

Production of sialylated oligosaccharides in *Bacillus* cells

The present invention relates to the technical field of genetic engineering, in particular to genetic engineering of *Bacillus* cells for the production of sialylated oligosaccharides in said *Bacillus* cells, to the fermentative production of sialylated oligosaccharides using said *Bacillus* cells, and to the use of thus produced sialylated oligosaccharides.

Background

Human milk provides young infants with all nutrients they need for healthy growth and development. The saccharides that are present in human milk represent its main component, ahead of fats and proteins. In addition to lactose, which serves as an energy source, human milk contains more complex saccharide molecules, i.e. oligosaccharides. Approximately 200 structurally distinct oligosaccharides have been identified in human milk to date. These oligosaccharides are found at significant concentrations only in human milk, and they are collectively known as human milk oligosaccharides (HMOs). Said HMOs are based on the disaccharide lactose (consisting of a glucose (Glc) moiety and a galactose (Gal) moiety) and bear additional monosaccharide residues which are based on *N*-acetyl-glucosamine (GlcNAc), fucose (Fuc), sialic acid/*N*-acetylneuraminic acid (NeuNAc), and/or galactose (Gal). The concentration and composition of HMOs in human milk varies between individuals and during the lactation period from up to 20 g/L in colostrum to 5-10 g/L in mature milk.

A considerable number of HMOs bear at least one NeuNAc moiety. Among these sialylated human milk oligosaccharides (SHMOs), 3'-sialyllactose, 6'-sialyllactose, sialyllacto-*N*-tetraose a, sialyllacto-*N*-tetraose b, sialyllacto-*N*-tetraose c and disialyllacto-*N*-tetraose are the most prevalent members in human milk.

Sialic acids (Sia) are a family of negatively charged monosaccharides with a nine-carbon backbone. More than 50 forms of these α -keto acids have been found in nature. The most abundant sialic acid appears to be *N*-acetylneuraminic acid (NANA, NeuNAc, Neu5Ac).

Sialic acids are present as terminal monosaccharide moieties of glycans that are present in glycoconjugates (glycoproteins and glycolipids) on the surface of cells of vertebrates and higher invertebrates. Sialic acids are components of the lipopolysaccharides and capsular polysaccharides of pathogenic bacteria including *Escherichia coli* K1, *Haemophilus influenzae*, *Haemophilus ducreyi*, *Pasteurella multocida*,
5 *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Campylobacter jejuni* and *Streptococcus agalactiae*.

Sialylated HMOs were observed to support the infant's resistance to enteropathogenic bacteria and viruses. Interestingly, recent studies further demonstrated a protective effect of long-chained SHMOs against necrotizing enterocolitis, which is one
10 of the most common and lethal disease in preterm infants. Also, sialylated oligosaccharides have been shown to neutralize enterotoxins of various pathogenic microbes including *Escherichia coli*, *Vibrio cholerae* and *Salmonella*. Further, it was found that sialylated oligosaccharides interfere with the colonization of the gut by
15 *Helicobacter pylori* and thereby prevent or inhibit gastric and duodenal ulcers. In addition, SHMOs are believed to support an infant's brain development and its cognitive capabilities.

Due to the known benefits of HMOs, especially of sialylated HMOs, an economically worthwhile process for their production is desired such that these sialylated oligosaccharides or at least some of these sialylated oligosaccharides become available
20 as supplement for infant formulae.

The limited availability of human milk for other purposes than breast feeding an infant and the difficulties to obtain pure fractions of individual human milk oligosaccharides from natural sources led to the development of chemical routes for their
25 synthesis. However, neither chemical synthesis nor biocatalytic *in-vitro* approaches proved to be commercially sustainable. Moreover, chemical synthesis of human milk oligosaccharides involves the use of several noxious chemicals which impose the risk of contaminating the final product.

As an alternative to chemical and biocatalytic *in-vitro* synthesis, fermentative
30 production of HMOs has been developed. To date, recombinant *Escherichia coli* cells are used for microbial production of some HMOs in an industrial scale.

