	α -1,2-fucosyl-transferase	2'-FL, DFL, LNFP-I, LNDFH-I
<i>FucT2_HpUA802</i>	AAC99764.1	α -1,2-fucosyl-transferase	2'-FL, DFL, LNFP-I, LNDFH-I
<i>FucT2_EcO126t</i>	ABE98421.1	α -1,2-fucosyl-transferase	2'-FL, DFL, LNFP-I, LNDFH-I
<i>FucT2_Hm12198</i>	CBG40460.1	α -1,2-fucosyl-transferase	2'-FL, DFL, LNFP-I, LNDFH-I
<i>FucT2_Pm9515</i>	ABM71599.1	α -1,2-fucosyl-transferase	2'-FL, DFL, LNFP-I, LNDFH-I
<i>FucT2_HpF57</i>	BAJ59215.1	α -1,2-fucosyl-transferase	2'-FL, DFL, LNFP-I, LNDFH-I
<i>FucT6_3_Bf</i>	CAH09151.1	α -1,3-fucosyl-transferase	2'-FL, 3-FL, DFL, LNFP-I, LNFP-III, LNFP-V, LNFP-VI, LNDFH-II, F-pLNH I
<i>FucT7_3_Bf</i>	CAH09495.1	α -1,3-fucosyl-transferase	2'-FL, 3-FL, DFL, LNFP-I, LNFP-III, LNFP-V, LNFP-VI, LNDFH-II, F-pLNH I
<i>FucT_3_Am</i>	ACD04596.1	α -1,3-fucosyl-transferase	2'-FL, 3-FL, DFL, LNFP-I, LNFP-III, LNFP-V, LNFP-VI, LNDFH-II, F-pLNH I
<i>MAMA_R764</i>	AGC02224.1	α -1,3-fucosyl-transferase	2'-FL, 3-FL, DFL, LNFP-I, LNFP-III, LNFP-V, LNFP-VI, LNDFH-II, F-pLNH I
<i>Mg791</i>	AEQ33441.1	α -1,3-fucosyl-transferase	2'-FL, 3-FL, DFL, LNFP-I, LNFP-III, LNFP-V, LNFP-VI, LNDFH-II, F-pLNH I
<i>Moumou_00703</i>	YP_007354660	α -1,3-fucosyl-transferase	2'-FL, 3-FL, DFL, LNFP-I, LNFP-III, LNFP-V, LNFP-VI, LNDFH-II, F-pLNH I
<i>futA</i>	NP_207177.1	α -1,3-fucosyl-transferase	2'-FL, 3-FL, DFL, LNFP-I, LNFP-III, LNFP-V, LNFP-VI, LNDFH-II, F-pLNH I
<i>fucT</i>	AAB81031.1	α -1,3-fucosyl-transferase	2'-FL, 3-FL, DFL, LNFP-I, LNFP-III, LNFP-V, LNFP-VI, LNDFH-II, F-pLNH I
<i>fucTIII</i>	AY450598.1	α -1,4-fucosyl-transferase	LNDFH-I, LNDFH-II
<i>fucTa</i>	AF194963.1	α -1,3/4-fucosyl-transferase	LNFP-II, LNDFH-I, LNDFH-II

Gene	Protein Sequence ID (GenBank)	Description	HMO example
<i>Pd2,6ST</i>	<i>BAA25316.1</i>	α -2,6-sialyltransferase	6'-SL
<i>PspST6</i>	<i>BAF92026.1</i>	α -2,6-sialyltransferase	6'-SL
<i>PiST6_145</i>	<i>BAF91416.1</i>	α -2,6-sialyltransferase	6'-SL
<i>PiST6_119</i>	<i>BAI49484.1</i>	α -2,6-sialyltransferase	6'-SL
<i>NST</i>	<i>AAC44541.1</i>	α -2,3-sialyltransferase	3'-SL

An aspect of the present invention is the provision of a nucleic acid construct comprising a heterologous nucleic acid sequence(s) encoding a protein capable of sugar transportation which is a major facilitator superfamily (MFS) protein as shown in

5 SEQ ID NO: 1, or a functional homologue thereof which amino acid sequence is at least 80 % identical to SEQ ID NO: 1, wherein the nucleic acid sequence encoding the MFS protein has at least 70 % sequence identity to SEQ ID NO: 2.

By the term "heterologous nucleic acid sequence", "recombinant

10 gene/nucleic acid/DNA encoding" or "coding nucleic acid sequence" is meant an artificial nucleic acid sequence (i.e. produced *in vitro* using standard laboratory methods for making nucleic acid sequences) that comprises a set of consecutive, non-overlapping triplets (codons) which is transcribed into mRNA and translated into a polypeptide when placed under the control of the appropriate control sequences,

15 i.e. promoter. The boundaries of the coding sequence are generally determined by a ribosome binding site located just upstream of the open reading frame at the 5' end of the mRNA, a transcriptional start codon (AUG, GUG or UUG), and a translational stop codon (UAA, UGA or UAG). A coding sequence can include, but is not limited to, genomic DNA, cDNA, synthetic, and recombinant nucleic acid sequences. The

20 term "nucleic acid" includes RNA, DNA and cDNA molecules. It is understood that, as a result of the degeneracy of the genetic code, a multitude of nucleotide sequences encoding a given protein may be produced. The term nucleic acid is used interchangeably with the term "polynucleotide". An "oligonucleotide" is a short chain nucleic acid molecule. "Primer" is an oligonucleotide, whether occurring naturally as

25 in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is induced,

(i.e., in the presence of nucleotides and an inducing agent such as DNA polymerase and at a suitable temperature and pH). The primer is preferably single stranded for maximum efficiency in amplification but may alternatively be double stranded. If double stranded, the primer is first treated to separate its strands before being used
5 to prepare extension products. Preferably, the primer is a deoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the inducing agent. The exact lengths of the primers will depend on many factors, including temperature, source of primer and the use of the method.

The recombinant nucleic sequence of the invention may be a coding DNA
10 sequence, e.g. a gene, or non-coding DNA sequence, e.g. a regulatory DNA, such as a promoter sequence. One aspect of the invention relates to providing a recombinant cell comprising recombinant DNA sequences encoding enzymes necessary for the production of one or more HMOs and a DNA sequence encoding Nec transporter. Accordingly, in one embodiment the invention relates to a nucleic
15 acid construct comprising a coding nucleic sequence, i.e. recombinant DNA sequence of a gene of interest, e.g. a glycosyltransferase gene or the *nec* gene, and a non-coding DNA sequence, e.g. a promoter DNA sequence, e.g. a recombinant promoter sequence derived from the promoter of *lac* operon or an *glp* operon, or a promoter sequence derived from another genomic promoter DNA sequence, or a
20 synthetic promoter sequence, wherein the coding and promoter sequences are operably linked. The term "operably linked" refers to a functional relationship between two or more nucleic acid (e.g., DNA) segments. Typically, it refers to the functional relationship of a transcriptional regulatory sequence to a transcribed sequence. For example, a promoter sequence is operably linked to a coding
25 sequence if it stimulates or modulates the transcription of the coding sequence in an appropriate host cell or other expression system. Generally, promoter transcriptional regulatory sequences that are operably linked to a transcribed sequence are physically contiguous to the transcribed sequence, i.e., they are cis-acting.

