

KDO-free production hosts for oligosaccharide synthesis

The present invention relates to the technical field of synthetic biology and metabolic engineering. More particularly, the present invention relates to the technical field of fermentation of
5 metabolically engineered microorganisms. The present invention describes engineered microorganisms which produce oligosaccharides which are free of KDO-lactose impurities and/or KDO-oligosaccharide impurities.

Background

10 Oligosaccharides can be produced via enzymatic, chemical as well as fermentative production. However, all of them have significant disadvantages. For instance, chemical synthesis requires many sequential chemical steps and enzymatic synthesis requires expensive precursors, whereas the fermentative process is still under heavy development. Nonetheless, the latter has the highest industrial production potential.

15 One problem observed with fermentative production of oligosaccharides, e.g. sialylated oligosaccharides, is that in some instances KDO-lactose and/or KDO-oligosaccharide is produced instead of the desired oligosaccharide.

Another problem that arises when adapting a microbial host for oligosaccharide synthesis is that an enormous burden is created on the cell wall integrity of the microbial host, since the host
20 (partially) shifts its cell wall glycan synthesis machinery into the synthesis of the desired oligosaccharide. Both cell wall glycan synthesis and oligosaccharide synthesis need specific nucleotide-activated sugars and glycosyltransferases that transfer the monosaccharide building block from a nucleotide-activated sugar onto a growing nascent saccharide chain. Often both cell wall glycan synthesis and oligosaccharide synthesis conflict with each other for the same
25 nucleotide-activated sugars and/or glycosyltransferases. As such, cells adapted for oligosaccharide synthesis are more vulnerable due to a weaker and/or altered cell wall composition, and, confer higher osmotic stress compared to the native microbial host.

The microbe's cytoplasm is usually hypertonic to its surrounding environment and the net flow of free water is into the microbial cell. Without a strong cell wall, the microbial cell would burst from
30 the osmotic pressure of the water flowing into the cell. To protect the cell from the environment and osmotic lysis, microbial cells are coated with an impressive array of distinct glycan structures that comprise their cell wall (Campanero-Rhodes et al., Front Microbiol. 2020 (doi.org/10.3389/fmicb.2019.02909); Tra and Dube, Chem. Commun. (Camb.) 2014, 50(36), 4659-4673; Vollmer et al., FEMS Microb. Rev. 2008, 32(2), 149-167).

35 Gram-negative bacteria (e.g. Escherichia coli) have a cell wall which consists of a double membrane enclosing a peptidoglycan (PG) containing periplasmic space. PG is a glycosidic structure being composed of alternating GlcNAc and MurNAc sugars that are cross-linked by short peptide bridges. The inner membrane (IM) or cytoplasmic membrane is a phospholipid

bilayer whereas the outer membrane (OM) is asymmetrical. The OM of Gram-negative bacteria is also a lipid bilayer, but its surface exposed outer leaflet is composed of lipopolysaccharides or LPS. The outer membrane structure protects the bacteria against harsh environmental conditions and forms a barrier against numerous stress factors. LPS is a glycopospholipid consisting of an antigenic, variable-size, carbohydrate chain covalently linked to lipid A, the conserved hydrophobic region structurally defined as N,O-acyl beta-1,6-D-glucosamine 1,4'-bisphosphate. The hydrophobic anchor of LPS is a glucosamine-based phospholipid backbone called 2-keto 3-deoxy-D-manno-octulosonate (KDO)-LipidA. The biogenesis of KDO-LipidA involves nine enzymes and was first uncovered by Christian R.H. Raetz, hence is referred to as the Raetz pathway. The Δ KDO phenotype is lethal. This lethal Δ KDO phenotype can be suppressed by overexpression of mutant msbA as disclosed by Meredith et al. (ACS Chem. Biol. 2006, 1(1):33-42) and/or by overexpression of mutant YhjD as disclosed by Mamat et al. (Mol. Microbiol. 2008, 67(3): 633-648) and WO 2013/036756 and/or by overexpression of LpxL as disclosed by WO 2017/119628. Both Meredith et al. (ACS Chem. Biol. 2006, 1(1):33-42) and Mamat et al. (Mol. Microbiol. 2008, 67(3): 633-648) do not further modify the viable KDO KO strains for production of a compound of interest. Both WO 2013/036756 and WO 2017/119628 further modify the viable KDO KO strains for production of recombinant protein, yet, recombinant protein expression does not create a burden on the cell wall integrity nor on the cell's resistance to osmotic stress. However, the suppression as found in the prior art described above is only partially and leaves the cell with a lowered resistance towards osmotic stress, which, as explained above, would be problematic when this cell is adapted to produce oligosaccharide.

Removal of KDO-oligosaccharides is done using a wide range of techniques such as ultracentrifugation, two-phase extraction or affinity chromatography, but there is no universal removal technique, and all depends highly on the product type and application. Drouillard et al. (Carbohydr. Res. 2010 Jul 2;345(10):1394-9) and WO2014153253 describe that a substantially KDO-lactose-free sialyllactose production can be obtained by fermentation of a sialyllactose producing micro-organism having a reduced expression of the sialyltransferase. This reduced expression was obtained by reducing the strength of the promoter or ribosome binding site, limiting transcription or translation of the transferase. WO2019/118829 overcomes the unwanted synthesis of KDO-lactose, as example of a KDO-oligosaccharide, by a microbial host by expressing specific exogenous lactose-utilizing sialyltransferase enzymes that have more preference for CMP-sialic acid than for CMP-KDO as a substrate.

Based on the above, it can be concluded that the production of the KDO-lactose, and in a broader sense also KDO-oligosaccharides, is due to the glycosyltransferase present in the production host having affinity to the CMP-KDO produced by the microbial cell naturally producing KDO. Such glycosyltransferases accept CMP-KDO thereby producing the KDO-oligosaccharide side product.

There is thus a need in the art to reduce the cost of downstream processing in respect of removal

of KDO-oligosaccharide and to create an expression host to be used in such a biotechnological production process for the production of oligosaccharides. Such expression platform should offer a way of oligosaccharides production while minimizing the cost for purifications and elimination.

5 **Description**

Surprisingly, we have been able to create a microbial cell naturally synthesizing KDO and genetically modified for the production of oligosaccharide wherein the KDO biosynthesis of the cell is knocked out or rendered less functional, such that the cell no longer produces KDO-lactose and/or KDO-oligosaccharides as unwanted side-product.

- 10 Gram-negative microbial cells contain a pathway for the synthesis of 3-deoxy-D-manno-octulosonate, so called KDO. This is an integral part of the outer membrane structure and forms a part of the endotoxins in e.g. the Enterobacteriaceae. The deletion of the synthesis of the endotoxins leads to cell death. On the other hand, the presence of CMP-KDO in the cell can interfere in other pathways and already proved to lead to unwanted impurities in fermentative
- 15 sialylated oligosaccharide production. Taking away the KDO-synthesis will destabilize the cell wall and cells will grow slower.

- In the present invention we have reduced and eliminated KDO synthesis in oligosaccharide production hosts wherein production of the oligosaccharide is not hampered significantly by the reduced cell growth when producing oligosaccharides. Oligosaccharides pose osmotic stress on
- 20 the cell, which is mediated by the outer membrane, KDO synthesis knockouts reduce said resistance. To our surprise still ample cell fitness was maintained after deletion of the KDO biosynthesis route. No KDO moieties are transferred anymore on said oligosaccharide. By doing so, the purity of said oligosaccharide is increased, by reducing KDO based side products. Said reduced KDO synthesis further also enhanced downstream processing of the oligosaccharide
- 25 production as no additional purification step would be needed anymore.

Definitions

- The words used in this specification to describe the invention and its various embodiments are to be understood not only in the sense of their commonly defined meanings, but to include by special
- 30 definition in this specification structure, material or acts beyond the scope of the commonly defined meanings. Thus, if an element can be understood in the context of this specification as including more than one meaning, then its use in a claim must be understood as being generic to all possible meanings supported by the specification and by the word itself.

- The various embodiments and aspects of embodiments of the invention disclosed herein are to be understood not only in the order and context specifically described in this specification, but to
- 35 include any order and any combination thereof. Whenever the context requires, all words used in the singular number shall be deemed to include the plural and vice versa. Unless defined otherwise, all technical and scientific terms used herein generally have the same meaning as

commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures in cell culture, molecular genetics, organic chemistry and nucleic acid chemistry and hybridization described herein are those well-known and commonly employed in the art. Standard techniques are used for nucleic acid and peptide synthesis. Generally, enzymatic reactions and purification steps are performed according to the manufacturer's specifications.

In the drawings and specification, there have been disclosed embodiments of the invention, and although specific terms are employed, the terms are used in a descriptive sense only and not for purposes of limitation, the scope of the invention being set forth in the following claims. It must be understood that the illustrated embodiments have been set forth only for the purposes of example and that it should not be taken as limiting the invention. It will be apparent to those skilled in the art that alterations, other embodiments, improvements, details and uses can be made consistent with the letter and spirit of the disclosure herein and within the scope of this disclosure, which is limited only by the claims, construed in accordance with the patent law, including the doctrine of equivalents. In the claims which follow, reference characters used to designate claim steps are provided for convenience of description only, and are not intended to imply any particular order for performing the steps.

"Isolated" means altered "by the hand of man" from its natural state, i.e., if it occurs in nature, it has been changed or removed from its original environment, or both. For example, a polynucleotide or a polypeptide naturally present in a living organism is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein. Similarly, a "synthetic" sequence, as the term is used herein, means any sequence that has been generated synthetically and not directly isolated from a natural source. "Synthesized", as the term is used herein, means any synthetically generated sequence and not directly isolated from a natural source.

The term "endogenous" within the context of the present disclosure refers to any polynucleotide, polypeptide or protein sequence which is a natural part of a cell and is occurring at its natural location in the cell chromosome. The term "exogenous" refers to any polynucleotide, polypeptide or protein sequence which originates from outside the cell under study and is not a natural part of the cell or which is not occurring at its natural location in the cell chromosome or plasmid.

"Recombinant" means genetically engineered DNA prepared by transplanting or splicing genes from one species into the cells of a host organism of a different species. Such DNA becomes part of the host's genetic make-up and is replicated. "Mutant" cell or microorganism as used within the context of the present disclosure refers to a cell or microorganism which is genetically engineered or has an altered genetic make-up.

The term "modified expression" of a gene relates to a change in expression compared to the wild type expression of said gene in any phase of the production process of the oligosaccharide. Said modified expression is either a lower or higher expression compared to the wild type, wherein the

term "higher expression" is also defined as "overexpression" of said gene in the case of an endogenous gene; or "expression" or "overexpression" in the case of a heterologous gene that is not present in the wild type strain. Lower expression or reduced expression or 'genes which are rendered less-functional or non-functional' is/are obtained by means of common well-known technologies for a skilled person (such as the usage of siRNA, CRISPR, CRISPRi, riboswitch, recombineering, homologous recombination, ssDNA mutagenesis, RNAi, miRNA, asRNA, mutating genes, knocking-out genes, transposon mutagenesis, ...) which are used to change the genes in such a way that they are less-able (i.e. statistically significantly 'less-able' compared to a functional wild-type gene) or completely unable (such as knocked-out genes) to produce functional final products. Next to changing the gene of interest in such a way that lower expression is obtained as described above, lower expression can also be obtained by changing the transcription unit, the promoter, an untranslated region, the ribosome binding site, the Shine Dalgarno sequence or the transcription terminator. Lower expression or reduced expression or 'genes which are rendered less-functional or non-functional' can for instance be obtained by mutating one or more base pairs in the promoter sequence or changing the promoter sequence fully to a constitutive promoter with a lower expression strength compared to the wild type or an inducible promoter which result in regulated expression or a repressible promoter which results in regulated expression. Overexpression or expression is obtained by means of common well-known technologies for a skilled person, wherein e.g. said gene is part of an "expression cassette" which relates to any sequence in which a promoter sequence, untranslated region sequence (containing either a ribosome binding sequence or Kozak sequence), a coding sequence (for instance a membrane protein gene sequence) and optionally a transcription terminator is present, and leading to the expression of a functional active protein. Said expression is either constitutive or conditional or regulated.

The term "riboswitch" as used herein is defined to be part of the messenger RNA that folds into intricate structures that block expression by interfering with translation. Binding of an effector molecule induces conformational change(s) permitting regulated expression post-transcriptionally.

The term "wild type" refers to the commonly known genetic or phenotypical situation as it occurs in nature.

The terms "cells" and "host cells" and "recombinant host cells", which are used interchangeably herein, refer to cells that are capable of or have been transformed with a vector, typically an expression vector. The host cells used herein are cells naturally comprising KDO biosynthesis. Such cells can be Gram-negative bacterial cells for example Bacteriaceae. It is understood that such terms refer not only to the particular subject cell, but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

The term "defective" as used herein, with regard to a gene or gene expression, means that the gene is not a wild type gene and that the organism does not have a wild type genotype and/or a wild type phenotype. The defective gene, genotype or phenotype may be the consequence of a mutation in that gene, or of a gene that regulates the expression of that gene (e.g., transcriptional or post-transcriptional), such that its normal expression is disrupted or extinguished. "Disrupted gene expression" is intended to include both complete inhibition and decreased gene expression (e.g., as in a leaky mutation), below wild type gene expression.

The terms "conditional", "regulated", "inducible", or similar terms, refer in particular to gene expression which is not constitutive, but which takes place in response to a stimulus (e.g., temperature, heavy metals or other medium additive).

The term "nucleic acid" refers to polynucleotides or oligonucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include, as equivalents, analogs of either RNA or DNA made from nucleotide analogs and as applicable to the embodiment being described, single (sense or antisense) and double-stranded polynucleotides.

As used herein, the term "transfection" means the introduction of a nucleic acid (e.g., via an expression vector) into a recipient cell by nucleic acid-mediated gene transfer.

"Transformation", as used herein, refers to a process in which a cell's genotype is changed as a result of the cellular uptake of exogenous DNA or RNA. A transformed cell can also be one that expresses a nucleic acid that interferes with the expression of an endogenous nucleic acid.

As used herein, the term "transgene" means a nucleic acid that has been introduced into a cell. A transgene could be partly or entirely heterologous, i.e., foreign, to the transgenic cell into which it is introduced, or, can be homologous to an endogenous gene of the cell into which it is introduced, but which is designed to be inserted, or is inserted, into the cell's genome in such a way as to alter the genome of the cell into which it is inserted. A transgene can also be present in a cell in the form of an episome.

The term "treating" a subject for a condition or disease, as used herein, is intended to encompass curing, as well as ameliorating at least one symptom of the condition or disease.