However, the genus *Escherichia coli* comprises pathogenic members as well as non-pathogenic members. Notwithstanding that non-pathogenic *E. coli* strains are employed for the microbial production of HMOs, such non-pathogenic *E. coli* are not recognized as safe for the manufacturing of products that are intended for human consumption in a variety of jurisdictions. This hinders regulatory approval of HMOs being manufactured by current biotechnological methods for human consumption in said jurisdictions. Therefore, microbial cells of genera that are recognized as safe for human consumption or recognized as safe when used in the production of compounds for human consumption in such jurisdictions are needed for the manufacturing saccharides that are intended for human consumption, e.g. human milk oligosaccharides, in particular for consumption by infants. The use of production strains recognized as safe reduces the – at least alleged – concerns with respect to the likelihood of the saccharide's risks for human health, and will facilitate their regulatory approval in most jurisdictions.

The problem is solved by using bacterial cells of the genus *Bacillus* for the production of sialylated oligosaccharides, in particular of sialylated HMOs. Bacterial cells of some species of the genus *Bacillus* are already recognized as being safe for human consumption or for the production of compounds/food for human consumption. Hence, provided are *Bacillus* cells of species and/or strains that are generally recognized as safe, for the production of sialylated oligosaccharides, in particular for the production of sialylated human milk oligosaccharides.

Bacteria of the genus *Bacillus* are gram-positive, rod-shaped, endospore-forming microbial cells of either aerobic or facultatively anaerobic species. The genus *Bacillus* belongs to the phylum Firmicutes. The genome of the members of the genus *Bacillus* possesses a bias towards A-T base pairs in its codon usage. *Bacillus* species are almost ubiquitously present in nature. They can for example be found in soil (*B. subtilis*), but also occur in extreme environments such as high pH (*B. alcalophilus*), high temperature (*B. thermophilus*), or high salt (*B. halodurans*).

The genus *Bacillus* includes 266 named species which include free living species as well as parasitic pathogenic species. Two *Bacillus* species are considered of being medically significant: *B. anthracis*, which causes anthrax, and *B. cereus*, which causes food poisoning. A third species, *B. thuringiensis*, is an important insect pathogen

producing a toxin that can kill insects. Thus, it is used as an insecticide to control insect pests.

Because of their GRAS (Generally Recognized as Safe) status, several *Bacillus* species, e.g. *B. amyloliquefaciens*, *B. licheniformis* and *B. subtilis*, are used in the
5 biotechnological production of various proteins and compounds to be used in food and pharmaceutical industry.

Bacillus amyloliquefaciens is the source of restriction enzyme BamHI, and also synthesizes the natural antibiotic protein barnase. In addition, *B. amyloliquefaciens* produces plantazolicin, an antibiotic with selective activity against *B. anthracis*. Alpha-amylase from *B. amyloliquefaciens* is often used in starch hydrolysis. *B. amylolique-*
10 *faciens* is also a source of subtilisin, which catalyzes the breakdown of proteins.

Bacillus amyloliquefaciens is a root-colonizing bacterium that is used to fight some plant root pathogens in agriculture, aquaculture and hydroponics as it acts against bacterial and fungal pathogens, and may prevent infection by competitive exclusion
15 or out-competing the unwanted pathogen.

Its high capacity of secreting alkaline serine protease has made *B. licheniformis* one of the most important bacteria in industrial enzyme production. Subtilisin Carlsberg secreted by *B. licheniformis* is used as a detergent protease, and is sold under the trade name Alcalase®.

20 *Bacillus subtilis* is a catalase positive bacterium that is found in soil and the gastrointestinal tract of ruminants and humans. *B. subtilis* and substances derived from this endotoxin-free bacterium have been evaluated by different authoritative bodies for their safe and beneficial use in food. In the United States carbohydrase and protease enzymes from *B. subtilis* are recognized as generally safe (GRAS) by the
25 US Food and Safety Administration (FDA). *Bacillus subtilis* has also been granted the "Qualified Presumption of Safety" status by the European Food Safety Authority.

Moreover, nontoxigenic and nonpathogenic strains of *B. subtilis* strain are commonly used in food. For example, fermented soy beans in the form of Natto are frequently consumed in Japan and contain as many as 10^8 viable *B. subtilis* cells per gram.
30 Natto is recognized for its contribution to a healthy gut flora and vitamin K₂ intake.

The Natto product and the *B. subtilis* var. *natto* as its principal component are FOSHU (Foods for Specified Health Use) approved by the Japanese Ministry of Health, Labor and Welfare as effective for preservation of health.

