

Production of bioproduct in a host cell

Field of the invention

The present invention is in the technical field of synthetic biology and metabolic engineering. More particularly, the present invention is in the technical field of fermentation of metabolically engineered host cells. The present invention describes a method of producing bioproducts by fermentation with a genetically modified cell, as well as to the genetically modified cell used in the method. The cell is genetically modified to produce a bioproduct and is further genetically modified by reducing the expression of at least one endogenous membrane protein encoding gene and/or mutating the expression of the endogenous membrane protein.

Background

Industrial production of bioproducts can be performed by fermentation of cells enabled to produce the bioproducts. The preparation of cultures is labour intensive, occupying much space and equipment, and there is a considerable risk of contamination with spoilage bacteria and/or phages during the step of propagation. The failure of bacterial cultures by bacteriophage (phage) infection and multiplication is a major problem with the industrial use of bacterial cultures. There are many different types of phages with varying mechanisms to attack bacteria. Moreover, new strains of bacteriophages appear.

Strategies used in industry to minimise bacteriophage infection, and thus failure of a bacterial culture, include the use of: (i) mixed starter cultures; and (ii) the alternate use of strains having different phage susceptibility profiles (strain rotation).

The complex composition of mixed starter cultures ensures that a certain level of resistance to phage attack is present. However, repeated sub-culturing of mixed strain cultures leads to unpredictable changes in the distribution of individual strains and eventually undesired strain dominance. This in turn may lead to increased susceptibility to phage attack and risk of fermentation failures.

Rotation of selected bacterial strains which are sensitive to different phages is another approach to limit phage development. However, it is difficult and cumbersome to identify and select a sufficient number of strains having different phage type profiles to provide an efficient and reliable rotation program. In addition, the continuous use of strains requires careful monitoring for new infectious phages and the need to quickly substitute a strain which is infected by the new bacteriophage by a resistant strain, in manufacturing plants where large quantities of bulk starter cultures are made ahead of time, such a quick response is usually not possible.

There is a continuing need in the art to provide improved bacterial strains for use in fermentative production industry - such as bacterial strains producing a desired product. Improved bacterial strains that are phage resistant are particularly desirable.

The evolutionary pressure imposed by phage predation on bacteria has led to the development of efficient resistance systems to protect bacteria from phage infection. Makarova et al. (J Bacteriol. 2011, Nov, 193(21): 6039-6056) disclosed that a substantial fraction of bacterial and archaeal genomes is dedicated to phage defense and that the defense genes are typically clustered in genomic islands termed defense islands. Some of these systems include restriction-modification systems, abortive infection mechanisms, CRISPR/Cas adaptive defense system, prokaryotic argonaute system, BREX system (see WO2015/059690) and DISARM system (WO2018/142416). Others found that for specific bacterial strains specific deletion of a membrane protein encoding gene makes the cell more resistant to phage attack (e.g. fhuA gene as disclosed in V. Braun (2009) J Bacteriol. 191(11):3431-3436 and Link et al., 1997, J. Bact. 179: 6228- 8237). However, in the fermentative production of specific bioproducts, more in particular monosaccharide, phosphorylated monosaccharide, activated monosaccharide, disaccharide, oligosaccharide or glycolipid, hesitation exists for engineering other genetic loci than strictly needed for the production of the bioproduct as such further engineering steps might have a negative effect on growth of the cell and/or production of the desired bioproduct. Furthermore, full gene deletions may hamper normal cell function, depending on the importance of the role of the membrane protein.

Description

Summary of the invention

The present inventors have uncovered a novel phage resistance system to be used in E. coli producing bioproducts such as monosaccharide, phosphorylated monosaccharide, activated monosaccharide, disaccharide, oligosaccharide or glycolipid. The phage resistance system comprises a reduced expression of at least one endogenous membrane protein encoding gene and/or a mutation of the endogenous membrane protein encoding gene and more preferably a reduced expression and/or mutation of an endogenous outer membrane protein encoding gene. The newly discovered system effectively and efficiently protects against phages and at the same time is not negatively influencing the bioproduct productivity and/or growth of the fermenting E. coli bacteria.

Specifically, the present inventors have uncovered that the phage resistance system confers complete or partial resistance against E. coli phages spanning a wide phylogeny of phage types, including lytic and temperate (also referred lysogenic) phages, even in the first cycle of infection. The invention also provides methods for enhanced production of at least one desired bioproduct. The bioproduct is obtained with a genetically modified host cell comprising the phage resistance system of the present invention.

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 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 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.

According to the present invention, the term "membrane protein" refers to a protein found in biological membranes or cell envelope and commonly known by a person skilled in the art (Lodish H, Berk A, Zipursky SL, et al., 2000 and Silhavy et al 2010). It is the protein component of the cytoplasmic membrane, the outer membrane or the cell wall. Membrane proteins may be integral, peripheral or lipid anchored proteins or combinations thereof. The term refers to proteins that are part of or interact with the cell membrane and can for instance control the flow of molecules, information across the cell or form a structural part of the membrane. The membrane proteins are preferably involved in transport, be it import into or export out of the cell.

The term "membrane protein encoding gene(s)" as used herein encompasses polynucleotides that include a sequence encoding a membrane protein of the invention. The term also encompasses polynucleotides that include a single continuous region or discontinuous regions encoding the membrane protein (for example, interrupted by integrated phage or an insertion sequence or editing) together with additional regions that also may contain coding and/or non-coding sequences.

According to the present invention, the term "polynucleotide(s)" generally refers to any polyribonucleotide or polydeoxyribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. "Polynucleotide(s)" include, without limitation, single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions or single-, double- and triple-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded, or triple-stranded regions, or a mixture of single- and double-stranded regions. In addition, "polynucleotide" as used herein refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The strands in such regions may be from the same molecule or from different molecules. The regions may include all of one or more of the molecules, but more typically involve only a region of some of the molecules. One of the molecules of a triple-helical region often is an oligonucleotide. As used herein, the term "polynucleotide(s)" also includes DNAs or RNAs as described above that contain one or more modified bases. Thus, DNAs or RNAs with backbones modified for stability or for other reasons are "polynucleotide(s)" according to the present invention. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritylated bases, are to be understood to be covered by the term "polynucleotides". It will be appreciated that a great variety of modifications have been made to DNA and RNA that serve many useful purposes known to those of skill in the art. The term "polynucleotide(s)" as it is employed herein embraces such chemically, enzymatically or metabolically modified forms of polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including, for example, simple and complex cells. The term "polynucleotide(s)" also embraces short polynucleotides often referred to as oligonucleotide(s).

"Polypeptide(s)" refers to any peptide or protein comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds. "Polypeptide(s)" refers to both short chains, commonly referred to as peptides, oligopeptides and oligomers and to longer chains generally referred to as proteins. Polypeptides may contain amino acids other than the 20 gene encoded amino acids. "Polypeptide(s)" include those modified either by natural processes, such as processing and other post-translational modifications, but also by chemical modification techniques. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature, and they are well known to the skilled person. The same type of modification may be present in the same or varying degree at several

5 sites in a given polypeptide. Furthermore, a given polypeptide may contain many types of modifications. Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains, and the amino or carboxyl termini. Modifications include, for example, acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation, selenoylation, transfer-RNA mediated addition of amino acids to proteins, such as arginylation, and ubiquitination. Polypeptides may be branched or cyclic, with or without branching. Cyclic, branched and branched circular polypeptides may result from post-translational natural processes and may be made by entirely synthetic methods, as well.

15 The term "polynucleotide encoding a polypeptide" as used herein encompasses polynucleotides that include a sequence encoding a polypeptide of the invention. The term also encompasses polynucleotides that include a single continuous region or discontinuous regions encoding the polypeptide (for example, interrupted by integrated phage or an insertion sequence or editing) together with additional regions that also may contain coding and/or non-coding sequences.

20 "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" or "synthetic", as the term is used herein, means any synthetically generated sequence and not directly isolated from a natural source.

30 The term "recombinant" or "transgenic" or "genetically modified", as used herein with reference to a cell or host cell indicates that the bacterial cell replicates a heterologous nucleic acid, or expresses a peptide or protein encoded by a heterologous nucleic acid (i.e., a sequence "foreign to said cell" or a sequence "foreign to said location or environment in said cell"). Such cells are described to be transformed with at least one heterologous or exogenous gene, or are described to be transformed by the introduction of at least one heterologous or exogenous gene. Recombinant or transgenic cells can contain genes that are not found within the native (non-recombinant) form of the cell. Recombinant cells can also contain genes found in the native form of the cell wherein the genes are modified and re-introduced into the cell by artificial means. The term also encompasses cells that contain a nucleic acid endogenous to the cell that has been

modified or its expression has been modified without removing the nucleic acid from the cell; such modifications include those obtained by gene replacement, replacement of a promoter; site-specific mutation; and related techniques. Accordingly, a "recombinant polypeptide" is one which has been produced by a recombinant cell. A "heterologous sequence" or a "heterologous nucleic acid", as used herein, is one that originates from a source foreign to the particular cell (e.g. from a different species), or, if from the same source, is modified from its original form or place in the genome. Thus, a heterologous nucleic acid operably linked to a promoter is from a source different from that from which the promoter was derived, or, if from the same source, is modified from its original form or place in the genome. The heterologous sequence may be stably introduced, e.g. by transfection, transformation, conjugation or transduction, into the genome of the host microorganism cell, wherein techniques may be applied which will depend on the host cell and the sequence that is to be introduced. Various techniques are known to a person skilled in the art and are, e.g., disclosed in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

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 "heterologous" or "exogenous" when used in reference to a polynucleotide, gene, nucleic acid, polypeptide, or enzyme refers to a polynucleotide, gene, nucleic acid, polypeptide, or enzyme that is from a source or derived from a source other than the host organism species. In contrast a "homologous" polynucleotide, gene, nucleic acid, polypeptide, or enzyme is used herein to denote a polynucleotide, gene, nucleic acid, polypeptide, or enzyme that is derived from the host organism species. When referring to a gene regulatory sequence or to an auxiliary nucleic acid sequence used for maintaining or manipulating a gene sequence (e.g. a promoter, a 5' untranslated region, 3' untranslated region, poly A addition sequence, intron sequence, splice site, ribosome binding site, internal ribosome entry sequence, genome homology region, recombination site, etc.), "heterologous" means that the regulatory sequence or auxiliary sequence is not naturally associated with the gene with which the regulatory or auxiliary nucleic acid sequence is juxtaposed in a construct, genome, chromosome, or episome. Thus, a promoter operably linked to a gene to which it is not operably linked to in its natural state (i.e. in the genome of a non-genetically engineered organism) is referred to herein as a "heterologous promoter," even though the promoter may be derived from the same species (or, in some cases, the same organism) as the gene to which it is linked.

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 encoded protein. 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" in the case of a heterologous gene that is not present in the

wild type strain. Lower expression or reduced expression is obtained by means of common well-known technologies for a skilled person (such as the usage of siRNA, CrispR, CrispRi, 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 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 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 Shine Dalgarno 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 "constitutive expression" is defined as expression that is not regulated by transcription factors other than the subunits of RNA polymerase (e.g. the bacterial sigma factors) under certain growth conditions. Non-limiting examples of such transcription factors are CRP, LacI, ArcA, Cra, IclR in *E. coli*. These transcription factors bind on a specific sequence and may block or enhance expression in certain growth conditions. RNA polymerase binds a specific sequence to initiate transcription, for instance via a sigma factor in prokaryotic hosts.

The term "regulated expression" is defined as expression that is regulated by transcription factors other than the subunits of RNA polymerase (e.g. bacterial sigma factors) under certain growth conditions. Examples of such transcription factors are described above. Commonly expression regulation is obtained by means of an inducer or repressor, such as but not limited to IPTG, arabinose, rhamnose, fucose, allo-lactose or pH shifts, or temperature shifts or carbon depletion or substrates or the produced product or chemical repression.

The term "control sequences" refers to sequences recognized by the host cells transcriptional and translational systems, allowing transcription and translation of a polynucleotide sequence to a polypeptide. Such DNA sequences are thus necessary for the expression of an operably linked coding sequence in a particular host cell or organism. Such control sequences can be, but are not limited to, promoter sequences, ribosome binding sequences, Shine Dalgarno sequences, Kozak sequences, transcription terminator sequences. The control sequences that are suitable

for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers. DNA for a presequence or secretory leader may be operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Said control sequences can furthermore be controlled with external chemicals, such as, but not limited to, IPTG, arabinose, lactose, allo-lactose, rhamnose or fucose via an inducible promoter or via a genetic circuit that either induces or represses the transcription or translation of said polynucleotide to a polypeptide.

Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous.

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

"Variant(s)" as the term is used herein, is a polynucleotide or polypeptide that differs from a reference polynucleotide or polypeptide respectively but retains essential properties. A typical variant of a polynucleotide differs in nucleotide sequence from another, reference polynucleotide.

Changes in the nucleotide sequence of the variant may or may not alter the amino acid sequence of a polypeptide encoded by the reference polynucleotide. Nucleotide changes may result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference sequence, as discussed below. A typical variant of a polypeptide differs in amino acid sequence from another, reference polypeptide. Generally, differences are limited so that the sequences of the reference polypeptide and the variant are closely similar overall and, in many regions, identical. A variant and reference polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions in any combination. A substituted or inserted amino acid residue may or may not be one encoded by the genetic code. A variant of a polynucleotide or polypeptide may be naturally occurring such as an allelic variant, or it may be a variant that is not known to occur naturally. Non-naturally occurring variants of polynucleotides and polypeptides may be made by mutagenesis techniques, by direct synthesis, and by other recombinant methods known to the persons skilled in the art.

In some embodiments, the present disclosure contemplates making functional variants by modifying the structure of a membrane protein as used in the present invention. Variants can be produced by amino acid substitution, deletion, addition, or combinations thereof. For instance, it is reasonable to expect that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid (e.g., conservative mutations) will not have a major effect

on the biological activity of the resulting molecule. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Whether a change in the amino acid sequence of a polypeptide of the disclosure results in a functional homolog can be readily determined by assessing the ability of the variant polypeptide to produce a response in cells in a fashion similar to the wild-type polypeptide, and in case of the present invention to provide better yield, productivity, and/or growth speed than a cell without the variant.

The term "functional homolog" as used herein describes those molecules that have sequence similarity and also share at least one functional characteristic such as a biochemical activity. Functional homologs will typically give rise to the same characteristics to a similar, but not necessarily the same, degree. Functionally homologous proteins give the same characteristics where the quantitative measurement produced by one homolog is at least 10 percent of the other; more typically, at least 20 percent, between about 30 percent and about 40 percent; for example, between about 50 percent and about 60 percent; between about 70 percent and about 80 percent; or between about 90 percent and about 95 percent; between about 98 percent and about 100 percent, or greater than 100 percent of that produced by the original molecule. Thus, where the molecule has enzymatic activity the functional homolog will have the above-recited percent enzymatic activities compared to the original enzyme. A functional homolog and the reference polypeptide may be naturally occurring polypeptides, and the sequence similarity may be due to convergent or divergent evolutionary events. Functional homologs are sometimes referred to as orthologs, where "ortholog", refers to a homologous gene or protein that is the functional equivalent of the referenced gene or protein in another species. Functional homologs can be identified by analysis of nucleotide and polypeptide sequence alignments. For example, performing a query on a database of nucleotide or polypeptide sequences can identify homologs of interesting polypeptides. Sequence analysis can involve BLAST, Reciprocal BLAST, or PSI-BLAST analysis of non-redundant databases using amino acid sequence of an interesting polypeptide as the reference sequence. Amino acid sequence is, in some instances, deduced from the nucleotide sequence. Typically, those polypeptides in the database that have greater than 40 percent sequence identity are candidates for further evaluation for suitability as an interesting polypeptide. Amino acid sequence similarity allows for conservative amino acid substitutions, such as substitution of one hydrophobic residue for another or substitution of one polar residue for another. If desired, manual inspection of such candidates can be carried out in order to narrow the number of candidates to be further evaluated. Manual inspection can be performed by selecting those candidates that appear to have domains present in interesting polypeptides, e.g., conserved functional domains.

"Fragment", with respect to a polynucleotide, refers to a clone or any part of a polynucleotide molecule, particularly a part of a polynucleotide that retains a usable, functional characteristic or in other cases the fragment is non-functional. Useful fragments include oligonucleotides and polynucleotides that may be used in hybridization or amplification technologies or in the regulation

of replication, transcription or translation. A "polynucleotide fragment" refers to any subsequence of a polynucleotide, typically, of at least about 9 consecutive nucleotides, for example at least about 30 nucleotides or at least about 50 nucleotides of any of the sequences provided herein. Exemplary fragments can additionally or alternatively include fragments that comprise, consist essentially of, or consist of a region that encodes a conserved family domain of a polypeptide. Exemplary fragments can additionally or alternatively include fragments that comprise a conserved domain of a polypeptide.

Fragments may additionally or alternatively include subsequences of polypeptides and protein molecules, or a subsequence of the polypeptide. In some cases, the fragment or domain is a subsequence of the polypeptide which performs at least one biological function of the intact polypeptide in substantially the same manner, or to a similar extent, as does the intact polypeptide. For example, a polypeptide fragment can comprise a recognizable structural motif or functional domain such as a DNA-binding site or domain that binds to a DNA promoter region, an activation domain, or a domain for protein-protein interactions, and may initiate transcription.

Fragments can vary in size from as few as 3 amino acid residues to the full length of the intact polypeptide, for example at least about 20 amino acid residues in length, for example at least about 30 amino acid residues in length. Preferentially a fragment is a functional fragment that has at least one property or activity of the polypeptide from which it is derived, such as, for example, the fragment can include a functional domain or conserved domain of a polypeptide. A domain can be characterized, for example, by a Pfam or Conserved Domain Database (CDD) designation. According to the present invention such functional fragment has a reduced and/or abolished bacteriophage binding capacity but still performs another of its properties or activities as the original, full length polypeptide.

The term "bioproduct" as used herein refers to the group of molecules comprising at least one monosaccharide as defined herein. More in particular the term bioproduct is chosen from the list comprising, preferably consisting of, monosaccharide, phosphorylated monosaccharide, activated monosaccharide, disaccharide, oligosaccharide and glycolipid.