The term "vector" refers to a nucleic acid molecule, which is capable of transporting another nucleic acid to which it has been linked. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression vectors."

The term "expression system" as used herein refers to an expression vector under conditions whereby an mRNA may be transcribed and/or an mRNA may be translated into protein, structural RNA, or other cellular component. The expression system may be an in vitro expression system, which is commercially available or readily made according to art known techniques, or may be an in vivo expression system, such as a eukaryotic or prokaryotic cell containing the expression vector. In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids" which refer generally to circular double stranded DNA loops that, in their vector

form, are not bound to the chromosome. In the present specification, "plasmid" and "vector" are used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and are well known in the art or which become known in the art subsequently hereto (e.g., cosmid, phagemid and bacteriophage vectors).

The term "oligosaccharide" as used herein refers to a saccharide polymer containing a small number, typically two to fifteen, preferably three to fifteen, of simple sugars, i.e. monosaccharides. Examples of oligosaccharides include but are not limited to mammalian milk oligosaccharides, glycosaminoglycans, chitosans, chondroitins, heparosans, glucuronylated oligosaccharides, fucosylated oligosaccharides, neutral oligosaccharides and/or sialylated oligosaccharides.

The term "monosaccharide" as used herein refers to saccharides containing only one simple sugar. Examples of monosaccharides comprise Hexose, D-Glucopyranose, D-Galactofuranose, D-Galactopyranose, L-Galactopyranose, D-Mannopyranose, D-Allopyranose, L-Altropyranose, D-Gulopyranose, L-Idopyranose, D-Talopyranose, D-Ribofuranose, D-Ribopyranose, D-Arabinofuranose, D-Arabinopyranose, L-Arabinofuranose, L-Arabinopyranose, D-Xylopyranose, D-Lyxopyranose, D-Erythofuranose, D-Threofuranose, Heptose, L-glycero-D-manno-Heptopyranose (LDmanHep), D-glycero-D-manno-Heptopyranose (DDmanHep), 6-Deoxy-L-altropyranose, 6-Deoxy-D-gulopyranose, 6-Deoxy-D-talopyranose, 6-Deoxy-D-galactopyranose, 6-Deoxy-L-galactopyranose, 6-Deoxy-D-mannopyranose, 6-Deoxy-L-mannopyranose, 6-Deoxy-D-glucopyranose, 2-Deoxy-D-arabino-hexose, 2-Deoxy-D-erythro-pentose, 2,6-Dideoxy-D-arabino-hexopyranose, 3,6-Dideoxy-D-arabino-hexopyranose, 3,6-Dideoxy-L-arabino-hexopyranose, 3,6-Dideoxy-D-xylo-hexopyranose, 3,6-Dideoxy-D-ribo-hexopyranose, 2,6-Dideoxy-D-ribo-hexopyranose, 3,6-Dideoxy-L-xylo-hexopyranose, 2-Amino-2-deoxy-D-glucopyranose, 2-Amino-2-deoxy-D-galactopyranose, 2-Amino-2-deoxy-D-mannopyranose, 2-Amino-2-deoxy-D-allopyranose, 2-Amino-2-deoxy-L-altropyranose, 2-Amino-2-deoxy-D-gulopyranose, 2-Amino-2-deoxy-L-idopyranose, 2-Amino-2-deoxy-D-talopyranose, 2-Acetamido-2-deoxy-D-glucopyranose, 2-Acetamido-2-deoxy-D-galactopyranose, 2-Acetamido-2-deoxy-D-mannopyranose, 2-Acetamido-2-deoxy-D-allopyranose, 2-Acetamido-2-deoxy-L-altropyranose, 2-Acetamido-2-deoxy-D-gulopyranose, 2-Acetamido-2-deoxy-L-idopyranose, 2-Acetamido-2-deoxy-D-talopyranose, 2-Acetamido-2,6-dideoxy-D-galactopyranose, 2-Acetamido-2,6-dideoxy-L-galactopyranose, 2-Acetamido-2,6-dideoxy-L-mannopyranose, 2-Acetamido-2,6-dideoxy-D-glucopyranose, 2-Acetamido-2,6-dideoxy-L-altropyranose, 2-Acetamido-2,6-dideoxy-D-talopyranose, D-Glucopyranuronic acid, D-Galactopyranuronic acid, D-Mannopyranuronic acid, D-Allopyranuronic acid, L-Altropyranuronic acid, D-Gulopyranuronic acid, L-Gulopyranuronic acid, L-Idopyranuronic acid, D-Talopyranuronic acid, Sialic acid, 5-Amino-3,5-dideoxy-D-glycero-D-galacto-non-2-ulosonic acid, 5-Acetamido-3,5-dideoxy-D-glycero-D-galacto-non-2-ulosonic acid, 5-Glycolylamido-3,5-dideoxy-D-glycero-D-galacto-non-2-ulosonic acid, Erythritol, Arabinitol, Xylitol, Ribitol, Glucitol, Galactitol, Mannitol, D-ribo-Hex-2-ulopyranose, D-arabino-Hex-2-

- ulofuranose (D-fructofuranose), D-arabino-Hex-2-ulopyranose, L-xylo-Hex-2-ulopyranose, D-lyxo-Hex-2-ulopyranose, D-threo-Pent-2-ulopyranose, D-altro-Hept-2-ulopyranose, 3-C-(Hydroxymethyl)-D-erythofuranose, 2,4,6-Trideoxy-2,4-diamino-D-glucopyranose, 6-Deoxy-3-O-methyl-D-glucose, 3-O-Methyl-D-rhamnose, 2,6-Dideoxy-3-methyl-D-ribo-hexose, 2-Amino-3-O-
- 5 [(R)-1-carboxyethyl]-2-deoxy-D-glucopyranose, 2-Acetamido-3-O-[(R)-1-carboxyethyl]-2-deoxy-D-glucopyranose, 2-Glycolylamido-3-O-[(R)-1-carboxyethyl]-2-deoxy-D-glucopyranose, 3-Deoxy-D-lyxo-hept-2-ulopyranosaric acid, 3-Deoxy-D-manno-oct-2-ulopyranosonic acid, 3-Deoxy-D-glycero-D-galacto-non-2-ulopyranosonic acid, 5,7-Diamino-3,5,7,9-tetradecoxy-L-glycero-L-manno-non-2-ulopyranosonic acid, 5,7-Diamino-3,5,7,9-tetradecoxy-L-glycero-L-altro-non-2-
- 10 ulopyranosonic acid, 5,7-Diamino-3,5,7,9-tetradecoxy-D-glycero-D-galacto-non-2-ulopyranosonic acid, 5,7-Diamino-3,5,7,9-tetradecoxy-D-glycero-D-talo-non-2-ulopyranosonic acid, glucose, galactose, N-acetylglucosamine, glucosamine, mannose, xylose, N-acetylmannosamine, N-acetylneuraminic acid, N-glycolylneuraminic acid, a sialic acid, N-acetylgalactosamine, galactosamine, fucose, rhamnose, glucuronic acid, gluconic acid, fructose and polyols.
- 15 The term "mammalian milk oligosaccharide" as used herein refers to oligosaccharides found in mammalian milk, such as but not limited to 3-fucosyllactose, 2'-fucosyllactose, 6-fucosyllactose, 2',3-difucosyllactose, 2',2-difucosyllactose, 3,4-difucosyllactose, 6'-sialyllactose, 3'-sialyllactose, 3,6-disialyllactose, 6,6'-disialyllactose, 3,6-disialyllacto-N-tetraose, lactodifucotetraose, lacto-N-tetraose, lacto-N-neotetraose, lacto-N-fucopentaose II, lacto-N-fucopentaose I, lacto-N-
- 20 fucopentaose III, sialyllacto-N-tetraose c, sialyllacto-N-tetraose b, sialyllacto-N-tetraose a, lacto-N-difucohexaose I, lacto-N-difucohexaose II, lacto-N-hexaose, lacto-N-neohexaose, para-lacto-N-hexaose, monofucosylmonosialyllacto-N-tetraose c, monofucosyl para-lacto-N-hexaose, monofucosyllacto-N-hexaose III, isomeric fucosylated lacto-N-hexaose III, isomeric fucosylated lacto-N-hexaose I, sialyllacto-N-hexaose, sialyllacto-N-neohexaose II, difucosyl-para-lacto-N-
- 25 hexaose, difucosyllacto-N-hexaose, difucosyllacto-N-hexaose a, difucosyllacto-N-hexaose c, galactosylated chitosan.
- A 'sialylated oligosaccharide' as used herein refers to a charged sialic acid containing oligosaccharide, i.e. an oligosaccharide having a sialic acid residue. It has an acidic nature. Some examples are 3'-SL (3'-sialyllactose), 3'-sialyllactosamine, 6'-SL (6'-sialyllactose), 6'-
- 30 sialyllactosamine, oligosaccharides comprising 6'-sialyllactose, SGG hexasaccharide (Neu5Aca-2,3Gal beta -1,3GalNAc beta -1,3Gala-1,4Gal beta -1,4Gal), sialylated tetrasaccharide (Neu5Aca-2,3Gal beta -1,4GlcNAc beta -1,4GlcNAc), pentasaccharide LSTD (Neu5Aca-2,3Gal beta -1,4GlcNAc beta -1,3Gal beta -1,4Glc), sialylated lacto-N-biose, sialylated lacto-N-triose, sialylated lacto-N-tetraose, sialyllacto-N-neotetraose, monosialyllacto-N-hexaose, disialyllacto-N-hexaose
- 35 I, monosialyllacto-N-neohexaose I, monosialyllacto-N-neohexaose II, disialyllacto-N-neohexaose, disialyllacto-N-tetraose, disialyllacto-N-hexaose II, sialyllacto-N-tetraose a, disialyllacto-N-hexaose I, sialyllacto-N-tetraose b, 3'-sialyl-3-fucosyllactose, disialomonofucosyllacto-N-neohexaose, monofucosylmonosialyllacto-N-octaose (sialyl Lea),

- sialyllacto-N-fucohexaose II, disialyllacto-N-fucopentaose II, monofucosylsialyllacto-N-tetraose and oligosaccharides bearing one or several sialic acid residu(s), including but not limited to: oligosaccharide moieties of the gangliosides selected from GM3 (3'sialyllactose, Neu5Aca-2,3Gal β -4Glc) and oligosaccharides comprising the GM3 motif, GD3 Neu5Aca-2,8Neu5Aca-2,3Gal β -1,4Glc GT3 (Neu5Aca-2,8Neu5Aca-2,8Neu5Aca-2,3Gal β -1,4Glc); GM2 GalNAc β -1,4(Neu5Aca-2,3)Gal β -1,4Glc, GM1 Gal β -1,3GalNAc β -1,4(Neu5Aca-2,3)Gal β -1,4Glc, GD1a Neu5Aca-2,3Gal β -1,3GalNAc β -1,4(Neu5Aca-2,3)Gal β -1,4Glc GT1a Neu5Aca-2,8Neu5Aca-2,3Gal β -1,3GalNAc β -1,4(Neu5Aca-2,3)Gal β -1,4Glc GD2 GalNAc β -1,4(Neu5Aca-2,8Neu5Aca2,3)Gal β -1,4Glc GT2 GspalNAc β -1,4(Neu5Aca-2,8Neu5Aca-2,8Neu5Aca2,3)Gal β -1,4Glc GD1b, Gal β -1,3GalNAc β -1,4(Neu5Aca-2,8Neu5Aca2,3)Gal β -1,4Glc GT1b Neu5Aca-2,3Gal β -1,3GalNAc β -1,4(Neu5Aca-2,8Neu5Aca2,3)Gal β -1,4Glc GQ1b Neu5Aca-2,8Neu5Aca-2,3Gal β -1,3GalNAc β -1,4(Neu5Aca-2,8Neu5Aca2,3)Gal β -1,4Glc GT1c Gal β -1,3GalNAc β -1,4(Neu5Aca-2,8Neu5Aca-2,8Neu5Aca2,3)Gal β -1,4Glc GQ1c, Neu5Aca-2,3Gal β -1,3GalNAc β -1,4(Neu5Aca-2,8Neu5Aca-2,8Neu5Aca2,3)Gal β -1,4Glc GP1c Neu5Aca-2,8Neu5Aca-2,3Gal β -1,3GalNAc β -1,4(Neu5Aca-2,8Neu5Aca-2,8Neu5Aca2,3)Gal β -1,4Glc GD1a Neu5Aca-2,3Gal β -1,3(Neu5Aca-2,6)GalNAc β -1,4Gal β -1,4Glc Fucosyl-GM1 Fuca-1,2Gal β -1,3GalNAc β -1,4(Neu5Aca-2,3)Gal β -1,4Glc; all of which may be extended to the production of the corresponding gangliosides by reacting the above oligosaccharide moieties with ceramide or synthesizing the above oligosaccharides on a ceramide;
- A 'fucosylated oligosaccharide' as used herein is generally understood in the state of the art as an oligosaccharide that is carrying a fucose-residue. Examples comprise 2'-fucosyllactose, 3-fucosyllactose, difucosyllactose, lactodifucotetraose (LDFT), Lacto-N-fucopentaose I (LNF I), Lacto-N-fucopentaose II (LNF II),), Lacto-N-fucopentaose III (LNF III), lacto-N-fucopentaose V (LNF V), lacto-N-neofucopentaose I, lacto-N-difucohexaose I (LDFH I), lacto-N-difucohexaose II (LDFH II), Monofucosyllacto-N-hexaose III (MFLNH III), Difucosyllacto-N-hexaose (DFLNHa), difucosyl-lacto-N-neohexaose.
- A 'neutral oligosaccharide' as used herein is generally understood in the state of the art as an oligosaccharide that has no negative charge originating from a carboxylic acid group. Examples of such neutral oligosaccharide are 2'-fucosyllactose, 3-fucosyllactose, 2', 3- difucosyllactose, lacto-N-triose II, lacto-N-tetraose, lacto-N-neotetraose, lacto-N-fucopentaose I, lacto-N-neofucopentaose I, lacto-N-fucopentaose II, lacto-N-fucopentaose III, lacto-N-fucopentaose V, lacto-N-neofucopentaose V, lacto-N-difucohexaose I, lacto-N-difucohexaose II, 6'-galactosyllactose, 3'- galactosyllactose, lacto-N-hexaose, lacto-N-neohexaose, para-lacto-N-hexaose, para-lacto-N-neohexaose, difucosyl-lacto-N-hexaose and difucosyl-lacto-N-neohexaose.
- The term "KDO-oligosaccharide" as used herein refers to KDO linked to a monosaccharide unit within an oligosaccharide or an acceptor as defined herein. Examples include, but are not limited to, KDO-lactose, KDO-LacNAc, KDO-N-Acetyl-D-lactosamine, KDO-LNB, KDO-Lacto-N-biose.