Bacillus subtilis is easy to handle, grows rapidly and the culture conditions are simple. Recombinant *B. subtilis* strains are used in the production of polyhydroxy-
5 alkanolates, hyaluronic acid and various enzymes such as amylase and proteases.

Wild-type natural isolates of *B. subtilis* are difficult to work with as compared to laboratory strains that have undergone domestication processes of mutagenesis and selection. These domesticated strains often have improved capabilities to
10 undergo natural competence development (uptake and integration of environmental DNA), improved capabilities of growth and loss of abilities needed "in the wild". In *B. subtilis*, linear DNA as well as multimeric forms of plasmid DNA are actively taken up by natural competent cells.

Under certain physiological conditions, a small subpopulation of *B. subtilis* cells
15 becomes competent. In *B. subtilis*, natural competence is regulated by a complex regulatory network. Key regulators in this network are, amongst others, the master regulator of competence ComK and the transcriptional master regulator of sporulation Spo0A. Transformation efficiency of *B. subtilis* cells and probably the efficacy for integrating DNA into their genome can be improved by genetic engineering. This
20 can be achieved by ectopic integration of an expression cassette, comprising a regulated promoter (e.g. the mannitol-inducible P_{mtlA} promoter) and the genes *comK* and *comS*, into the genome of *B. subtilis*. Additionally, this strategy allows transformation of *B. subtilis* by natural competence using a complex medium (e.g. LB).

For the production of sialylated oligosaccharides, *Bacillus* cells can be genetically
25 engineered by various methods.

The term "genetically engineered" as used herein refers to the modification of the *Bacillus* cell's genetic make-up using molecular biological methods. The modification of the *Bacillus* cell's genetic make-up may include the transfer of genes within and/or across species boundaries, inserting, deleting, replacing and/or modifying
30 nucleotides, triplets, genes, open reading frames, promoters, enhancers, terminators and other nucleotide sequences mediating and/or controlling gene expres-

- sion. The modification of the *Bacillus* cell's genetic make-up aims to generate a genetically modified *Bacillus* cell possessing particular, desired properties. Genetically engineered *Bacillus* cells can contain one or more genes that are not present in the native (not genetically engineered) form of the cell. Techniques for introducing
- 5 exogenous nucleic acid molecules and/or inserting exogenous nucleic acid molecules (recombinant, heterologous) into a *Bacillus* cell's hereditary information for inserting, deleting or altering the nucleotide sequence of a cell's genetic information are known to the skilled artisan. Genetically engineered *Bacillus* cells can contain one or more genes that are present in the native form of the cell, wherein said genes
- 10 are modified and re-introduced into the *Bacillus* cell by artificial means. The term "genetically engineered" also encompasses *Bacillus* cells that contain a nucleic acid molecule being endogenous to the cell, and that has been modified without removing the nucleic acid molecule from the cell. Such modifications include those obtained by gene replacement, site-specific mutations, and related techniques.
- 15 Gene integration and/or (simultaneous) gene inactivation by disruption or deletion can be achieved by homologous recombination. For an efficient homologous recombination, at least 400-500 bp of homology arms are necessary in *B. subtilis*.

- Another method for targeted genome engineering is the CRISPR-Cas9 system. This rapid and markerless genome-editing tool can be used for large scale genomic
- 20 deletions, small and large DNA insertions, gene silencing by introduction of a stop codon as well as introduction of point mutations. No previous genome modifications are required for the scarless genome editing by CRISPR-Cas9.

- Random chromosomal integration of genes and insertional mutagenesis can be performed using a modified mariner-derived transposon. This system is not biased
- 25 towards hotspots in *B. subtilis* while showing high efficiency in random ectopic integration.

- Although *Bacillus* species are used for industrial scale production of enzymes, *Bacillus* cells have not been implemented to date for industrial scale production of oligosaccharides, in particular of industrial scale production of sialylated oligo-
- 30 saccharides.

Chinese patent application CN 108 410 787 A discloses recombinant *Bacillus subtilis* cells which synthesize lactyl-N-neotetraose. Said recombinant *B. subtilis* cells possess a lactose permease gene which has been integrated into the cell's genome. In addition, said *Bacillus* cell which bears a plasmid comprising a β -1,3-*N*-glucosamine transferase gene and a β -1,4-galactosyltransferase gene. The *B. subtilis* cells may be cultivated in the presence of exogenous lactose, and synthesizes lactyl-N-neotetraose at titers of up to about 1 g/L which is too little for economically reasonable industrial scale production.