In one embodiment, the nucleic acid construct of the invention may be a
30 part of the vector DNA, in another embodiment the construct it is an expression cassette/cartridge that is integrated in the genome of a host cell. Accordingly, the term "nucleic acid construct" means an artificially constructed segment of nucleic acid, in particular a DNA segment, which is intended to be 'transplanted' into a target cell, e.g. a bacterial cell, to modify expression of a gene of the genome or express a
35 gene/coding DNA sequence which may be included in the construct. In the context of the invention, the nucleic acid construct contains a recombinant DNA sequence

comprising two or more recombinant DNA sequences: essentially, a non-coding DNA sequence comprising a promoter DNA sequence and a coding DNA sequence encoding a gene of interest, e.g. Nec protein, a glycosyltransferase, of another gene useful for production of an HMO in a host cell. Preferably, the construct comprises

5 further non-coding DNA sequences that either regulate transcription or translation of the coding DNA of the construct, e.g. a DNA sequence facilitating ribosome binding to the transcript, a leading DNA sequence that stabilize the transcript.

Integration of the recombinant nucleic acid of interest comprised in the construct (expression cassette) into the bacterial genome can be achieved by

10 conventional methods, e.g. by using linear cartridges that contain flanking sequences homologous to a specific site on the chromosome, as described for the attTn7-site (Waddell C.S. and Craig N.L., *Genes Dev.* (1988) Feb;2(2):137-49.); methods for genomic integration of nucleic acid sequences in which recombination is mediated by the Red recombinase function of the phage λ or the RecE/RecT

15 recombinase function of the λ prophage (Murphy, *J Bacteriol.* (1998);180(8):2063-7; Zhang *et al.*, *Nature Genetics* (1998) 20: 123-128 Muirers *et al.*, *EMBO Rep.* (2000) 1(3): 239-243); methods based on Red/ET recombination (Wenzel *et al.*, *Chem Biol.* (2005), 12(3):349-56.; Vetcher *et al.*, *Appl Environ Microbiol.* (2005);71(4):1829-35); or positive clones, i.e. clones that carry the

20 expression cassette, can be selected e.g. by means of a marker gene, or loss or gain of gene function.

A single copy of the expression cassette comprising a gene of interest may be sufficient to secure production of a desired HMO and achieve the desired effects according to the invention. Accordingly, in some preferred embodiments, the

25 invention relates to a recombinant HMO producing cell that comprises one, two or three copies of a gene of interest integrated in the genomic DNA of the cell. In some embodiments the single copy of the gene is preferred.

In one preferred embodiment, recombinant coding nucleic acid sequence of the nucleic acid construct of the invention is heterologous with respect to the

30 promoter, which means that in the equivalent native coding sequence in the genome of species of origin is transcribed under control of another promoter sequence (i.e. not the promoter sequence of the construct). Still, with respect to the host cell, the coding DNA may be either heterologous (i.e. derived from another biological species or genus), such as e.g. the DNA sequence encoding Nec protein expressed in

35 *Escherichia coli* host cells, or homologous (i.e. derived from the host cell), such as e.g. genes of the colonic acid operon, the *wca* genes.

The term, a "regulatory element" or "promoter" or "promoter region" or "promoter element" is a nucleic acid sequence that is recognized and bound by a DNA dependent RNA polymerase during initiation of transcription. The promoter, together with other transcriptional and translational regulatory nucleic acid sequences (also termed "control sequences") is necessary to express a given gene or group of genes (an operon). In general, the transcriptional and translational regulatory sequences include, but are not limited to, promoter sequences, ribosomal binding sites, transcriptional start and stop sequences, translational start and stop sequences, and enhancer or activator sequences. The "transcription start site" means the first nucleotide to be transcribed and is designated +1. Nucleotides downstream of the start site are numbered +2, +3, +4 etc., and nucleotides in the 5' opposite (upstream) direction are numbered -1, -2, -3 etc. The promoter DNA sequence of the construct can derive from a promoter region of any gene of the genome of a selected species, preferably, a promoter region of the genomic DNA of *E. coli*. Accordingly, any promoter DNA sequence that is able to bind to an RNA polymerase and initiate transcription is suitable for practicing the invention. In principle, any promoter DNA sequence can be used to control transcription of the recombinant gene of interest of the construct, different or same promoter sequences may be used to drive transcription of different genes of interest integrated in the genome of the host cell or in expression vector DNA. To have an optimal expression of the recombinant genes included in the construct, the construct may comprise further regulatory sequences, e.g. a leading DNA sequence, such as a DNA sequence derived from 5'-untranslated region (5'UTR) of a *glp* gene of *E. coli*, a sequence for ribosomal binding. Examples of the later sequences are described in WO2019123324 (incorporated herein by reference) and illustrated in non-limiting working examples herein.

In some preferred embodiments, the regulatory element for the regulation of the expression of a recombinant gene included in the construct of the invention is *glpFKX* operon promoter, *PglpF*, in other preferred embodiments, the promoter is *lac* operon promoter, *P_{lac}*.

In a further aspect the regulatory element for the regulation of the expression of a recombinant gene included in the construct of the invention is the *mgIBAC*; galactose/methyl-galactoside transporter promoter *PmgIB* or variants thereof such as but not limited to *PmgIB_70UTR* of SEQ ID NO: 15, or *PmgIB_70UTR_SD4* of SEQ ID NO: 16.

In a further aspect, the regulatory element for the regulation of the expression of a recombinant gene included in the construct of the invention is the *gatYZABCD*; tagatose-1,6-bisP aldolase promoter *PgatY* or variants thereof such as but not limited to *PgatY_U70UTR* of SEQ ID NO: 17.

5 The preferred regulatory element present in a genetically modified cell or in a nucleic acid construct of the present invention, is selected from the group consisting of *PgatY_70UTR*, *PglpF*, *PglpF_SD1*, *PglpF_SD10*, *PglpF_SD2*, *PglpF_SD3*, *PglpF_SD4*, *PglpF_SD5*, *PglpF_SD6*, *PglpF_SD7*, *PglpF_SD8*, *PglpF_SD9*, *Plac_16UTR*, *Plac*, *PmglB_70UTR* and *PmglB_70UTR_SD4*.

10 Especially preferred regulatory elements present in a genetically modified cell or in a nucleic acid construct of the present invention, is selected from the group consisting of *PglpF* and *Plac*.

However, any promoter enabling transcription and/or regulation of the level of transcription of one or more recombinant nucleic acids that encode one or more
15 proteins (or one or more regulatory nucleic acids) that are either necessary or beneficial to achieve an optimal level of biosynthetic production of one or more HMOs in the host cell, e.g. proteins involved in transmembrane transport of HMO, or HMO precursors, degradation of by-products of the HMO production, gene expression regulatory proteins, etc, and allowing to achieve the desired effects
20 according to the invention is suitable for practicing the invention.

Preferably, the construct of the invention comprising a gene related to biosynthetic production of an HMO, a promoter DNA sequence, and other regulatory sequences, such as a ribosomal binding site sequence (e.g. Shine-Dalgarno sequence), expressed in the host cell enables production of the HMO at the level of
25 at least 0,03 g/OD (optical density) of 1 liter of the fermentation media comprising a suspension of host cells, e.g., at the level of around 0.05 g/l/OD to around 0,1 g/l/OD. For the purposes of the invention, the later level of HMO production is regarded as "sufficient" and the host cell capable of producing this level of a desired HMO is regarded as "suitable host cell", i.e. the cell can be further modified to
30 express the HMO transporter protein, e.g. Nec, to achieve at least one effect described herein that is advantageous for the HMO production.

The genetically modified cell or the nucleic acid construct of the present invention comprises a nucleic acid sequence such as a heterologous gene encoding a putative MFS (major facilitator superfamily) transporter protein.

35 A MFS transport protein of particular interest in the present invention is Nec protein. A nucleic acid construct of the present invention therefore contains a nucleic

acid sequence having at least 70% sequence identity to the gene, *nec*, SEQ ID NO: 2.