The term "precursor" as used herein refers to substances which are taken up or synthesized by the cell for the specific production of an oligosaccharide. In this sense a precursor can be an acceptor as defined herein, but can also be another substance, metabolite, which is first modified within the cell as part of the biochemical synthesis route of the oligosaccharide. Examples of such precursors comprise the acceptors as defined herein, and/or glucose, galactose, fructose, glycerol, sialic acid, fucose, mannose, maltose, sucrose, lactose glucose-1-phosphate, galactose-1-phosphate, UDP-glucose, UDP-galactose, glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate, glycerol-3-phosphate, dihydroxyacetone, glyceraldehyde-3-phosphate, dihydroxyacetone-phosphate, glucosamine-6-phosphate, glucosamine, N-acetyl-glucosamine-6-phosphate, N-acetyl-glucosamine, N-acetyl-mannosamine, N-acetylmannosamine-6-phosphate, UDP-N-acetylglucosamine, N-acetylglucosamine-1-phosphate, N-acetylneuraminic acid (sialic acid), N-acetyl-Neuraminic acid – 9 phosphate, CMP-sialic acid, mannose-6-phosphate, mannose-1-phosphate, GDP-mannose, GDP-4-dehydro-6-deoxy- α -D-mannose, and/or GDP-fucose.

The term "acceptor" as used herein refers to oligosaccharides which can be modified by a glycosyltransferase. Examples of such acceptors are lactose, lacto-N-biose (LNB), lacto-N-triose, lacto-N-tetraose (LNT), lacto-N-neotetraose (LNnT), N-acetyl-lactosamine (LacNAc), lacto-N-pentaose (LNP), lacto-N-neopentaose, para lacto-N-pentaose, para lacto-N-neopentaose, lacto-N-novopentaose I, lacto-N-hexaose (LNH), lacto-N-neohexaose (LNnH), para lacto-N-neohexaose (pLNnH), para lacto-N-hexaose (pLNH), lacto-N-heptaose, lacto-N-neoheptaose, para lacto-N-neoheptaose, para lacto-N-heptaose, lacto-N-octaose (LNO), lacto-N-neooctaose, iso lacto-N-octaose, para lacto-N-octaose, iso lacto-N-neooctaose, novo lacto-N-neooctaose, para lacto-N-neooctaose, iso lacto-N-nonaose, novo lacto-N-nonaose, lacto-N-nonaose, lacto-N-decaose, iso lacto-N-decaose, novo lacto-N-decaose, lacto-N-neodecaose, galactosyllactose, a lactose extended with 1, 2, 3, 4, 5, or a multiple of N-acetylglucosamine units and/or 1, 2, 3, 4, 5, or a multiple of, Lacto-N-biose units, and oligosaccharide containing 1 or multiple N-acetylglucosamine units and/or 1 or multiple lacto-N-biose units or an intermediate or fucosylated or sialylated versions thereof.

The term "KDO" or "3-Deoxy- d-manno-oct-2-ulosonic acid" or "keto-deoxyoctulosonate" is an ulosonic acid of a 2-ketooctose formed in many bacteria as part of their lipo-oligosaccharide structure.

The term "purified" refers to material that is substantially or essentially free from components which interfere with the activity of the biological molecule. For cells, saccharides, nucleic acids, and polypeptides, the term "purified" refers to material that is substantially or essentially free from components which normally accompany the material as found in its native state. Typically, purified saccharides, oligosaccharides, proteins or nucleic acids of the invention are at least about 50 %, 55 %, 60 %, 65 %, 70 %, 75 %, 80 % or 85 % pure, usually at least about 90 %, 91 %, 92 %, 93 %, 94 %, 95 %, 96 %, 97 %, 98 %, or 99 % pure as measured by band intensity on a silver stained

gel or other method for determining purity. Purity or homogeneity can be indicated by a number of means well known in the art, such as polyacrylamide gel electrophoresis of a protein or nucleic acid sample, followed by visualization upon staining. For certain purposes high resolution will be needed and HPLC or a similar means for purification utilized. For oligosaccharides, e.g., 3-sialyllactose, purity can be determined using methods such as but not limited to thin layer chromatography, gas chromatography, NMR, HPLC, capillary electrophoresis or mass spectroscopy.

The terms "identical" or percent "identity" or % "identity" in the context of two or more nucleic acid or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same, when compared and aligned for maximum correspondence, as measured using sequence comparison algorithms or by visual inspection. For sequence comparison, one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are inputted into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters. Percent identity may be calculated globally over the full-length sequence of the reference sequence, resulting in a global percent identity score. Alternatively, percent identity may be calculated over a partial sequence of the reference sequence, resulting in a local percent identity score. Using the full-length of the reference sequence in a local sequence alignment results in a global percent identity score between the test and the reference sequence.

Percent identity can be determined using different algorithms like for example BLAST and PSI-BLAST (Altschul et al., 1990, J Mol Biol 215:3, 403- 410; Altschul et al., 1997, Nucleic Acids Res 25: 17, 3389-402), the Clustal Omega method (Sievers et al., 2011, Mol. Syst. Biol. 7:539), the MatGAT method (Campanella et al., 2003, BMC Bioinformatics, 4:29) or EMBOSS Needle (<https://galaxy-iuc.github.io/emboss-5.0-docs/needle.html>).

The BLAST (Basic Local Alignment Search Tool) method of alignment is an algorithm provided by the National Center for Biotechnology Information (NCBI) to compare sequences using default parameters. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance. PSI-BLAST (Position-Specific Iterative Basic Local Alignment Search Tool) derives a position-specific scoring matrix (PSSM) or profile from the multiple sequence alignment of sequences detected above a given score threshold using protein-protein BLAST (BLASTp). The BLAST method can be used for pairwise or multiple sequence alignments. Pairwise Sequence Alignment is used to identify regions of similarity that may indicate functional, structural and/or evolutionary relationships between two biological sequences (protein or nucleic acid). The web interface for BLAST is available at: <https://blast.ncbi.nlm.nih.gov/Blast.cgi>.

Clustal Omega (Clustal W) is a multiple sequence alignment program that uses seeded guide trees and HMM profile-profile techniques to generate alignments between three or more sequences. It produces biologically meaningful multiple sequence alignments of divergent sequences. The web interface for Clustal W is available at

5 <https://www.ebi.ac.uk/Tools/msa/clustalo/>. Default parameters for multiple sequence alignments and calculation of percent identity of protein sequences using the Clustal W method are: enabling de-alignment of input sequences: FALSE; enabling mbed-like clustering guide-tree: TRUE; enabling mbed-like clustering iteration: TRUE; Number of (combined guide-tree/HMM) iterations: default(0); Max Guide Tree Iterations: default [-1]; Max HMM Iterations: default [-1]; order: aligned.

10 MatGAT (Matrix Global Alignment Tool) is a computer application that generates similarity/identity matrices for DNA or protein sequences without needing pre-alignment of the data. The program performs a series of pairwise alignments using the Myers and Miller global alignment algorithm, calculates similarity and identity, and then places the results in a distance matrix. The user may specify which type of alignment matrix (e.g. BLOSUM50, BLOSUM62, and PAM250) to employ

15 with their protein sequence examination.

EMBOSS Needle (<https://galaxy-iuc.github.io/emboss-5.0-docs/needle.html>) uses the Needleman-Wunsch global alignment algorithm to find the optimal alignment (including gaps) of two sequences when considering their entire length. The optimal alignment is ensured by dynamic programming methods by exploring all possible alignments and choosing the best. The

20 Needleman-Wunsch algorithm is a member of the class of algorithms that can calculate the best score and alignment in the order of mn steps, (where 'n' and 'm' are the lengths of the two sequences). The gap open penalty (default 10.0) is the score taken away when a gap is created. The default value assumes you are using the EBLOSUM62 matrix for protein sequences. The gap extension (default 0.5) penalty is added to the standard gap penalty for each base or residue

25 in the gap. This is how long gaps are penalized.

For the purposes of this invention, percent identity is determined using MatGAT2.01 (Campanella et al., 2003, BMC Bioinformatics 4:29). The following default parameters for protein are employed: (1) Gap cost Existence: 12 and Extension: 2; (2) The Matrix employed was BLOSUM50.

30 **Detailed description of the invention**

The present invention provides a microbial host cell. In particular, the present invention provides a microbial cell naturally synthesizing KDO genetically modified to produce an oligosaccharide. The KDO biosynthesis of said cell is knocked out or rendered less functional.

In a preferred embodiment, the cell comprises at least one glycosyltransferase with affinity for

35 CMP-KDO. In a further preferred embodiment, said glycosyltransferase is overexpressed.

In a specific preferred embodiment, the cell according to the invention is capable of synthesizing a nucleotide sugar selected from the group: GDP-fucose, GDP-mannose, GDP-rhamnose, CMP-N-acetylneuraminic acid, CMP-N-glycolylneuraminic acid, UDP-glucose, dTDP-glucose, UDP-

galactose, UDP-N-acetylmannosamine, UDP-N-acetylglucosamine, UDP-N-acetylgalactosamine, UDP-glucuronic acid, UDP-xylose, UDP-arabinose, and/or UDP-galacturonic acid.

The cell according to the invention can comprise i) an overexpressed ATP-dependent translocator encoding gene; and/or ii) an overexpressed inner membrane protein encoding gene; and/or iii) an overexpressed lauroyl acyltransferase encoding gene. Preferably, said inner membrane protein is a transmembrane transporter. Alternatively or additionally, said cell can comprise a modified endogenous ATP-dependent translocator encoding gene; and/or a modified endogenous inner membrane protein encoding gene. Preferably, said inner membrane protein is a transmembrane transporter.

In a preferred embodiment, said overexpression of the ATP-dependent translocator, the inner membrane protein and/or lauroyl acyltransferase is an overexpression of the endogenous gene encoding said protein. Alternatively, said overexpression is obtained by introducing and expressing said protein(s). It has been shown that overexpression of any of the above proteins provides for a reduced or abolished KDO-biosynthesis. This reduced and/or abolished KDO-biosynthesis provides for production of oligosaccharide without at the same time producing KDO-by-products or KDO-side products, such as KDO-lactose, in general KDO-oligosaccharide or KDO containing lipopolysaccharides.

In another preferred embodiment, said modification of said endogenous ATP-dependent translocator encoding gene and/or said endogenous inner membrane protein encoding gene is a point-mutation. It has been shown herein that also a point mutation in any of the genes encoding said endogenous ATP-dependent translocator or inner membrane protein provides for a reduced or abolished KDO-biosynthesis.

In preferred embodiments of the present invention, the ATP-dependent translocator has 80% or more sequence identity to SEQ ID NO: 4 and has ATP-dependent translocator activity; the inner membrane protein has 80% or more sequence identity to SEQ ID NO: 21 and has transmembrane transporter activity and/or said lauroyl acyltransferase has 80% or more sequence identity to SEQ ID NO: 1 and has lauroyl acyltransferase activity.

The amino acid sequence of the polypeptide used herein can be a sequence chosen from SEQ ID NO 4, 21 or 1 of the attached sequence listing. The amino acid sequence of the polypeptide can also be an amino acid sequence that has 80% or more sequence identity, 80 %, 81 %, 82 %, 83 %, 84 %, 85 %, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 95,5%, 96%, 96,5%, 97%, 97,5%, 98%, 98,5%, 99%, 99,5%, 99,6%, 99,7%, 99,8%, 99,9% sequence identity to the full length amino acid sequence of any one of SEQ NO 4, 21 or 1.

In another preferred embodiment, the cell comprises a KDO-independent lauroyl acyltransferase encoding gene and/or an ATP-binding cassette multidrug transporter encoding gene. Preferably, said KDO-independent lauroyl acyltransferase encoding gene and/or said ATP-binding cassette multidrug transporter is/are i) introduced and expressed or ii) overexpressed in said cell. This type

of LpxL proteins is independent of KDO modified Lipid IVa, allowing full acylation of the lipidA structure when the KDO biosynthesis pathway is knocked out.

Preferably, the cell comprises expression of the gene encoding the KDO-independent lauroyl acyltransferase of SEQ ID NO: 2 or SEQ ID NO: 3, or a polypeptide having at least 80% sequence identity thereto and having KDO-independent lauroyl acyltransferase activity. The amino acid sequence of the polypeptide used herein can be a sequence chosen from SEQ ID NO 2 or 3 of the attached sequence listing. The amino acid sequence can also be an amino acid sequence that has 80% or more sequence identity, 80 %, 81 %, 82 %, 83 %, 84 %, 85 %, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 95,5%, 96%, 96,5%, 97%, 97,5%, 98%, 98,5%, 99%, 99,5%, 99,6%, 99,7%, 99,8%, 99,9% sequence identity to the full length amino acid sequence of any one of SEQ ID NO: 2 or 3.

In a preferred embodiment of the present invention, the ATP-binding cassette multidrug transporter is LmrA from *Lactococcus lactis*, preferably comprising SEQ ID NO: 22. In another preferred embodiment, the cell comprises expression of a gene encoding a polypeptide having 80% or more sequence identity to SEQ ID NO: 22 and having transmembrane transporter activity. The amino acid sequence of the polypeptide can also be an amino acid sequence that has 80% or more sequence identity, 80 %, 81 %, 82 %, 83 %, 84 %, 85 %, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 95,5%, 96%, 96,5%, 97%, 97,5%, 98%, 98,5%, 99%, 99,5%, 99,6%, 99,7%, 99,8%, 99,9% sequence identity to the full length amino acid sequence of SEQ ID NO: 22.

In a specific embodiment of the invention, the cell is an *Escherichia coli* wherein an *msbA* encoding gene is overexpressed and/or an *yhjD* encoding gene is overexpressed and/or a LpxL encoding gene is overexpressed and/or an endogenous *msbA* encoding gene is modified and/or an endogenous *yhjD* encoding gene is modified.

In a further preferred embodiment the microbial cell naturally synthesizing KDO and genetically modified to produce an oligosaccharide, as described herein additionally or alternatively comprises at least one of the genes selected from the group of genes encoding for D-arabinose 5-phosphate isomerase, 3-deoxy-D-*manno*-octulosonate 8-phosphate synthase, 3-deoxy-D-*manno*-octulosonate 8-phosphate phosphatase, and/or 3-deoxy-*manno*-octulosonate cytidyltransferase which is rendered less functional or knocked out.

The microbial cell according to the invention is genetically modified to produce an oligosaccharide. Such oligosaccharide can be any oligosaccharide as described herein. Preferably, the cell produces a neutral, sialylated or fucosylated oligosaccharide. More preferably a mammalian milk oligosaccharide, even more preferably chosen from fucosylated milk oligosaccharides, neutral milk oligosaccharides and/or sialylated milk oligosaccharides.