Chinese patent application CN 109 735 479 A discloses recombinant *Bacillus subtilis* cells for synthesizing 2'-fucosyllactose, wherein the expression of a lactose transport enzyme is enhanced, and which cell expresses a fucose kinase, a phosphate guanine transferase and a fucosyltransferase. The yield of 2'-fucosyllactose in the fermentation medium was reported to be between 0.424 g/L and 1.042 g/L.

Although a variety of patent applications mention *Bacillus* as a genus which is deemed to be suitable for the production of neutral oligosaccharides such as lacto-*N*-neotetraose or 2'-fucosyllactose, use of *Bacillus* for the productions of sialylated oligosaccharides, in particular of sialylated human milk oligosaccharides, has not been implemented yet, presumably because of the significant efforts in metabolic engineering that are required to implement the necessary biosynthetic pathways for HMO production in *Bacillus*. Whereas above-referenced *B. subtilis* for the production of LNnT relies on donor substrates that are naturally occurring in *B. subtilis* cells, production of a sialylated oligosaccharide in *Bacillus* requires implementation of a heterologous metabolic pathway in the cell for providing a donor substrate, i.e. CMP-NeuNAc, that is essential for the synthesis of sialylated oligosaccharides.

The object has been achieved by providing a *Bacillus* cell that possesses a lactose permease for import of exogenous lactose into the cell, a CMP-NeuNAc biosynthesis pathway for the intracellular formation of a nucleotide-activated sialic acid, i.e. cytidine monophosphate *N*-acetylneuraminic acid (CMP-NeuNAc), as donor substrate for a sialic acid moiety, and a sialyltransferase for transfer of the sialic acid moiety from CMP-NeuNAc to an acceptor substrate. Cultivation of such *Bacillus* cells in the presence of exogenous lactose allows production of a desired sialylated oligosaccharide.

Summary

According to a first aspect, provided is a non-sporulating bacterial cell of the genus *Bacillus* for the production of a sialylated oligosaccharide, wherein the *Bacillus* cell possesses a lactose permease, a CMP-NeuNAc biosynthesis pathway and a sialyltransferase.

According to a second aspect, provided is the use of the non-sporulating bacterial cell of the genus *Bacillus* according to the first aspect for the production of a sialylated oligosaccharide.

According to a third aspect, provided is a method for the production of a sialylated oligosaccharide, the method comprising:

- providing a non-sporulating bacterial cell of the genus *Bacillus*, wherein said *Bacillus* cell possesses a lactose permease, a CMP-NeuNAc biosynthesis pathway and a sialyltransferase;
- cultivating the *Bacillus* cell in a culture medium containing lactose and under conditions that are permissive for the *Bacillus* cell to produce the sialylated oligosaccharide, and
- optionally retrieving the sialylated oligosaccharide from the culture medium and/or the *Bacillus* cell.

According to a fourth aspect, provided are sialylated oligosaccharides that were produced by a *Bacillus* cell which possesses a lactose permease, a CMP-NeuNAc biosynthesis pathway and a sialyltransferase.

According to a fifth aspect, provided is the use of a sialylated oligosaccharide that was produced by a *Bacillus* cell which possesses a lactose permease, a CMP-NeuNAc biosynthesis pathway and a sialyltransferase for the manufacturing of a nutritional composition.

According to a sixth aspect, provided are nutritional compositions containing at least one sialylated oligosaccharide that had been produced by a *Bacillus* cell which possesses a lactose permease, a CMP-NeuNAc biosynthesis pathway and a sialyltransferase.

Description of the figures

Fig. 1 illustrates different embodiments of the CMP-NeuNAc biosynthesis pathway that is implemented in a *Bacillus* cell for the production of a sialylated oligosaccharide.

5 Detailed description

According to the first aspect, provided is a non-sporulating bacterial cell of the genus *Bacillus* for the production of a sialylated oligosaccharide. For being able to produce a sialylated oligosaccharide, the *Bacillus* cell has to provide a donor substrate comprising a sialic acid moiety, and an acceptor substrate being a disaccharide or an
10 oligosaccharide to a sialyltransferase such that the sialyltransferase can transfer the sialic acid moiety from the donor substrate to said acceptor substrate thereby generating the sialylated oligosaccharide.