The nucleic acid sequence contained in the genetically modified cell or in nucleic acid construct encodes for a protein of SEQ ID NO: 1, or a functional homologue thereof which amino acid sequence is at least 80 % identical to SEQ ID NO: 1.

A functional homologue of the protein of SEQ ID NO: 1, may be obtained by mutagenesis. The functional homologue should have a remaining functionality of at least 50 %, such as 60 %, 70 %, 80 %, 90 % or 100 % compared to the functionality of amino acid sequence of SEQ ID NO: 1. The functional homologue can have a higher functionality compared to the functionality of amino acid sequence of SEQ ID NO: 1. The functional homologue of SEQ ID NO: 1, should be able to enhance HMO production of the genetically modified cell according to the invention.

The genetically modified cell (host cell or recombinant cell) may be e.g. a bacterial or yeast cell. In one preferred embodiment, the genetically modified bacterial cell. Regarding the bacterial host cells, there are, in principle, no limitations; they may be eubacteria (gram-positive or gram-negative) or archaeobacteria, as long as they allow genetic manipulation for insertion of a gene of interest and can be cultivated on a manufacturing scale. Preferably, the host cell has the property to allow cultivation to high cell densities. Non-limiting examples of bacterial host cells that are suitable for recombinant industrial production of an HMO(s) according to the invention could be *Erwinia herbicola* (*Pantoea agglomerans*), *Citrobacter freundii*, *Pantoea citrea*, *Pectobacterium carotovorum*, or *Xanthomonas campestris*. Bacteria of the genus *Bacillus* may also be used, including *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus coagulans*, *Bacillus thermophilus*, *Bacillus laterosporus*, *Bacillus megaterium*, *Bacillus mycoides*, *Bacillus pumilus*, *Bacillus lentus*, *Bacillus cereus*, and *Bacillus circulans*. Similarly, bacteria of the genera *Lactobacillus* and *Lactococcus* may be modified using the methods of this invention, including but not limited to *Lactobacillus acidophilus*, *Lactobacillus salivarius*, *Lactobacillus plantarum*, *Lactobacillus helveticus*, *Lactobacillus delbrueckii*, *Lactobacillus rhamnosus*, *Lactobacillus bulgaricus*, *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus casei*, *Lactobacillus reuteri*, *Lactobacillus jensenii*, and *Lactococcus lactis*. *Streptococcus thermophiles* and *Propionibacterium freudenreichii* are also suitable bacterial species for the invention described herein. Also included as part of this invention are strains, modified as described here, from the genera *Enterococcus* (e.g., *Enterococcus faecium* and *Enterococcus thermophiles*), *Bifidobacterium* (e.g., *Bifidobacterium longum*, *Bifidobacterium infantis*, and *Bifidobacterium bifidum*),

Sporolactobacillus spp., *Micromonospora* spp., *Micrococcus* spp., *Rhodococcus* spp., and *Pseudomonas* (e.g., *Pseudomonas fluorescens* and *Pseudomonas aeruginosa*). Bacteria comprising the characteristics described herein are cultured in the presence of lactose, and an oligosaccharide, such as an HMO, produced by the
5 cell is retrieved, either from the bacterium itself or from a culture supernatant of the bacterium. In one preferred embodiment, the genetically modified cell of the invention is an *Escherichia coli* cell.

In another preferred embodiment the host cell is a yeast cell e.g. *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Pichia pastoris*,
10 *Kluyveromyces lactis*, *Kluyveromyces marxianus*, etc.

The HMOs produced by recombinant cells of the invention may be purified using a suitable procedure available in the art (e.g. such as described in WO2015188834, WO2017182965 or WO2017152918).

Genetically modified cells of the invention can be provided using standard
15 methods of the art e.g. those described in the manuals by Sambrook *et al.*, Wilson & Walker, "Maniatis *et al.*", and Ausubel *et al.*

A host suitable for the HMO production, e.g. *E. coli*, may comprise an endogenous β -galactosidase gene or an exogenous β -galactosidase gene, e.g. *E. coli* comprises an endogenous *lacZ* gene (e.g., GenBank Accession Number V00296
20 (GI:41901)). For the purposes of the invention, an HMO-producing host cell is genetically manipulated to either comprise any β -galactosidase gene or to comprise the gene that is inactivated. The gene may be inactivated by a complete or partial deletion of the corresponding nucleic acid sequence from the bacterial genome, or the gene sequence is mutated in the way that it is transcribed, or, if transcribed, the
25 transcript is not translated or if translated to a protein (i.e. β -galactosidase), the protein does not have the corresponding enzymatic activity. In this way the HMO-producing bacterium accumulates an increased intracellular lactose pool which is beneficial for the production of HMOs.

In some embodiments, the the engineered cell, e.g. bacterium, contains a
30 deficient sialic acid catabolic pathway. By "sialic acid catabolic pathway" is meant a sequence of reactions, usually controlled and catalyzed by enzymes, which results in the degradation of sialic acid. An exemplary sialic acid catabolic pathway described herein is the *E. coli* pathway. In this pathway, sialic acid (Neu5Ac; N-acetylneuraminic acid) is degraded by the enzymes NanA (N-acetylneuraminic acid
35 lyase) and NanK (N-acetylmannosamine kinase) and NanE (N-acetylmannosamine-6-phosphate epimerase), all encoded from the *nanATEK-yhcH* operon, and

repressed by NanR (<http://ecocyc.org/ECOLI>). A deficient sialic acid catabolic pathway is rendered in the *E. coli* host by introducing a mutation in the endogenous *nanA* (N-acetylneuraminase lyase) (e.g., GenBank Accession Number D00067.1 (GL216588)) and/or *nanK* (N-acetylmannosamine kinase) genes (e.g.,

5 GenBank Accession Number (amino acid) BAE77265.1 (GL85676015)), and/or *nanE* (N-acetylmannosamine-6-phosphate epimerase, GI: 947745, incorporated herein by reference). Optionally, the *nanT* (N-acetylneuraminase transporter) gene is also inactivated or mutated. Other intermediates of sialic acid metabolism include:

(ManNAc-6-P) N-acetylmannosamine-6-phosphate; (GlcNAc-6-P) N-

10 acetylglucosamine-6-phosphate; (GlcN-6-P) Glucosamine-6-phosphate, and (Fruc-6-P) Fructose-6-phosphate. In some preferred embodiments, *nanA* is mutated. In other preferred embodiments, *nanA* and *nanK* are mutated, while *nanE* remains functional. In another preferred embodiment, *nanA* and *nanE* are mutated, while *nanK* has not been mutated, inactivated or deleted. A mutation is one or more changes in the

15 nucleic acid sequence coding the gene product of *nanA*, *nanK*, *nanE*, and/or *nanT*. For example, the mutation may be 1, 2, up to 5, up to 10, up to 25, up to 50 or up to 100 changes in the nucleic acid sequence. For example, the *nanA*, *nanK*, *nanE*, and/or *nanT* genes are mutated by a null mutation. Null mutations as described herein encompass amino acid substitutions, additions, deletions, or insertions, which

20 either cause a loss of function of the enzyme (i.e. reduced or no activity) or loss of the enzyme (i.e. no gene product). By "deleted" is meant that the coding region is removed completely or in part such that no (functional) gene product is produced. By inactivated is meant that the coding sequence has been altered such that the resulting gene product is functionally inactive or encodes for a gene product with less

25 than 100 %, e.g. 90 %, 80 %, 70 %, 60 %, 50 %, 40 %, 30 % or 20 % of the activity of the native, naturally occurring, endogenous gene product. A "not mutated" gene or protein does not differ from a native, naturally-occurring, or endogenous coding sequence by 1, 2, up to 5, up to 10, up to 20, up to 50, up to 100, up to 200 or up to 500 or more codons, or to the corresponding encoded amino acid sequence.