In a preferred embodiment, the glycosyltransferase is a sialyltransferase. Preferably, the cell is then producing sialylated oligosaccharide, preferably sialylated mammalian milk oligosaccharide as described herein.

The cell according to the invention described herein can further comprise a deleted or inactivated endogenous beta-galactosidase gene. In case the cell is an E. coli cell, preferably the deleted or inactivated beta-galactosidase gene is E. coli lacZ gene.

The cell according to the invention described herein can further comprise a deleted, inactivated,
5 or mutated galactoside-O-acyltransferase encoding gene. In case the cell is an E. coli cell, preferably the deleted, inactivated, or mutated galactoside-O-acyltransferase encoding gene is E. coli lacA gene.

The cell according to the invention described herein can further comprise a nucleic acid sequence encoding at least one of the following additional proteins: an exporter protein or a permease
10 exporting the synthesized oligosaccharide from the microbial cell. Such exporter or permease will enable the export of the oligosaccharide out of the cell.

The cell according to the invention described herein can further be genetically modified to contain a nucleic acid sequence encoding a glycosidase for the specific degradation of interfering oligosaccharides, such as intermediates, side products or endogenous oligosaccharides
15 generated by bacterial host cell, wherein the expression of said glycosidase is under control of a regulatory sequence.

The cell according to the invention described herein can further be an isolated microbial cell according to any invention described herein.

The present invention also provides for the use of a cell according to any one of embodiment
20 described herein, for the production of oligosaccharides substantially free of KDO-lactose and/or KDO-oligosaccharide.

The present invention provides for a method for the fermentative production of an oligosaccharide substantially free of KDO-oligosaccharide, said method comprising: providing a microbial cell according to any one of the embodiments described herein, cultivating said cells in favourable
25 growing conditions and optionally separating or isolating the oligosaccharide from the culture.

In a specific embodiment, the present invention provides for a method for producing a sialylated oligosaccharide by fermentation through a genetically modified microbial cell naturally synthesizing KDO, the method comprising the steps of:

- 30 a) Obtaining a microbial cell naturally synthesizing KDO and being capable to produce sialylated oligosaccharides and expressing a sialyltransferase with affinity for CMP-KDO, and wherein the KDO-biosynthesis route of said cell is knocked out or rendered less functional;
- b) Culturing the host cell from step a) in favourable growing conditions, thus producing said
35 sialylated oligosaccharide and
- c) Optionally, separating or isolating the sialylated oligosaccharide from the culture medium.

The culture medium used in the methods described herein preferably comprises precursor for the

production of said oligosaccharide, preferably said culture medium comprises any one or more of lactose, galactose, N-acetylglucosamine, N-acetyl-D-lactosamine, Lacto-N-biose, sialic acid, N-acetylneuraminic acid, fucose. Alternatively or additionally, the cell produces the precursor internally, preferably said cell produces any one or more of lactose, N-acetyl-D-lactosamine,

5 Lacto-N-biose, sialic acid, N-acetylneuraminic acid, fucose.

According to a preferred embodiment of the invention described herein the microbial cell produces a sialylated oligosaccharide, preferably chosen from the group comprising 3'-sialyllactose, 6'-sialyllactose, disialyl lacto-N-tetraose, sialylated lacto-N-triose, sialylated lacto-N-tetraose, sialylated lacto-N-neotetraose, 3-fucosyl-3'-sialyllactose, lacto-N-sialylpentaose, LSTa, LSTb,
10 LSTc, LSTd. Alternatively or additionally, the cell produces a fucosylated and/or neutral oligosaccharide as described herein.

According to a preferred embodiment of the invention described herein the microbial cell is a Gram-negative microbial cell, preferably said cell is an *Escherichia coli* strain, preferably an *Escherichia coli* strain which is a K12 strain, more preferably the *Escherichia coli* K12 strain is
15 *Escherichia coli* K12 substr. MG1655.

The present invention however contemplates the use of any type of Gram-negative bacterial cell in the construction of microbial cell naturally synthesizing KDO, but wherein the KDO biosynthesis is knocked out or rendered less functional. Examples of Gram-negative bacteria useful in the present invention include, but are not limited to of *Escherichia* spp., *Shigella* spp., *Salmonella* spp., *Campylobacter* spp., *Neisseria* spp., *Haemophilus* spp., *Aeromonas* spp., *Francisella* spp.,
20 *Yersinia* spp., *Klebsiella* spp., *Bordetella* spp., *Legionella* spp., *Citrobacter* spp., *Chlamydia* spp., *Brucella* spp., *Pseudomonas* spp., *Helicobacter* spp., *Moraxella* spp., *Stenotrophomonas* spp., *Bdellovibrio* spp., *Acinetobacter* spp., *Enterobacter* spp. and *Vibrio* spp.. Even more preferably, the host cell is selected from *Escherichia* spp., *Salmonella* spp., and *Pseudomonas* spp.. In preferred embodiments, *Escherichia coli* is used. Examples of *Escherichia* strains which can be used include, but are not limited to, *Escherichia coli* B, *Escherichia coli* C, *Escherichia coli* W, *Escherichia coli* K12, *Escherichia coli* Nissle. More specifically, the latter term relates to cultivated *Escherichia coli* strains – designated as *E. coli* K12 strains – which are well-adapted to the laboratory environment, and, unlike wild type strains, have lost their ability to thrive in the intestine.

Well-known examples of the *E. coli* K12 strains are K12 Wild type, W3110, MG1655, M182, MC1000, MC1060, MC1061, MC4100, JM101, NZN111 and AA200. Hence, the present invention specifically relates to a mutated and/or transformed *Escherichia coli* strain as indicated above wherein said *E. coli* strain is a K12 strain. More specifically, the present invention relates to a mutated and/or transformed *Escherichia coli* strain as indicated above wherein said K12 strain is
30 *E. coli* K12 substr. MG1655.

Alternatively, the *E. coli* is selected from the group consisting of K-12 strain, W3110, MG1655, B/r, BL21, O157:h7, 042, 101-1, 1180, 1357, 1412, 1520, 1827-70, 2362-75, 3431, 53638, 83972, 929-78, 98NK2, ABU 83972, B, B088, B171, B185, B354, B646, B7A, C, c7122, CFT073, DH1,

DH5a, E110019, E128010, E74/68, E851/71, EAEC 042, EPECa11, EPECa12, EPECa14, ETEC, H10407, F11, F18+, FVEC1302, FVEC1412, GEMS_EPEC1, HB101, HT115, KO11, LF82, LT-41, LT-62, LT-68, MS107-1, MS119-7, MS124-1, MS 145-7, MS 79-2, MS 85-1, NCTC 86, Nissle 1917, NT:H19, NT:H40, NU14, O103:H2, O103:HNM, O103:K+, O104:H12, O108:H25, O109:H9, O111H-, O111:H19, O111:H2, O111:H21, O111:Nm, O115:H-, O115:HMN, O115:K+, O119:H6, O119:UT, O124:H40, O127a:H6, O127:H6, O128:H2, O131:H25, O136:H-, O139:H28 (strain E24377A/ETEC), O13:H11, O142:H6, O145:H-, O153:H21, O153:H7, O154:H9, O157:12, O157:H-, O157:H12, O157:H43, O157:H45, O157:H7 EDL933, O157:Nm, O15:Nm, O177:H11, O17:K52:H18 (strain UMN026/ExPEC), O180:H-, O1:K1/APEC, O26, O26:H-, O26:H11, O26:H11:K60, O26:Nm, O41:H-, O45:K1 (strain S88/ExPEC), O51:H-, O55:H51, O55:H6, O55:H7, O5:H-, O6, O63:H6, O63:HNM, O6:K15:H31 (strain 536/UPEC), O7:K1 (strain IAI39/ExPEC), O8 (strain IAI1), O81 (strain ED1a), O84:H-, O86a:H34, O86a:H40, O90:H8, O91:H21, O9:H4 (strain HS), O9:H51, ONT:H-, ONT:H25, OP50, Orough:H12, Orough:H19, Orough:H34, Orough:H37, Orough:H9, OUT:H12, OUT:H45, OUT:H6, OUT:H7, OUT:HNM, OUT:Nm, RN587/1, RS218, 55989/EAEC, B/BL21, B/BL21-DE3, SE11, SMS-3-5/SECEC, UTI89/UPEC, TA004, TA155, TX1999, and Vir68.

In the methods of the invention the oligosaccharide can be isolated from the culture medium by means of unit operation selected from the group comprising centrifugation, filtration, microfiltration, ultrafiltration, nanofiltration, ion exchange, electrodialysis, chromatography, simulated moving bed chromatography, simulated moving bed ion exchange, evaporation, precipitation, crystallisation, spray drying and any combination thereof.

In a preferred embodiment of the methods of the invention the produced oligosaccharide or mix of oligosaccharides is separated from the culture.

As used herein, the term "separating" means harvesting, collecting or retrieving the oligosaccharide from the host cell and/or the medium of its growth as explained herein.

Oligosaccharide can be separated in a conventional manner from the culture or aqueous culture medium, in which the mixture was made. In case the oligosaccharide is still present in the cells producing the oligosaccharide, conventional manners to free or to extract the oligosaccharide out of the cells can be used, such as cell destruction using high pH, heat shock, sonication, French press, homogenisation, enzymatic hydrolysis, chemical hydrolysis, solvent hydrolysis, detergent, hydrolysis,... The culture medium, reaction mixture and/or cell extract, together and separately called oligosaccharide containing mixture or culture, can then be further used for separating the oligosaccharide.

Typically oligosaccharides are purified by first removing macro components, i.e. first the cells and cell debris, then the smaller components, i.e. proteins, endotoxins and other components between 1000 Da and 1000 kDa and then the oligosaccharide is desalted by means of retaining the oligosaccharide with a nanofiltration membrane or with electrodialysis in a first step and ion exchange in a second step, which consists of a cation exchange resin and anion exchange resin,

wherein most preferably the cation exchange chromatography is performed before the anion exchange chromatography. These steps do not separate sugars with a small difference in degree of polymerization from each other. Said separation is done for instance by chromatographical separation.

- 5 Separation preferably involves clarifying the oligosaccharide containing mixtures to remove suspended particulates and contaminants, particularly cells, cell components, insoluble metabolites and debris produced by culturing the genetically modified cell and/or performing the enzymatic reaction. In this step, the oligosaccharide containing mixture can be clarified in a conventional manner. Preferably, the oligosaccharide containing mixture is clarified by
- 10 centrifugation, flocculation, decantation and/or filtration. A second step of separating the oligosaccharide from the oligosaccharide containing mixture preferably involves removing substantially all the proteins, as well as peptides, amino acids, RNA and DNA and any endotoxins and glycolipids that could interfere with the subsequent separation step, from the oligosaccharide containing mixture, preferably after it has been clarified. In this step, proteins and related
- 15 impurities can be removed from the oligosaccharide containing mixture in a conventional manner. Preferably, proteins, salts, by-products, colour and other related impurities are removed from the oligosaccharide containing mixture by ultrafiltration, nanofiltration, reverse osmosis, microfiltration, activated charcoal or carbon treatment, tangential flow high-performance filtration, tangential flow ultrafiltration, affinity chromatography, ion exchange chromatography (such as but
- 20 not limited to cation exchange, anion exchange, mixed bed ion exchange), hydrophobic interaction chromatography and/or gel filtration (i.e., size exclusion chromatography), particularly by chromatography, more particularly by ion exchange chromatography or hydrophobic interaction chromatography or ligand exchange chromatography. With the exception of size exclusion chromatography, proteins and related impurities are retained by a chromatography
- 25 medium or a selected membrane, while oligosaccharide remains in the oligosaccharide containing mixture.

- Contaminating compounds with a molecular weight above 1000 Da (dalton) are removed by means of ultrafiltration membranes with a cut-off above 1000 Da to approximately 1000 kDa. The membrane retains the contaminant and the oligosaccharide goes to the filtrate. Typical
- 30 ultrafiltration principles are well known in the art and are based on Tubular modules, Hollow fibre, spiral-wound or plates. These are used in cross flow conditions or as a dead-end filtration. The membrane composition is well known and available from several vendors, and are composed of PES (Polyethylene sulfone), polyvinylpyrrolidone, PAN (Polyacrylonitrile), PA (Poly-amide), Polyvinylidene difluoride (PVDF), NC (Nitrocellulose), ceramic materials or combinations thereof.
- 35 Components smaller than the oligosaccharide, for instance monosaccharides, salts, disaccharides, acids, bases, medium constituents are separated by means of a nano-filtration or/and electrodialysis. Such membranes have molecular weight cut-offs between 100 Da and 1000 Da. For an oligosaccharide such as 3'-sialyllactose or 6'-sialyllactose the optimal cut-off is

between 300 Da and 500 Da, minimizing losses in the filtrate. Typical membrane compositions are well known and are for example polyamide (PA), TFC, PA-TFC, Polypiperazine-amide, PES, Cellulose Acetate or combinations thereof.

The oligosaccharide is further isolated from the culture medium and/or cell with or without further
5 purification steps by evaporation, lyophilization, crystallization, precipitation, and/or drying, spray drying. Said further purification steps allow the formulation of oligosaccharide in combination with other oligosaccharides and/or products, for instance but not limited to the co-formulation by means of spray drying, drying or lyophilization or concentration by means of evaporation in liquid form.

10 In an even further aspect, the present invention also provides for a further purification of the oligosaccharide. A further purification of said oligosaccharide may be accomplished, for example, by use of (activated) charcoal or carbon, nanofiltration, ultrafiltration or ion exchange to remove any remaining DNA, protein, LPS, endotoxins, or other impurity. Alcohols, such as ethanol, and aqueous alcohol mixtures can also be used. Another purification step is accomplished by
15 crystallization or precipitation of the product. Another purification step is to spray dry or lyophilize oligosaccharide.

The separated and preferably also purified oligosaccharide can be used as a supplement in infant formulas and for treating various diseases in new-born infants.

In a specific embodiment an oligosaccharide is produced by the cell according to any one of
20 embodiments described herein and/or according to the method described in any one of embodiments described herein. Said oligosaccharide is added to food formulation, feed formulation, pharmaceutical formulation, cosmetic formulation, or agrochemical formulation.