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-Erythrofuranose, 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-[(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-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.

The term "phosphorylated monosaccharide" as used herein refers to one of the above listed monosaccharides which is phosphorylated. Examples of phosphorylated monosaccharides include but are not limited to glucose-1-phosphate, glucose-6-phosphate, glucose-1,6-bisphosphate, galactose-1-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate, fructose-1-phosphate, glucosamine-1-phosphate, glucosamine-6-phosphate, N-acetylglucosamine-1-phosphate, mannose-1-phosphate, mannose-6-phosphate or fucose-1-

phosphate. Some, but not all, of these phosphorylated monosaccharides are precursors or intermediates for the production of activated monosaccharide.

The term "activated monosaccharide" as used herein refers to activated forms of monosaccharides, such as the monosaccharides as listed here above. Examples of activated monosaccharides include but are not limited to GDP-fucose, GDP-mannose, CMP-N-acetylneuraminic acid, CMP-N-glycolylneuraminic acid, UDP-glucuronate, UDP-N-acetylgalactosamine,, UDP-glucose, UDP-galactose, CMP-sialic acid; and UDP-N-acetylglucosamine. Activated monosaccharides, also known as nucleotide sugars, act as glycosyl donors in glycosylation reactions. Those reactions are catalyzed by a group of enzymes called glycosyltransferases.

The term "disaccharide" as used herein refers to a saccharide polymer containing two simple sugars, i.e. monosaccharides. Such disaccharides contain monosaccharides as described above and are preferably selected from the list of monosaccharides as used herein above. Examples of disaccharides comprise lactose, N-acetyllactosamine, and Lacto-N-biose.

"Oligosaccharide" as the term is used herein and as generally understood in the state of the art, refers to a saccharide polymer containing a small number, typically three to fifteen, of simple sugars, i.e. monosaccharides. Preferably the oligosaccharide as described herein contains monosaccharides selected from the list as used herein above. Examples of oligosaccharides include but are not limited to Lewis-type antigen oligosaccharides, mammalian milk oligosaccharides and human milk oligosaccharides.

As used herein, "mammalian milk oligosaccharide" refers to oligosaccharides 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-fucopentaose III, lacto-N-fucopentaose V, lacto-N-fucopentaose VI, 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-hexaose, difucosyllacto-N-hexaose, difucosyllacto-N-hexaose a, difucosyllacto-N-hexaose c, galactosylated chitosan, fucosylated oligosaccharides, neutral oligosaccharide and/or sialylated oligosaccharides.

As used herein the term "Lewis-type antigens" comprise the following oligosaccharides: H1 antigen, which is $\text{Fuca1-2Gal}\beta\text{1-3GlcNAc}$, or in short 2'FLNB; Lewis^a, which is the trisaccharide $\text{Gal}\beta\text{1-3[Fuca1-4]GlcNAc}$, or in short 4-FLNB; Lewis^b, which is the tetrasaccharide $\text{Fuca1-2Gal}\beta\text{1-3[Fuca1-4]GlcNAc}$, or in short DiF-LNB; sialyl Lewis^a which is 5-acetylneuraminy-(2-3)-galactosyl-(1-3)-(fucopyranosyl-(1-4))-N-acetylglucosamine, or written in short Neu5Ac α 2-

3Gal β 1-3[Fuc α 1-4]GlcNAc; H2 antigen, which is Fuc α 1-2Gal β 1-4GlcNAc, or otherwise stated 2'fucosyl-N-acetyl-lactosamine, in short 2'FLacNAc; Lewis^x, which is the trisaccharide Gal β 1-4[Fuc α 1-3]GlcNAc, or otherwise known as 3-Fucosyl-N-acetyl-lactosamine, in short 3-FLacNAc, Lewis^y, which is the tetrasaccharide Fuc α 1-2Gal β 1-4[Fuc α 1-3]GlcNAc and sialyl Lewis^x which is 5-acetylneuraminy-(2-3)-galactosyl-(1-4)-(fucopyranosyl-(1-3))-N-acetylglucosamine, or written in short Neu5Ac α 2-3Gal β 1-4[Fuc α 1-3]GlcNAc.

As used herein, a 'sialylated oligosaccharide' is to be understood as 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'-sialyllactosamine, oligosaccharides comprising 6'-sialyllactose, SGG hexasaccharide (Neu5Ac α -2,3Gal β -1,3GalNAc β -1,3Gal α -1,4Gal β -1,4Gal), sialylated tetrasaccharide (Neu5Ac α -2,3Gal β -1,4GlcNAc β -1,4GlcNAc), pentasaccharide LSTD (Neu5Ac α -2,3Gal β -1,4GlcNAc β -1,3Gal β -1,4Glc), sialylated lacto-N-triose, sialylated lacto-N-tetraose, sialyllacto-N-neotetraose, monosialyllacto-N-hexaose, disialyllacto-N-hexaose 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, monofucosyldisialyllacto-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, Neu5Ac α -2,3Gal β -4Glc) and oligosaccharides comprising the GM3 motif, GD3 Neu5Ac α -2,8Neu5Ac α -2,3Gal β -1,4Glc GT3 (Neu5Ac α -2,8Neu5Ac α -2,8Neu5Ac α -2,3Gal β -1,4Glc); GM2 GalNAc β -1,4(Neu5Ac α -2,3)Gal β -1,4Glc, GM1 Gal β -1,3GalNAc β -1,4(Neu5Ac α -2,3)Gal β -1,4Glc, GD1a Neu5Ac α -2,3Gal β -1,3GalNAc β -1,4(Neu5Ac α -2,3)Gal β -1,4Glc, GT1a Neu5Ac α -2,8Neu5Ac α -2,3Gal β -1,3GalNAc β -1,4(Neu5Ac α -2,3)Gal β -1,4Glc, GD2 GalNAc β -1,4(Neu5Ac α -2,8Neu5Ac α -2,3)Gal β -1,4Glc, GT2 GalNAc β -1,4(Neu5Ac α -2,8Neu5Ac α -2,8Neu5Ac α -2,3)Gal β -1,4Glc, GD1b, Gal β -1,3GalNAc β -1,4(Neu5Ac α -2,8Neu5Ac α -2,3)Gal β -1,4Glc, GT1b Neu5Ac α -2,3Gal β -1,3GalNAc β -1,4(Neu5Ac α -2,8Neu5Ac α -2,3)Gal β -1,4Glc, GQ1b Neu5Ac α -2,8Neu5Ac α -2,3Gal β -1,3GalNAc β -1,4(Neu5Ac α -2,8Neu5Ac α -2,3)Gal β -1,4Glc, GQ1c Neu5Ac α -2,3Gal β -1,3GalNAc β -1,4(Neu5Ac α -2,8Neu5Ac α -2,8Neu5Ac α -2,3)Gal β -1,4Glc, GP1c Neu5Ac α -2,8Neu5Ac α -2,3Gal β -1,3GalNAc β -1,4(Neu5Ac α -2,8Neu5Ac α -2,8Neu5Ac α -2,3)Gal β -1,4Glc, GD1a Neu5Ac α -2,3Gal β -1,3(Neu5Ac α -2,6)GalNAc β -1,4Gal β -1,4Glc, Fucosyl-GM1 Fuc α -1,2Gal β -1,3GalNAc β -1,4(Neu5Ac α -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 and as generally understood in the state of the art is an oligosaccharide that is carrying a fucose-residue. Examples comprise 2'-fucosyllactose (2'FL), 3-fucosyllactose (3FL), 4-fucosyllactose (4FL), 6-fucosyllactose (6FL), difucosyllactose (diFL), 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-fucopentaose VI (LNF VI), 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 and as generally understood in the state of the art is an oligosaccharide that has no negative charge originating from a carboxylic acid group. Examples of such neutral oligosaccharide are 2'-fucosyllactose (2'FL), 3-fucosyllactose (3FL), 2', 3-difucosyllactose (diFL), 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-fucopentaose VI, 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.

A 'fucosylation pathway' as used herein is a biochemical pathway consisting of the enzymes and their respective genes, mannose-6-phosphate isomerase, phosphomannomutase, mannose-1-phosphate guanylyltransferase, GDP-mannose 4,6-dehydratase, GDP-L-fucose synthase and/or the salvage pathway L-fucokinase/GDP-fucose pyrophosphorylase, combined with a fucosyltransferase leading to α 1,2; α 1,3 α 1,4 or α 1,6 fucosylated oligosaccharides or fucosylated oligosaccharide containing bioproduct.

A 'sialylation pathway' is a biochemical pathway consisting of the enzymes and their respective genes, L-glutamine—D-fructose-6-phosphate aminotransferase, glucosamine-6-phosphate deaminase, phosphoglucosamine mutase, N-acetylglucosamine-6-phosphate deacetylase, N-acetylglucosamine epimerase, UDP-N-acetylglucosamine 2-epimerase, N-acetylglucosamine-6P 2-epimerase, Glucosamine 6-phosphate N-acetyltransferase, N-AcetylGlucosamine-6-phosphate phosphatase, N-acetylmannosamine-6-phosphate phosphatase, N-acetylmannosamine kinase, phosphoacetylglucosamine mutase, N-acetylglucosamine-1-phosphate uridylyltransferase, glucosamine-1-phosphate acetyltransferase, sialic acid synthase, N-acetylneuraminate lyase, N-acylneuraminate-9-phosphate synthase, N-acylneuraminate-9-phosphate phosphatase, and/or CMP-sialic acid synthase, combined with a sialyltransferase leading to α 2,3; α 2,6 α 2,8 sialylated oligosaccharides or sialylated oligosaccharide containing bioproduct.

A 'galactosylation pathway' as used herein is a biochemical pathway consisting of the enzymes and their respective genes, galactose-1-epimerase, galactokinase, glucokinase, galactose-1-phosphate uridylyltransferase, UDP-glucose 4-epimerase, glucose-1-phosphate

uridylyltransferase, and/or glucophosphomutase, combined with a galactosyltransferase leading to an alpha or beta bound galactose on the 2, 3, 4, 6 hydroxyl group of a mono, di, oligo or polysaccharide containing bioproduct.

5 An 'N-acetylglucosamine carbohydrate pathway' as used herein is a biochemical pathway consisting of the enzymes and their respective genes, L-glutamine—D-fructose-6-phosphate aminotransferase, glucosamine-6-phosphate deaminase, phosphoglucosamine mutase, N-acetylglucosamine-6-phosphate deacetylase, glucosamine 6-phosphate N-acetyltransferase, N-acetylglucosamine-1-phosphate uridylyltransferase, glucosamine-1-phosphate acetyltransferase, and/or glucosamine-1-phosphate acetyltransferase, combined with a
10 glycosyltransferase leading to an alpha or beta bound N-acetylglucosamine on the 3, 4, 6 hydroxylgroup of a mono, di, oligo or polysaccharide containing bioproduct.

As used herein, the term "glycolipid" refers to any of the glycolipids which are generally known in the art. Glycolipids (GLs) can be subclassified into Simple (SGLs) and Complex (CGLs) glycolipids. Simple GLs, sometimes called saccharolipids, are two-component (glycosyl and lipid
15 moieties) GLs in which the glycosyl and lipid moieties are directly linked to each other. Examples of SGLs include glycosylated fatty acids, fatty alcohols, carotenoids, hopanoids, sterols or paraconic acids. Bacterially produced SGLs can be classified into rhamnolipids, glucolipids, trehalolipids, other glycosylated (non-trehalose containing) mycolates, trehalose-containing oligosaccharide lipids, glycosylated fatty alcohols, glycosylated macro-lactones and macro-
20 lactams, glycomacrodilides (glycosylated macrocyclic dilactones), glyco-carotenoids and glyco-terpenoids, and glycosylated hopanoids/sterols. Complex glycolipids (CGLs) are, however, structurally more heterogeneous, as they contain, in addition to the glycosyl and lipid moieties, other residues like for example glycerol (glycoglycerolipids), peptide (glycopeptidolipids), acylated-sphingosine (glycosphingolipids), or other residues (lipopolysaccharides, phenolic
25 glycolipids, nucleoside lipids).

The term "phage insensitive" or "phage resistant" or "phage resistance" or "phage resistant profile" is understood to mean a bacterial strain that is less sensitive, and preferably insensitive to infection and/ or killing by phage and/ or growth inhibition.

As used herein, the terms "anti-phage activity" or "resistant to infection by at least one phage"
30 refers to an increase in resistance of a bacterial cell expressing a functional phage resistance system to infection by at least one phage family in comparison to a bacterial cell of the same species under the same developmental stage (e.g. culture state) which does not express a functional phage resistance system, as may be determined by e.g. bacterial viability, phage lysogeny, phage genomic replication and phage genomic degradation. The phage can be a lytic
35 phage or a temperate (lysogenic) phage as further described hereinbelow. According to specific embodiments the cell is 100% resistant as described above.

According to other specific embodiments the increase is by at least 5 %, by at least a 10 %, at least 20 %, at least 30 %, at least 40 %, at least 50 %, at least 60 %, at least 70 %, at least 80 %, at least 90 % or more than 99 % as compared to a fully phage resistant cell.

Assays for testing phage resistance are well known in the art and described hereinbelow.

- 5 As used herein "abortive infection (Abi)" refers to a controlled cell death of an infected bacterial cell which takes place prior to the production of phage progeny, thus protecting the culture from phage propagation. Methods of analyzing Abi include, but are not limited to cell survival assays using high multiplicity of infection, one step growth assays and determination of phage DNA replication by e.g. DNA sequencing and southern blot analysis as further described hereinbelow.
- 10 As used herein "adsorption" refers to the attachment to the host (e.g. bacteria) cell surface via plasma membrane proteins and glycoproteins. Methods of analyzing phage adsorption include, but are not limited to enumerating free phages in bacterial cultures infected with the phages immediately after phage addition and at early time points (e.g. 30 minutes) following phage addition as further described hereinbelow.
- 15 As used herein, the term "prevent" or "preventing" refers to a decrease in activity (e.g. phage genomic replication, phage lysogeny) in bacteria expressing a functional phage resistance system in comparison to bacteria of the same species under the same developmental stage (e.g. culture state) which does not express a functional phage resistance system. According to specific embodiments the decrease is at least 1.5 fold, at least 2 fold, at least 3 fold, at least 5 fold, at
- 20 least 10 fold, or at least 20 fold as compared to same in the absence of the functional phage resistance system. According to other specific embodiments the decrease is by at least 5 %, by at least a 10 %, at least 20 %, at least 30 %, at least 40 %, at least 50 %, at least 60 %, at least 70 %, at least 80 %, at least 90 %, at least 95 % or 99 % or 100 % as compared to same in the absence of the functional phage resistance system.
- 25 As used herein "phage genomic replication" refers to production of new copies of the phage genome which can be dsDNA or ssDNA. Methods of analyzing phage genomic replication are well known in the art and described e.g. in Goldfarb et al., EMBO J, 34, 169-183.
- As used herein, the term "lysogeny" refers to the incorporation of the phage genetic material inside the genome of the host (e.g. bacteria). Methods of analyzing phage lysogeny are well
- 30 known in the art and include, but not limited to, DNA sequencing and PCR analysis. Typically, when a temperate phage infects a bacterium, its genetic material becomes circular before it incorporates into the bacterial genome. Circularization of phage genome can be analyzed by methods well known in the art including, but not limited to, PCR analysis as described in the art.
- When referring to "degradation of phage genome" the meaning is the cleavage of the foreign
- 35 phage genome by the host bacteria. Method of analyzing genomic degradation are well known in the art including, but not limited to, DNA sequencing and PCR analysis.

As used herein, the phrase "reducing and/or abolishing the bacteriophage binding capacity" refers to a reduced or decreased ability of the membrane protein to bind bacteriophage, such decrease is by at least 5 %, by at least 10 %, at least 20 %, at least 30 %, at least 40 %, at least 50 %, at least 60 %, at least 70 %, at least 80 %, at least 90 %, at least 95 %, at least 96%, at least 97%,
5 at least 98%, at least 99 % or 100 % as compared to same in the absence of the functional phage resistance system.

The term "non-native" as used herein with reference to a bioproduct indicates that the bioproduct is i) not naturally produced or ii) when naturally produced not in the same amounts by the cell; and that the cell has been genetically modified to be able to produce said bioproduct or have a
10 higher production of the bioproduct.

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
15 saccharides, oligosaccharides, glycolipids, 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
20 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 example, for oligosaccharides, e.g., 3-fucosyllactose, 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
30 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.
35 Percent identity can be determined using 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). 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.

As used herein, the term "cell productivity index (CPI)" refers to the mass of the product produced by the recombinant cells divided by the mass of the recombinant cells produced in the culture.

- 5 As used herein, the term "normalised production" or "normalised productivity" refers to the mass of the product produced by the recombinant cells divided by the mass of the recombinant cells produced in the culture (CPI), and further normalised to a particular reference value (which is unless otherwise stated the averaged CPI value of a reference strain in the same experiment).

10 Detailed description of the invention

In a first range of embodiments, the present invention provides a transgenic Escherichia coli cell genetically modified to produce at least one bioproduct of the list comprising, preferably consisting of, monosaccharide, phosphorylated monosaccharide, activated monosaccharide, disaccharide, oligosaccharide or glycolipid. The cell comprises an endogenous membrane protein encoding gene that has a reduced expression and/or said endogenous membrane protein encoding gene is mutated. The endogenous membrane protein is any one of a protein as described in table 1. Table 1 further also comprises lists of exemplary genes conforming to the description of the respective membrane protein.