30 Furthermore, the bacterium (e.g., *E. coli*) also comprises a sialic acid synthetic capability. For example, the bacterium comprises a sialic acid synthetic capability through provision of an exogenous UDP-GlcNAc 2-epimerase (e.g., *neuC* of *Campylobacter jejuni* (GenBank AAK91727.1) or equivalent (e.g. (GenBank CAR04561.1), a Neu5Ac synthase (e.g., *neuB* of *C. jejuni* (GenBank AAK91726.1)

35 or equivalent, (e.g. *Flavobacterium limnosediminis* sialic acid synthase, GenBank WP_023580510.1), and/or a CMP-Neu5Ac synthetase (e.g., *neuA* of *C. jejuni*

(GenBank AAK91728.1) or equivalent, (e.g. *Vibrio brasiliensis* CMP-sialic acid synthase, GenBank WP_006881452.1).

Production of neutral N-acetylglucosamine-containing HMOs in engineered bacteria is also known in the art (see e.g. Gebus C *et al.* (2012) Carbohydrate

5 Research 363 83-90).

For the production of N-acetylglucosamine-containing HMOs, such as Lacto-N-triose 2 (LNT-2), Lacto-N-tetraose (LNT), Lacto-N-neotetraose (LNnT), Lacto-N-fucopentaose I (LNFP-I), Lacto-N-fucopentaose II (LNFP-II), Lacto-N-fucopentaose III (LNFP-III), Lacto-N-fucopentaose V (LNFP-V), Lacto-N-difucohexaose I (LDFH-I), Lacto-N-difucohexaose II (LDFH-II), and Lacto-N-neodifucohexaose II (LNDFH-III), the bacterium comprises a functional *lacY* and a dysfunctional *lacZ* gene, as described above, and it is engineered to comprise an exogenous UDP-GlcNAc:Gal α / β -R beta-3-N-acetylglucosaminyltransferase gene, or a functional variant or fragment thereof. This exogenous UDP-GlcNAc:Gal α / β -R

10 beta-3-N-acetylglucosaminyltransferase gene may be obtained from any one of a number of sources, e.g., the *lgtA* gene described from *N. meningitidis* (Genbank protein Accession AAF42258.1) or *N. gonorrhoeae* (Genbank protein Accession ACF31229.1). Optionally, an additional exogenous glycosyltransferase gene may be co-expressed in the bacterium comprising an exogenous UDP-GlcNAc:Gal α / β -R

20 beta-3-N-acetylglucosaminyltransferase. For example, a beta-1,4-galactosyltransferase gene is co-expressed with the UDP-GlcNAc:Gal α / β -R beta-3-N-acetylglucosaminyltransferase gene. This exogenous beta-1,4-galactosyltransferase gene can be obtained from any one of a number of sources, e.g., the one described from *N. meningitidis*, the *lgtB* gene (Genbank protein

25 Accession AAF42257.1), or from *H. pylori*, the *HP0826/galT* gene (Genbank protein Accession NP_207619.1). Optionally, the additional exogenous glycosyltransferase gene co-expressed in the bacterium comprising an exogenous UDP-GlcNAc:Gal α / β -R beta-3-N-acetylglucosaminyltransferase gene is a P-1,3-galactosyltransferase gene, e.g., that described from *E. coli* 055:H7, the *wbgO* gene (Genbank protein

30 Accession WP_000582563.1), or from *H. pylori*, the *jhp0563* gene (Genbank protein Accession AEZ55696.1), or from *Streptococcus agalactiae* type *Ib* O12 the *cpsIBJ* gene (Genbank protein Accession AB050723). Functional variants and fragments of any of the enzymes described above are also encompassed by the disclosed invention.

35 A N-acetylglucosaminyltransferase gene and/or a galactosyltransferase gene, can also be operably linked to a *Pglp* and be expressed from the

corresponding genome-integrated cassette. In one embodiment, the gene that is genome integrated is a gene encoding for a galactosyltransferase, e.g. *HP0826* gene encoding for the GalT enzyme from *H. pylori* (Genbank protein Accession NP_207619.1); in another embodiment, the gene that is genome integrated is a gene
5 encoding a beta-1,3-N-acetylglucosaminyltransferase, e.g. *lgtA* gene from *N. meningitidis* (Genbank protein Accession AAF42258.1). In these embodiments, the second gene, i.e. a gene encoding a beta-1,3-N-acetylglucosaminyltransferase or galactosyltransferase, correspondingly, may either be expressed from a genome-integrated or plasmid borne cassette. The second gene may optionally be expressed
10 either under the control of a *glp* promoter or under the control of any other promoter suitable for the expression system, e.g. *Plac*.

Unless otherwise specified, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Singleton *et al.* (1994) Dictionary of Microbiology and
15 Molecular Biology, second edition, John Wiley and Sons (New York) provides one of skill with a general dictionary of many of the terms used in this invention. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described. Most of the nomenclature and general laboratory
20 procedures required in this application can be found in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (2012); Wilson K. and Walker J., Principles and Techniques of Biochemistry and Molecular Biology (2010), Cambridge University Press; or in Maniatis *et al.*, Molecular Cloning A laboratory Manual, Cold Spring Harbor
25 Laboratory (2012); or in Ausubel *et al.*, Current protocols in molecular biology, John Wiley and Sohns (2010). The manuals are hereinafter referred to as "Sambrook *et al.*", "*Wilson & Walker*", "*Maniatis et al.*", "*Ausubel et al.*", correspondingly.

A second aspect of the invention related to a method for the production of
30 one or more HMOs, the method comprising the steps of:

- (i) providing a genetically modified cell capable of producing an HMO, wherein said cell comprises a recombinant nucleic acid encoding a protein of SEQ ID NO: 1, or a functional homologue thereof which amino acid sequence is at least 80 % identical, preferably at least
35 85 % identical, more preferably at least 90 % identical to SEQ ID NO: 1;

- (ii) culturing the cell of (i) in a suitable cell culture medium to allow the HMO production and expression of the DNA sequence to produce the protein having the amino acid sequence of SEQ ID NO: 1, or a functional thereof which amino acid sequence is at least 80 % identical, preferably at least 85 % identical, more preferably at least 90 % identical to SEQ ID NO: 1;
- (iii) harvesting the HMOs produced in step (ii).

According to the invention, the term "culturing" (or "cultivating" or "cultivation", also termed "fermentation") relates to the propagation of bacterial expression cells in a controlled bioreactor according to methods known in the industry.

To produce one or more HMOs, the HMO-producing bacteria as described herein are cultivated according to the procedures known in the art in the presence of a suitable carbon source, e.g. glucose, glycerol, lactose, etc., and the produced HMO is harvested from the cultivation media and the microbial biomass formed during the cultivation process. Thereafter, the HMOs are purified according to the procedures known in the art, e.g. such as described in WO2015188834, WO2017182965 or WO2017152918, and the purified HMOs are used as nutraceuticals, pharmaceuticals, or for any other purpose, e.g. for research.

Manufacturing of HMOs is typically accomplished by performing cultivation in larger volumes. The term "manufacturing" and "manufacturing scale" in the meaning of the invention defines a fermentation with a minimum volume of 5 L culture broth. Usually, a "manufacturing scale" process is defined by being capable of processing large volumes of a preparation containing the HMO or HMOs of interest and yielding amounts of the protein of interest that meet, e.g. in the case of a therapeutic compound or composition, the demands for clinical trials as well as for market supply. In addition to the large volume, a manufacturing scale method, as opposed to simple lab scale methods like shake flask cultivation, is characterized by the use of the technical system of a bioreactor (fermenter) which is equipped with devices for agitation, aeration, nutrient feeding, monitoring and control of process parameters (pH, temperature, dissolved oxygen tension, back pressure, etc.). To a large extent, the behavior of an expression system in a lab scale method, such as shake flasks, benchtop bioreactors or the deep well format described in the examples of the disclosure, does allow to predict the behavior of that system in the complex environment of a bioreactor.