Moreover, the present invention relates to the following specific embodiments:

- 25 1. Microbial cell naturally synthesizing KDO genetically modified to produce an oligosaccharide characterised in that the KDO biosynthesis of said cell is knocked out or rendered less functional.
2. A cell according to embodiment 1 wherein said cell comprises at least one glycosyltransferase with affinity for CMP-KDO, preferably said glycosyltransferase is overexpressed.
- 30 3. A cell according to any one of embodiment 1 to 2, wherein said cell is capable of synthesizing a nucleotide sugar selected from the group: GDP-fucose, GDP-mannose, GDP-rhamnose, CMP-N-acetylneuraminic acid, CMP-N-glycolylneuraminic acid, UDP-glucose, dTDP-glucose, UDP-galactose, UDP-N-acetylmannosamine, UDP-N-acetylglucosamine, UDP-N-acetylgalactosamine, UDP-glucuronic acid, UDP-xylose, UDP-arabinose, and/or UDP-
35 galacturonic acid
4. The cell according to any one of embodiment 1 to 3, wherein an ATP-dependent translocator encoding gene is overexpressed and/or an inner membrane protein encoding gene is overexpressed and/or an lauroyl acyltransferase encoding gene is overexpressed and/or an

endogenous ATP-dependent translocator encoding gene is modified and/or an endogenous inner membrane protein encoding gene is modified, preferably said inner membrane protein is a transmembrane transporter.

5. The cell according to any one of embodiment 1 to 4, wherein the cell comprises a KDO-independent lauroyl acyltransferase encoding gene and/or wherein the cell comprises an ATP-binding cassette multidrug transporter encoding gene.
6. Cell according to any one of embodiment 1 to 5, wherein the cell comprises expression of a gene encoding a KDO-independent lauroyl acyltransferase of SEQ ID NO: 2 or SEQ ID NO: 3, or a protein having at least 80% sequence identity thereto and having KDO-independent lauroyl acyltransferase activity.
7. Cell according to any one of embodiment 1 to 6, wherein the cell is an Escherichia coli and wherein an msbA encoding gene is overexpressed and/or an yhjD encoding gene is overexpressed and/or a LpxL encoding gene is overexpressed and/or an endogenous msbA encoding gene is modified and/or an endogenous yhjD encoding gene is modified.
8. Cell according to any one of embodiment 1 to 7, wherein said ATP-dependent translocator has 80% or more sequence identity to SEQ ID NO: 4 and has ATP-dependent translocator activity; said inner membrane protein has 80% or more sequence identity to SEQ ID NO: 21 and has transmembrane transporter activity and said lauroyl acyltransferase has 80% or more sequence identity to SEQ ID NO: 1 and has lauroyl acyltransferase activity.
9. Cell according to any one of embodiment 1 to 8, wherein said overexpression of the ATP-dependent translocator, the inner membrane protein and/or lauroyl acyltransferase is an overexpression of the endogenous gene encoding said protein or obtained by introducing and expressing said protein.
10. Cell according to any one of embodiment 1 to 9, wherein said modification of said endogenous ATP-dependent translocator encoding gene and/or said endogenous inner membrane protein encoding gene is a point-mutation.
11. Cell according to any one of embodiment 1 to 10, wherein said KDO-independent lauroyl acyltransferase encoding gene and/or wherein said ATP-binding cassette multidrug transporter is i) introduced and expressed or ii) overexpressed in said cell.
12. Cell according to any one of embodiment 1 to 11, wherein the cell comprises expression of the gene encoding the KDO-independent lauroyl acyltransferase of SEQ ID NO: 2 or SEQ ID NO: 3, or a protein having at least 80% sequence identity thereto and having KDO-independent lauroyl acyltransferase activity.
13. Cell according to any one of embodiment 1 to 12, wherein the cell comprises expression of the gene encoding the ATP-binding cassette multidrug transporter of SEQ ID NO: 22, or a protein having at least 80% sequence identity thereto and having transmembrane transporter activity.
14. The cell according to any one of embodiment 1 to 13, wherein at least one of the genes

selected from the group of genes encoding for D-arabinose 5-phosphate isomerase, 3-deoxy-D-manno-octulosonate 8-phosphate synthase, 3-deoxy-D-manno-octulosonate 8-phosphate phosphatase, and/or 3-deoxy-manno-octulosonate cytidyltransferase is rendered less functional or knocked out.

- 5 15. Cell according to any one of embodiment 1 to 14, wherein said cell produces a neutral, sialylated and/or fucosylated oligosaccharide.
16. Cell according to any one of embodiment 1 to 15, wherein said produces a mammalian milk oligosaccharide, preferably a neutral, sialylated and/or fucosylated milk oligosaccharide.
17. Cell according to any one of embodiment 1 to 16, wherein said glycosyltransferase is a
10 sialyltransferase.
18. Cell according to any one of embodiment 1 to 17, wherein said cell comprises a deleted or inactivated endogenous beta-galactosidase gene.
19. Cell according to embodiment 18, wherein said deleted or inactivated beta-galactosidase gene comprises E. coli lacZ gene.
- 15 20. Cell according to any one of the preceding embodiments, wherein said microbial cell further comprises a deleted, inactivated, or mutated galactoside-O-acyltransferase (lacA) encoding gene.
21. Cell according to any one of the preceding embodiments, wherein said cell further comprises a nucleic acid sequence encoding at least one of the following additional proteins: an exporter
20 protein or a permease exporting the synthesized oligosaccharide from the microbial cell.
22. Cell according to any one of the preceding embodiments, wherein said cell is further genetically modified to contain a nucleic acid sequence encoding a glycosidase for the specific degradation of interfering oligosaccharides, such as intermediates, side products or endogenous oligosaccharides generated by bacterial host cell, wherein the expression of said
25 glycosidase is under control of a regulatory sequence.
23. An isolated microbial cell according to any one of embodiment 1 to 22.
24. Use of a cell according to any one of embodiment 1 to 23, for the production of sialylated oligosaccharides substantially free of KDO-lactose and/or KDO-oligosaccharide.
25. Method for the fermentative production of an oligosaccharide substantially free of KDO-
30 oligosaccharide, said method comprising: providing a microbial cell according to any one of embodiment 1 to 23, cultivating said cells in favourable growing conditions and optionally separating or isolating the oligosaccharide from the culture.
26. Method for producing a sialylated oligosaccharide by fermentation through genetically modified microbial cell naturally synthesizing KDO, the method comprising the steps of:
35 a) Obtaining a microbial cell naturally synthesizing KDO and being capable to produce sialylated oligosaccharides and expressing a sialyltransferase with affinity for CMP-KDO, and wherein the KDO-biosynthesis route of said cell is knocked out or rendered less functional;

- b) Culturing the cell from step a) in favourable growing conditions, thus producing said sialylated oligosaccharide and
- c) Optionally, separating or isolating the sialylated oligosaccharide from the culture medium.
27. The method according to any one of embodiment 25 or 26, wherein said culture medium comprises precursor for the production of said oligosaccharide, preferably said culture medium comprises any one or more of lactose, galactose, N-acetylglucosamine, N-acetyl-D-lactosamine, Lacto-N-biose, sialic acid, N-acetylneuraminic acid, fucose.
28. Method according to any one of embodiment 25 to 27, wherein said cell produces the precursor internally, preferably said cell produces any one or more of lactose, N-acetyl-D-lactosamine, Lacto-N-biose, sialic acid, N-acetylneuraminic acid, fucose.
29. Method according to any one of embodiment 25 to 28, wherein said sialylated oligosaccharide is 3'-sialyllactose, 6'-sialyllactose, disialyl lacto-N-tetraose, sialylated lacto-N-triose, sialylated lacto-N-tetraose, sialylated lacto-N-neotetraose, 3-fucosyl-3'-sialyllactose, lacto-N-sialylpentaose LSTa, LSTb, LSTc, LSTd.
30. Microbial cell according to any one of embodiment 1 to 23, wherein said sialylated oligosaccharide is 3'-sialyllactose, 6'-sialyllactose, disialyl lacto-N-tetraose, sialylated lacto-N-triose, sialylated lacto-N-tetraose, sialylated lacto-N-neotetraose, 3-fucosyl-3'-sialyllactose, lacto-N-sialylpentaose LSTa, LSTb, LSTc, LSTd.
31. Method according to any one of embodiment 25 to 29, wherein said cell is a Gram-negative microbial cell, preferably said cell is an *Escherichia coli* strain, preferably an *Escherichia coli* strain which is a K12 strain, more preferably the *Escherichia coli* K12 strain is *Escherichia coli* K12 substr. MG1655.
32. Microbial cell according to any one of claims 1 to 23, or 30, wherein said cell is a Gram-negative microbial cell, preferably said cell is an *Escherichia coli* strain, preferably an *Escherichia coli* strain which is a K12 strain, more preferably the *Escherichia coli* K12 strain is *Escherichia coli* K12 substr. MG1655.
33. The method according to any one of embodiment 25 to 29, or 31, wherein said oligosaccharide is isolated from said culture medium by means of unit operation selected from the group comprising centrifugation, filtration, microfiltration, ultrafiltration, nanofiltration, ion exchange, electrodialysis, chromatography, simulated moving bed chromatography, simulated moving bed ion exchange, evaporation, precipitation, crystallisation, spray drying and any combination thereof.
34. An oligosaccharide produced by the cell according to any one of embodiment 1 to 23, 30 or 32 and/or according to the method described in any one of embodiment 25 to 29, 31 or 33, wherein said oligosaccharide is added to food formulation, feed formulation, pharmaceutical formulation, cosmetic formulation, or agrochemical formulation.

The following examples will serve as further illustration and clarification of the present invention and are not intended to be limiting.

Examples

5 Example 1: Material and methods *Escherichia coli*

Media

Three different media were used, namely a rich Luria Broth (LB), a minimal medium for shake flask (MMsf) and a minimal medium for fermentation (MMf). Both minimal media use a trace element mix.

- 10 Trace element mix consisted of 3.6 g/L $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 5 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.3 g/L $\text{MnCl}_2 \cdot 2\text{H}_2\text{O}$, 0.38 g/L $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 0.5 g/L $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.94 g/L ZnCl_2 , 0.0311 g/L H_3BO_4 , 0.4 g/L $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ and 1.01 g/L thiamine.HCl. The molybdate solution contained 0.967 g/L $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$. The selenium solution contained 42 g/L SeO_2 .

- The Luria Broth (LB) medium consisted of 1% tryptone peptone (Difco, Erembodegem, Belgium),
 15 0.5% yeast extract (Difco) and 0.5% sodium chloride (VWR, Leuven, Belgium). Luria Broth agar (LBA) plates consisted of the LB media, with 12 g/L agar (Difco, Erembodegem, Belgium) added. The minimal medium for the shake flasks (MMsf) experiments contained 2.00 g/L NH_4Cl , 5.00 g/L $(\text{NH}_4)_2\text{SO}_4$, 2.993 g/L KH_2PO_4 , 7.315 g/L K_2HPO_4 , 8.372 g/L MOPS, 0.5 g/L NaCl, 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 14.26 g/L sucrose or another carbon source when specified in the examples, 1
 20 ml/L trace element mix, 100 $\mu\text{L/L}$ molybdate solution, and 1 mL/L selenium solution. The medium was set to a pH of 7 with 1M KOH. Depending on the experiment lactose, LNB or LacNAc could be added as a precursor.

- The minimal medium for fermentations (MMf) contained 6.75 g/L NH_4Cl , 1.25 g/L $(\text{NH}_4)_2\text{SO}_4$, 2.93 g/L KH_2PO_4 and 7.31 g/L K_2HPO_4 , 0.5 g/L NaCl, 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 14.26 g/L sucrose, 1 mL/L
 25 trace element mix, 100 $\mu\text{L/L}$ molybdate solution, and 1 mL/L selenium solution with the same composition as described above.

- Complex medium, e.g. LB, was sterilized by autoclaving (121°C, 21') and minimal medium by filtration (0.22 μm Sartorius). When necessary, the medium was made selective by adding an antibiotic (e.g. ampicillin (100 mg/L), chloramphenicol (20 mg/L), carbenicillin (100 mg/L),
 30 spectinomycin (40 mg/L) and/or kanamycin (50 mg/L)).

Plasmids

- pKD46 (Red helper plasmid, Ampicillin resistance), pKD3 (contains an FRT-flanked chloramphenicol resistance (cat) gene), pKD4 (contains an FRT-flanked kanamycin resistance (kan) gene), and pCP20 (expresses FLP recombinase activity) plasmids were obtained from Prof.
 35 R. Cunin (Vrije Universiteit Brussel, Belgium in 2007).

Plasmids for additional sialyltransferase expression were constructed in a pSC101 or a p15A ori containing backbone vector, respectively, using Golden Gate assembly. The sialyltransferases

used in the enclosed examples are an alpha-2,6-sialyltransferases from Photobacterium sp. JT-ISH-224 and an alpha-2,3-sialyltransferase from Neisseria meningitidis with protein sequence SEQ ID 06 and 07 respectively. Table 1 gives an overview of the genes used to allow the deletion of the KDO biosynthetic pathway and to allow production of sialylated oligosaccharides. Genes that needed to be expressed, be it from a plasmid or from the genome were synthetically synthesized with one of the following companies: DNA2.0, Gen9, IDT or Twist Bioscience, or cloned in the genome of the original organism E. coli K12 and wherein specific genetic mutations are introduced via kits such as Quickchange Site directed mutagenesis (NEB). Expression could be further facilitated by optimizing the codon usage to the codon usage of the expression host. Genes were optimized using the tools of the supplier.

Transcription units

Heterologous genes allowing a deletion of the KDO biosynthetic pathway genes and sialyltransferase genes were expressed in different transcriptional units (TUs) using specific promoter, UTR and terminator combinations as enlisted in Table 2. The genes were expressed using promoters from Mutalik et al. (Nat. Methods 2013, No. 10, 354-360), as described herein as "PROM0005", "PROM0006", "PROM0012", "PROM0035" and "PROM0052" and a promoter from the Anderson promoter library (<http://parts.igem.org/Promoters/Catalog/Anderson>), as described herein as "PROM0024", a IPTG inducible promoter described herein as "PROM0017" and an inducible arabinose promoter as described herein as "PROM0018". UTRs used as described herein as "UTR0002", "UTR0006", "UTR0010", "UTR0017", "UTR0018", "UTR0029" and "UTR0049" were obtained from Mutalik et al. (Nat. Methods 2013, No. 10, 354-360). Terminators used in the examples are described as "TER0002" (Orosz et al. (Eur. J. Biochem. 1991, 201, 653-59)) and as "TER0004" obtained from Kim et al. (Mol. Cells 1997 Feb 28; 7(1):110-4). Table 2 shows the overview of the transcriptional units used in the examples by combination of the above promoters, UTRs and terminators.