20 **Table 1:**

Description membrane protein	exemplary genes	Example in Uniprot
outer membrane porin A	Tut, Con, TolG, OmpA, outer membrane protein II*, polypeptide II*, protein II*, outer membrane protein D, outer membrane protein 3a, outer membrane protein 0-11, outer membrane protein B	P0A910
outer membrane porin C	Par, MeoA, outer membrane protein A ₂ , outer membrane protein Ib, outer membrane protein 4, outer membrane protein 0-8, OmpC	P06996
outer membrane porin F	NfxB, TolF, Cry, CmlB, outer membrane protein F, colB, outer membrane protein A ₁ , outer membrane protein 1a, outer membrane protein 4, outer membrane protein b, outer membrane protein 0-9, OmpF	P02931
outer membrane protease VII (outer membrane protein 3b)	OmpT, omptin, protease 7	P09169
cobalamin/cobinamide outer membrane transporter	Cer, Bfe, BtuB	P06129

outer membrane channel	WeeA, Toc, Refl, MukA, MtcB, TolC	P02930
maltose outer membrane channel / phage lambda receptor protein	MalL, MalB, LamB	P02943
ferrichrome outer membrane transporter/phage receptor	TonA, FhuA	P06971
Ton complex subunit TonB	ExbA, TonB	P02929
long-chain fatty acid outer membrane channel / bacteriophage T2 receptor	Ttr, FadL	P10384
nucleoside-specific channel-forming protein	NupA, Tsx, nucleoside channel; receptor of phage T6 and colicin K	P0A927
ferric enterobactin outer membrane transporter	Fep, Cbt, Cbr, FeuB, FepA	P05825
putative TonB-dependent outer membrane receptor	YncD	P76115
outer membrane porin, outer membrane phosphoporin	OmpE, outer membrane pore protein E (E,Ic,NmpAB), PhoE	P02932
bacteriophage N4 receptor, outer membrane protein	NfrA	P31600
L-methionine/D-methionine ABC transporter ATP binding subunit	MetD, Abc, metN	P30750
cell division protein FtsX	FtsS, FtsX	P0AC30
cytochrome c menaquinol dehydrogenase TorC	TorC	P33226
cytochrome c quinol dehydrogenase TorY	YecK, TorY	P52005
soluble lytic murein transglycosylase	SltY, Slt, Slt70	P0AGC3
outer membrane lipoprotein Blc	YjeL, Blc, outer membrane lipoprotein (lipocalin)	P0A901
surface-exposed outer membrane lipoprotein	YaiW	P77562
DNA-binding transcriptional activator EnvY	EnvY, envelope protein; thermoregulation of porin biosynthesis	P10805
multidrug efflux pump accessory protein AcrZ	AcrZ, YbhT, small membrane protein that interacts with the AcrAB-TolC multidrug efflux pump	P0AAW9
adhesin-like autotransporter YpjA	YpjA	P52143
inner membrane protein IgaA	YrfF, IgaA	P45800
Type II secretion system protein GspL	YheK, GspL	P45763
peptidoglycan glycosyltransferase MtgA	Mgt, YrbM, MtgA	P46022

UDP-N-acetylglucosamine—undecaprenyl-phosphate N-acetylglucosaminephosphotransferase	WecA, Rfe	P0AC78
myristoyl-acyl carrier protein-dependent acyltransferase	WaaN, Mlt, LpxM, MsbB	P24205
bifunctional (p)ppGpp synthase/hydrolase SpoT	SpoT	P0AG24
GDP/GTP pyrophosphokinase	RelA, stringent factor, ppGpp synthetase I, PSI, ppGpp synthase I, (p)ppGpp synthetase I	P0AG20
putative ferritin-like protein	YecI, FtnB	P0A9A2
ferritin iron storage protein	RsgA, FtnA, Ftn	P0A998
phosphatidylglycerophosphatase B	PgpB	P0A924
lipoprotein Nlpl	YhbM, Nlpl	P0AFB1
PF13488 family lipoprotein YiaD	YiaD	P37665
murein hydrolase activator NlpD	NlpD, NlpD divisome associated factor; activates peptidoglycan hydrolase	P0ADA3
lipoprotein YghG	YghG, GspS _{<sub><math>\beta</math></sub>}	Q46835
small protein AppX	CbdX, AppX, YccB	P24244
membrane-bound lytic murein transglycosylase A	YgdM, Mlt, MltA, Mlt38	P0A935
outer membrane phospholipase A	PldA, OMPLA, Outer Membrane Phospholipase A	P0A921
outer membrane porin family protein UidC	GusC, UidC	Q47706
DUF1283 domain-containing protein YnfB	YnfB	P76170
putative outer membrane porin YfeN	YfeN	P45564
putative fimbrial usher protein EcpC	MatD, YagX, ecpC	P77802
inverse autotransporter adhesin	YeeJ	P76347
outer membrane lipoprotein YhbC	YhbC	P46130
DUF1471 domain-containing multiple stress resistance outer membrane protein BhsA	ComC, YofR, BhsA, outer membrane protein involved in copper permeability, stress resistance and biofilm formation	P0AB40
putative fimbrial usher protein HtrE	HtrE	P33129
putative uncharacterized protein YddL	YddL, putative truncated Porin_1 family protein YddL	P77519
putative fimbrial usher protein ElfC	ElfC, YcbS	P75857
DUF1375 domain-containing lipoprotein YceK	YceK	P0AB31

translocation and assembly module subunit TamA	TamA, YtfM	P0ADE4
chitobiose outer membrane channel	ChiP, YbfM	P75733
metalloprotease LoiP	LoiP, YggG	P25894
Type II secretion system protein GspD	YheF, GspD	P45758
lytic murein transglycosylase E	YcgP, SltZ, EmtA, MltE	P0C960
Lipid IV_A palmitoyltransferase	YbeG, CrcA, PagP	P37001
acid-inducible putative outer membrane protein YdiY	YdiY	P76206
DUF5508 domain-containing protein YpjB	YpjB	P76612
DLP12 prophage; putative prophage lysis lipoprotein RzoD	RzoD	P58041
peptidoglycan-associated outer membrane lipoprotein Pal	ExcC, protein 21K, Pal - outer membrane lipoprotein of the Tol-Pal system	P0A912
putative fimbrial usher protein SfmD	SfmD	P77468
outer membrane protein YaiO	YaiO	Q47534
<i>N</i> -acetylmuramoyl-L-alanine amidase D	YbjR, AmiD	P75820
putative iron siderophore outer membrane transporter	Fiu, YbiL	P75780
EG10155-MONOMER	Cir, FeuA, CirA, colicin I receptor	P17315
inhibitor of c-type lysozyme, putative lipoprotein	YdhA, MliC	P28224
putative fimbrial usher protein YhcD	YhcD	P45420
poly β -1,6- <i>N</i> -acetyl-D-glucosamine <i>N</i> -deacetylase and β -1,6 glycoside hydrolase	YcdR, HmsF, PgaB	P75906
lipopolysaccharide assembly protein LptD	LptD, YabG, OstA, Imp	P31554
fimbrial usher domain-containing protein YdeT	FmlC, YdeT, fimbrial usher protein, C-terminal fragment	P76137
DUF1190 domain-containing protein YgiB	YgiB	P0ADT2
outer membrane protein W	YciD, OmpW	P0A915
outer membrane protein assembly factor BamA	YzzN, YzzY, EcfK, YaeT, BamA	P0A940
putative exopolysaccharide export lipoprotein GfcE	YccZ, GfcE	P0A932
protein RhsD	RhsD	P16919

copper/silver export system outer membrane channel	IbeB, CusC, YlcB	P77211
outer membrane protein assembly factor BamC	DapX, NlpB, lipoprotein-34, BamC	P0A903
type I fimbriae usher protein	FimD_1, FimD, FimD_2	P30130
curli assembly component	CsgF	P0AE98
putative fimbrial usher protein YehB	YehB	P33341
outer membrane porin N	YnaG, OmpN	P77747
outer membrane protein assembly factor BamE	SmqA, small membrane protein A, BamE, SmpA	P0A937
MltA-interacting protein	YeaF, MipA, scaffolding protein that interacts with murein polymerase and murein hydrolase	P0A908
putative fimbrial usher protein YbgQ	YbgQ	P75750
starvation lipoprotein	Slp	P37194
outer membrane porin G	OmpG	P76045
cellulose biosynthesis protein BcsC	BcsC, YhjL	P37650
protein YjgL	YjgL	P39336
DNA utilization protein HofQ	HopQ, HofQ, protein involved in utilization of DNA as a carbon source	P34749
putative multidrug efflux pump outer membrane channel	MdtP, YjcP, SdsP	P32714
intermembrane phospholipid transport system - outer membrane lipoprotein MlaA	MlaA, VacJ	P76506
putative porin YfaZ	YfaZ	P76471
outer membrane polysaccharide export protein Wza	Wza	P0A930
partially deacetylated poly β -1,6- <i>N</i> -acetyl-D-glucosamine outer membrane porin	YcdS, HmsH, PgaA	P69434
Rac prophage; putative lipoprotein	RzoR	P58042
outer membrane lipoprotein SlyB	SlyB	P0A905
ferric citrate outer membrane transporter	FecA	P13036
lipoprotein YqhH	YqhH	P65298
sensor lipoprotein RcsF	RcsF, RcsF outer membrane lipoprotein - activates the Rcs pathway during envelope stress	P69411
outer membrane protein assembly factor BamD	EcfD, YfiO, BamD	P0AC02

putative outer membrane porin L	YshA, OmpL	P76773
putative TonB-dependent receptor	YddB	P31827
outer membrane lipoprotein - activator of MrcB activity	LpoB, YcfM	P0AB38
outer membrane protein YiaT	YiaT	P37681
N-acetylneuraminic acid outer membrane channel	NanC, YjhA	P69856
rhs element protein RhsB	RhsB	P16917
protein YzcX	YzcX	P11291
peptidoglycan DD-endopeptidase/peptidoglycan LD-carboxypeptidase	YeiV, Spr, MepS	P0AFV4
outer membrane protein X	YbiG, OmpP, OmpX	P0A917
carbohydrate-specific outer membrane porin, cryptic	YieC, BglH	P26218
outer membrane protein assembly factor BamB	YfgL, BamB	P77774
putative fimbrial usher protein YraJ	YraJ	P42915
ferric coprogen/ferric rhodotorulic acid outer membrane transporter	FhuE, ferric coprogen outer membrane receptor	P16869
flagellar L-ring protein	FlaY, FlgH	P0A6S0
flagellar basal-body rod protein FlgG	FlaL, FlgG	P0ABX5
rare lipoprotein RlpA	RlpA	P10100
bacteriolytic entericidin B lipoprotein	YjeU, EcnB	P0ADB7
LysM domain-containing putative peptidase lipoprotein YgeR	YgeR, putative DNA-binding transcriptional regulator	Q46798
DLP12 prophage; prophage lipoprotein BorD	Iss, BorD, Bor, YbcU, VboR, lipoprotein <i>bor</i> homolog from lambdoid prophage DLP12	P77330
lipoprotein YfiB	YfiB	P07021
YjbH family protein	YjbH	P32689
translocation and assembly module subunit TamB	TamB, YtfO, YtfN	P39321
membrane-bound lytic murein transglycosylase D	YafG, MltD, DniR	P0AEZ7
lipoprotein YfgH	YfgH	P65290
putative lipoprotein GfcD	YmcA, GfcD	P75882

SoxR [2Fe-2S] reducing system protein RsxG	YdgP, RsxG	P77285
entericidin A lipoprotein, antidote to entericidin B	EcnA	P0ADB4
polyphosphate kinase	Ppk	P0A7B1
intermembrane transport lipoprotein PqiC	PqiC, YmbA	P0AB10
putative invasin YchO	YchO, YchP	P39165
murein lipoprotein	MlpA, Lpp	P69776
outer membrane lipoprotein QseG	QseG, YfhG	P0AD44
periplasmic chaperone Skp	Skp, OmpH, HlpA, HLP-I	P0AEU7
lipopolysaccharide transport system protein LptA	YhbN, LptA	P0ADV1
membrane-bound lytic murein transglycosylase B	MltB, Sit, Sit35	P41052
outer membrane lipoprotein LolB	YchC, LolB, HemM	P61320
aldose sugar dehydrogenase YliI	YliI	P75804
curli transport specificity factor	CsgE	P0AE95
CP4-44 prophage; self recognizing antigen 43 (Ag43) autotransporter	Agn, YeeQ, YzzX, Flu, Ag43	P39180
curli secretion channel	CsgG	P0AEA2
lipopolysaccharide assembly protein LptE	LptE, RlpB	P0ADC1
divisome-associated lipoprotein YraP	EcfH, YraP	P64596
membrane-bound lytic murein transglycosylase C	YggZ, MltC	P0C066
membrane-bound lytic murein transglycosylase F	YfhD, MltF	P0AGC5
outer membrane lipoprotein - activator of MrcA activity	LpoA, YraM	P45464
putative autotransporter adhesin YfaL	YfaJ, YfaK, YfaF, YfaL	P45508
curlin, minor subunit	CsgB	P0ABK7

In further embodiments, the present invention provides a method for conferring bacteriophage resistance in an E. coli cell. First an E. coli cell which is genetically modified to produce at least one bioproduct as described herein is provided. At least one endogenous membrane protein encoding gene of said cell is mutated and/or has a reduced expression. The membrane protein is any one of a protein as described in Table 1.

The present invention also provides a method for producing at least one bioproduct as described herein with an E. coli cell. First an E. coli cell which is genetically modified to produce at least one

bioproduct as described herein is provided. At least one endogenous membrane protein encoding gene of said cell has been mutated and/or has a reduced expression. The membrane protein is any one of a protein as described in Table 1. The cell is cultivated in a medium under conditions permissive for the production of the desired bioproduct. Preferably the bioproduct is separated from the cultivation. More preferably, the bioproduct is purified after separation from the cultivation.

In a further embodiment, the present invention provides a method for increasing the production of at least one bioproduct as described herein with an *E. coli* cell which is genetically modified to produce at least one bioproduct as compared to an *E. coli* cell genetically modified to produce said bioproduct(s) but lacking the extra reduced expression and/or mutation described hereafter. An *E. coli* cell which is genetically modified to produce at least one bioproduct is further altered by providing a mutation in and/or a reduced expression of an endogenous membrane protein encoding gene. The cell is cultivated in a medium under conditions permissive for the production of the desired bioproduct. Preferably the bioproduct is separated from the cultivation. The bioproduct can also be purified as described herein. The membrane protein is any one of the proteins as described in Table 1.

According to the present invention, *Escherichia coli* (abbreviated herein as *E. coli*) can be, but not limited to, *Escherichia coli* B, *Escherichia coli* BL21, *Escherichia coli* C, *Escherichia coli* W, *Escherichia coli* Nissle, *Escherichia coli* K12. 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, JM109, DH1, M182, MC1000, MC1060, MC1061, MC4100, JM101, NZN111 and AA200. Hence, the present invention preferably relates to a mutated and/or transformed *Escherichia coli* strain as indicated above wherein said *E. coli* strain is a K12 strain. More preferably, the present invention relates to a mutated and/or transformed *Escherichia coli* strain as indicated above wherein said K12 strain is *E. coli* MG1655.

In a further embodiment, the membrane protein is chosen from the list consisting of: COG groups COG4206, COG2067, COG4771, COG1629, COG4580, COG2885, COG3203, COG4571, COG1538, COG3248, COG0810, COG0457; an outer membrane porin, an outer membrane protease 7, a cobalamin/cobinamide outer membrane transporter, an outer membrane channel, a maltose outer membrane channel, a ferrichrome outer membrane transporter, a Ton complex subunit, a long-chain fatty acid outer membrane channel, a nucleoside-specific channel-forming protein, a ferric enterobactin outer membrane transporter, a putative TonB-dependent outer membrane receptor, an outer membrane protein, a phage receptor.

Preferably the membrane protein is chosen from the list comprising, more preferably consisting, of: OmpA (SEQ ID NO: 2), OmpC (SEQ ID NO: 4), OmpF (SEQ ID NO: 6), OmpT (SEQ ID NO: 8), BtuB (SEQ ID NO: 10), TolC (SEQ ID NO: 12), LamB (SEQ ID NO: 14), FhuA (SEQ ID NO:

16), TonB (SEQ ID NO: 18), FadL (SEQ ID NO: 20), Tsx (SEQ ID NO: 22), FepA (SEQ ID NO: 24), YncD (SEQ ID NO: 26), PhoE (SEQ ID NO: 28), and NfrA (SEQ ID NO: 30), a functional homolog of any one of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, or a membrane protein having at least 70% sequence identity to the full length amino acid sequence of any one of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30.

As used herein, a membrane protein having an amino acid sequence having at least 70% sequence identity to any of the enlisted membrane proteins, is to be understood as that the sequence has 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 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 of the amino acid sequence of the respective membrane protein.

The amino acid sequence of such membrane protein can be a sequence chosen from SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28 or 30 of the attached sequence listing, a functional homolog of any one of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, or an amino acid sequence that has at least 70% sequence identity, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 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 NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28 or 30.

According to a preferred embodiment of the invention, the mutation and/or reduced expression of the membrane protein encoding gene confers bacteriophage resistance to a bacteriophage selected from the bacteriophage families listed in table 2.