With regard to the suitable cell medium used in the fermentation process, there are no limitations. The culture medium may be semi-defined, i.e. containing complex media compounds (e.g. yeast extract, soy peptone, casamino acids, etc.), or it may be chemically defined, without any complex compounds.

- 5 By the term "one or more HMOs" is meant that an HMO production cell may be able to produce a single HMO structure (a first HMO) or multiple HMO structures (a second, a third, etc. HMO). In some embodiments, it may be preferred a host cell that produces a single HMO, in other preferred embodiments, a host cell producing multiple HMO structures may be preferred. Non-limiting examples for host cells
- 10 producing single HMO structures are 2'-FL, 3-FL, 3'-SL, 6'-SL or LNT-2 producing cells. Non-limiting examples of host cells capable of producing multiple HMO structures can be DFL, FSL, LNT, LNnT, LNFP I, LNFP II, LNFP III, LNFP IV, LNFP V, pLNnH, pLNH2 producing cells.

- The term "harvesting" in the context in the invention relates to collecting the
- 15 produced HMO(s) following the termination of fermentation. In different embodiments it may include collecting the HMO(s) included in both the biomass (i.e. the host cells) and cultivation media, i.e. before/without separation of the fermentation broth from the biomass. In other embodiments the produced HMOs may be collected separately from the biomass and fermentation broth, i.e. after/following the separation of
- 20 biomass from cultivation media (i.e. fermentation broth). The separation of cells from the medium can be carried out with any of the methods well known to the skilled person in the art, such as any suitable type of centrifugation or filtration. The separation of cells from the medium can follow immediately after harvesting the fermentation broth or be carried out at a later stage after storing the fermentation
- 25 broth at appropriate conditions. Recovery of the produced HMO(s) from the remaining biomass (or total fermentation) include extraction thereof from the biomass (the production cells). It can be done by any suitable methods of the art, e.g. by sonication, boiling, homogenization, enzymatic lysis using lysozyme, or freezing and grinding.

- 30 After recovery from fermentation, HMO(s) are available for further processing and purification.

Purification of HMOs produced by fermentation can be done using a suitable procedure described in WO2016095924, WO2015188834, WO2017152918, WO2017182965, US20190119314 (all incorporated by reference).

In some embodiments of the invention, a host cell may produce several HMOs, wherein one HMO is the "product" HMO and some/all the other HMOs are "by-product" HMOs. Typically, by-product HMOs are either the major HMO precursors or products of further modification of the major HMO. In some
5 embodiments, it may be desired to produce the product HMO in abundant amounts and by-product HMOs in minor amounts. Cells and methods for HMO production described herein allow for controlled production of an HMO product with a defined HMO profile, e.g. in one embodiment, the produced HMO mixture wherein the product HMO is a dominating HMO compared to the other HMOs (i.e. by-product
10 HMOs) of the mixture, i.e. the product HMO is produced in higher amounts than other by-product HMOs; in other embodiments, the cell producing the same HMO mixture may be tuned to produce one or more by-product HMOs in higher amount than product HMO. For example, during the production of 2'-FL, the product HMO, often a significant amount of DFL, the by-product HMO, is produced. With the
15 genetically modified cells of the present invention the level of DFL in the 2'-FL product can be significantly reduced.

Advantageously, the invention provides both a decreased ratio of by-product to product and an increased overall yield of the product (and/or HMOs in total). This, less by-product formation in relation to product formation facilitates an elevated
20 product production and increases efficiency of both the production and product recovery process, providing superior manufacturing procedure of HMOs.

In different preferred embodiments, different host cells producing either/both 2'-FL, 3-FL, 3'-SL, 6'-SL, LNT-2, DFL, FSL, LNT, LNnT, DFL, FSL, LNT, LNnT, LNFP I, LNFP II, LNFP III, LNFP IV, LNFP V, pLNnH, pLNH2, as the product or by-
25 product HMO, may be selected. In one preferred embodiment, the product is 3-FL and by-product is DFL. In another preferred embodiment, the product is 2'-FL and by-product is DFL. In another preferred embodiment, the product is LNT-2, and by-products are LNT and LNFP I.

30 The invention also relates to the use of a genetically modified cell or a nucleic acid construct according to the invention, for the production of one or more oligosaccharides, preferably one or more human milk oligosaccharide(s). In one embodiment, the genetically modified cell or the nucleic acid construct according to the invention is used in the production of a specific HMO selected from the group
35 consisting of 2'-FL, 3-FL, DLF, LNT, LNT-II, LNnT, LNFP-1, pLNnH and pLNH-II. In a preferred embodiment the genetically modified cell or the nucleic acid construct

according to the invention is used in the production of a specific HMO selected from the group consisting of 2'-FL, 3-FL, DLF, LNT, LNT-II, LNnT, pLNnH and pLNH-II.

In an especially preferred embodiment the genetically modified cell or the nucleic acid construct according to the invention is used in the production of a
5 specific HMO selected from the group consisting of 2'-FL, 3-FL, LNT, LNT-II, LNnT and pLNH-II.

The invention is further illustrated by non-limiting examples and embodiments below.

10 EXAMPLES

Materials and Methods

Unless otherwise noted, standard techniques, vectors, control sequence elements, and other expression system elements known in the field of molecular
15 biology are used for nucleic acid manipulation, transformation, and expression. Such standard techniques, vectors, and elements can be found, for example, in: Ausubel *et al.* (eds.), *Current Protocols in Molecular Biology* (1995) (John Wiley & Sons); Sambrook, Fritsch, & Maniatis (eds.), *Molecular Cloning* (1989) (Cold Spring Harbor Laboratory Press, NY); Berger & Kimmel, *Methods in Enzymology* 152: Guide to
20 Molecular Cloning Techniques (1987) (Academic Press); Bukhari *et al.* (eds.), *DNA Insertion Elements, Plasmids and Episomes* (1977) (Cold Spring Harbor Laboratory Press, NY); Miller, J.H. *Experiments in molecular genetics* (1972.) (Cold spring Harbor Laboratory Press, NY)

25 Strains

The bacterial strain used, MDO, was constructed from *Escherichia coli* K12 DH1. The *E. coli* K12 DH1 genotype is: F^- , λ^- , *gyrA96*, *recA1*, *relA1*, *endA1*, *thi-1*, *hsdR17*, *supE44*. Strains utilized in the present Examples are described in Table 2.

Table 2

Strain IDs	Product	Relevant Genotype
DH1	-	<i>F⁻ λ endA1 recA1 relA1 gyrA96 thi-1 glnV44 hsdR17(r_K m_K)</i>
MDO	-	<i>E coli DH1 ΔlacZ, ΔlacA, ΔnanKETA, ΔmelA, ΔwcaJ, ΔmdoH</i>
MPA1	2'-FL	<i>MDO CA -futC</i>
MPA2		<i>MDO CA futC nec</i>
MPA3	3-FL	<i>MDO CA futA</i>
MPA4		<i>MDO CA futA nec</i>
MP4002	LNT2	<i>MDO lgtA lgtA</i>
MP4039		<i>MDO lgtA nec</i>
MP4473	LNT	<i>MDO lgtA galTK</i>
MP4537		<i>MDO -lgtA galTK nec</i>
MP2789	LNFP-I	<i>MDO lgtA galTK futC CA</i>
MP4597		<i>MDO gtA galTK futC nec</i>

Media

The Luria Broth (LB) medium was made using LB Broth Powder, Millers (Fisher Scientific) and LB agar plates were made using LB Agar Powder, Millers (Fisher Scientific). When appropriated ampicillin ((100 μ g/mL) or any appropriate antibiotic), and/or chloramphenicol (20 μ g/mL) was added.