Table 1:

SEQ ID NO (protein)	Name / TCDB group	Organism	Origin	Country of origin of digital sequence information
01	EcLpxL	E. coli K12 substr. MG1655	Synthetic	USA
02	FtLpxL1	Francisella tularensis subsp. Novicida	Synthetic	USA
03	FtLpxL2	Francisella tularensis subsp. Novicida	Synthetic	USA
04	EcMsbA	E. coli K12 substr. MG1655	Synthetic	USA
05	NmNeuA	N. meningitidis	Synthetic	UK

25

06	PbST224	Photobacterium sp. JT-ISH-224	Synthetic	Japan
07	NmST	N. meningitidis	Synthetic	UK
08	EclacY	Escherichia coli K12	Synthetic	US
09	EwcscB	Escherichia coli W	Synthetic	US
10	BaSP	Bifidobacterium adolescentis	Synthetic	Germany
11	Zmfrk	Zymomonas mobilis	Synthetic	UK
12	EcglmS	Escherichia coli K12	Synthetic	US
13	ScGNA1	Saccharomyces cerevisiae	Synthetic	US
14	BoAGE	Bacteroides ovatus	Synthetic	US
15	CjneuC	Campylobacter jejuni	Synthetic	US
16	NmlgtA	Neisseria meningitidis	Synthetic	UK
17	NmlgtB	Neisseria meningitidis	Synthetic	UK
18	EcwbgO	E. coli O55:H7	Synthetic	Germany
19	CjneuC	Campylobacter jejuni	Synthetic	US
20	kdsA	E. coli K12 substr. MG1655	Synthetic	US
21	yhjD	E. coli K12 substr. MG1655	Synthetic	US
22	LmrA	Lactococcus lactis	Synthetic	NA

Table 2:

TU number	Promoter part	CDS part	UTR part	Terminator part
TU 01	PROM0012	EcLpxL	UTR0049	TER0004
TU 02	PROM0012	EcLpxL	UTR0002	TER0004
TU 03	PROM0012	EcLpxL	UTR0017	TER0004
TU 04	PROM0006	EcLpxL	UTR0049	TER0004
TU 05	PROM0006	EcLpxL	UTR0002	TER0004
TU 06	PROM0006	EcLpxL	UTR0017	TER0004
TU 07	PROM0052	EcLpxL	UTR0029	TER0004
TU 08	PROM0024	EcLpxL	UTR0049	TER0004
TU 09	PROM0024	EcLpxL	UTR0002	TER0004
TU 10	PROM0024	EcLpxL	UTR0017	TER0004
TU 11	PROM0017	EcLpxL	UTR0006	TER0004
TU 12	PROM0018	EcLpxL	UTR0002	TER0004
TU 13	PROM0012	FtLpxL1	UTR0049	TER0004
TU 14	PROM0012	FtLpxL1	UTR0002	TER0004
TU 15	PROM0012	FtLpxL1	UTR0017	TER0004
TU 16	PROM0006	FtLpxL1	UTR0049	TER0004
TU 17	PROM0006	FtLpxL1	UTR0002	TER0004

TU 18	PROM0006	FtLpxL1	UTR0017	TER0004
TU 19	PROM0052	FtLpxL1	UTR0029	TER0004
TU 20	PROM0024	FtLpxL1	UTR0049	TER0004
TU 21	PROM0024	FtLpxL1	UTR0002	TER0004
TU 22	PROM0024	FtLpxL1	UTR0017	TER0004
TU 23	PROM0012	FtLpxL2	UTR0049	TER0004
TU 24	PROM0012	FtLpxL2	UTR0002	TER0004
TU 25	PROM0012	FtLpxL2	UTR0017	TER0004
TU 26	PROM0006	FtLpxL2	UTR0049	TER0004
TU 27	PROM0006	FtLpxL2	UTR0002	TER0004
TU 28	PROM0006	FtLpxL2	UTR0017	TER0004
TU 29	PROM0052	FtLpxL2	UTR0029	TER0004
TU 30	PROM0024	FtLpxL2	UTR0049	TER0004
TU 31	PROM0024	FtLpxL2	UTR0002	TER0004
TU 32	PROM0024	FtLpxL2	UTR0017	TER0004
TU 33	PROM0017	FtLpxL2	UTR0006	TER0004
TU 34	PROM0018	FtLpxL2	UTR0002	TER0004
TU 35	PROM0035	NmNeuA	UTR0018	TER0002
TU 36	PROM0005	PbST224	UTR0010	TER0002
TU 37	PROM0005	NmST	UTR0010	TER0002

Strains and mutations

Escherichia coli K12 substr. MG1655 [λ +, F⁻, rph-1] was obtained from the Coli Genetic Stock Center (US), CGSC Strain#: 7740, in March 2007. Gene disruptions as well as gene

5 introductions were performed using the technique published by Datsenko and Wanner (PNAS 97 (2000), 6640- 6645). This technique is based on antibiotic selection after homologous recombination performed by lambda Red recombinase. Subsequent catalysis of a flippase

recombinase ensures removal of the antibiotic selection cassette in the final production strain. Transformants carrying a Red helper plasmid pKD46 were grown in 10 mL LB media with

10 ampicillin, (100 mg/L) and L-arabinose (10 mM) at 30 °C to an OD_{600nm} of 0.6. The cells were made electrocompetent by washing them with 50 mL of ice-cold water, a first time, and with 1 mL ice cold water, a second time. Then, the cells were resuspended in 50 μ L of ice-cold water. Electroporation was done with 50 μ L of cells and 10-100 ng of linear double-stranded-DNA

15 product by using a Gene Pulser™ (BioRad) (600 Ω , 25 μ FD, and 250 volts). After electroporation, cells were added to 1 mL LB media incubated 1 h at 37 °C, and finally spread onto LB-agar containing 25 mg/L of chloramphenicol or 50 mg/L of kanamycin to select antibiotic resistant transformants. The selected mutants were verified by PCR with primers

upstream and downstream of the modified region and were grown in LB-agar at 42 °C for the loss of the helper plasmid. The mutants were tested for ampicillin sensitivity.

The linear ds-DNA amplicons were obtained by PCR using pKD3, pKD4 and their derivatives as template. The primers used had a part of the sequence complementary to the template and another part complementary to the side on the chromosomal DNA where the recombination must take place. For the genomic knockout, the region of homology was designed 50-nt upstream and 50-nt downstream of the start and stop codon of the gene of interest. For the genomic knock-in, the transcriptional starting point (+1) had to be respected. PCR products were PCR-purified, digested with DpnI, repurified from an agarose gel, and suspended in elution buffer (5 mM Tris, pH 8.0).

The selected mutants (chloramphenicol or kanamycin resistant) were transformed with pCP20 plasmid, which is an ampicillin and chloramphenicol resistant plasmid that shows temperature-sensitive replication and thermal induction of FLP synthesis. The ampicillin-resistant transformants were selected at 30 °C, after which a few were colony purified in LB at 42 °C and then tested for loss of all antibiotic resistance and of the FLP helper plasmid. The gene knockouts and knock-ins are checked with control primers (Fw/Rv-gene-out).

A sialic acid producing base strain derived from *E. coli* K12 substr. MG1655 was created by knocking out the genes *asl*, *ldhA*, *poxB*, *atpI-gidB* and *ackA-pta*, and knocking out the operons *lacZYA*, *nagAB* and the genes *nanA*, *nanE* and *nanK*. Additionally, the *E. coli lacY* gene (SEQ ID NO: 08) was introduced at the location of *lacZYA*. A fructose kinase gene (*frk*, SEQ ID NO: 11) originating from *Zymomonas mobilis*, an *E. coli W* sucrose transporter (*cscB*, SEQ ID NO: 09), a sucrose phosphorylase (*SP*, SEQ ID NO: 10) originating from *Bifidobacterium adolescentis*, an *E. coli* mutant fructose-6-P-aminotransferase (*EcglmS**54, as described by Deng et al. (Biochimie 88, 419-29 (2006), SEQ ID NO: 12), glucosamine-6-P-aminotransferase from *Saccharomyces cerevisiae* (*ScGNA1*, SEQ ID NO: 13), an N-acetylglucosamine-2-epimerase from *Bacteroides ovatus* (*BoAGE*, SEQ ID NO: 14) and a sialic acid synthase from *Campylobacter jejuni* (*Cjneub*, SEQ ID NO: 15) were knocked in into the genome.

All strains are stored in cryovials at -80°C (overnight LB culture mixed in a 1:1 ratio with 70% glycerol).

Cultivation conditions

A preculture of 96-well microtiter plate experiments was started from a cryovial, in 150 µL LB and was incubated overnight at 37 °C on an orbital shaker at 800 rpm. This culture was used as inoculum for a 96-well square microtiter plate, with 400 µL MMs medium by diluting 400x. Each strain was grown in multiple wells of the 96-well plate as biological replicates. These final 96-well culture plates were then incubated at 37°C on an orbital shaker at 800 rpm for 72h, or shorter, or longer.

Alternatively, a preculture for shake flask experiments was started from a cryovial, in 5 mL LB medium and was incubated for 8h at 37°C on an orbital shaker at 200 rpm. From this preculture, 1 mL was transferred to 100 mL minimal medium (MMsf) in a 500 mL shake flask and incubated at 37°C on an orbital shaker at 200 rpm for 72h, or shorter, or longer. This setup is used for shake flask experiments.

At the end of the cultivation experiment samples were taken from each well to measure the supernatant concentration (extracellular sugar concentrations, after 5 min. spinning down the cells), or the whole broth concentration (by boiling the culture broth for 15 min at 60°C before spinning down the cells (= intra- and extracellular sugar concentrations together).

- Also, a dilution of the cultures was made to measure the optical density at 600 nm. The cell performance index or CPI is determined by dividing the oligosaccharide concentrations, e.g. sialyllactose concentrations, measured in the whole broth by the biomass, in relative percentages compared to the reference strain. The biomass is empirically determined to be approximately 1/3rd of the optical density measured at 600 nm.
- A preculture for the bioreactor was started from an entire 1 mL cryovial of a certain strain, inoculated in 250 mL or 500 mL of MMsf medium in a 1 L or 2.5 L shake flask and incubated for 24 h at 37°C on an orbital shaker at 200 rpm. A 5 L bioreactor was then inoculated (250 mL inoculum in 2 L batch medium); the process was controlled by MFCS control software (Sartorius Stedim Biotech, Melsungen, Germany). Culturing condition were set to 37 °C, and maximal stirring; pressure gas flow rates were dependent on the strain and bioreactor. The pH was controlled at 6.8 using 0.5 M H₂SO₄ and 20% NH₄OH. The exhaust gas was cooled. 10% solution of silicone antifoaming agent was added when foaming raised during the fermentation.

Analytical methods

Optical density

Cell density of the cultures was frequently monitored by measuring optical density at 600 nm (Implen Nanophotometer NP80, Westburg, Belgium or with a Spark 10M microplate reader, Tecan, Switzerland).

Productivity

The specific productivity Q_p is the specific production rate of the oligosaccharide product, typically expressed in mass units of product per mass unit of biomass per time unit (= g oligosaccharide / g biomass / h). The Q_p value has been determined for each phase of the fermentation runs, i.e. Batch and Fed-Batch phase, by measuring both the amount of product and biomass formed at the end of each phase and the time frame each phase lasted.

The specific productivity Q_s is the specific consumption rate of the substrate, e.g. sucrose, typically expressed in mass units of substrate per mass unit of biomass per time unit (= g sucrose / g biomass / h). The Q_s value has been determined for each phase of the fermentation runs, i.e.

Batch and Fed-Batch phase, by measuring both the total amount of sucrose consumed and biomass formed at the end of each phase and the time frame each phase lasted.

The yield on sucrose Y_s is the fraction of product that is made from substrate and is typically expressed in mass unit of product per mass unit of substrate (= g oligosaccharide / g sucrose).

- 5 The Y_s has been determined for each phase of the fermentation runs, i.e. Batch and Fed-Batch phase, by measuring both the total amount of oligosaccharide produced and total amount of sucrose consumed at the end of each phase.

- The yield on biomass Y_x is the fraction of biomass that is made from substrate and is typically expressed in mass unit of biomass per mass unit of substrate (= g biomass / g sucrose). The Y_p has been determined for each phase of the fermentation runs, i.e. Batch and Fed-Batch phase, by measuring both the total amount of biomass produced and total amount of sucrose consumed at the end of each phase.

- The rate is the speed by which the product is made in a fermentation run, typically expressed in concentration of product made per time unit (= g oligosaccharide / L / h). The rate is determined by measuring the concentration of oligosaccharide that has been made at the end of the Fed-Batch phase and dividing this concentration by the total fermentation time.

- The lactose conversion rate is the speed by which lactose is consumed in a fermentation run, typically expressed in mass units of lactose per time unit (= g lactose consumed / h). The lactose conversion rate is determined by measurement of the total lactose that is consumed during a fermentation run, divided by the total fermentation time. Similar conversion rates can be calculated for other precursors such as Lacto-N-biose, N-acetyl-lactosamine, Lacto-N-tetraose, or Lacto-N-neotetraose.

Growth rate/speed measurement

- 25 The maximal growth rate (μ_{Max}) was calculated based on the observed optical densities at 600 nm using the R package grofit.

Liquid chromatography

- Standards for 6'-sialyllactose, 3'-sialyllactose, LNT and LNnT were synthesized in house. Other standards such as but not limited to lactose, sucrose, glucose, glycerol, fructose were purchased from Sigma, and LST, LacNAc and LNB were purchased from Carbosynth.

TLC analysis for oligosaccharide measurement was carried out on silica gels and the oligosaccharides were eluted with butanol–acetic acid–water (2:1:1 two runs). Sugars were detected by dipping the plate in orcinol sulfuric reagent and heating.

- 35 Carbohydrates were also analysed via an UPLC-RI (Waters, USA) method, whereby RI (Refractive Index) detects the change in the refraction index of a mobile phase when containing a sample. The sugars were separated in an isocratic flow using an Acquity BEH Amide column (Waters, USA) and a mobile phase containing 70 % acetonitrile, 26 % ammonium acetate buffer

and 4 % methanol. The column size was 2.1 x 100mm with 1.7 µm particle size. The temperature of the column was set at 25°C and the pump flow rate was 0.13 mL/min.

KDO-oligosaccharides and sialylated oligosaccharides were also measured by LC MS. Separation was performed on a Waters Acquity LC system with column heater set to 30°C and a PGC column (Hypercarb 100x2.1 mm, 3 µm, Thermo Scientific). The injection volume was 5 µL. The mobile phases consisted of Milli-Q ultrapure water (A) and CH₃CN (B), both containing 0.1% CH₂O₂ and delivered at a flow rate of 200 µL/min. The gradient consisted of an initial increase from 0 to 12% B over 21 min, from 12 to 40% B over 11 min, from 40 to 100% B over 5 min. A washing step was conducted at 100% B for 5 min. The gradient was then decreased to 0% B over 1 min and maintained at 0% B for 12 min for column equilibration. Total run time was 55 min. As mass-spectrometer, we used a Xevo TQ-MS with ESI in negative ionisation mode, a cone voltage of 20V, desolvation temperature of 350 °C and nitrogen gas flow of 650 l/h. KDO (MW: 237 g/mol) containing oligosaccharides differ in mass from N-acetylneuraminate (MW: 309 g/mol) containing oligosaccharides and can be discriminated as such.