Table 2: bacteriophage families and their receptors

Phage	Family	Main host	Receptor	References
434	Siphoviridae	Escherichia coli	OmpC	Hantke 1978
BF23	Siphoviridae	Escherichia coli	BtuB	Bradbeer, Woodrow and Khalifah 1976
K3	Myoviridae	Escherichia coli	OmpA	Skurray, Hancock and Reeves 1974; Manning and Reeves 1976; Van Alphen, Havekes and Lugtenberg 1977
K10	Siphoviridae	Escherichia coli	LamB	Roa 1979
Me1	Myoviridae	Escherichia coli	OmpC	Verhoef, de Graaff and Lugtenberg 1977
M1	Myoviridae	Escherichia coli	OmpA	Hashemolhosseini et al. 1994
Ox2	Myoviridae	Escherichia coli	OmpA	Morona and Henning 1984

TLS	Siphoviridae	Escherichia coli	TolC	German and Misra 2001
Tula	Myoviridae	Escherichia coli	OmpF	Datta, Arden and Henning 1977
Tulb	Myoviridae	Escherichia coli	OmpC	Datta, Arden and Henning 1977
Tull*	Myoviridae	Escherichia coli	OmpA	Datta, Arden and Henning 1977
T1	Siphoviridae	Escherichia coli	FhuA // TonB	Hantke and Braun 1975; Hancock and Braun 1976; Hantke and Braun 1978
T2	Myoviridae	Escherichia coli	OmpF // FadL	Hantke 1978; Morona and Henning 1986; Black 1988
T4	Myoviridae	Escherichia coli	OmpC	Prehm et al. 1976; Mutoh, Furukawa and Mizushima 1978; Goldberg, Grinius and Letellier 1994
T5	Siphoviridae	Escherichia coli	FhuA	Braun, Schaller and Wolff 1973; Braun and Wolff 1973; Heller and Braun 1982
T6	Myoviridae	Escherichia coli	Tsx	Manning and Reeves 1976; Manning and Reeves 1978
λ	Siphoviridae	Escherichia coli	LamB	Randall-Hazelbauer and Schwartz 1973
ϕ 80	Siphoviridae	Escherichia coli	FhuA // TonB	Hantke and Braun 1975; Wayne and Neilands 1975; Hancock and Braun 1976; Hantke and Braun 1978
TC45 TC23		Escherichia coli	PhoE	Chai and Foulds, 1978 (PMID: 97266)
T2	Myoviridae	Escherichia coli	OmpT	Hashemolhosseini et al, 1994 (PMID: 8027994)
N4		Escherichia coli	NfrA	McPartland et al, 2009 (PMID: 19011026)
H8	Siphoviridae	Escherichia coli	FepA	Rabsch et al, 2007 (PMID:17526714)
IME253		Escherichia coli	FepA	Li et al, 2019 (PMID:31105661)
IME347 (T1-like)		Escherichia coli	YncD	Li et al, 2019 (PMID:31105661); Li et al, 2018 (PMID:30146706)

According to specific embodiments, the bacteriophage resistance is characterized by at least one of:

- (a) not causing an abortive bacteriophage infection;
- (b) preventing phage genomic replication in an E. coli cell;
- (c) preventing phage lysogeny in an E. coli cell;

- (d) reducing and/or abolishing the bacteriophage binding capacity of the membrane protein;
- (e) not impairing bioproduct production;
- (f) enhancing bioproduct production;
- (g) enhancing productivity in a fermentation
- 5 (h) not impairing growth or growth speed of the cells;
- (i) enhancing growth or growth speed of the cells;
- (j) not impairing biomass production in a fermentation using the cell;
- (k) enhancing biomass production in a fermentation using the cell; and/or
- (l) reducing biomass production in a fermentation using the cell
- 10 each possibility represents a separate embodiment of the present invention.

The functional phage resistance may be characterized by one, two, three, four, five, six, seven, eight, nine, ten, eleven or all of (a) - (l).

- 15 According to specific embodiments, the functional phage resistance is characterized by at least (a) + (b), (a) + (c), (a) + (d), (a) + (e), (a) + (f), (a) + (g), (a) + (h), (a) + (i), (a) + (j), (a) + (k), (a) + (l), (b) + (c), (b) + (d), (b) + (e), (b) + (f), (b) + (g), (b) + (h), (b) + (i), (b) + (j), (b) + (k), (b) + (l), (c) + (d), (c) + (e), (c) + (f), (c) + (g), (c) + (h), (c) + (i), (c) + (j), (c) + (k), (c) + (l), (d) + (e), (d) + (f), (d) + (g), (d) + (h), (d) + (i), (d) + (j), (d) + (k), (d) + (l), (e) + (f), (e) + (g), (e) + (i), (e) + (j), (e) + (k), (e) + (l), (f) + (g), (f) + (h), (f) + (i), (f) + (j), (f) + (k), (f) + (l), (g) + (h), (g) + (i), (g) + (j), (g) + (k), (g) + (l), (i) + (j), (i) + (k), (i) + (l), (j) + (k), (j) + (l), and/or (k) + (l).

According to specific embodiments, the functional phage resistance system is characterized by at least (d) + (e), (d) + (f), (d) + (g), (d) + (h), (d) + (i), (d) + (j), (d) + (k), and/or (d) + (l).

- 25 According to a specific embodiment, the functional phage resistance system is characterized by (d) + (f) + (g), (d) + (g) + (i), (d) + (g) + (k), (d) + (f) + (j), (d) + (g) + (l), (d) + (f) + (k), (d) + (f) + (l), (d) + (e) + (i), (d) + (e) + (k), (d) + (e) + (h) + (j), (d) + (f) + (h) + (k).

According to a specific embodiment, the functional phage resistance system is characterized by (d) + (e) + (h) + (j), (d) + (f) + (g) + (i), (d) + (g) + (e) + (k) + (i), (d) + (g) + (f) + (i) + (l), (d) + (g) + (e) + (k), (d) + (g) + (f) + (l).

30

In some embodiments of the present invention the mutation and/or reduced expression of the membrane protein encoding gene confers unaffected bioproduct production wherein similar or the same levels of bioproduct are produced as is produced by a cell having the same genetic make-up but lacking the modified expression of the membrane protein encoding gene. Similar or

35 the same levels of bioproduct produced is to be understood to be at least 75% of the levels of bioproduct as produced by a cell having the same genetic make-up but lacking the modified expression of the membrane protein encoding gene. A production of at least 75% is to be understood as to be 75%, 76%, 77%, 78%, 79%, 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%, 100% of the levels produced by a cell having the same genetic make-up but lacking the modified expression of the membrane protein encoding gene. Preferably, the mutation and/or reduced expression of the membrane protein encoding gene confers enhanced bioproduct formation in or by the cell wherein the cell produces more bioproduct in comparison to a cell having the same genetic make-up but lacking the mutation and/or reduced expression of the membrane protein encoding gene. In some other embodiments of the present invention the mutation and/or reduced expression of the membrane protein encoding gene confers unaffected cell growth, or cell growth speed, productivity and/or biomass production wherein similar or the same levels of cell growth speed and/or biomass is produced as the cell growth speed, productivity and or biomass produced by a cell having the same genetic make-up but lacking the mutation and/or reduced expression of the membrane protein encoding gene. Preferably, the mutation and/or reduced expression of the membrane protein encoding gene confers enhanced cell growth speed, productivity and/or biomass production in or by the cell wherein the cell produces more biomass, has a higher productivity and/or has an enhanced cell growth speed in comparison to a cell having the same genetic make-up but lacking the mutation and/or reduced expression of the membrane protein encoding gene.

According to some embodiments of the present invention the mutation and/or reduced expression of the membrane protein encoding gene confers reduced and/or abolished bacteriophage binding capacity of the membrane protein and/or to the cell.

According to specific embodiments of the present invention the reduced expression of the membrane protein encoding gene comprises any one or more of:

- i) mutating the transcription unit of the membrane protein encoding gene;
- ii) mutating the endogenous/homologous promoter of the membrane protein encoding gene;
- iii) mutating the ribosome binding site of the membrane protein encoding gene;
- iv) mutating an UTR of the membrane protein encoding gene and/or
- v) mutating the transcription terminator.

In some embodiments of the present invention the mutation of the membrane protein encoding gene is a point mutation. Such point mutation can result in either i) a membrane protein of the same length; ii) a shorter membrane protein due to the mutation creating a premature stop codon in the membrane protein encoding gene; iii) a shorter membrane protein being a fragment as defined herein; or iv) a longer membrane protein due to the mutation changing the normal stop codon to a codon coding for an amino acid and translation continuing till the next stop.

In some embodiments of the present invention the mutation of the membrane protein encoding gene renders the membrane protein shorter. This can be obtained by i) a point-mutation due to

the mutation creating a premature stop codon in the membrane protein encoding gene, ii) other mutations creating a premature stop codon in the membrane protein encoding gene, iii) a fragment as defined herein, or iv) deletion of part of the membrane protein encoding gene's polynucleotide sequence. Such shorter proteins in some instances result in the same phenotype as a knock-out mutant.

In some embodiments of the present invention the mutation of the membrane protein encoding gene completely knocks out the membrane protein encoding gene to be obtained in ways as known by the person skilled in the art.

In other embodiments of the present invention the mutation of the membrane protein encoding gene renders the membrane protein longer. This can be obtained by an insertion or a C- or N-terminal addition of at least one base in the membrane protein encoding gene. Preferably the mutation confers an insertion or addition of at least 2 amino acids into the encoded membrane protein's amino acid sequence. More preferably the mutation confers an insertion of more than 2, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 amino acids. Even more preferred, the mutation confers an insertion ranging between 15 and 45 amino acids, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45 amino acids. Preferably, the insertion extends the extracellular loops in the 3 dimensional space of the protein, and that mutation confers resistance to any bacteriophage that is able to infect the cell by binding to said phage receptor protein. Preferably, the mutation does not decrease i) bioproduct production; ii) growth of the cell, iii) productivity and/or iv) biomass production. More preferably, the mutation increases and/or enhances i) bioproduct production; ii) growth of the cell, iii) productivity and/or iv) biomass production.

According to the present invention the mutation of the membrane protein encoding gene is any one of an in-frame mutation, an out-of-frame mutation or a partial or complete knock-out mutation. In a preferred embodiment, a cell is provided according to the present invention wherein the mutation occurs in a tolC (SEQ ID NO: 12) encoding gene or a gene encoding a functional homolog of SEQ ID NO: 12 or a gene encoding a protein having at least 70% sequence identity of the full length of SEQ ID NO: 12, and wherein said mutation comprises an 11 amino acid duplication of the amino acid sequence VGLSFSLPIYQ (SEQ ID NO: 31).

In a further preferred embodiment, the cell and/or the method comprises at least two endogenous membrane protein encoding genes which are mutated and/or have a reduced expression. The endogenous membrane proteins are at least any two of the proteins as described in table 1. More preferably, at least 2, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 endogenous membrane protein encoding genes are mutated and/or have a reduced expression.

It is to be understood that a person skilled in the art will, upon reading the invention, be able to identify any other mutation to the membrane protein encoding gene, such as the exemplary publicly available mutations as listed in Table 3. However, not all of the mutations will prove useful in the production of the bioproduct by the genetically modified cell. The skilled person will however learn from the present invention which membrane proteins and what kind of mutation (knock out, elongation, truncation, fragment of the protein) and/or reduced expression will provide unimpaired, or even enhanced i) bioproduct production; ii) growth of the cell, iii) productivity and/or iv) biomass production, when compared to production by a cell having the same genetic make-up but lacking the mutation and/or reduced expression in the membrane protein encoding gene.

10

Table 3: mutations conferring resistance to bacteriophage

omp	mutation	Resistance to phage families	reference
tolC	full_deletion	TLS	German and Misra et al, 2001 (PMID:11350161)
tolC	Insertion_AA73 (YS)	TLS	German and Misra et al, 2001 (PMID:11350161)
tolC	Insertion_AA99 (SYRDANGINSNATSASLOLTQSIF.)	TLS	German and Misra et al, 2001 (PMID:11350161)
tolC	Deletion_AA80-86	TLS	German and Misra et al, 2001 (PMID:11350161)
tolC	G75V	TLS	German and Misra et al, 2001 (PMID:11350161)
tolC	S279P	TLS	German and Misra et al, 2001 (PMID:11350161)
tolC	G302C	TLS	German and Misra et al, 2001 (PMID:11350161)
tolC	G302D	TLS	German and Misra et al, 2001 (PMID:11350161)
tolC	Q303P	TLS	German and Misra et al, 2001 (PMID:11350161)
tolC	Deletion_AA295-303, N304D	TLS	German and Misra et al, 2001 (PMID:11350161)
tolC	Y283D	TLS	German and Misra et al, 2001 (PMID:11350161)
fepA	full_deletion	H8	Rabsch et al, 2007 (PMID:17526714)
tonB	full_deletion	H8	Rabsch et al, 2007 (PMID:17526714)

fepA	Deletion_AA1-150	H8	Rabsch et al, 2007 (PMID:17526714)
fepA	Deletion_AA199-206	H8	Rabsch et al, 2007 (PMID:17526714)
fepA	Deletion_AA315-326	H8	Rabsch et al, 2007 (PMID:17526714)
fepA	Deletion_AA467-497	H8	Rabsch et al, 2007 (PMID:17526714)
fepA	Deletion_AA592-603	H8	Rabsch et al, 2007 (PMID:17526714)
fepA	Deletion_AA681-708	H8	Rabsch et al, 2007 (PMID:17526714)
fepA	R313A R316A	H8	Rabsch et al, 2007 (PMID:17526714)
fepA	Y260A F329A	H8	Rabsch et al, 2007 (PMID:17526714)
fepA	Y260A Y272A	H8	Rabsch et al, 2007 (PMID:17526714)
yncD	full_deletion	T1-like (IME347)	Li et al, 2018 (PMID:30146706)
fhuA	Deletion_AA675-704	IME18	Li et al, 2019 (PMID:31105661)
fhuA	T629-fs	IME18	Li et al, 2019 (PMID:31105661)
fhuA	F519-fs	IME18	Li et al, 2019 (PMID:31105661)
fepA	full_deletion	IME253	Li et al, 2019 (PMID:31105661)
ompF	Deletion_AA79-128	IME281	Li et al, 2019 (PMID:31105661)
ompF	D76-fs	IME281	Li et al, 2019 (PMID:31105661)
ompF	full_deletion	IME281	Li et al, 2019 (PMID:31105661)
tsx	F18-fs	IME339	Li et al, 2019 (PMID:31105661)
tsx	L149E	IME339	Li et al, 2019 (PMID:31105661)
tsx	full_deletion	IME339	Li et al, 2019 (PMID:31105661)
ompA	V122-fs	IME340	Li et al, 2019 (PMID:31105661)
ompA	full_deletion	IME340	Li et al, 2019 (PMID:31105661)
ompA	Q38*	IME340	Li et al, 2019 (PMID:31105661)
fadL	D34*	IME341	Li et al, 2019 (PMID:31105661)
fadL	L161V	IME341	Li et al, 2019 (PMID:31105661)
fadL	L394E	IME341	Li et al, 2019 (PMID:31105661)
yncD	full_deletion	IME347	Li et al, 2019 (PMID:31105661)
LamB	G43V	Lambda	Chatterjee and Rothenberg, 2012 (PMID:23202520)
LamB	E173K	Lambda	Chatterjee and Rothenberg, 2012 (PMID:23202520)
LamB	G176D	Lambda	Chatterjee and Rothenberg, 2012 (PMID:23202520)
LamB	S177F	Lambda	Chatterjee and Rothenberg, 2012 (PMID:23202520)
LamB	S179F	Lambda	Chatterjee and Rothenberg, 2012 (PMID:23202520)

LamB	F180S	Lambda	Chatterjee and Rothenberg, 2012 (PMID:23202520)
LamB	Y188D	Lambda	Chatterjee and Rothenberg, 2012 (PMID:23202520)
LamB	T189P	Lambda	Chatterjee and Rothenberg, 2012 (PMID:23202520)
LamB	G270R	Lambda	Chatterjee and Rothenberg, 2012 (PMID:23202520)
LamB	G270V	Lambda	Chatterjee and Rothenberg, 2012 (PMID:23202520)
LamB	S272L	Lambda	Chatterjee and Rothenberg, 2012 (PMID:23202520)
LamB	G274D	Lambda	Chatterjee and Rothenberg, 2012 (PMID:23202520)
LamB	S275F	Lambda	Chatterjee and Rothenberg, 2012 (PMID:23202520)
LamB	F284V	Lambda	Chatterjee and Rothenberg, 2012 (PMID:23202520)
LamB	G407D	Lambda	Chatterjee and Rothenberg, 2012 (PMID:23202520)
LamB	G407V	Lambda	Chatterjee and Rothenberg, 2012 (PMID:23202520)
LamB	G426D	Lambda	Chatterjee and Rothenberg, 2012 (PMID:23202520)
PhoE	R179H	TC45	Korteland et al, 1985 (PMID:2414105)
ompF	full_deletion	bacteriophage K20	Taurig and Misra, 1999 (PMID:10564794)
ompF	Deletion_AA222-229	bacteriophage K20	Taurig and Misra, 1999 (PMID:10564794)
ompF	N264K	bacteriophage K20	Taurig and Misra, 1999 (PMID:10564794)
ompF	G271R	bacteriophage K20	Taurig and Misra, 1999 (PMID:10564794)
ompF	G307D	bacteriophage K20	Taurig and Misra, 1999 (PMID:10564794)
ompF	G307D G69D	bacteriophage K20	Taurig and Misra, 1999 (PMID:10564794)

ompF	L227F	bacteriophage K20	Taurig and Misra, 1999 (PMID:10564794)
ompF	G271S	bacteriophage K20	Taurig and Misra, 1999 (PMID:10564794)
ompF	G307S	bacteriophage K20	Taurig and Misra, 1999 (PMID:10564794)
ompF	G307R	bacteriophage K20	Taurig and Misra, 1999 (PMID:10564794)
btuB	Deletion_AA464-468	phage BF23	Full-Schaefer et al, 2005 (PMID:15716445)
btuB	Deletion_AA514-519	phage BF23	Full-Schaefer et al, 2005 (PMID:15716445)
btuB	Deletion_AA353-358	phage BF23	Full-Schaefer et al, 2005 (PMID:15716445)
ompA	G175D	Ox2, Ox4, Ox5, M1, Ox2h5, Ox2h20	Morona et al, 1985 (PMID:3902787)
ompA	G175S	Ox2, Ox4, Ox5, Ox2h5, Ox2h20	Morona et al, 1985 (PMID:3902787)
ompA	G49V	Ox2, Ox4, Ox5, Ox2h20	Morona et al, 1985 (PMID:3902787)
ompA	G91D	Tull*-46, Tull*-60, Tull*-24, Tull*-6, K3, K4, K5, Ac3, Ox3, Tull*-26, Ox2, Ox4, Ox5, Ox2h20	Morona et al, 1985 (PMID:3902787)
ompA	G86D	Tull*-46, Tull*-60, Tull*-6, Ox2, Ox4, Ox5	Morona et al, 1985 (PMID:3902787)
ompA	S129F	Tull*-60, Tull*-6, Ac3, Ox3	Morona et al, 1985 (PMID:3902787)
ompA	S129P	Tull*-46, Tull*-60, Tull*-6, Ac3, Ox3, Ox2, Ox4, Ox5, M1	Morona et al, 1985 (PMID:3902787)
ompA	V131D	Tull*-46, Tull*-60, Tull*-24, Tull*-6, K3, K4, K5, Ac3, Ox3, Tull*-26	Morona et al, 1985 (PMID:3902787)
ompA	Deletion_AA89	Tull*-46, Tull*-60, Tull*-24, Tull*-6, K3, K4, K5, K3h1, Ac3, Ox3, Tull*-26, Ox2, Ox4, Ox5, Ox2h5, Ox2h20	Morona et al, 1985 (PMID:3902787)

ompA	E89K	Tull*-46, Tull*-60, Tull*-24, Tull*-6, K3, K4, K5, K3h1, Ac3, Ox3, Tull*-26, Ox2, Ox4, Ox5, Ox2h5, Ox2h20	Morona et al, 1985 (PMID:3902787)
ompA	I45N	Tull*-60, Ox2, Ox4, Ox5	Morona et al, 1985 (PMID:3902787)
ompA	G91V	Tull*-46, Tull*-60, Tull*-24, Tull*-6, K3, K4, K5, Ac3, Ox3, Tull*-26, Ox2, Ox4, Ox5, Ox2h5, Ox2h20	Morona et al, 1985 (PMID:3902787)
ompA	G91C	Tull*-60, Ac3, Ox3, Ox2, Ox4, Ox5, Ox2h20	Morona et al, 1985 (PMID:3902787)
fhuA	Deletion_AA381	T1, T5, phi80	Killmann et al, 1992 (PMID:1534324)
fhuA	Deletion_AA364-374	T1, T5, phi80	Killmann et al, 1995 (PMID:7836303)
fhuA	Deletion_AA380-386	T1, T5, phi80	Killmann et al, 1995 (PMID:7836303)
fhuA	Deletion_AA349-358	phi80	Killmann et al, 1995 (PMID:7836303)
ompC	Deletion_AA172-200	?	Vakharia and Misra, 1996 (PMID:8820656)
tsx	N276Y	T6, H1, H3, H8, K9, K18, Ox1	Maier et al, 1990 (PMID:2199819)
tsx	full_deletion	T6, T6h3.1, Ox1, H1, H3, H8, K18	Schneider et al, 1993 (PMID:8491700)
tsx	N271K	(T6), T6h3.1, Ox1, H1, H3, H8, K18	Schneider et al, 1993 (PMID:8491700)
tsx	N276K	(T6), Ox1, H1, H3, H8, K18	Schneider et al, 1993 (PMID:8491700)
tsx	Deletion_AA261-266	T6, T6h3.1, Ox1, H1, H3, H8, K18	Schneider et al, 1993 (PMID:8491700)
tsx	Duplication_AA251-259	(T6), T6h3.1, Ox1, H1, H3, H8, K18	Schneider et al, 1993 (PMID:8491700)

According to the invention, the cell is genetically modified for the production of at least one bioproduct. Such bioproduct can be a monosaccharide, a phosphorylated monosaccharide, an activated monosaccharide, a disaccharide, an oligosaccharide or a glycolipid.