It is to be understood that the sialylated oligosaccharide said *Bacillus* cell is intended to produce is the desired sialylated oligosaccharide, whereas other sialylated oligo-
15 saccharides that may be generated due to the promiscuity of the sialyltransferase during production of the desired sialylated oligosaccharide in the *Bacillus* cell are considered as undesired sialylated oligosaccharides or by-products.

The *Bacillus* cell for producing a sialylated oligosaccharide is a non-sporulating *Bacillus* cell. Wild-type *Bacillus* cells are capable of forming spores. Sporulation, i.e.
20 the process of forming spores, in bacteria is considered to be a reaction of the bacterial cell which initiates a developmental program leading to the formation of daughter cells of distinct morphology and fate. Sporulation of *Bacillus* was studied as a basic model for cell differentiation. During sporulation, a rod-shaped *Bacillus* cell divides asymmetrically, thereby resulting in two genetically identical cells with
25 different morphology and fates.

However, in industrial production it is not desired if the bacterial production strain sporulates during fermentative production of the desired product. Therefore, it is preferred to use *Bacillus* cells for the production of sialylated oligosaccharides that are not capable of forming spores. Such *Bacillus* cells are called "non-sporulating".

In an embodiment, the non-sporulating *Bacillus* cell for the production of a sialylated oligosaccharide originated from one of the *B. subtilis* strains listed in Table 1.

Strain	Manufacturer/Reference	Product	Application
<i>Bacillus subtilis</i> 168	BGSCID 1A1	-	reference strain
<i>Bacillus subtilis</i> 168 Δ spoII $GA::erm$	BGSCID BKE15310	-	reference strain
<i>Bacillus subtilis</i> 168 Δ sigE $::erm$	BGSCID BKE15320	-	reference strain
<i>Bacillus subtilis</i> 168 Δ sigG $::erm$	BGSCID BKE15330	-	reference strain
<i>Bacillus subtilis</i> 168 Δ sigF $::erm$	BGSCID BKE23450	-	reference strain
<i>Bacillus subtilis</i> 3NA	BGSCID 1S1	-	reference strain
<i>Bacillus subtilis</i> W23	BGSCID 2A9	-	reference strain
<i>Bacillus subtilis</i> PY79	BGSCID 1A747	-	reference strain
<i>Bacillus subtilis</i>	Garden of Life®	Primal Defense®	Human use
<i>Bacillus subtilis</i>	Roux-Ocefa Laboratorios	Totalflora	Human use
<i>Bacillus subtilis</i>	Ist. Bioch. Italiano	Lactipan Plus	Human use
<i>Bacillus subtilis</i> 2335	Biopharma	Biosporin®	Human use
LifeinU™ <i>Bacillus subtilis</i> CU1	Lesaffre Human Care	Super Smart®	Human use
<i>Bacillus subtilis</i> PXN/21	Probiotics International Ltd	Bio-Kult®	Human use
<i>Bacillus subtilis</i> HU58	Microbiome LABS	HU58™	Human use
<i>Bacillus subtilis</i> R0179	Hanmi Pharmaceutical Co. Ltd.	Medilac®	Human use
<i>Bacillus subtilis</i> DE111	Deerland Enzymes & Probiotics	NutriCommit	Human use
<i>Bacillus subtilis</i> DSM21097	MorinagaGarden B.V.	Morinaga Plus+	Human use
<i>Bacillus subtilis</i> IDCC1101	Ildong Pharmaceutical	Biobaby®	Human use
<i>Bacillus subtilis</i> KATMIRA1933	JSL Foods	YoguFarm	Human use
<i>Bacillus subtilis</i> spp. Natto	various	Natto	Human use
<i>Bacillus subtilis</i> SC-8	various	Cheonggukjang	Human use
<i>Bacillus subtilis</i> CH201/DSM5750	Christian Hansen	BioPlus2B®	Veterinary Use
<i>Bacillus subtilis</i> PB6	Kenim®	CLOSTAT®	Veterinary Use

Table 1: Non-limiting list of *Bacillus subtilis* strains.