Basal Minimal medium had the following composition: NaOH (1 g/L), KOH (2.5 g/L), KH₂PO₄ (7 g/L), NH₄H₂PO₄ (7 g/L), Citric acid (0.5 g/l), Trace mineral solution (5 mL/L). The trace mineral stock solution contained: ZnSO₄*7H₂O 0.82 g/L, Citric acid 20 g/L, MnSO₄*H₂O 0.98 g/L, FeSO₄*7H₂O 3.925 g/L, CuSO₄*5H₂O 0.2 g/L. The pH of the Basal Minimal Medium was adjusted to 7.0 with 5 N NaOH and autoclaved. Before inoculation the Basal Minimal medium was supplied with 1 mM MgSO₄, 4 μ g/mL thiamin, 0.5 % of a given carbon source (glycerol (Carbosynth)), and when appropriated Isopropyl- β -D-Thiogalactoside (IPTG) (0.2 mM) was added. Thiamin, antibiotics, and IPTG were sterilized by filtration. All percentage concentrations for glycerol are expressed as v/v and for glucose as w/v.

M9 plates containing 2-deoxy-galactose had the following composition: 15 g/L agar (Fisher Scientific), 2.26 g/L 5x M9 Minimal Salt (Sigma-Aldrich), 2 mM MgSO₄, 4 μ g/mL thiamine, 0.2 % glycerol, and 0.2 % 2-deoxy-D-galactose (Carbosynth).

MacConkey indicator plates had the following composition: 40 g/L MacConkey agar Base (BD Difco™) and a carbon source at a final concentration of 1 %.

Cultivation

Unless otherwise noted, *E. coli* strains were propagated in Luria-Bertani (LB) medium containing 0.2 % glucose at 37°C with agitation. Agar plates were incubated at 37°C overnight.

5 *Chemical competent cells and transformations*

- E. coli* was inoculated from LB plates in 5 mL LB containing 0.2 % glucose at 37°C with shaking until OD₆₀₀ ~0.4. 2 mL culture was harvested by centrifugation for 25 seconds at 13.000 g. The supernatant was removed, and the cell pellet resuspended in 600 µL cold TB solutions (10 mM PIPES, 15 mM CaCl₂, 250 mM KCl).
- 10 The cells were incubated on ice for 20 minutes followed by pelleting for 15 seconds at 13.000 g. The supernatant was removed, and the cell pellet resuspended in 100 µL cold TB solution. Transformation of plasmids were done using 100 µL competent cells and 1 to 10 ng plasmid DNA. Cells and DNA were incubated on ice for 20 minutes before heat shocking at 42°C for 45 seconds. After 2 min incubation on ice 400 µL
- 15 SOC (20 g/L tryptone, 5 g/L Yeast extract, 0.5 g/L NaCl, 0.186 g/L KCl, 10 mM MgCl₂, 10 mM MgSO₄ and 20 mM glucose) was added and the cell culture was incubated at 37°C with shaking for 1 hour before plating on selective plates.

Plasmid were transformed into TOP10 chemical competent cells at conditions recommended by the supplier (ThermoFisher Scientific).

20

DNA techniques

- Plasmid DNA from *E. coli* was isolated using the QIAprep Spin Miniprep kit (Qiagen). Chromosomal DNA from *E. coli* was isolated using the QIAamp DNA Mini Kit (Qiagen). PCR products were purified using the QIAquick PCR Purification Kit
- 25 (Qiagen). DreamTaq PCR Master Mix (ThermoFisher), Phusion U hot start PCR master mix (ThermoFisher), USER Enzyme (New England Biolab) were used as recommended by the supplier. Primers were supplied by Eurofins Genomics, Germany. PCR fragments and plasmids were sequenced by Eurofins Genomics. Colony PCR was done using DreamTaq PCR Master Mix in a T100™ Thermal Cycler
- 30 (Bio-Rad).

Table 3: Oligos used for amplification of plasmid backbones, promoter elements, and *nec*

Name	SEQ ID NO	Oligonucleotide Sequence 5' – 3'	Description
O40	5	ATTAACCCUCCAGGCATCAAATAAAACGAAAGG C	Backbone.for
O68	6	ATGCGCAAAUTGTGAGTTAGCTCACTCATTAG	Plac.for
O79	7	ATTTGCGCAUCACCAATCAAATTCACGCGGCC	Backbone.rev
O113	8	AGCTGTTUCCTCCTTAGGTACCCAGCTTTTGTTCC	Plac.rev
O261	9	ATGCGCAAAUGCGGCACGCCTTGCAGATTACG	PglpF.for
O262	10	AGCTGTTUCCTCCTTGGTTAATGTTTGTGTATGCG	PglpF.rev
O741	11	AAACAGCUATGCAGAGCTTCACCCCGCC	nec.for
O742	12	AGGGTTAAUTTACGCCTGCTCTTTAACACGCAGC	nec.rev
O48	13	CCCAGCGAGACCTGACCGCAGAAC	galK.for
O49	14	CCCCAGTCCATCAGCGTGACTACC	galK.rev

Table 4: The heterologous proteins expressed in the HMO producing cells

Gene	Origin of Genes	Protein Accession Number	Protein Function
<i>futC</i>	<i>Helicobacter pylori</i> 26695	EF452503	<i>alpha-1,2-fucosyl-transferase</i>
<i>lgtA</i>	<i>Neisseria meningitidis</i> 053442	CP000381	<i>beta-1,3-N-acetylglucosaminyltransferase</i>
<i>galT</i>	<i>Helicobacter pylori</i> 26695	AE000511	<i>beta-1,4-galactosyltransferase</i>
<i>galTK</i>	<i>Helicobacter pylori</i> 43504	homologous to BD182026	<i>beta-1,3-galactosyltransferase</i>
<i>nec</i>	<i>Rosenbergiella nectarea</i>	WP_092672081.1	MFS transporter

Table 5: The synthetic DNA elements utilized for expression of *nec*

Sequence name	SEQ ID NO	Sequence (5'-3')	Description
<i>PglpF</i>	3	GCGGCACGCCTTGCAGATTACGGTTT GCCACACTTTTCATCCTTCTCCTGGT GACATAATCCACATCAATCGAAAATGT TAATAAATTTGTTGCGCGAATGATCTA ACAAACATGCATCATGTACAATCAGAT GGAATAAATGGCGCGATAACGCTCAT TTTATGACGAGGCACACACATTTTAA GTTTCGATATTTCTCGTTTTTGCTCGTT AACGATAAGTTTACAGCATGCCTACA AGCATCGTGGAGGTCCGTGACTTTCA CGCATACAACAAACATTAACCAAGGA GGAAACAGCT	300-nucleotide DNA expression element
<i>Plac</i>	4	ATGCGCAAATTGTGAGTTAGCTCACT CATTAGGCACCCCAGGCTTTACACTT TATGCTTCCGGCTCGTATGTTGTGTG GAATTGTGAGCGGATAACAATTTTAC ACAGGAAACAGCTATGACCATGATTA CGCCAAGCGCGCAATTAACCCCTCACT AAAGGGAACAAAAGCTGGGTACCTAA GGAGGAAACAGCT	195-nucleotide DNA expression element