- 5 In some embodiments, the bioproduct is a monosaccharide as described herein. Preferably, the monosaccharide is selected from the group comprising glucosamine, mannose, xylose, N-acetylmannosamine, N-acetylneuraminic acid, N-glycolylneuraminic acid, sialic acid, N-acetylgalactosamine, galactosamine, fucose, rhamnose, glucuronic acid, gluconic acid.

In some embodiments, the bioproduct is a phosphorylated monosaccharide as described herein. Preferably, said phosphorylated monosaccharide is selected from the group comprising glucose-1-phosphate, glucose-6-phosphate, glucose-1,6-bisphosphate, galactose-1-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate, fructose-1-phosphate, glucosamine-1-phosphate, glucosamine-6-phosphate, N-acetylglucosamine-1-phosphate, mannose-1-phosphate, mannose-6-phosphate or fucose-1-phosphate.

In other embodiments of the present invention the bioproduct is an activated monosaccharide as described herein. Preferably the activated monosaccharide is selected from the group comprising GDP-fucose, UDP-glucose, UDP-galactose, UDP-N-acetylglucosamine, CMP-sialic acid.

In other embodiments of the invention the bioproduct is a disaccharide as described herein. Preferably such disaccharide is lactose or N-acetyllactosamine (LacNAc). An example of fermentative production of lactose by the cell is provided in the examples. Fermentative production of LacNAc is possible by feeding the cell N-acetyllactosamine (GlcNAc) as described by Ruffing and Chen, *Microb Cell Fact.* 2006, 5: 25.

In some embodiments of the invention the bioproduct is an oligosaccharide as defined herein. Preferably, the oligosaccharide is selected from the group of fucosyllactoses, sialyllactoses, Lacto-N-tetraoses, difucosyllacto-N-tetraose, sialyl-lacto-N-tetraoses, lacto-N-fucopentaoses, lewis-type antigens. More preferably, the oligosaccharide is selected from the group comprising 2'FL, 3FL, DiFL, Lacto-N-triose, LNT, LNnT, 3'SL, 6'SL, LSTa, LSTb, LSTc, LSTd, DFLNT, lacto-N-fucopentaose I, lacto-N-fucopentaose II, lacto-N-fucopentaose III, lacto-N-fucopentaose V, lacto-N-fucopentaose VI, H1 antigen, Lewis^a, Lewis^b, sialyl Lewis^a, H2 antigen, Lewis^x, Lewis^y; sialyl-Lewis^x. Examples of cells enabled to produce such oligosaccharides are described herein. In other embodiments, the bioproduct is a glycolipid as described herein.

In one embodiment the *E. coli* cell is transformed with at least one heterologous gene to produce a sialic acid pathway or sialylation pathway, or fucosylation pathway or galactosylation pathway or N-acetylglucosamine carbohydrate pathway. This cell is transformed by introduction of a heterologous gene, genetic cassette or set of genes as described in the art.

A further embodiment of the present invention provides a method to produce a fucosylated, sialylated, galactosylated oligosaccharide, N-acetylglucosamine containing oligosaccharide, or sialic acid with a cell as described herein, respectively.

In one embodiment of the present invention the methods as described herein are producing the bioproduct LNnT and the membrane protein is preferably any one or more of LamB (SEQ ID NO: 14), FhuA (SEQ ID NO: 16), FadL (SEQ ID NO: 20), and NfrA (SEQ ID NO: 30), a functional homolog of any one of SEQ ID NO: 14, 16, 20 or 30, or a membrane protein having at least 70% sequence identity to the full length amino acid sequence of any one of SEQ ID NOs: 14, 16, 20, 30 and wherein preferably said mutation results in a knock-out phenotype of said gene.

In another embodiment of the present invention the methods as described herein are producing sialyllactose, preferably 6'SL, and preferably the membrane protein is FhuA (SEQ ID NO: 16), a

functional homolog of SEQ ID NO: 16, or a membrane protein having at least 70% sequence identity to the full length amino acid sequence of SEQ ID NO: 16. Preferably the mutation results in a knock-out phenotype of the gene.

In a further embodiment, the present invention provides for the use of a cell as described herein
5 for the production of a bioproduct, and preferably in the methods as described herein.

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

1. An Escherichia coli cell genetically modified to produce at least one bioproduct of the list comprising monosaccharide, phosphorylated monosaccharide, activated monosaccharide,
10 disaccharide, oligosaccharide or glycolipid, wherein i) the expression of an endogenous membrane protein encoding gene is reduced and/or ii) wherein the endogenous membrane protein encoding gene is mutated, preferably, said mutation results in reduced expression of the membrane protein encoding gene, and wherein said membrane protein is any one of a protein as described in Table 1.
- 15 2. Cell according to embodiment 1 wherein said membrane protein is chosen from the list consisting of: COG groups COG4206, COG2067, COG4771, COG1629, COG4580, COG2885, COG3203, COG4571, COG1538, COG3248, COG0810, COG0457; an outer membrane porin, an outer membrane protease 7, a cobalamin/cobinamide outer membrane transporter, an outer membrane channel, a maltose outer membrane channel, a ferrichrome
20 outer membrane transporter, a Ton complex subunit, a long-chain fatty acid outer membrane channel, a nucleoside-specific channel-forming protein, a ferric enterobactin outer membrane transporter, a putative TonB-dependent outer membrane receptor, an outer membrane protein, a phage receptor.
- 25 3. Cell according to any one of embodiment 1 or 2 wherein said membrane protein is chosen from the list consisting of: OmpA (SEQ ID NO: 2), OmpC (SEQ ID NO: 4), OmpF (SEQ ID NO: 6), OmpT (SEQ ID NO: 8), BtuB (SEQ ID NO: 10), TolC (SEQ ID NO: 12), LamB (SEQ ID NO: 14), FhuA (SEQ ID NO: 16), TonB (SEQ ID NO: 18), FadL (SEQ ID NO: 20), Tsx (SEQ ID NO: 22), FepA (SEQ ID NO: 24), YncD (SEQ ID NO: 26), PhoE (SEQ ID NO: 28), and NfrA (SEQ ID NO: 30), a functional homolog of any one of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16,
30 18, 20, 22, 24, 26, 28, 30, or a membrane protein having at least 70% sequence identity to the full length amino acid sequence of any one of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30.
- 35 4. Cell according to any one of embodiment 1, 2 or 3 wherein said reduced expression of the membrane protein encoding gene and/or mutation of the membrane protein encoding gene confers bacteriophage resistance and wherein said bacteriophage is selected from the bacteriophage families listed in table 2.
5. Cell according to any one of the previous embodiments, wherein said reduced expression of the membrane protein encoding gene and/or mutation of the membrane protein encoding

gene confers unaffected and/or enhanced i) bioproduct production, ii) productivity, iii) biomass production, and/or iv) cell growth.

6. Cell according to any one of the previous embodiments, wherein said mutation and/or reduced expression comprises reducing and/or abolishing the bacteriophage binding capacity of said membrane protein.
7. Cell according to any one of the previous embodiments, wherein said the E. coli cell is transformed with at least one heterologous gene to produce at least any one of a sialic acid pathway or sialylation pathway, or fucosylation pathway or galactosylation pathway or N-acetylglucosamine carbohydrate pathway, preferably the cell is transformed by introduction of a heterologous gene, genetic cassette or set of genes as described in the art.
8. Cell according to any one of the previous embodiments wherein the mutation and/or reduced expression of the endogenous membrane protein comprises any one or more of:
 - i) mutating the transcription unit of the membrane protein encoding gene;
 - ii) mutating the endogenous/homologous promoter of the membrane protein encoding gene;
 - iii) mutating the ribosome binding site of the membrane protein encoding gene;
 - iv) mutating an UTR of the membrane protein encoding gene and/or
 - v) mutating the transcription terminator.
9. Cell according to any one of the previous embodiments wherein the mutation of the membrane protein encoding gene comprises rendering said membrane protein shorter, longer and/or completely knocks out the membrane protein.
10. Cell according to any one of embodiments 1 to 9 wherein said mutation of the membrane protein encoding gene is an in-frame mutation of the membrane protein encoding gene.
11. Cell according to embodiment 10, wherein said in-frame mutation is an insertion of at least 2 amino acids into the encoded membrane protein's amino acid sequence, preferably wherein said mutation comprises an insertion of more than 2 amino acids.
12. Cell according to any one of embodiments 9 to 11 wherein said mutation occurs in the tolC encoding gene, and wherein said mutation comprises an 11 amino acid duplication of the amino acid sequence VGLSFSLPIYQ (SEQ ID NO: 31).
13. Cell according to any one of the previous embodiments, wherein at least two of said membrane protein encoding genes are mutated and/or have a reduced expression.
14. Cell according to any one of the preceding embodiments wherein said bioproduct is an oligosaccharide, preferably said oligosaccharide is selected from the group of fucosyllactoses, sialyllactoses, Lacto-N-tetraoses, difucosyllacto-N-tetraose, sialyl-lacto-N-tetraoses, lacto-N-fucopentaoses, lewis-type antigens, more preferably selected from the group comprising 2'FL, 3FL, DiFL, Lacto-N-triose, LNT, LNnT, 3'SL, 6'SL, LSTa, LSTb, LSTc, LSTd, DFLNT, lacto-N-fucopentaose I, lacto-N-fucopentaose II, lacto-N-fucopentaose III,

lacto-N-fucopentaose V, lacto-N-fucopentaose VI, H1 antigen, Lewis^a, Lewis^b, sialyl Lewis^a, H2 antigen, Lewis^x, Lewis^y; sialyl-Lewis^x.

15. Cell according to any one of the embodiments 1 to 13, wherein said bioproduct is a disaccharide preferably selected from the group comprising N-acetyllactosamine, lactose; or
 5 wherein said bioproduct is a activated monosaccharide preferably selected from the group comprising GDP-fucose, UDP-glucose, UDP-galactose, UDP-N-acetylglucosamine, CMP-sialic acid; or wherein said bioproduct is a monosaccharide preferably selected from the group comprising glucosamine, mannose, xylose, N-acetylmannosamine, N-acetylneuraminic acid, N-glycolylneuraminic acid, sialic acid, N-acetylgalactosamine, galactosamine, fucose,
 10 rhamnose, glucuronic acid, gluconic acid, or wherein said bioproduct is a phosphorylated monosaccharide preferably selected from the group comprising glucose-1-phosphate, glucose-6-phosphate, glucose-1,6-bisphosphate, galactose-1-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate, fructose-1-phosphate, glucosamine-1-phosphate, glucosamine-6-phosphate, N-acetylglucosamine-1-phosphate, mannose-1-phosphate,
 15 mannose-6-phosphate or fucose-1-phosphate.
16. A method for conferring bacteriophage resistance in an E. coli cell, said method comprising:
 - providing an E. coli cell genetically modified to produce at least one bioproduct of the list comprising monosaccharide, phosphorylated monosaccharide, activated monosaccharide, disaccharide, oligosaccharide or glycolipid,
 - 20 - reducing the expression of and/or mutating a membrane protein encoding gene of said E. coli cell,
 - wherein said membrane protein is any one of a protein as described in Table 1.
17. A method for producing at least one bioproduct of the list comprising monosaccharide, phosphorylated monosaccharide, activated monosaccharide, disaccharide, oligosaccharide
 25 or glycolipid with an E. coli cell, said method comprising:
 - providing an E. coli cell genetically modified to produce at least one bioproduct of the list comprising monosaccharide, phosphorylated monosaccharide, activated monosaccharide, disaccharide, oligosaccharide or glycolipid,
 - reducing the expression of and/or mutating a membrane protein encoding gene of said
 30 E. coli cell,
 - cultivating the cell in a medium under conditions permissive for the production of the desired bioproduct
 - preferably separating the bioproduct from the cultivation;

wherein said membrane protein is any one of the proteins as described in Table 1.
- 35 18. A method for increasing the production of at least one bioproduct of the list comprising monosaccharide, phosphorylated monosaccharide, activated monosaccharide, disaccharide, oligosaccharide or glycolipid with an E. coli cell as compared to an E. coli cell genetically modified to produce said bioproduct(s), said method comprising:

- providing an E. coli cell genetically modified to produce at least one bioproduct of the list comprising monosaccharide, phosphorylated monosaccharide, activated monosaccharide, disaccharide, oligosaccharide or glycolipid,
 - reducing the expression of and/or mutating a membrane protein encoding gene of said E. coli cell,
 - cultivating the cell in a medium under conditions permissive for the production of the desired bioproduct
 - preferably separating the bioproduct from the cultivation;
- wherein said membrane protein is any one of the proteins as described in Table 1.
19. Method according to any one of embodiments 16 to 18 wherein said membrane protein is chosen from the list consisting of: COG groups COG4206, COG2067, COG4771, COG1629, COG4580, COG2885, COG3203, COG4571, COG1538, COG3248, COG0810, COG0457; an outer membrane porin, an outer membrane protease 7, a cobalamin/cobinamide outer membrane transporter, an outer membrane channel, a maltose outer membrane channel, a ferrichrome outer membrane transporter, a Ton complex subunit, a long-chain fatty acid outer membrane channel, a nucleoside-specific channel-forming protein, a ferric enterobactin outer membrane transporter, a putative TonB-dependent outer membrane receptor, an outer membrane protein, a phage receptor.
20. Method according to any one of embodiments 16 to 19 wherein said membrane protein is chosen from the list consisting of: OmpA (SEQ ID NO: 2), OmpC (SEQ ID NO: 4), OmpF (SEQ ID NO: 6), OmpT (SEQ ID NO: 8), BtuB (SEQ ID NO: 10), TolC (SEQ ID NO: 12), LamB (SEQ ID NO: 14), FhuA (SEQ ID NO: 16), TonB (SEQ ID NO: 18), FadL (SEQ ID NO: 20), Tsx (SEQ ID NO: 22), FepA (SEQ ID NO: 24), YncD (SEQ ID NO: 26), PhoE (SEQ ID NO: 28), and NfrA (SEQ ID NO: 30), a functional homolog of any one of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, or a membrane protein having at least 70% sequence identity to the full length amino acid sequence of any one of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30.
21. Method according to any one of embodiments 16 to 20, wherein said modified expression and/or mutation of the membrane protein encoding gene confers bacteriophage resistance and wherein said bacteriophage is selected from the bacteriophage families listed in table 2.
22. Method according to any one of embodiments 16 to 21, wherein said modified expression and/or mutation of the membrane protein encoding gene confers unaffected and/or enhanced bioproduct production.
23. Method according to any one of embodiments 16 to 22, wherein said modified expression and/or mutation comprises reducing and/or abolishing the bacteriophage binding capacity of said membrane protein.
24. Method according to any one of the embodiments 16 to 23, wherein said E. coli cell is genetically modified to produce at least one bioproduct chosen from a fucosylated, sialylated,

galactosylated oligosaccharide, N-acetylglucosamine containing oligosaccharide, or sialic acid.