- 5 In some embodiments, the *Bacillus* cell has been genetically engineered to become non-sporulating, for example by deletion or functional inactivation of Spo0A. Spo0A is a DNA-binding protein which influences, directly or indirectly, the expression of more than 500 genes during the early stage of sporulation. Suitable functional inactivation of Spo0A includes deletion of the phosphorylation site at which Spo0F and Spo0B phosphotransferases phosphorylate Spo0A. Deletion or functional inactivation of the Spo0F and Spo0B phosphotransferase phosphorylation site leads
- 10

to a functional inactivation of Spo0A and thereby impairs the *Bacillus* cell's ability to sporulate. Alternatively, the gene or nucleotide sequence encoding Spo0A, or part of the gene or nucleotide sequence encoding Spo0A may be deleted from the *Bacillus* cell's genome.

- 5 In an additional and/or alternative embodiment, the *Bacillus* cell has been genetically engineered by deletion or functional inactivation of the gene(s) encoding sigma factor SigE (*sigE*) and/or sigma factor SigF (*sigF*). Sigma factors SigE and SigF are transcription factors that are involved in the expression of genes which encode proteins that are involved in the early phase of sporulation.

- 10 Deletion or functional inactivation of Spo0A, SigE and/or SigF impairs the sporulation ability of a *Bacillus* cell to sporulate. Such non-sporulating *Bacillus* cell may be used as a progenitor for generating a *Bacillus* cell that is able to produce a sialylated oligosaccharide.

- Wild-type *Bacillus* cells neither synthesize lactose intracellularly nor do they internalize exogenous lactose. However, lactose is the acceptor substrate for a sialic acid moiety by a lactose-accepting sialyltransferase in the formation of some sialylated oligosaccharides such as 3'-sialyllactose (3-SL) or 6'-sialyllactose (6-SL). Hence, to be able to produce such sialylated oligosaccharides, the *Bacillus* cell has to have the capability of providing lactose to the lactose-accepting sialyltransferase,
15 either by generating lactose intracellularly and/or by internalizing exogenous lactose. Moreover, lactose is typically the disaccharide moiety most if - not all - sialylated HMOs bear. Hence, internalization of lactose is also required for the biosynthesis of other sialylated HMOs than trisaccharides, i.e. when the acceptor substrate for the NeuNAc moiety is an oligosaccharide rather than the disaccharide lactose.

- 25 In an embodiment, the *Bacillus* cell for producing a sialylated oligosaccharide possesses a lactose permease for internalization of exogenous lactose. Hence, the *Bacillus* cell is able to internalize exogenous lactose. Alternatively, the *Bacillus* cell may be genetically engineered to synthesize lactose intracellularly from glucose and galactose such that an exogenous supply of lactose will not be necessary for the
30 production of a sialylated oligosaccharide.

The term "exogenous" with respect to lactose as used herein refers to lactose that does not originate from the *Bacillus* cell for the production of the sialylated oligosaccharide, i.e. being intracellularly synthesized by said *Bacillus* cell, but which originates from outside of said *Bacillus* cell and which is added to the culture
5 medium in which the *Bacillus* cell for producing the sialylated oligosaccharide is grown to produce the sialylated oligosaccharide.

In some embodiments, the *Bacillus* cell has been genetically engineered to be able to internalize exogenous lactose, i.e. it has been genetically engineered to possess a lactose permease. Thus, the *Bacillus* cell for producing a sialylated oligosaccha-
10 ride possesses a heterologous lactose permease. A suitable heterologous lactose permease is *E. coli* LacY or a functional variant thereof.

The term "heterologous" as used herein with respect to proteins, polypeptides, enzymes and transporters, but also with respect to nucleic acid molecules, nucleotide sequences and/or genes, refers to a molecule that is non-native to the species
15 of the *Bacillus* cell which contains said molecule. The term "non-native" indicates that said molecule is not present in the naturally occurring or wild-type progenitor *Bacillus* cell, i.e. in the *Bacillus* cell of the same species that is most commonly occurring in nature. Thus, a "heterologous sequence" or a "heterologous nucleic acid" or "heterologous polypeptide", as used herein, is one that originates from a
20 source foreign to the *Bacillus* cell (e.g. from a different species), or, if from the same source, is modified from its original form. The heterologous sequence may be stably introduced, e.g. by transfection, transformation, conjugation or transduction, into the genome of the host microbial host cell, thus representing a genetically modified host cell. Techniques may be applied which will depend on the host cell the sequence is
25 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). Accordingly, a "heterologous polypeptide" is a polypeptide that does not naturally occur in the cell, and a "heterologous sialyltransferase" is a sialyltransferase that
30 does not naturally occur in the microbial cell.