<i>nec</i>	2	ATGCAGAGCTTCACCCCGCCGGCGC CGAAGGGTGGCAACCCGGTGTTTAT GATGTTTATGCTGGTGACCTTCTTTGT GAGCATTGCGGGTGCCTGCAGGCG CCGACCCTGAGCCTGTACCTGAGCC AAGAGCTGGCGGCGAAACCGTTCAT GGTGGGCCTGTTCTTTACCATTAAACG CGGTTACCGGTATCATTATCAGCTTTA TCCTGGCGAAGCGTAGCGACCGTAA AGGTGACCGTCGTCGTCTGCTGATGT TCTGCTGCGCGATGGCGATCGCGAA CGCGCTGATGTTTCGCGTTTGTTCTGC AGTACGTGGTTCTGATTACCCTGGGC CTGATCCTGAGCGCGCTGACCAGCG TGGTTATGCCGCAACTGTTTCGCGCTG GCGCGTGAGTATGCGGACCGTACCG GTCGTGAAGTGGTTATGTTTAGCAGC GTGATGCGTACCCAAATGAGCCTGGC GTGGGTTATTGGCCCGCCGATCAGCT TCGCGCTGGCGCTGAACTACGGTTTT ATTACCCTGTATCTGGTGGCTGCGGC GCTGTTTCTGCTGAGCCTGATTCTGA TCAAGACCACCCTGCCGAGCGTTCC GCGTCTGTATCCGGCGGAAGACCTG GCGAAGAGCGCGGCGAGCGGTTGGA AACGTACCGATGTGCGTTTTCTGTTT GCGGCGAGCGTGCTGATGTGGGTTT GCAACCTGATGTACATTATCGATATG CCGCTGTATATCAGCAAAAGCCTGGG TATGCCGGAGAGCTTCGCGGGTGTT CTGATGGGCACCGCGGCGGGTCTGG AAATTCCGGTGATGCTGCTGGCGGG CTACCTGGCGAAGCGTGTTGGTAAAC GTCCGCTGGTGATTGTTGCGGCGGT GTGCGGCCTGGCGTTCTATCCGGCG ATGCTGGTTTTTACCAGCAAACCGG TCTGCTGATTATCCAGCTGCTGAACG CGGTGTTTATTGGCATCGTGGCGGG TCTGGTTATGCTGTGGTTTCAAGACC TGATGCCGGGTAAAGCGGGTGCGGC GACCACCCTGTTACCAACAGCGTTA GCACCGGCATGATCTTTGCGGGCCT GTGCCAGGGTCTGCTGAGCGATCTG CTGGGTCACCAAGCGATTTACGTGCT GGCGACCGTGCTGATGGTTATCGCG CTGCTGCTGCTGCTGCGTGTTAAAGA GCAGGCGTAA	DNA encoding putative MFS transporter WP_092672081 .1 (Nec)
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Construction of Plasmids

Plasmid backbones containing two *I-SceI* endonuclease sites, separated by two DNA fragments appropriated for homologous recombination into the *E. coli*

genome and a T1 transcriptional terminator sequence were synthesized. For example, in one plasmid backbone the *gal* operon (required for homologous recombination in *galK*), and a T1 transcriptional terminator sequence (pUC57::*gal*) was synthesized (GeneScript). The DNA sequences used for homologous recombination in the *gal* operon covered base pairs 3.628.621-3.628.720 and 3.627.572-3.627.671 in sequence *Escherichia coli* K-12 MG155 complete genome GenBank: ID: CP014225.1. Insertion by homologous recombination would result in a deletion of 949 base pairs of *galK* and a *galK*- phenotype. In similar ways backbones based on pUC57 (GeneScript) or an any other appropriated vector containing two *I-SceI* endonuclease sites, separated by two DNA fragments appropriated for homologous recombination into the *E. coli* genome and a T1 transcriptional terminator sequence could be synthesized. Standard techniques well-known in the field of molecular biology were used for designing of primers and amplification of specific DNA sequences of the *Escherichia coli* K-12 DH1 chromosomal DNA. Such standard techniques, vectors, and elements can be found, for example, in: Ausubel *et al.* (eds.), *Current Protocols in Molecular Biology* (1995) (John Wiley & Sons); Sambrook, Fritsch, & Maniatis (eds.), *Molecular Cloning* (1989) (Cold Spring Harbor Laboratory Press, NY); Berger & Kimmel, *Methods in Enzymology* 152: Guide to Molecular Cloning Techniques (1987) (Academic Press); Bukhari *et al.* (eds.).

Chromosomal DNA obtained from *E. coli* DH1 was used to amplify a 300 bp DNA fragment containing the promoter *PglpF* using oligos O261 and O262, and a 195 bp DNA fragment containing *Plac* using oligos O68 and O113 (Table 3).

A 1.185 bp DNA fragment containing a codon optimized version of the *nec* gene originating from *Rosenbergiella nectarea* was synthesized by GeneScript (Table 5). The *nec* gene was amplified by PCR using oligos O741 and O742.

All PCR fragments (plasmid backbones, promoter containing elements and the *nec* gene) were purified, and plasmid backbones, promoter elements (*PglpF*, or *Plac*), and a *nec* containing DNA fragment were assembled. The plasmids were cloned by standard USER cloning. Cloning in any appropriated plasmid could be done using any standard DNA cloning techniques. The plasmids were transformed into TOP10 cells and selected on LB plates containing 100 µg/mL ampicillin (or any appropriated antibiotic) and 0.2 % glucose. The constructed plasmids were purified and the promoter sequence and the 5'end of the *nec* gene was verified by DNA sequencing (MWG Eurofins Genomics). In this way, a genetic cassette containing any promoter of interest linked to the *nec* gene was constructed.

Table 6: Examples of Helper and Donor plasmids used for strain construction

Plasmid	Relevant genotype	Marker gene
pACBSR	<i>Para-I-SceI-λ Red, p15A ori, cam*</i>	cam
pUC57	<i>pMB1, bla</i>	bla
pUC57:: <i>gal</i>	<i>pUC57::<i>galTK'</i> / T1-<i>galKM'</i></i>	bla

Construction of strains

The bacterial strain used, MDO, was constructed from *Escherichia coli* K-12 DH1. The *E. coli* K-12 DH1 genotype is: *F⁻, λ ⁻, gyrA96, recA1, relA1, endA1, thi-1, hsdR17, supE44*. In addition to the *E. coli* K-12 DH1 genotype MDO has the following modifications: *lacZ*: deletion of 1.5 kbp, *lacA*: deletion of 0.5 kbp, *nanKETA*: deletion of 3.3 kbp, *meIA*: deletion of 0.9 kbp, *wcaJ*: deletion of 0.5 kbp, *mdoH*: deletion of 0.5 kbp, and insertion of *Plac* promoter upstream of the *gmd* gene.

Insertion of an expression cassette containing a promoter linked to the *nec* gene and to a T1 transcriptional terminator sequence was performed by Gene Gorging essentially as described by Herring *et al.* (Herring, C.D., Glasner, J.D. and Blattner, F.R. (2003). *Gene* (311). 153-163). Briefly, the donor plasmid and the helper plasmid were co-transformed into MDO and selected on LB plates containing 0.2 % glucose, ampicillin (100 μ g/mL) or kanamycin (50 mg/mL) and chloramphenicol (20 μ g/mL). A single colony was inoculated in 1 mL LB containing chloramphenicol (20 μ g/mL) and 10 μ L of 20 % L-arabinose and incubated at 37°C with shaking for 7 to 8 hours. For integration in the *galK* loci of *E. coli* cells were then plated on M9-DOG plates and incubated at 37°C for 48 hours. Single colonies formed on MM-DOG plates were re-streaked on LB plates containing 0.2 % glucose and incubated for 24 hours at 37°C. Colonies that appeared white on MacConkey-galactose agar plates and were sensitive for both ampicillin and chloramphenicol were expected to have lost the donor and the helper plasmid and contain an insertion in the *galK* loci. Insertions in the *galK* site was identified by colony PCR using primers O48 (SEQ ID NO: 13) and O49 (SEQ ID NO: 14) and the inserted DNA was verified by sequencing (Eurofins Genomics, Germany).