25. Method according to any one of the embodiments 16 to 24, wherein the modified expression of the endogenous membrane protein encoding gene is a lower or reduced expression, preferably said lower expression comprises any one or more of:
- i) mutating the transcription unit of the membrane protein encoding gene;
 - ii) mutating the endogenous/homologous promoter of the membrane protein encoding gene;
 - iii) mutating the ribosome binding site of the membrane protein encoding gene;
 - iv) mutating an UTR of the membrane protein encoding gene and/or
 - v) mutating the transcription terminator
26. Method according to any one of the embodiments 16 to 25, wherein the mutation of the membrane protein encoding gene comprises rendering said membrane protein shorter, longer or completely knocks out said membrane protein.
27. Method according to any one of embodiments 16 to 26 wherein said mutation is an in-frame mutation of the membrane protein encoding gene, preferably said in-frame mutation is an insertion of at least 2 amino acids into the encoded membrane protein's amino acid sequence, more preferably wherein said mutation comprises an insertion of more than 2 amino acids.
28. Method according to any one of embodiments 16 to 27 wherein said mutation occurs in the *tolC* gene of *Escherichia coli* or in a functional homolog of the *tolC* gene in an *E.coli*, and wherein said mutation provides resistance against the TLS family of bacteriophages, and wherein said mutation gives rise to an 11 amino acid duplication of the amino acid sequence VGLSFSLPIYQ (SEQ ID NO: 31)
29. Method according to any one of embodiments 16 to 28, wherein at least two of said membrane protein encoding genes are mutated and/or have a reduced expression.
30. Method according to any one of embodiments 16 to 29, wherein said bioproduct is an oligosaccharide, preferably said oligosaccharide is selected from the group of fucosyllactoses, sialyllactoses, Lacto-N-tetraoses, difucosyllacto-N-tetraose, sialyl-lacto-N-tetraoses, lacto-N-fucopentaoses, lewis-type antigens, more preferably, 2'FL, 3FL, DiFL, Lacto-N-triose, LNT, LNnT, 3'SL, 6'SL, LSTa, LSTb, LSTc, LSTd, DFLNT, lacto-N-fucopentaose I, lacto-N-fucopentaose II, lacto-N-fucopentaose III, lacto-N-fucopentaose V, lacto-N-fucopentaose VI, H1 antigen, Lewis^a, Lewis^b, sialyl Lewis^a, H2 antigen, Lewis^x, Lewis^y; sialyl-Lewis^x.
31. Method according to any one of embodiments 16 to 29, wherein said bioproduct is a disaccharide preferably selected from the group comprising LacNAc, lactose; or wherein said bioproduct is an activated monosaccharide preferably selected from the group comprising GDP-fucose, UDP-glucose, UDP-galactose, UDP-N-acetylglucosamine, CMP-sialic acid; or wherein said bioproduct is a monosaccharide preferably selected from the group comprising

- glucosamine, mannose, xylose, N-acetylmannosamine, N-acetylneuraminic acid, N-glycolylneuraminic acid, sialic acid, N-acetylgalactosamine, galactosamine, fucose, rhamnose, glucuronic acid, gluconic acid, or wherein said bioproduct is a phosphorylated monosaccharide preferably selected from the group comprising glucose-1-phosphate, glucose-6-phosphate, glucose-1,6-bisphosphate, galactose-1-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate, fructose-1-phosphate, glucosamine-1-phosphate, glucosamine-6-phosphate, N-acetylglucosamine-1-phosphate, mannose-1-phosphate, mannose-6-phosphate or fucose-1-phosphate.
- 5 32. Method for fermentative production of at least one bioproduct of the list comprising monosaccharide, phosphorylated monosaccharide, activated monosaccharide, disaccharide, oligosaccharide or glycolipid using genetically modified cells to produce said bioproduct(s), comprising the steps of:
- 10 - providing a cell as described in any one of the embodiments 1 to 15;
- cultivating the cell in a medium under conditions permissive for the production of the desired bioproduct;
- 15 - preferably separating the bioproduct from the cultivation.
33. Method according to any one of the embodiments 16 to 32, wherein said bioproduct is LNnT, wherein preferably said membrane protein is any one or more of LamB (SEQ ID NO: 14), FhuA (SEQ ID NO: 16), FadL (SEQ ID NO: 20), and NfrA (SEQ ID NO: 30), a functional
- 20 homolog of any one of SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 20 and SEQ ID NO: 30, or a membrane protein having at least 70% sequence identity to the full length amino acid sequence of any one of SEQ ID NOs 14, 16, 20, 30 and wherein preferably said mutation results in a knock-out phenotype of said gene.
34. Method according to any one of the embodiments 16 to 32, wherein said bioproduct is sialyllactose, preferably 6'SL, wherein preferably said membrane protein is FhuA (SEQ ID NO: 16), a functional homolog thereof or a membrane protein having at least 70% sequence identity to the full length amino acid sequence of SEQ ID NO: 16 and wherein preferably said mutation and/or reduced expression of the membrane protein encoding gene results in a knock-out phenotype of said gene.
- 25 35. Use of a cell as described in any one of the embodiments 1 to 15.
- 30

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

35 **Description of the figures**

Figure 1 shows the normalised absorbance measured at 600 nm after 72 hours of cultivation of a 2'FL and DiFL production strain with the wild type tolC gene or the tolC_IS1 or tolC_2 mutation.

Figure 2 shows the normalised production of 2'FL and DiFL after 72 hours of cultivation of a 2'FL and DiFL production strain with the wild type tolC gene or the tolC_IS1 or tolC_2 mutation.

Figure 3 shows the normalised growth speed of a 2'FL and DiFL production strain with the wild type tolC gene or the tolC_IS1 or tolC_2 mutation.

- 5 **Figure 4** shows the normalised production of 2'FL or 3FL after 72 hours of cultivation by strains with the wild type tolC or the tolC_2 mutation.

Figure 5 shows the normalised production of LNnT after 72 hours of cultivation of a strain with the wild type fhuA gene (= reference strain, Ref) or the fhuA-fs mutation.

- 10 **Figure 6** shows the normalised growth speed of both strains after 72 hours of cultivation of a strain with the wild type fhuA gene (= reference strain, Ref) or the fhuA-fs mutation.

Figure 7 shows the normalised production of 6'SL after 72 hours of cultivation of a strain with the wild type fhuA gene (= reference strain, Ref) or the fhuA::IS2 mutation.

Figure 8 shows the normalised growth speed of both strains after 72 hours of cultivation of a strain with the wild type fhuA gene (= reference strain, Ref) or the fhuA::IS2 mutation.

- 15 **Figure 9** shows the normalised production of LNnT after 72 hours of cultivation of the reference and mutant strains.

Figure 10 shows the normalised production of 2'FL after 72 hours of cultivation, and the normalized growth speed of the reference and mutant strains where various OMP genes were deleted.

- 20 **Figure 11** shows the normalised production of 3FL after 72 hours of cultivation, and the normalized growth speed of the reference and mutant strains where various OMP genes were deleted.

- 25 **Figure 12** shows the normalised production of DiFL after 72 hours of cultivation, and the normalized growth speed of the reference and mutant strains where various OMP genes were deleted.

Figure 13 shows the normalised production of 6'SL after 72 hours of cultivation, and the normalized growth speed of the reference and mutant strains where various OMP genes were deleted.

- 30 **Figure 14** shows the normalised production of 3'SL after 72 hours of cultivation, and the normalized growth speed of the reference and mutant strains where various OMP genes were deleted.

Figure 15 shows the normalised production of LNnT after 72 hours of cultivation of the reference and mutant strains where various OMP genes were deleted.

- 35 **Figure 16** shows the normalised production of LN3 and LNT after 72 hours of cultivation of the reference and mutant strains where various OMP genes were deleted.

Examples

Example 1: Material and methods Escherichia coli

Media

The Luria Broth (LB) medium consisted of 1% tryptone peptone (Difco, Erembodegem, Belgium), 0.5% yeast extract (Difco) and 0.5% sodium chloride (VWR, Leuven, Belgium). The medium for the shake flasks experiments contained 2.00 g/L NH₄Cl, 5.00 g/L (NH₄)₂SO₄, 2.993 g/L KH₂PO₄, 7.315 g/L K₂HPO₄, 8.372 g/L MOPS, 0.5 g/L NaCl, 0.5 g/L MgSO₄·7H₂O, 14.26 g/L sucrose or another carbon source when specified in the examples, 1 ml/L vitamin solution, 100 µl/L molybdate solution, and 1 mL/L selenium solution. The medium was set to a pH of 7 with 1M KOH. Vitamin solution consisted of 3.6 g/L FeCl₂·4H₂O, 5 g/L CaCl₂·2H₂O, 1.3 g/L MnCl₂·2H₂O, 0.38 g/L CuCl₂·2H₂O, 0.5 g/L CoCl₂·6H₂O, 0.94 g/L ZnCl₂, 0.0311 g/L H₃BO₄, 0.4 g/L Na₂EDTA·2H₂O and 1.01 g/L thiamine·HCl. The molybdate solution contained 0.967 g/L NaMoO₄·2H₂O. The selenium solution contained 42 g/L SeO₂.

The minimal medium for fermentations contained 6.75 g/L NH₄Cl, 1.25 g/L (NH₄)₂SO₄, 2.93 g/L KH₂PO₄ and 7.31 g/L K₂HPO₄, 0.5 g/L NaCl, 0.5 g/L MgSO₄·7H₂O, 14.26 g/L sucrose or another carbon source as specified in the respective examples, 1 mL/L vitamin solution, 100 µL/L molybdate solution, and 1 mL/L selenium solution with the same composition as described above. Complex medium 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. chloramphenicol (20 mg/L), carbenicillin (100mg/L), spectinomycin (40mg/L) and/or kanamycin (50mg/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. R. Cunin (Vrije Universiteit Brussel, Belgium in 2007).

Plasmids were maintained in the host *E. coli* DH5alpha (F⁻, phi80d/lacZΔM15, Δ(lacZYA-argF) U169, *deoR*, *recA1*, *endA1*, *hsdR17*(rk⁻, mk⁺), *phoA*, *supE44*, lambda⁻, *thi-1*, *gyrA96*, *relA1*) bought from Invitrogen.

Strains and mutations

Escherichia coli K12 MG1655 [λ⁻, F⁻, rph-1] was obtained from the Coli Genetic Stock Center (US), CGSC Strain#: 7740, in March 2007. Gene disruptions, gene introductions and gene replacements 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 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 1ml ice cold water, a second time. Then, the cells were resuspended in 50 µl of ice-cold water.

- 5 Electroporation was done with 50 µl of cells and 10-100 ng of linear double-stranded-DNA product by using a Gene Pulser™ (BioRad) (600 Ω, 25 µFD, 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
10 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 derivates 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
15 take place. For the genomic knock-out, 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).

- 20 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 knock
25 outs and knock ins are checked with control primers (Fw/Rv-gene-out).

For 2'FL, 3FL and diFL production, the mutant strains derived from *E. coli* K12 MG1655 have knock-outs of the genes *lacZ*, *lacY*, *lacA*, *glgC*, *agp*, *pfkA*, *pfkB*, *pgi*, *arcA*, *iclR*, *wcaJ*, *pgi*, *lon* and *thyA* and additionally genomic knock-ins of constitutive expression constructs containing the *E. coli lacY* gene, a fructose kinase gene (*frk*) originating from *Zymomonas mobilis* and a sucrose
30 phosphorylase (*SP*) originating from *Bifidobacterium adolescentis*. These genetic modifications are also described in WO2016075243 and WO2012007481. In addition, an alpha-1,2- and/or alpha-1,3-fucosyltransferase expression plasmid is added to the strains.

For LNT and LNT production, the strain has a genomic knock out of the *lacZ* gene and *nagB* gene and knock-ins of constitutive expression constructs containing a galactoside beta-1,3-N-acetylglucosaminyltransferase (*lgtA*) from *Neisseria meningitidis* and either an N-acetylglucosamine beta-1,3-galactosyltransferase (*wbgO*) from *Escherichia coli* O55:H7 for LNT
35 production or an N-acetylglucosamine beta-1,4-galactosyltransferase (*lgtB*) from *Neisseria meningitidis* for LNT production.

For 3'SL and 6'SL production the strains are described in WO18122225. The mutant strain has the following gene knock-outs: *lacZ*, *nagABCDE*, *nanATEK*, *manXYZ*. Additionally, the strain has genomic knock-ins of constitutive expression constructs containing a mutated variant of the L-glutamine—D-fructose-6-phosphate aminotransferase (*glmS*) from *Escherichia coli*, a
5 glucosamine 6-phosphate N-acetyltransferase (*GNA1*) from *Saccharomyces cerevisiae*, an N-acetylglucosamine 2-epimerase (BoAGE) from *Bacteroides ovatus*, an N-acetylneuraminate synthase (NeuB) from *Campylobacter jejuni*, a CMP-Neu5Ac synthetase (NeuA) from *Campylobacter jejuni*, and either a beta-galactoside alpha-2,3-sialyltransferase from *Pasteurella multocida* for 3'SL production or a beta-galactoside alpha-2,6-sialyltransferase from
10 *Photobacterium damsela* for 6'SL production.

All constitutive promoters and UTRs originate from the libraries described by De Mey *et al.* (BMC Biotechnology, 2007) and Mutalik *et al.* (Nat. Methods 2013, No. 10, 354-360). All genes were ordered synthetically at Twist Bioscience (twistbioscience.com) or IDT (eu.idtdna.com) and the codon usage was adapted using the tools of the supplier.

15 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 96well microtiter plate experiments was started from a cryovial, in 150 µL LB and
20 was incubated overnight at 37 °C on an orbital shaker at 800 rpm. This culture was used as inoculum for a 96well square microtiter plate, with 400 µL MMsf medium by diluting 400x. 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. At the end of the cultivation experiment samples were taken from each well to measure sugar concentrations in the broth supernatant (extracellular sugar concentrations,
25 after spinning down the cells), or by boiling the culture broth for 15 min at 90°C before spinning down the cells (= whole broth measurements, average of intra- and extracellular sugar concentrations).

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
30 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
35 of silicone antifoaming agent was added when foaming raised during the fermentation.

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).

Liquid chromatography

Standards for 2'-fucosyllactose, 3-fucosyllactose, difucosyllactose, Lacto-N-tetraose, Lacto-N-neotetraose, 3'-sialyllactose and 6'-sialyllactose were synthesized in house. Other standards such as but not limited to lactose, sucrose, glucose, fructose were purchased from Sigma.

- 10 Carbohydrates were analyzed 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. All sugars were separated in an isocratic flow using an Acquity UPLC BEH Amide column (Waters, USA) and a mobile phase containing 75 mL acetonitrile, 25 mL Ultrapure water and 0.25 mL triethylamine (for 2'FL, 3FL, DiFL, LNT and LnnT) or containing 70 mL acetonitrile, 26 mL 150 mM ammonium acetate and 4 mL methanol with 0.05% pyrrolidine (for 3'SL and 6'SL). The column size was 2.1 x 50 mm with 1.7 µm particle size. The temperature of the column was set at 50°C (for 2'FL, 3FL, DiFL, LNT, LnnT) or 25°C (for 3'SL and 6'SL) and the pump flow rate was 0.130 mL/min.

20 ***Example 2: strain resistant to a "T1-like" or "TLS" bacteriophage***

An E. coli MG1655 K-12 strain modified to produce 2'-fucosyllactose and difucosyllactose containing the alpha-1,2-fucosyltransferase HpFutC from Helicobacter pylori (SEQ ID NO: 36) was further mutated with two distinct mutations, both in the tolC gene.

- 25 One mutation comprised an insertion of the E. coli IS1 element 374 bp downstream of the start codon and thus completely abolished the gene function of tolC (tolC_IS1, SEQ ID NO: 34).

- A second mutation comprised a 33 bp duplication of the sequence (gttggcctgagcttctcgctgccgattatcag, bp 916 to 948 of SEQ ID No: 32), causing a direct repeat in the tolC ORF (tolC_2, SEQ ID NO: 32). This insertion causes an in-frame 11 amino acids extension in the tolC protein sequence (V306 to Q316, SEQ ID NO: 31), which in the wild type sequence is partially overlapping with the beta-strand transmembrane region (M301 to S311) and extending into the periplasmic domain of the protein.

- 30 Both above E. coli mutants showed to be resistant to a phage belonging to the order Caudovirales, family Siphoviridae, genus "T1-like viruses", related to bacteriophage TLS as described in German and Misra (2001), as no lysis of the isolated cells could be detected after overnight incubation with the phage sample (shake flask culture with fermentation medium as described in example 1), while a control strain, the original 2'FL E. coli production strain, clearly was lysed (low biomass and high phage particle density)).

Without wishing to be bound by theory, it has been hypothesized that because of the 11 amino acid duplication, as a consequence, in the 3-dimensional protein structure model, the beta-sheets in the second region of the beta-barrel domain re-align and extend the outer loop in between the two beta-strands. It has further been hypothesized that this extended outer loop has an increased flexibility and hinders bacteriophage binding.

Example 3: Evaluation of growth and 2'FL and DiFL production of wild-type tolC vs mutated tolC variants in Escherichia coli

The novel "TLS" bacteriophage resistant strains described in Example 2 were evaluated in a growth experiment according to the cultivation conditions provided in Example 1. These strains contain an alpha-1,2-fucosyltransferase enzyme (HpFutC, SEQ ID NO: 36), and are able to produce 2-fucosyllactose and difucosyllactose, but differ in the tolC gene sequence present in their genome (tolC_WT: SEQ ID NO: 11; tolC_2, SEQ ID NO: 32; tolC_IS1: SEQ ID NO: 34). Each strain was grown in multiple wells of a 96-well plate. In all figures each datapoint corresponds to data from one well. The dashed horizontal line indicates the setpoint to which all datapoints were normalized. As shown in figure 1, the biomass obtained is clearly lower in samples of strains containing a completely inactivated tolC gene (tolC_IS1), while for strains with wild type tolC and the tolC gene variant with the 33 bp duplication (tolC_2) the obtained amount of biomass is comparable.

Figure 2 shows that the production of both sugars is clearly lower in samples of strains containing a completely inactivated tolC gene (tolC_IS1), while for strains with wild type tolC and the tolC gene variant with the 33 bp duplication (tolC_2) the productivity is comparable.

As can be seen in figure 3, the average growth speed is slightly lower in samples of strains containing a completely inactivated tolC gene (tolC_IS1), while for strains with wild type tolC and the tolC gene variant with the 33 bp duplication (tolC_2) this is comparable.

Altogether, these results suggest that the protein encoded by the tolC_2 gene variant is at least still partially active as we see a similar growth speed and 2'FL production capacity as the strain with wild type tolC, while these parameters are drastically reduced in a strain carrying a completely inactivated tolC variant (tolC_IS1).