Normalization of the data

For all types of cultivation conditions, data obtained from the mutant strains was normalized against data obtained in identical cultivation conditions with reference strains having an identical genetic background as the mutant strains but having an active KDO biosynthesis pathway. The dashed horizontal line on each plot that is shown in the examples, indicates the setpoint to which all adaptations were normalized. All data is given in relative percentages to that setpoint.

Example 2: Production hosts for the synthesis of an oligosaccharide

A production host was created capable to synthesize a sialylated oligosaccharide. To do so, a sialic acid producing base strain as described in Example 1 was used for the overexpression on plasmid and/or on the genome of a CMP-sialic acid synthetase from *Neisseria meningitidis* (NmneuA, SEQ ID NO 05, TU 35) and an α-2,6-sialyltransferase from *Photobacterium* sp. JT-ISH-224 (PbST224, SEQ ID NO 06, TU 36). This transferase is known to accept both CMP-sialic acid and CMP-KDO. The production host was cultivated in lactose containing medium as described in Example 1 and formed KDO-lactose and 6'-SL. Oversialylation is avoided by adding lactose in excess to the medium, eliminating the formation of 6,6'-disialyllactose.

An alternative biosynthetic route towards sialylated oligosaccharides makes use of the internally available UDP-GlcNAc, by expressing a heterologous UDP-GlcNAc-2-epimerase (neuC), converting UDP-GlcNAc to N-acetylmannosamine which is further converted into sialic acid by means of overexpression of a sialic acid synthase (NeuB) and to CMP-sialic acid by means of the overexpression of a CMP-sialic acid synthase (NeuA). To do so, a sialic acid producing base strain as described in Example 1 without a glucosamine-6-P-aminotransferase from *Saccharomyces cerevisiae* (ScGNA1, SEQ ID NO: 13) and without an N-acetylglucosamine-2-

- epimerase from *Bacteroides ovatus* (BoAGE, SEQ ID NO: 14), but with an UDP-GlcNAc-2-epimerase from *Campylobacter jejuni* (CjneuC, SEQ ID NO: 19) and a sialic acid synthase from *Campylobacter jejuni* (CjneuD, SEQ ID NO: 15) was created with the methods as described in Example 1. Further on, this sialic acid producing strain was used for the overexpression on plasmid and/or on the genome of a CMP-sialic acid synthetase from *Neisseria meningitidis* (NmneuA, SEQ ID NO 05, TU 35) and an α -2,6-sialyltransferase from *Photobacterium* sp. JT-ISH-224 (PbST224, SEQ ID NO 06, TU 36). This transferase is known to accept both CMP-sialic acid and CMP-KDO. The production host was cultivated in lactose containing medium as described in Example 1 and formed KDO-lactose and 6'-SL. Oversialylation is avoided by adding lactose in excess to the medium, eliminating the formation of 6,6'-disialyllactose.
- Also, another production host was created as described in Example 1 capable to synthesize a sialylated oligosaccharide such as sialylated LacNAc (sLacNAc). To allow production of sLacNAc, a sialic acid base strain as described above is modified by the overexpression on plasmid and/or on the genome of a CMP-sialic acid synthetase from *Neisseria meningitidis* (NmneuA, SEQ ID NO 05, TU 35) and an α -2,6-sialyltransferase from *Photobacterium* sp. JT-ISH-224 (PbST224, SEQ ID NO 06, TU 36). This transferase is known to accept both CMP-sialic acid and CMP-KDO. The production host was cultivated in LacNAc containing medium as described in Example 1 and formed KDO-LacNAc and 6'-sLacNAc. Oversialylation is avoided by adding LacNAc in excess to the medium, eliminating the formation of 6,6'-disialyllacNAc.
- Also, another production host was created as described in Example 1 capable to synthesize a sialylated oligosaccharide such as sialylated LNB (sLNB). To allow production of sLNB, a sialic acid base strain as described above is modified by the overexpression on plasmid and/or on the genome of a CMP-sialic acid synthetase from *Neisseria meningitidis* (NmneuA, SEQ ID NO 05, TU 35) and an α -2,6-sialyltransferase from *Photobacterium* sp. JT-ISH-224 (PbST224, SEQ ID NO 06, TU 36). This transferase is known to accept both CMP-sialic acid and CMP-KDO. The production host was cultivated in LNB containing medium as described in Example 1 and formed KDO-LNB and 6'-sLNB. Oversialylation is avoided by adding LNB in excess to the medium, eliminating the formation of 6,6'-disialyllNB.
- Example 3: A first modified production host wherein the KDO biosynthesis route is knocked out
- A production host as described in Example 2 was further modified by introducing a point mutation in the endogenous msbA gene (SEQ ID NO 04) at nucleotide 52 causing a C:G to T:A transition, changing the amino acid from a proline to a serine. Said mutation allowed the deletion of the KDO biosynthesis pathway. In particular the KDO biosynthesis pathway genes coding for D-arabinose 5-phosphate isomerase, which is for *E. coli* coded by gutQ and kdsD, the genes coding for 3-deoxy-D-manno-octulosonate 8-phosphate synthase, which is for *E. coli* coded by kdsA, the genes coding for 3-deoxy-D-manno-octulosonate 8-phosphate phosphatase, which is for *E. coli* coded by kdsC, and/or the gene coding for 3-deoxy-manno-octulosonate cytidylyltransferase,

which is for E. coli coded by kdsB were deleted. Primers to construct these genetic deletions and the specific msbA mutation are listed in Table 3. Primers to check the specific KDO pathway gene knockouts are listed in Table 4.

Said modified organism was cultivated as described in Example 1 and the formation of KDO-
5 containing oligosaccharides was evaluated. No KDO-oligosaccharides were formed in this host, only 6'-SL, 6'-sLacNAc or 6'-sLNB depending on the used medium.

Table 3:

Primer sequence (5' - 3')	Knockout (KO) / mutation construction
<u>attgtgctgcattaattaatcgacattttactcaagattaaggcgatcctccggtgcggtgccagg</u>	<u>kdsA KO</u>
<u>atgaaaaaagtcttaacgcagaaacgctaatacttttttcaagcaaaaaagactacgcccccaactgagagaac</u>	<u>kdsA KO</u>
<u>tgtcggaacggaagcccgctgctgactgctgatgagagtaaatcatgaccggtgcggtgccagg</u>	<u>kdsB KO</u>
<u>taacggtacgacactcctccaaaattggctgaagtgctgaagtgaactacgcccccaactgagagaac</u>	<u>kdsB KO</u>
<u>catgatttactcgtgcaggcgtagttaaaagattcaaggataaacaacaccggtgcggtgccagg</u>	<u>kdsC KO</u>
<u>agaacgcccagtgatagcacaatgataaccaacgtctggcttactcatctacgcccccaactgagagaac</u>	<u>kdsC KO</u>
<u>gttgactggttatcgccaataactcgttgaataactggaacgcattatgcgcggtgcggtgccagg</u>	<u>kdsD KO</u>
<u>tttgctcattgttttaicctgaatctttacactacgctgcacgcagctacgcccccaactgagagaac</u>	<u>kdsD KO</u>
<u>agagagcaatgagtgaaagcactactgaacgcggaacgicagacgttaatgcgcggtgcggtgccag</u>	<u>gutQ KO</u>
<u>cggctggcgaaacgtctgggtgaaggattaataatccggcctgatactacgcccccaactgagag</u>	<u>gutQ KO</u>
<u>gcacgtctcaatctgggtcacaatgcataacgacaaagatctctacgtggcagacattccgacgtgtgtcaacc</u>	<u>msbA nt 52: C:G to T:A</u>
<u>tgcgctcactaagggtcacagcttattggccaaactgcattttgtg</u>	<u>msbA nt 52: C:G to T:A</u>
<u>gcacgtctcaatctgggtcacaatgcataacgacaaagatctctacg</u>	<u>msbA nt 148: C:G to T:A</u>
<u>tatgcgtctctgtgacttaaggagcgataacatg</u>	<u>msbA nt 148: C:G to T:A</u>
<u>actacgtctcactcttctgatgagctttgg</u>	<u>msbA nt 148: C:G to T:A</u>
<u>tgcgctcactaagggtcacagcttattggccaaactgcattttgtg</u>	<u>msbA nt 148: C:G to T:A</u>
<u>gcacgtctcaatctgggtcacaatgcacgaggaacgagatc</u>	<u>yhiD nt 400 C:G to T:A</u>
<u>tatgcgtctctacactgctgaacgcggtgttg</u>	<u>yhiD nt 400 C:G to T:A</u>
<u>actacgtctcatgtacgactgtaggcctgtcg</u>	<u>yhiD nt 400 C:G to T:A</u>
<u>atgccgtctcactaagggtcacagcttaaggctgcttttccc</u>	<u>yhiD nt 400 C:G to T:A</u>

10 **Table 4:**

Primer sequence (5' - 3')	Knockout (KO) check
<u>tccgatggcgaaatttggc</u>	<u>kdsA KO</u>
<u>tttactggcttctggtggc</u>	<u>kdsA KO</u>
<u>agatccctatgaaattcgcg</u>	<u>kdsA KO</u>
<u>cgcactggcactgtttggc</u>	<u>kdsA KO</u>

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ggtgacattaggatgctgcc	kdsB KO
tctttgagttgttcccagcg	kdsB KO
cgtctgcttgaaatcattgcc	kdsB KO
aatggcttaattcggcacgc	kdsB KO
atacgggcatgagatcccg	kdsC KO
ttctgggttatagacgagcg	kdsC KO
tgaggcactgaactaatgc	kdsC KO
tgccaagttgagagtctggc	kdsC KO
gtgagtgatccccaatgtggc	kdsD KO
cccgcgctgataacgccagg	kdsD KO
ctgattgcagaagctgccg	gutQ KO
gaagtggcggcggtgcccg	gutQ KO

Example 4: A second modified production host wherein the KDO biosynthesis route is knocked out

- A production host as described in Example 2 was further modified by introducing a point mutation in the endogenous msbA gene (SEQ ID NO 04) at base 148 causing a C:G to T:A transition, changing the amino acid from a proline to a serine. Said mutation allowed the deletion of the KDO biosynthesis pathway. In particular the KDO biosynthesis pathway genes coding for D-arabinose 5-phosphate isomerase, which is for E. coli coded by gutQ and kdsD, the genes coding for 3-deoxy-D-manno-octulosonate 8-phosphate synthase, which is for E. coli coded by kdsA, the genes coding for 3-deoxy-D-manno-octulosonate 8-phosphate phosphatase, which is for E. coli coded by kdsC, and/or the gene coding for 3-deoxy-manno-octulosonate cytidyltransferase, which is for E. coli coded by kdsB were deleted. Primers to construct these genetic deletions and the specific msbA mutation are listed in Table 3. Primers to check the specific KDO pathway gene knockouts are listed in Table 4.
- Said modified organism was cultivated as described in Example 1 and the formation of KDO-containing oligosaccharides was evaluated. No KDO-oligosaccharides were formed in this host, only 6'-SL, 6'-sLacNAc or 6'-sLNB depending on the used medium.

Example 5: A third modified production host wherein the KDO biosynthesis route is knocked out

- A production host as described in Example 2 was further modified by overexpressing the endogenous msbA (SEQ ID NO 04) in the production host. Said overexpression allowed the deletion of the KDO biosynthesis pathway. In particular the KDO biosynthesis pathway genes coding for D-arabinose 5-phosphate isomerase, which is for E. coli coded by gutQ and kdsD, the genes coding for 3-deoxy-D-manno-octulosonate 8-phosphate synthase, which is for E. coli coded by kdsA, the genes coding for 3-deoxy-D-manno-octulosonate 8-phosphate phosphatase, which is for E. coli coded by kdsC, and/or the gene coding for 3-deoxy-manno-octulosonate

cytidyltransferase, which is for E. coli coded by kdsB were deleted. Primers to construct these genetic deletions and the specific msbA mutation are listed in Table 3. Primers to check the specific KDO pathway gene knockouts are listed in Table 4.

Said modified organism was cultivated as described in Example 1 and the formation of KDO-containing oligosaccharides was evaluated. No KDO-oligosaccharides were formed in this host, only 6'-SL, 6'-sLacNAc or 6'-sLNB depending on the used medium.

Example 6: A fourth modified production host wherein the KDO biosynthesis route is knocked out

A production host as described in Example 2 was further modified by introducing a point mutation in the endogenous yhjD gene causing a C:G to T:A transition at nucleotide number 400 leading to a change from arginine to cysteine at amino acid position 134. Said mutation allowed the deletion of the KDO biosynthesis pathway. In particular the KDO biosynthesis pathway genes coding for D-arabinose 5-phosphate isomerase, which is for E. coli coded by gutQ and kdsD, the genes coding for 3-deoxy-D-manno-octulosonate 8-phosphate synthase, which is for E. coli coded by kdsA, the genes coding for 3-deoxy-D-manno-octulosonate 8-phosphate phosphatase, which is for E. coli coded by kdsC, and/or the gene coding for 3-deoxy-manno-octulosonate cytidyltransferase, which is for E. coli coded by kdsB were deleted. Primers to construct these genetic deletions and the specific msbA mutation are listed in Table 3. Primers to check the specific KDO pathway gene knockouts are listed in Table 4.

Said modified organism was cultivated as described in Example 1 and the formation of KDO-containing oligosaccharides was evaluated. No KDO-oligosaccharides were formed in this host, only 6'-SL, 6'-sLacNAc or 6'-sLNB depending on the used medium.

Example 7: A fifth modified production host wherein the KDO biosynthesis route is knocked out

A production host as described in Example 2 was further modified by introducing additional copies of the endogenous LpxL (SEQ ID NO 01). Said LpxL gene was overexpressed and evaluated in different transcription units (TU 01 – TU 12). This overexpressed LpxL gene allowed the deletion of the KDO biosynthesis pathway. In particular the KDO biosynthesis pathway genes coding for D-arabinose 5-phosphate isomerase, which is for E. coli coded by gutQ and kdsD, the genes coding for 3-deoxy-D-manno-octulosonate 8-phosphate synthase, which is for E. coli coded by kdsA, the genes coding for 3-deoxy-D-manno-octulosonate 8-phosphate phosphatase, which is for E. coli coded by kdsC, and/or the gene coding for 3-deoxy-manno-octulosonate cytidyltransferase, which is for E. coli coded by kdsB were deleted. Primers to construct these genetic deletions and the specific msbA mutation are listed in Table 3. Primers to check the specific KDO pathway gene knockouts are listed in Table 4.