Insertion of genetic cassettes at other loci in the *E. coli* chromosomal DNA was done in a similar way using different selection marker genes.

Deep Well Assay

A single colony from an LB-plate was pre-cultured in 1 mL Basal Minimum media containing 5 g/L glucose, 1 M MgSO₄ and 4 mg/L thiamine in a 10 mL 24 Deep

well plate (Axygen). The plate was sealed before culturing with a Hydrophobic Gas Permeable Adhesive Seal (Axygen) and incubated for 24 hours at 34°C with shaking at 700 rpm in an orbital shaker (Edmund Bühler GmbH). Cell density of the culture was monitored at 600 nm using an S-20 spectrophotometer (Boeco, Germany). 40 µl of the overnight culture was used for inoculation in 2 mL Basal Minimum media containing 0.1 g/L glucose, 5 g/L lactose, 20 g/L sucrose, 1 M MgSO₄, 4 mg/L thiamine and 0.25 mg/L SUH (Sigma). IPTG were added if appropriated. The Deep well plates were covered with sealing foil and incubated for 48 or 72 hours at 28°C with orbital shaking at 700 rpm. After incubation, OD₆₀₀ was measured and the plate covered with sealing tape for heating (Saveen Werner) and incubated in a Thermomixer for 1 hour at 100°C with shaking at 400 rpm.

For the analysis of total samples, the cell lysate prepared by boiling was pelleted by centrifugation for 10 minutes at 4.700 rpm. The HMO concentration in the supernatant was determined by HPLC or HPAC methods.

After an initial centrifugation of the deep well plate for 10 minutes at 4.700 rpm, supernatant samples were kept for analytical measurements. For the analysis of the cell pellet, the remaining supernatant was thrown away and cells were washed with 2 mL cold PBS. After this washing step and re-centrifugation, the pellet was re-suspended in 500 mL MQ water and the whole deep well plate was boiled in a Thermomixer for 1 hour with shaking at 400 rpm. The cell lysate prepared by boiling was pelleted by centrifugation for 10 minutes at 4.700 rpm. The HMO concentration in the supernatant was determined by HPLC or HPAC methods.

Results

Example 1. Engineering of *Escherichia coli* for 2'-FL production expressing the *nec* gene

The *Escherichia coli* K-12 (DH1) MDO strains can be manipulated to express heterologous genes of interest. For instance, the strain MPA1 is a 2'-FL production strain overexpressing the alpha-1,2-fucosyltransferase gene, *futC*, and the colonic acid genes (*gmd-fcl-gmm-wcaI-cpsB-cpsG*). Insertion of an expression cassette containing a promoter element (*PglpF*) linked to a *nec* gene into the MPA2 chromosomal DNA resulted in i) relative higher titers of 2'-FL (Fig. 1A), ii) lower amount of 2'-FL in the cell fraction and higher amounts of 2'-FL in the media (Fig. 1B), iii) relative lower ratio of DFL to 2'-FL (Fig. 1C), and iv) relative lower optical density when measuring cell density at 600 nm (Fig. 1D).

Example 2. Engineering of *Escherichia coli* for 3-FL production expressing the *nec* gene

The *Escherichia coli* K-12 (DH1) MDO strains can be manipulated to express
5 heterologous genes of interest. For instance, the strain MPA3 is a 3-FL production strain overexpressing the alpha-1,3-fucosyltransferase gene, *futA*, and the colonic acid genes (*gmd-fcl-gmm-wcaI-cpsB-cpsG*). Insertion of an expression cassette containing a promoter element (*Plac*) linked to *nec* gene in a single copy into the MPA3 background strain (see MPA4 strain) resulted in i) relatively higher amounts of 3-FL in
10 the media fraction and ii) relative lower amounts of 3-FL found inside the cells.

Example 3. Engineering of *Escherichia coli* for LNT2 production expressing the *nec* gene

The *Escherichia coli* K-12 (DH1) MDO strains can be manipulated to express
15 heterologous genes of interest. For instance, the strain MP4002 is a LNT2 production strain optimally overexpressing the beta-1,3-N-acetylglucosaminyltransferase gene, *lgtA* (Table 4).

Analysis of total samples (Figure 3) showed that LNT2 titer can be markedly improved when an expression cassette containing a promoter element (*PglpF*) linked
20 to a *nec* gene is integrated into the chromosomal DNA of strain MP4002 to generate the strain MP4039 (Table 2). Interestingly, the extracellular fraction of LNT2 is also much larger for strain MP4039 than for strain MP4002, a fact that is also reflected in the lower trisaccharide levels that are detected in the pellet fraction of the *nec*-expressing strain MP4039 compared to the corresponding levels in the non-
25 transporter expressing strain MP4002 (Figure 3).

Example 4. Engineering of *Escherichia coli* for LNT production using the *nec* gene

The *Escherichia coli* K-12 (DH1) MDO strains can be manipulated to express
30 heterologous genes of interest. For instance, the strain MP4473 is a LNT production strain overexpressing the beta-1,3-N-acetylglucosaminyltransferase gene, *lgtA*, and the beta-1,3-galactosyltransferase gene, *galTK* (Table 4).

Insertion of an expression cassette containing a promoter element (*PglpF*) linked to a *nec* gene into the chromosomal DNA of strain MP4473 generate the strain
35 MP4537 (Table 2) resulted in i) more than 2-fold increase in the total LNT titer, ii) a

moderate increase in total LNT2 concentration, and iii) more than 2-fold higher total pLNH2 formation (Figure 4).

The LNT concentration in the supernatant fraction of cultures of strain MP4537 is increased by 2-fold compared to the one measured in the medium of MP4473 (Figure 4). Although limited Nec-mediated LNT2 export can be observed, the extracellular LNT2 fraction in the strain MP4537 is only slightly higher than the one in strain MP4473. The LNT2 transport event occurs presumably at a slower pace than the Nec-mediated LNT export. Interestingly, despite the large increase in total pLNH2 formation in transporter-expressing cells, pLNH2 is solely found in the cell pellet of the strain MP4537 (Figure 4).

Example 5. Engineering of *Escherichia coli* for LNFP-I production using the *nec* gene

The *Escherichia coli* K-12 (DH1) MDO strains can be manipulated to express heterologous genes of interest. For instance, the strain MP2789 is a LNFP-I production strain overexpressing the beta-1,3-N-acetylglucosaminyltransferase gene, *lgtA*, the beta-1,3-galactosyltransferase gene, *galTK*, the alpha-1,2-fucosyltransferase gene, *futC*, and the native colonic acid genes (*gmd-fcl-gmm-wcaI-cpsB-cpsG*) (Table 4). Insertion of an expression cassette containing a promoter element (*PglpF*) linked to a *nec* gene into the chromosomal DNA of strain MP2789 to generate the strain MP4597 (Table 2) resulted in i) similar LNFP-I titers, ii) more than 2-fold increase in total 2'-FL titer, and iii) a markedly higher total HMO sum (2'-FL, LNFP-I and LNT) (Figure 5). The data presented here indicate that 2'-FL rather than LNFP-I and LNT is efficiently transported out of the cell expressing Nec transporter, and in this manner 2'-FL formation and subsequent export is favored in *nec*-expressing LNFP-I production cells.