Example 4: Evaluation of Escherichia coli strains with a wild type or a mutated tolC gene in a batch fermentation for the production of 2'fucosyllactose

Mutant *E. coli* strains containing an alpha-1,2-fucosyltransferase (HpFutC, SEQ ID NO: 36) and either the wild type tolC gene sequence or the tolC variant with the 33 bp duplication conferring resistance to "TLS" bacteriophages as described in Examples 1 and 2 were evaluated in batch fermentations at bioreactor scale. The bioreactor runs were performed as described in Example 1. In these examples, sucrose was used as a carbon source. Lactose was added in the batch medium at 90 g/L as a precursor for 2'FL formation.

The batch length in time, the yield, the specific productivity and the 2'FL titer (concentration) at the end of the batch were similar for both strains. Strains with either wild type tolC or the 33 bp duplication variant of tolC (tolC_2) thus perform equally well in a biofermentation process.

5 **Example 5: Bacteriophage-resistance mutations in HMO-producing *E. coli* strains**

E. coli MG1655 K-12 strains modified to produce either Lacto-N-neotetraose, 2'-fucosyllactose or 6'-sialyllactose with genetic backgrounds as described in Example 1, were each further mutated with distinct mutations, all in the fhuA gene.

A first mutated strain contained an E555* point mutation introducing a premature stop codon (fhuA_E555*, SEQ ID NO: 42). A second mutated strain contained a 17 bp deletion (bp 1657 to 1673) (fhuA-fs, SEQ ID NO: 44). A third mutated strain contained an insertion of a transposon (fhuA::IS2, SEQ ID NO: 46). And a fourth mutated strain contained 75 bp in-frame deletion (bp 546 to 620) which only partially deleted a 25 amino acid region of the protein (fhuA_2, SEQ ID NO: 48).

15 All of the above *E. coli* mutants showed to be resistant to bacteriophage T5 and T1 family (no lysis of the isolated cells after overnight incubation (shake flask culture with fermentation medium as described in example 1) while a control strain, the original oligosaccharide *E. coli* production strain, clearly was lysed (low biomass and high phage particle density).

All strains with and without these mutations were evaluated for growth and HMO production in both MTP growth experiments and biofermentation processes and performed equally well as or better than the control strains without these mutations on both sucrose and glycerol as carbon source.

25 **Example 6: Evaluation of growth and 2'FL or 3FL production of wild-type tolC vs mutated tolC variants in *Escherichia coli***

The wild type tolC gene of the mutant *E. coli* K12 MG1655 strain background, in which the fhuA gene was already replaced by the fhuA-2 (SEQ ID NO: 48) mutant gene conferring resistance to infection by bacteriophage families T5 and T1, was replaced by the tolC gene variant with the 33 bp duplication conferring TLS bacteriophage resistance (tolC_2, SEQ ID NO: 32) by the gene replacement technique as described in Example 1. Additionally, plasmids with genes coding for alpha-1,2- fucosyltransferase (HpFutC, SEQ ID NO: 36) or alpha-1,3-fucosyltransferase enzymes (3FT_A: SEQ ID NO: 38; 3FT_B: SEQ ID NO: 40) were introduced in both strains (wild type vs mutated tolC) for the production of 2'FL or 3FL respectively. A growth experiment was performed with these strains according to the cultivation conditions provided in Example 1. Each strain was grown in multiple wells of a 96-well plate. As shown in figure 4, the production of both sugars is clearly similar for strains with wild type tolC vs tolC gene variant with the 33 bp duplication (tolC_2, SEQ ID NO: 32), and this for each fucosyltransferase that was tested. This tolC mutation conferring TLS bacteriophage resistance thus clearly does not impact the strain's production

capabilities. This combination of both mutations in *fhuA* and *tolC*, together conferring resistance to infection against bacteriophages of the T1, T5 and TLS family, thus clearly does not impact the strain's production capabilities.

5 **Example 7: Evaluation of a *fhuA* frame-shift mutation in LNNt-producing *E. coli* strains**

Lacto-N-neotetraose (LNNt) production strains with a genetic background as described in Example 1 with either a wild-type *fhuA* gene ("Ref", SEQ ID NO: 15) or with a frame-shift mutation (17 bp deletion, bp 1657 bp 1673, "*fhuA*-fs", SEQ ID NO: 44) were compared in a growth experiment according to the cultivation conditions provided in Example 1. Each strain was grown
10 in multiple wells of a 96-well plate, and this experiment was repeated independently for 5 times. In Figures 5 and 6 each boxplot represents data of 15 individual datapoints in total (5 independent experiments with 3 biological replicates each). The dashed horizontal line indicates the setpoint to which all datapoints were normalized.

The production of LNNt, as shown in figure 5, is similar for the strain with a wild type *fhuA* compared to a *fhuA* frame-shift variant. This *fhuA*-fs mutation conferring resistance to the T5 and
15 T1 family of phages thus clearly does not impact the strain's production capabilities. The growth speed, as shown in figure 6, is very similar for LNNt strains with a wild type *fhuA* or a *fhuA* frame-shift variant. This *fhuA*-fs mutation conferring resistance to the T5 and T1 family of phages thus clearly does not impact the strain's growth speed.

20

Example 8: Evaluation of a *fhuA::IS2* mutation in 6'SL-producing *E. coli* strains

6'SL production strains with a genetic background as described in Example 1 with either a wild-type *fhuA* gene ("Ref", SEQ ID NO: 15) or with a transposon insertion ("*fhuA::IS2*", SEQ ID NO: 46) were compared in a growth experiment according to the cultivation conditions provided in
25 Example 1. Each strain was grown in multiple wells of a 96-well plate, and this experiment was repeated independently for 5 times. In Figures 7 and 8, each boxplot represents data of 20 individual datapoints in total (5 independent experiments with 4 biological replicates each). The dashed horizontal line indicates the setpoint to which all datapoints were normalized. As can be seen in figure 7, the production of 6'SL is considerably higher for the strain with a *fhuA::IS2* mutation compared to the strain with a wild type *fhuA* gene. Figure 8 shows that the maximal
30 growth speed is considerably higher for the strain with a *fhuA::IS2* mutation compared to the strain with a wild type *fhuA* gene.

35 **Example 9: Evaluation of knock-outs of various outer membrane proteins in LNNt-producing *E. coli* strains**

A lacto-N-neotetraose (LNNt) production strain with genetic background as described in Example 1, referred to as "REF1", was further engineered to contain full gene knock-outs of the genes coding for the outer membrane proteins *fadL* (SEQ ID NO: 19), *fhuA* (SEQ ID NO: 15), *lamB* (SEQ

ID NO: 13) or *nfrA* (SEQ ID NO: 29). Depending on the specific outer membrane protein knock-out, the strains thus gain resistance against the respective phage families as described in Table 3. These strains were compared in a growth experiment according to the cultivation conditions provided in Example 1. Each strain was grown in multiple wells of a 96-well plate.

- 5 Figure 9 shows that the production of LNnT is slightly higher for strains which are knocked out in *fadL*, *fhuA*, *lamB* or *nfrA* compared to the reference strain.

Example 10: Evaluation of knock-outs of various outer membrane proteins in HMO-producing *E. coli* strains

- 10 Various strains for the production of 2'FL, 3FL, DiFL, LNT, LNnT, 3'SL and 6'SL, respectively (genetic backgrounds as described in Example 1) are engineered to contain full gene knock-outs of at least one of any one of the genes coding for the outer membrane proteins *ompF* (SEQ ID NO: 5), *fadL* (SEQ ID NO: 19), *btuB* (SEQ ID NO: 9), *nfrA* (SEQ ID NO: 29), *ompA* (SEQ ID NO: 1), *lamB* (SEQ ID NO: 13), *fepA* (SEQ ID NO: 23), *fhuA* (SEQ ID NO: 15), *ompC* (SEQ ID NO: 3),
 15 *yncD* (SEQ ID NO: 25), *tsx* (SEQ ID NO: 21), *tolC* (SEQ ID NO: 11), *tonB* (SEQ ID NO: 17), *ompT* (SEQ ID NO: 7), *phoE* (SEQ ID NO: 27). Depending on the specific outer membrane protein knock-out, the strains thus gain resistance against the respective phage families as described in Table 3. These strains are compared to their respective reference strains in a growth experiment according to the cultivation conditions provided in Example 1. Each strain is grown in multiple
 20 wells of a 96-well plate. The strains are evaluated on their fitness (maximal growth speed) and on their production capacity of the various HMOs as further described in Examples 18 to 22.

Example 11: Bacteriophage resistance in *E. coli* strains producing phosphorylated monosaccharides and/or activated monosaccharides

- 25 Mutations in membrane proteins conferring resistance to infection by certain families of bacteriophages as described herein, such as complete or partial knock-outs, in-frame or out-of-frame mutations in any one of *ompF* (SEQ ID NO: 5), *fadL* (SEQ ID NO: 19), *btuB* (SEQ ID NO: 9), *nfrA* (SEQ ID NO: 29), *ompA* (SEQ ID NO: 1), *lamB* (SEQ ID NO: 13), *fepA* (SEQ ID NO: 23), *fhuA* (SEQ ID NO: 15), *ompC* (SEQ ID NO: 3), *yncD* (SEQ ID NO: 25), *tsx* (SEQ ID NO: 21), *tolC* (SEQ ID NO: 11), *tonB* (SEQ ID NO: 17), *ompT* (SEQ ID NO: 7), *phoE* (SEQ ID NO: 27), are
 30 introduced in *E. coli* strains producing phosphorylated monosaccharides and/or activated monosaccharides. Examples of phosphorylated monosaccharides include but are not limited to glucose-1-phosphate, glucose-6-phosphate, glucose-1,6-bisphosphate, galactose-1-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate, fructose-1-phosphate,
 35 glucosamine-1-phosphate, glucosamine-6-phosphate, N-acetylglucosamine-1-phosphate, mannose-1-phosphate, mannose-6-phosphate or fucose-1-phosphate. Some but not all of these phosphorylated monosaccharides are precursors or intermediates for the production of activated monosaccharide. Examples of activated monosaccharides include but are not limited to GDP-

fucose, UDP-glucose, UDP-galactose and UDP-N-acetylglucosamine. These phosphorylated monosaccharides and/or activated monosaccharides can be produced in higher amounts than naturally occurring in *E. coli* e.g. by introducing some of the genetic modifications as described in Example 1. An *E. coli* strain with active expression units of the sucrose phosphorylase and fructokinase genes (BaSP SEQ ID NO: 54, ZmFrk SEQ ID NO: 53) is able to grow on sucrose as a carbon source and can produce high(er) amounts of glucose-1P, as described in WO2012/007481. Such a strain additionally containing a knock-out of the genes *pgi*, *pfkA* and *pfkB* accumulate fructose-6-phosphate in the medium when grown on sucrose. Alternatively, by knocking out genes coding for (a) phosphatase(s) (*agp*), glucose 6-phosphate-1-dehydrogenase (*zwf*), phosphoglucose isomerase (*pgi*), glucose-1-phosphate adenylyltransferase (*glgC*), phosphoglucomutase (*pgm*) a mutant is constructed which accumulates glucose-6-phosphate. Alternatively, the strain containing a sucrose phosphorylase and fructokinase with an additional overexpression of the wild type or variant protein of the L-glutamine—D-fructose-6-phosphate aminotransferase (*glmS*) from *E. coli* (SEQ ID NO: 57) can produce higher amounts of glucosamine-6P, glucosamine-1P and/or UDP-N-acetylglucosamine. Alternatively, by knocking out the *E. coli* gene *wcaJ* coding for the undecaprenyl-phosphate glucose phosphotransferase will have an increased pool of GDP-fucose. An increased pool of UDP-glucose and/or UDP-galactose could be achieved by overexpressing the *E. coli* enzymes glucose-1-phosphate uridylyltransferase (*galU*) and/or UDP-galactose-4-epimerase (*galE*). Alternatively, by overexpressing genes coding for galactokinase (*galK*) and galactose-1-phosphate uridylyltransferase (for example originating from *Bifidobacterium bifidum*) the formation of UDP-galactose is enhanced by additionally knocking out genes coding for (a) phosphatase(s) (*agp*), UDP-glucose, galactose-1P uridylyltransferase (*galT*), UDP-glucose-4-epimerase (*galE*) a mutant is constructed which accumulates galactose-1-phosphate.

Another example of an activated monosaccharide is CMP-sialic acid which is not naturally produced by *E. coli*. Production of CMP-sialic acid can e.g. be achieved by introducing genetic modifications as described in Example 1 for the 3'SL or 6'SL background strain (but without the necessity for a gene coding for a sialyltransferase enzyme).

Such strains can be used in a biofermentation process to produce these phosphorylated monosaccharides or activated monosaccharides in which the strains are grown on e.g. one or more of the following carbon sources: sucrose, glucose, glycerol, fructose, lactose, arabinose, maltotriose, sorbitol, xylose, rhamnose and mannose. Such strains additionally containing resistance mutations against one or more families of bacteriophages will have a serious advantage in industrial-scale fermentations as they will be less prone to bacteriophage infections.

Example 12: Bacteriophage resistance in *E. coli* strains producing monosaccharides

Mutations in outer membrane proteins conferring resistance to infection by certain families of bacteriophages as described herein, such as complete or partial knock-outs, in-frame or out-of-

frame mutations in *ompF* (SEQ ID NO: 5), *fadL* (SEQ ID NO: 19), *btuB* (SEQ ID NO: 9), *nfrA* (SEQ ID NO: 29), *ompA* (SEQ ID NO: 1), *lamB* (SEQ ID NO: 13), *fepA* (SEQ ID NO: 23), *fhuA* (SEQ ID NO: 15), *ompC* (SEQ ID NO: 3), *yncD* (SEQ ID NO: 25), *tsx* (SEQ ID NO: 21), *tolC* (SEQ ID NO: 11), *tonB* (SEQ ID NO: 17), *ompT* (SEQ ID NO: 7), *phoE* (SEQ ID NO: 27), can be introduced in *E. coli* production strains for monosaccharides. An example of such a monosaccharide is L-fucose. An *E. coli* fucose production strain can be created e.g. by starting from a strain that is able to produce 2'FL as described in Example 1 and by additionally knocking out the *E. coli* genes *fucK* and *fucI* (coding for an L-fucose isomerase and an L-fuculokinase) to avoid fucose degradation, and by expressing an 1,2-alpha-L-fucosidase (e.g. *afcA* from *Bifidobacterium bifidum* (genbank accession no. : AY303700)) to degrade 2'FL into fucose and lactose. Such a strain can be used in a biofermentation process to produce L-fucose in which the strain is grown on sucrose, glucose or glycerol and in the presence of catalytic amounts of lactose as an acceptor substrate for the alpha-1,2-fucosyltransferase. Such a strain additionally containing resistance mutations against one or more families of bacteriophages will have a serious advantage in industrial-scale fermentations as it will be less prone to bacteriophage infections.

Example 13: Bacteriophage resistance in *E. coli* strain producing disaccharides

Mutations in outer membrane proteins conferring resistance to infection by certain families of bacteriophages as described herein, such as complete or partial knock-outs, in-frame or out-of-frame mutations in *ompF* (SEQ ID NO: 5), *fadL* (SEQ ID NO: 19), *btuB* (SEQ ID NO: 9), *nfrA* (SEQ ID NO: 29), *ompA* (SEQ ID NO: 1), *lamB* (SEQ ID NO: 13), *fepA* (SEQ ID NO: 23), *fhuA* (SEQ ID NO: 15), *ompC* (SEQ ID NO: 3), *yncD* (SEQ ID NO: 25), *tsx* (SEQ ID NO: 21), *tolC* (SEQ ID NO: 11), *tonB* (SEQ ID NO: 17), *ompT* (SEQ ID NO: 7), *phoE* (SEQ ID NO: 27), can be introduced in *E. coli* strains aimed at producing disaccharides. An example of such a disaccharide is e.g. lactose (galactose-beta,1,4-glucose). An *E. coli* lactose production strain can be created e.g. by introducing in wild type *E. coli* at least one recombinant nucleic acid sequence encoding for a protein having a beta-1,4-galactosyltransferase activity and being able to transfer galactose on a free glucose monosaccharide to intracellularly generate lactose as e.g. described in WO2015150328. As such the sucrose is taken up or internalized into the host cell via a sucrose permease. Within the bacterial host cell, sucrose is degraded by invertase to fructose and glucose. The fructose is phosphorylated by fructokinase (e.g. *frk* from *Zymomonas mobilis* (SEQ ID NO: 53)) to fructose-6-phosphate, which can then be further converted to UDP-galactose by the endogenous *E. coli* enzymes phosphohexose isomerase (*pgi*), phosphoglucomutase (*pgm*), glucose-1-phosphate uridylyltransferase (*galU*) and UDP-galactose-4-epimerase (*galE*). A beta-1,4-galactosyltransferase (e.g. *lgtB* from *Neisseria meningitidis*, SEQ ID NO: 52) then catalyzes the reaction UDP-galactose + glucose => UDP + lactose.

Preferably, the strain is further modified to not express the *E. coli* lacZ enzyme, a beta-galactosidase which would otherwise degrade lactose.

Such a strain can be used in a biofermentation process to produce lactose in which the strain is grown on sucrose as the sole carbon source. Such a strain additionally containing resistance mutations against one or more families of bacteriophages will have a serious advantage in industrial-scale fermentations as it will be less prone to bacteriophage infections.

Example 14: Bacteriophage resistance in *E. coli* strains producing oligosaccharides and grown on carbon sources other than sucrose

Mutations in outer membrane proteins conferring resistance to infection by certain families of bacteriophages as described herein, such as complete or partial knock-outs, in-frame or out-of-frame mutations in *ompF* (SEQ ID NO: 5), *fadL* (SEQ ID NO: 19), *btuB* (SEQ ID NO: 9), *nfrA* (SEQ ID NO: 29), *ompA* (SEQ ID NO: 1), *lamB* (SEQ ID NO: 13), *fepA* (SEQ ID NO: 23), *fhuA* (SEQ ID NO: 15), *ompC* (SEQ ID NO: 3), *yncD* (SEQ ID NO: 25), *tsx* (SEQ ID NO: 21), *tolC* (SEQ ID NO: 11), *tonB* (SEQ ID NO: 17), *ompT* (SEQ ID NO: 7), *phoE* (SEQ ID NO: 27), can be introduced in *E. coli* strains aimed at producing non-native or increased amounts of native saccharides, monosaccharides, phosphorylated monosaccharides, activated monosaccharides or oligosaccharides, such as for example human milk oligosaccharides including but not limited to 2'FL, 3FL, DiFL, LNT, LNnT, 3'SL or 6'SL. Such *E. coli* HMO production strains can be created e.g. by introducing one or multiple genetic modifications as described in example 1. All such strains can originate from any *E. coli* strain and preferably have a genomic knock out of the *lacZ* gene to avoid lactose degradation.