Said modified organism was cultivated as described in Example 1 and the formation of KDO-containing oligosaccharides was evaluated. No KDO-oligosaccharides were formed in this host, only 6'-SL, 6'-sLacNAc or 6'-sLNB depending on the used medium.

Example 8: A sixth modified production host wherein the KDO biosynthesis route is knocked out

A production host as described in Example 2 was further modified by introducing an alternative LpxL gene originating from *Francisella tularensis* subsp. *novicida*, LpxL1 (SEQ ID NO 02). This group of LpxL proteins is independent of KDO modified Lipid IVa, allowing full acylation of the lipidA structure when the KDO biosynthesis pathway is knocked out. Said gene was overexpressed and evaluated in different transcription units (TU 13 – TU 22). This overexpressed FtLpxL1 gene allowed the deletion of the KDO biosynthesis pathway. In particular the KDO biosynthesis pathway genes coding for D-arabinose 5-phosphate isomerase, which is for *E. coli* coded by *gutQ* and *kdsD*, the genes coding for 3-deoxy-D-manno-octulosonate 8-phosphate synthase, which is for *E. coli* coded by *kdsA*, the genes coding for 3-deoxy-D-manno-octulosonate 8-phosphate phosphatase, which is for *E. coli* coded by *kdsC*, and/or the gene coding for 3-deoxy-manno-octulosonate cytidylyltransferase, which is for *E. coli* coded by *kdsB* were deleted. Primers to construct these genetic deletions and the specific *msbA* mutation are listed in Table 3. Primers to check the specific KDO pathway gene knockouts are listed in Table 4.

Said modified organism was cultivated as described in Example 1 and the formation of KDO-containing oligosaccharides was evaluated. No KDO-oligosaccharides were formed in this host, only 6'-SL, 6'-sLacNAc or 6'-sLNB depending on the used medium.

Example 9: A seventh modified production host wherein the KDO biosynthesis route is knocked out

A production host as described in Example 2 was further modified by introducing an alternative LpxL gene originating from *Francisella tularensis* subsp. *novicida*, LpxL2 (SEQ ID NO 03). This group of LpxL proteins is independent of KDO modified Lipid IVa, allowing full acylation of the lipidA structure when the KDO biosynthesis pathway is knocked out. Said gene was overexpressed and evaluated in different transcription units (TU 23 – TU 34). This overexpressed FtLpxL2 gene allowed the deletion of the KDO biosynthesis pathway. In particular the KDO biosynthesis pathway genes coding for D-arabinose 5-phosphate isomerase, which is for *E. coli* coded by *gutQ* and *kdsD*, the genes coding for 3-deoxy-D-manno-octulosonate 8-phosphate synthase, which is for *E. coli* coded by *kdsA*, the genes coding for 3-deoxy-D-manno-octulosonate 8-phosphate phosphatase, which is for *E. coli* coded by *kdsC*, and/or the gene coding for 3-deoxy-manno-octulosonate cytidylyltransferase, which is for *E. coli* coded by *kdsB* were deleted. Primers to construct these genetic deletions and the specific *msbA* mutation are listed in Table 3. Primers to check the specific KDO pathway gene knockouts are listed in Table 4.

Said modified organism was cultivated as described in Example 1 and the formation of KDO-containing oligosaccharides was evaluated. No KDO-oligosaccharides were formed in this host, only 6'-SL, 6'-sLacNAc or 6'-sLNB depending on the used medium.

Example 10: A production host for the synthesis of LSTc

A production host as described in Example 2 wherein the genes encoding for a beta-1,3-GlcNAc transferase from *Neisseria meningitidis* (NmIgtA, SEQ ID NO 16) and a beta-1,4-galactosyltransferase from *Neisseria meningitidis* (NmIgtB, SEQ ID NO 17) are introduced in the genome, allowing the synthesis of the precursor LNnT.

This organism produces in the culture conditions supplemented with lactose as described in Example 1 sialylated lacto-N-neotetraose (LSTc, a-NeuNAc-(2-6)-b-Gal-(1-4)-b-GlcNAc-(1-3)-b-Gal-(1-4)-Glc), and KDO containing oligosaccharides such as KDO-lactose.

10 Example 11: An eighth modified production host wherein the KDO biosynthesis route is knocked out

The production host as described in Example 10 was further modified by introducing additional copies of EcLpxL (SEQ ID NO 01), FtLpxL1 (SEQ ID NO 02) or FtLpxL2 (SEQ ID NO 03). Said LpxL genes were overexpressed and evaluated in different transcription units (TU 01 – TU 34).

15 These gene overexpressions allowed the deletion of the KDO biosynthesis pathway. In particular the KDO biosynthesis pathway genes coding for D-arabinose 5-phosphate isomerase, which is for *E. coli* coded by gutQ and kdsD, the genes coding for 3-deoxy-D-manno-octulosonate 8-phosphate synthase, which is for *E. coli* coded by kdsA, the genes coding for 3-deoxy-D-manno-octulosonate 8-phosphate phosphatase, which is for *E. coli* coded by kdsC, and/or the gene
20 coding for 3-deoxy-manno-octulosonate cytidylyltransferase, which is for *E. coli* coded by kdsB were deleted. Primers to construct these genetic deletions and the specific msbA mutation are listed in Table 3. Primers to check the specific KDO pathway gene knockouts are listed in Table 4.

This organism was cultured in the culture conditions supplemented with lactose as described in
25 Example 1 and produced the sialylated lacto-N-neotetraose (LSTc, a-NeuNAc-(2-6)-b-Gal-(1-4)-b-GlcNAc-(1-3)-b-Gal-(1-4)-Glc). No KDO-containing oligosaccharides were found in the culture medium.

Example 12: A ninth modified production host wherein the KDO biosynthesis route is knocked out

30 The production host as described in Example 10 was further modified by overexpressing the endogenous msbA (SEQ ID NO 04) in the production host. Said overexpression allowed the deletion of the KDO biosynthesis pathway. In particular the KDO biosynthesis pathway genes coding for D-arabinose 5-phosphate isomerase, which is for *E. coli* coded by gutQ and kdsD, the genes coding for 3-deoxy-D-manno-octulosonate 8-phosphate synthase, which is for *E. coli*
35 coded by kdsA, the genes coding for 3-deoxy-D-manno-octulosonate 8-phosphate phosphatase, which is for *E. coli* coded by kdsC, and/or the gene coding for 3-deoxy-manno-octulosonate cytidylyltransferase, which is for *E. coli* coded by kdsB were deleted. Primers to construct these genetic deletions and the specific msbA mutation are listed in Table 3. Primers to check the

specific KDO pathway gene knockouts are listed in Table 4.

This organism was cultured in the culture conditions supplemented with lactose as described in Example 1 and produced the sialylated lacto-N-neotetraose (LSTc, a-NeuNAc-(2-6)-b-Gal-(1-4)-b-GlcNAc-(1-3)-b-Gal-(1-4)-Glc). No KDO-containing oligosaccharides were found in the culture medium.

Example 13: A production host for the synthesis of LSTb

The production host as described in Example 2 wherein the genes encoding for a beta-1,3-GlcNAc transferase from *Neisseria meningitidis* (NmlgtA, SEQ ID NO 16) and a beta-1,3-galactosyltransferase from *E. coli* O55:H7 (Ecwbgo, SEQ ID NO 18) are introduced in the genome, allowing the synthesis of the precursor LNT. Said organism is further modified with the α -2,6-sialyltransferase ST6galI or ST6galII instead of the α -2,6-sialyltransferase from *Photobacterium* sp. JT-ISH-224.

This organism produces in the culture conditions supplemented with lactose as described in Example 1 sialylated lacto-N-tetraose (LSTb, a-NeuNAc-(2-6)-(b-D-Gal-[1-3])-b-D-GlcNAc-(1-3)-b-D-Gal-(1-4)-Glc), and KDO containing oligosaccharides such as KDO-lactose.

Example 14: A tenth modified production host wherein the KDO biosynthesis route is knocked out

The production host as described in Example 13 was further modified by introducing additional copies of EcLpxL (SEQ ID NO 01), FtLpxL1 (SEQ ID NO 02) or FtLpxL2 (SEQ ID NO 03). Said LpxL genes were overexpressed and evaluated in different transcription units (TU 01 – TU 34). These gene overexpressions allowed the deletion of the KDO biosynthesis pathway. In particular the KDO biosynthesis pathway genes coding for D-arabinose 5-phosphate isomerase, which is for *E. coli* coded by gutQ and kdsD, the genes coding for 3-deoxy-D-manno-octulosonate 8-phosphate synthase, which is for *E. coli* coded by kdsA, the genes coding for 3-deoxy-D-manno-octulosonate 8-phosphate phosphatase, which is for *E. coli* coded by kdsC, and/or the gene coding for 3-deoxy-manno-octulosonate cytidylyltransferase, which is for *E. coli* coded by kdsB were deleted. Primers to construct these genetic deletions and the specific msbA mutation are listed in Table 3. Primers to check the specific KDO pathway gene knockouts are listed in Table 4.

This organism was cultured in the culture conditions supplemented with lactose as described in Example 1 and produced sialylated lacto-N-tetraose (LSTb, a-NeuNAc-(2-6)-(b-D-Gal-[1-3])-b-D-GlcNAc-(1-3)-b-D-Gal-(1-4)-Glc). No KDO-containing oligosaccharides were found in the culture medium.

Example 15: Modified production hosts wherein the KDO biosynthesis route is knocked out for the synthesis of 3'-sialyllactose (3'-SL)

The production hosts as described in Example 3 to 9 were modified so that the 6'-SL forming

transferase is replaced by a 3'-SL forming transferase. Said α -2,3-sialyltransferase from *Neisseria meningitidis* (NmST, SEQ ID NO 07) was expressed on a plasmid and/or on the genome within TU 37.

- These organisms were cultured in the culture conditions supplemented with lactose, LacNAc or LNB as described in Example 1 and produced 3'-SL, 3'-sLacNAc or 3'-sLNB depending on the used medium. Within the culture fluid no KDO-containing oligosaccharides were found.

Example 16: Modified production hosts wherein the KDO biosynthesis route is knocked out for the synthesis of LSTd

- The production hosts as described in Example 15, wherein the genes encoding for a beta-1,3-GlcNAc transferase from *Neisseria meningitidis* (NmlgtA, SEQ ID NO 16) and a beta-1,4-galactosyltransferase from *Neisseria meningitidis* (NmlgtB, SEQ ID NO 17) are introduced in the genome, allowing the synthesis of the precursor LNnT.

- These organisms were cultured in the culture conditions supplemented with lactose as described in Example 1 and produced LSTd (a-NeuNAc-(2-3)-b-D-Gal-(1-4)-b-D-GlcNAc-(1-3)-b-D-Gal-(1-4)-Glc). Within the culture fluid no KDO-containing oligosaccharides were found.

Example 17: Modified production hosts wherein the KDO biosynthesis route is knocked out for the synthesis of LSTa

- The production hosts as described in Example 15, wherein the genes encoding for a beta-1,3-GlcNAc transferase from *Neisseria meningitidis* (NmlgtA, SEQ ID NO 16) and a beta-1,3-galactosyltransferase from *E. coli* O55:H7 (Ecwbgo, SEQ ID NO 18) are introduced in the genome, allowing the synthesis of the precursor LNT.

- These organisms were cultured in the culture conditions supplemented with lactose as described in Example 1 and produced LSTa (a-NeuNAc-(2-3)-b-D-Gal-(1-3)-b-D-GlcNAc-(1-3)-b-D-Gal-(1-4)-Glc). Within the culture fluid no KDO-containing oligosaccharides were found.

Example 18: Modified production hosts wherein the KDO biosynthesis route is knocked out for the synthesis of DSLNT

- The production hosts as described in Example 15, wherein the genes encoding for a beta-1,3-GlcNAc transferase from *Neisseria meningitidis* (NmlgtA, SEQ ID NO 16) and a beta-1,3-galactosyltransferase from *E. coli* O55:H7 (Ecwbgo, SEQ ID NO 18) are introduced in the genome, allowing the synthesis of the precursor LNT. Beside the α -2,3-sialyltransferase from *Neisseria meningitidis* (NmST, SEQ ID NO 07), an extra α -2,6-sialyltransferase like ST6gall or ST6GalII was expressed on a plasmid and/or on the genome.

These organisms were cultured in the culture conditions supplemented with lactose as described in Example 1 and produced DSLNT (a-NeuNAc-(2-3)-b-Gal-(1-3)-[a-NeuNAc-(2-6)]-b-GlcNAc-(1-3)-b-Gal-(1-4)-Glc). Within the culture fluid no KDO-containing oligosaccharides were found.

Example 19: Rendering genes less functional

So called rendering genes less functional is a common practice in biotechnology. As described above there are several techniques to lower expression or render a gene less functional (such as the usage of siRNA, CrispR interference, RNAi, miRNA, asRNA, mutating genes, knocking-out genes, transposon mutagenesis, ...).

CrispR interference is one of the newest techniques in rendering genes less functional. It entails the design of an sgRNA that recognizes the gene of interest (the gene to be rendered less functional) and the expression of a mutated RNA-guided DNA endonuclease enzyme that lost its endonuclease activity (e.g. the dCas9 protein). The sgRNA is composed of a base pairing region mostly existing of 20 nucleotides downstream or upstream next to a PAM region (e.g. NGG in the case of dCas9). The base pairing region is complementary to a region into the target gene. The closer the base pairing region binds to the 5' end of the gene target, the better the repression. To avoid off target repression, the base pairing region is BLASTed against the genome, ensuring there are no other regions complementary to the base pairing region apart from the gene of interest. An example of a design tool is described by Doench et. Al. 2016 [Nature Biotechnology volume 34, pages184–191(2016)] and is provided by most synthetic DNA providers.

Both dCas9 and the sgRNA are expressed in the cell according to the methods described in Example 1. Preferentially both are expressed from the genome, ensuring stable expression over several generations.

As an example, the kdsA gene with SEQ ID NO 20 was rendered less functional by using the CrispRi technique described above. The used sgRNA was CCGCTCCTCCATCCACTCTTATCGTGGACC with TGG as a PAM sequence. Said sequence was expressed by means of a constitutive promoter sequence ("PROM0012") as described in Example 1. Said expression cassette together with dCas9 were introduced in the strains described in Example 2. In the resulting strains the KDO biosynthesis pathway was rendered less functional and hence no detectable KDO oligosaccharides are formed.