For example, for 2'FL, 3FL and diFL production, such mutant strains are further modified to contain an alpha-1,2- and/or alpha-1,3-fucosyltransferase expression construct, on a plasmid or inserted into the genome.

Another example, for LNT and LNnT production, the *lacZ* knock-out strain can be further modified to contain a galactoside beta-1,3-N-acetylglucosaminyltransferase (e.g. *IgtA* from *Neisseria meningitidis*, SEQ ID NO: 50) expression construct and either an N-acetylglucosamine beta-1,3-galactosyltransferase (e.g. *wbgO* from *Escherichia coli* O55:H7, SEQ ID NO: 51) for LNT production or an N-acetylglucosamine beta-1,4-galactosyltransferase (e.g. *IgtB* from *Neisseria meningitidis*, SEQ ID NO: 52) for LNnT production.

Another example, for 3'SL and 6'SL production, the *lacZ* knock-out strain can be further modified to contain a glucosamine 6-phosphate N-acetyltransferase (e.g. *GNA1* from *Saccharomyces cerevisiae*, SEQ ID NO: 58), an N-acetylglucosamine 2-epimerase (e.g. BoAGE from *Bacteroides ovatus*, SEQ ID NO: 59), an N-acetylneuraminate synthase (e.g. NeuB from *Campylobacter jejuni*, SEQ ID NO: 60), a CMP-Neu5Ac synthetase (e.g. NeuA from *Campylobacter jejuni*, SEQ ID NO: 61), and either a beta-galactoside alpha-2,3-sialyltransferase for 3'SL production (e.g.

SEQ ID NO: 55) or a beta-galactoside alpha-2,6-sialyltransferase for 6'SL production (e.g. SEQ ID NO: 56).

These strains as exemplified above can further contain additional modifications to improve their productivity. Such strains can then be used in biofermentation processes to produce the desired oligosaccharide, after which the oligosaccharide is preferably purified from the broth. Such a biofermentation process needs lactose in the medium as an acceptor substrate and can be performed with any carbon source which *E. coli* is able to metabolize. Examples of such carbon sources include but are not limited to glucose, arabinose, maltotriose, glycerol, sorbitol, xylose, rhamnose and mannose, or any combination of two or more of these carbon sources. These strains additionally containing resistance mutations against one or more families of bacteriophages, as listed above, will have a serious advantage in industrial-scale fermentations as they will be less prone to bacteriophage infections.

Example 15: Combinations of mutations conferring resistance against bacteriophage infection in *E. coli* strains

Mutations in outer membrane proteins conferring resistance to infection by certain families of bacteriophages as described herein, such as complete or partial knock-outs, in-frame or out-of-frame mutations in *ompF* (SEQ ID NO: 5), *fadL* (SEQ ID NO: 19), *btuB* (SEQ ID NO: 9), *nfrA* (SEQ ID NO: 29), *ompA* (SEQ ID NO: 1), *lamB* (SEQ ID NO: 13), *fepA* (SEQ ID NO: 23), *fhuA* (SEQ ID NO: 15), *ompC* (SEQ ID NO: 3), *yncD* (SEQ ID NO: 25), *tsx* (SEQ ID NO: 21), *tolC* (SEQ ID NO: 11), *tonB* (SEQ ID NO: 17), *ompT* (SEQ ID NO: 7), *phoE* (SEQ ID NO: 27), can be introduced in *E. coli* strains aimed at producing non-native or increased amounts of native saccharides, monosaccharides, phosphorylated monosaccharides, activated monosaccharides or oligosaccharides, such as for example human milk oligosaccharides including but not limited to 2'FL, 3FL, DiFL, LNT, LNnT, 3'SL or 6'SL. Strains with any bacteriophage resistance mutation will have an advantage in industrial-scale fermentations as they will be less prone to bacteriophage infections. In addition, combinations of two or more of such mutations conferring bacteriophage resistance, in the same or in different outer membrane proteins, are possible. Preferably each mutation is selected in such a way that the combination of these individual mutations give rise to resistance against multiple families of bacteriophages. In addition, preferably each mutation individually as well as any combination of mutations increases or does not impair the strain's production as compared to a strain with the same genetic make-up but lacking the mutation in the membrane protein encoding genes. An example of two such mutations which can be combined in an HMO production strain is e.g. a 33 bp duplication in the *tolC* gene (SEQ ID NO: 32), which confers resistance against bacteriophages from the TLS family and any of the described mutations in *fhuA* (full knock-out, SEQ ID NO: 42, 44, 46 or 48) conferring resistance against bacteriophages from the T1, T5 and ϕ 80 family. These individual mutations and any combination thereof will increase or will not decrease the strain's productivity. Combined

in a single production strain, the strain will be resistant to infection by any bacteriophage of the TLS, T1, T5 and ϕ 80 family. Such a strain can be further modified to contain additional mutations (for example complete or partial knock-outs) in e.g. *lamB* (SEQ ID NO: 13) and/or *fadL* (SEQ ID NO: 19) and/or *nfrA* (SEQ ID NO: 29). These strains will in addition to their resistance against infection by bacteriophages of the TLS, T1, T5 and ϕ 80 family also have gained resistance against bacteriophages of family K10 and/or family I and/or family T2 and/or family N4. These strains can be used in biofermentation processes to produce any of the listed sugars and can be performed with any carbon source which *E. coli* is able to metabolize. Examples of such carbon sources include but are not limited to glucose, arabinose, maltotriose, glycerol, sorbitol, xylose, rhamnose and mannose, or any combination of two or more of these carbon sources.

Example 16: Identification of membrane protein families

Membrane proteins were classified based on the COG (Cluster of Orthologous Groups) numbers in the eggNOG database (<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6324079/>; <http://eggnogetool.embl.de/#/app/home>). The eggNOG database is a public database of orthology relationships, gene evolutionary histories and functional annotations. Identification of the COG group can be done by using a standalone version of eggNOG-mapper (<https://github.com/eggnogetool/eggnogetool-mapper>). For each of the COG groups an HMM-model can be downloaded on the eggNOG website and can be used for HMMsearch using the HMMER package (<http://hmmer.org/>) to protein databases.

Identification of COG group was done by using a standalone version of eggNOG-mapper, eggNOGv4.5 of eggNOG-mapperv1 (<http://eggnogetool.embl.de/#/app/home>).

The COG group of membrane proteins as used in the present invention are listed in Table 4.

Table 4:

Membrane protein	Membrane protein description	SEQ ID NOs	COG
btuB	cobalamin/cobinamide outer membrane transporter	09-10	COG4206
fadL	long-chain fatty acid outer membrane channel / bacteriophage T2 receptor	19-20	COG2067
fepA	ferric enterobactin outer membrane transporter	23-24	COG4771
fhuA	ferrichrome outer membrane transporter/phage receptor	16-16	COG1629
lamB	maltose outer membrane channel / phage lambda receptor protein	13-14	COG4580
ompA	outer membrane porin A	01-02	COG2885
ompC	outer membrane porin C	03-04	COG3203

ompF	outer membrane porin F	05-06	COG3203
ompT	outer membrane protease VII (outer membrane protein 3b)	07-08	COG4571
PhoE	outer membrane porin, outer membrane phosphoporin	27-28	COG3203
TolC	outer membrane channel	11-12	COG1538
tsx	nucleoside-specific channel-forming protein	21-22	COG3248
tonB	Ton complex subunit	17-18	COG0810
nfrA	bacteriophage N4 receptor, outer membrane protein	29-30	COG0457
yncD	putative TonB-dependent outer membrane receptor	25-26	COG1629

Example 17: Bacteriophage resistance in *E. coli* strains producing glycolipids

- Mutations in outer membrane proteins conferring resistance to infection by certain families of bacteriophages as described herein, such as complete or partial knock-outs, in-frame or out-of-frame mutations in ompF (SEQ ID NO: 5), fadL (SEQ ID NO: 19), btuB (SEQ ID NO: 9), nfrA (SEQ ID NO: 29), ompA (SEQ ID NO: 1), lamB (SEQ ID NO: 13), fepA (SEQ ID NO: 23), fhuA (SEQ ID NO: 15), ompC (SEQ ID NO: 3), yncD (SEQ ID NO: 25), tsx (SEQ ID NO: 21), tolC (SEQ ID NO: 11), tonB (SEQ ID NO: 17), ompT (SEQ ID NO: 7), phoE (SEQ ID NO: 27), can be introduced in *E. coli* production strains for glycolipids.
- An example of such a glycolipid is e.g. a rhamnolipid containing one or two rhamnose residues (mono- or dirhamnolipid). The production of monorhamnolipids can be catalyzed by the enzymatic complex rhamnosyltransferase 1 (Rt1), encoded by the rhlAB operon of *Pseudomonas aeruginosa*, using dTDP-L-rhamnose and beta-hydroxydecanoic acid precursors. Overexpression in an *E. coli* strain of this rhlAB operon, as well as overexpression of the *Pseudomonas aeruginosa* rmlBDAC operon genes to increase dTDP-L-rhamnose availability, allows for monorhamnolipids production, mainly containing a C10-C10 fatty acid dimer moiety. This can be achieved in various media such as rich LB medium or minimal medium with glucose as carbon source.
- Such a strain additionally containing resistance mutations against one or more families of bacteriophages will have a serious advantage in industrial-scale fermentations as it will be less prone to bacteriophage infections.

Example 18: Evaluation of knock-outs of various outer membrane proteins in 2'FL or 3FL producing *E. coli* strains

- A strain intended for 2'FL or 3FL production with genetic background as described in Example 1, containing the fhuA_2 (SEQ ID NO: 48) mutant gene conferring resistance to infection by bacteriophage families T5 and T1, and a tolC gene variant with the 33 bp duplication conferring TLS bacteriophage resistance (tolC_2, SEQ ID NO: 32), was further engineered to contain full

gene knock-outs of the genes coding for the outer membrane proteins (OMPs) *ompF* (SEQ ID NO: 5), *fadL* (SEQ ID NO: 19), *btuB* (SEQ ID NO: 9), *nfrA* (SEQ ID NO: 29), *lamB* (SEQ ID NO: 13), *fepA* (SEQ ID NO: 23), *fhuA* (SEQ ID NO: 15), *yncD* (SEQ ID NO: 25), *tsx* (SEQ ID NO: 21), *ompT* (SEQ ID NO: 7) or *phoE* (SEQ ID NO: 27). Depending on the specific outer membrane

5 protein knock-out, the mutant strains thus obtained gain resistance against the respective phage families as described in Table 3. Next, a plasmid with a gene coding for an alpha-1,2-fucosyltransferase (HpFutC, SEQ ID NO: 36) or for an alpha-1,3-fucosyltransferase (3FT_A, SEQ ID NO: 38) was added to all mutant strains for the production of 2'FL or 3FL, respectively.

A growth experiment was performed with these strains according to the cultivation conditions

10 provided in Example 1. Each strain was grown in multiple wells of a 96-well plate. As shown in figures 10 and 11, the production of 2'FL or 3FL, respectively, remained higher than 75% or was almost identical compared to a reference 2'- or 3-fucosyllactose production strain lacking the additional outer membrane protein knock-out (Ref strain). Also, all tested outer membrane protein gene deletions had no or only a moderate impact on the growth of the mutant strains, reaching

15 growth speed levels higher than 75% up till 100% of the growth speed of the reference strain. These additional OMP knock-outs together with both mutations in *fhuA* and *tolC* clearly do not impact the strain's production capabilities.

Example 19: Evaluation of knock-outs of various outer membrane proteins in DiFL-producing *E. coli* strains

20

In a next step, another experiment was set-up with a strain intended for DiFL production with genetic background as described in Example 1, containing the *fhuA_2* (SEQ ID NO: 48) mutant gene and the *tolC* gene variant with the 33 bp duplication (*tolC_2*, SEQ ID NO: 32). This strain was further engineered to contain full gene knock-outs of the genes coding for the outer

25 membrane proteins (OMPs) *ompF* (SEQ ID NO: 5), *fadL* (SEQ ID NO: 19), *btuB* (SEQ ID NO: 9), *nfrA* (SEQ ID NO: 29), *fepA* (SEQ ID NO: 23), *yncD* (SEQ ID NO: 25), *tsx* (SEQ ID NO: 21), *ompT* (SEQ ID NO: 7) or *phoE* (SEQ ID NO: 27). Depending on the specific outer membrane protein knock-out, the mutant strains gain resistance against the respective phage families as described in Table 3. Next, a plasmid with a gene coding for an alpha-1,2-fucosyltransferase (HpFutC, SEQ

30 ID NO: 36) and a plasmid with an alpha-1,3-fucosyltransferase (3FT_A, SEQ ID NO: 38) encoding gene were introduced to all mutant strains for the production of DiFL.

A growth experiment was performed with these strains according to the cultivation conditions provided in Example 1. Each strain was grown in multiple wells of a 96-well plate. As shown in figure 12, the production of DiFL remained higher than 75% or was almost identical compared to

35 a reference strain lacking the additional outer membrane protein knock-out. Also, all tested outer membrane protein gene deletions had no or only a moderate impact on the growth of the mutant strains, reaching growth speed levels higher than 75% up till 100% of the growth speed of the

reference strain. These additional OMP knock-outs together with both mutations in *fhuA* and *tolC* clearly do not impact the strain's production capabilities.

Example 20: Evaluation of knock-outs of various outer membrane proteins in 6'SL or 3'SL producing *E. coli* strains

A strain intended for 6'SL or 3'SL production with genetic background as described in Example 1 was further engineered to contain full gene knock-outs of the genes coding for the outer membrane proteins (OMPs) *ompF* (SEQ ID NO: 5), *fadL* (SEQ ID NO: 19), *btuB* (SEQ ID NO: 9), *nfrA* (SEQ ID NO: 29), *lamB* (SEQ ID NO: 13), *fepA* (SEQ ID NO: 23), *fhuA* (SEQ ID NO: 15), *yncD* (SEQ ID NO: 25), *tsx* (SEQ ID NO: 21), *ompT* (SEQ ID NO: 7), *phoE* (SEQ ID NO: 27) or *tonB* (SEQ ID NO: 17). Depending on the specific outer membrane protein knock-out, the strains thus gain resistance against the respective phage families as described in Table 3. Next, a plasmid with a gene coding for an alpha-2,6-sialyltransferase (PdbST, SEQ ID NO: 56) or an alpha-2,3-sialyltransferase (PmultST3, SEQ ID NO: 55) was added to all mutant strains for the production of 6'SL or 3'SL, respectively.

A growth experiment was performed with these strains according to the cultivation conditions provided in Example 1. Each strain was grown in multiple wells of a 96-well plate. As shown in figures 13 and 14, the production of 6'SL or 3'SL, respectively, remained higher than 75% or was almost identical compared to a reference 6'- or 3'-sialyllactose production strain lacking the additional outer membrane protein knock-out. Also, all tested outer membrane protein gene deletions had no or only a moderate impact on the growth of the mutant strains, reaching growth speed levels higher than 75% up till 100% of the growth speed of the reference strain. These OMP knock-outs clearly do not impact the strain's production capabilities.

Example 21: Evaluation of knock-outs of various outer membrane proteins in LNNt-producing *E. coli* strains

Additionally to the experiment described in Example 9, a mutant strain producing lacto-N-neotetraose (LNNt) and its intermediate compound lacto-N-triose (LN3) with genetic background as described in Example 1 was further engineered to contain full gene knock-outs of the genes coding for the outer membrane proteins (OMPs) *ompF* (SEQ ID NO: 5), *btuB* (SEQ ID NO: 9), *fepA* (SEQ ID NO: 23), *yncD* (SEQ ID NO: 25), *tsx* (SEQ ID NO: 21), *ompT* (SEQ ID NO: 7) or *phoE* (SEQ ID NO: 27). Depending on the specific outer membrane protein knock-out, the strains gain resistance against the respective phage families as described in Table 3.

A growth experiment was performed with these strains according to the cultivation conditions provided in Example 1. Each strain was grown in multiple wells of a 96-well plate. Figure 15 shows that the production of LNNt remained higher than 75% for all tested mutant strains as compared to the reference strain. As such, these new OMP knock-outs do not impact the strain's production

capabilities. Due to the high conversion rate of LN3 to LNnT, the LN3 levels could not be measured in this experiment.

Example 22: Evaluation of knock-outs of various outer membrane proteins in LNT-producing *E. coli* strains

In a next experiment, a mutant strain producing lacto-N-tetraose (LNT) and its intermediate compound lacto-N-triose (LN3) with genetic background as described in Example 1 was further engineered to contain full gene knock-outs of the genes coding for the outer membrane proteins (OMPs) *ompF* (SEQ ID NO: 5), *nfrA* (SEQ ID NO: 29), *lamB* (SEQ ID NO: 13), *fepA* (SEQ ID NO: 23), *fhuA* (SEQ ID NO: 15), *yncD* (SEQ ID NO: 25), *tsx* (SEQ ID NO: 21), *ompT* (SEQ ID NO: 7) or *phoE* (SEQ ID NO: 27). Depending on the specific outer membrane protein knock-out, the strains gain resistance against the respective phage families as described in Table 3.

A growth experiment was performed with these strains according to the cultivation conditions provided in Example 1. Each strain was grown in multiple wells of a 96-well plate. Figure 16 shows that the production of the intermediate LN3 compound as well as the final LNT product remained higher than 75% for all tested mutant strains as compared to the reference strain. The mutant strain having a knock-out in *ompT* even showed higher LN3 production with limited effect on LNT production as compared to the reference strain with a similar genetic make-up lacking the *ompT* knock-out. As such, these new OMP knock-outs do not impact the strain's production capabilities.