The term “functional variant” as used herein with respect to enzymes and/or transport molecules refers to proteins or polypeptides possessing the same activity (enzymatic, catalytic or translocating) as the referenced enzyme or transporter, but which has a different amino acid sequence than the referenced enzyme or transporter molecule. Thus, a typical variant of a protein/polypeptide differs in amino acid sequence from the reference protein/polypeptide. A variant and the reference protein/polypeptide may differ in amino acid sequence by one or more substitutions, additions, and/or deletions in any combination. Hence, the term “functional variant” comprises truncated versions of the referenced protein/polypeptide which possesses the same activity as the reference protein/polypeptide. A substituted or inserted amino acid residue may or may not be one encoded by the genetic code. A variant of a protein/ 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 proteins/polypeptides may be made by mutagenesis techniques, by direct synthesis, and by other recombinant methods known to the persons skilled in the art. Within the scope of the present disclosure, also proteins and interspecies homologs are comprised by the term “variant” that have an amino acid sequence that has greater than about 60% amino acid sequence identity, preferably 65%, 70%, 75%, 80%, 85%, 90%, more preferably 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% or greater amino acid sequence identity, preferably over a region of at least about 25, 50, 100, 200, 500, 1000, or more amino acids, to the referenced polypeptide.

The term “same activity” as used herein refers to an enzymatic, catalytic or transporting activity of a protein/polypeptide in qualitative manner only. Hence, “functional variant” also comprises variants which possess an increased or decreased activity as compared to the activity of the referenced protein/polypeptide.

In various embodiments, the *Bacillus* cell has been genetically engineered to contain and express a nucleotide sequence which encodes a lactose permease, preferably a nucleotide sequence that encodes the *E. coli* lactose permease LacY (UniProtKB – P02920) or a functional variant thereof.

The *E. coli* lactose permease LacY is encoded by the protein coding region (i.e. the open reading frame) of the *E. coli lacY* gene (GenBank acc. no: NP_414877.1).

Hence, in some embodiments, the *Bacillus* cell has been genetically engineered to contain and express a nucleotide sequence encoding the protein-coding region of *E. coli lacY*.

In some embodiments, the codon usage of the nucleotide sequence encoding the
5 heterologous lactose permease is adapted to the codon usage of *Bacillus*. The
codon usage of *B. subtilis* for example is peculiar in that the overall GC-content is
below about 45 %, the GC-content of the first letter of the codons above about 51 %, the
CG-content of the second letter of the codons below about 36.1 %, and the CG-
content of the third letter of the codons below about 46 %.

10 For expression of the lactose permease, the *Bacillus* cell contains a recombinant
lactose permease gene, wherein the nucleotide sequence which encodes the
lactose permease is operably linked to expression control sequences which mediate
expression of the nucleotide sequence encoding the lactose permease.

The term "operably linked" as used herein shall mean a functional linkage between
15 a nucleotide sequence which encodes a polypeptide or protein (typically referred to
as "protein coding region", "open reading frame", and sometimes even as "gene")
and a second nucleotide sequence (the expression control sequence such as a
promoter, signal sequence, or array of transcription factor binding sites) which
effects transcription and/or translation of the nucleotide sequence which encodes
20 the polypeptide or protein. Accordingly, the term "promoter" designates desoxyribo-
nucleic acid (DNA) sequences which usually "precede" an open reading frame in a
DNA polymer and provides a site for initiation of the transcription into mRNA.

"Regulator" DNA sequences, also usually "upstream" of (i.e., preceding) an open
reading frame in a given DNA polymer, bind proteins that determine the frequency
25 (or rate) of transcriptional initiation. Collectively referred to as "promoter/regulator" or
"control" DNA sequence, these sequences which precede a selected open reading
frame (or series of open reading frames) in a functional DNA polymer cooperate to
determine whether the transcription (and eventual expression) of an open reading
frame will occur. DNA sequences which "follow" a gene in a DNA polymer and
30 provide a signal for termination of the transcription into mRNA are referred to as
transcription "terminator" sequences.

The recombinant lactose permease gene may be present as being integrated into the *Bacillus* chromosome, or present as an episomal version on an additional plasmid within the *Bacillus* cell.

5 Expression of the heterologous lactose permease gene in the *Bacillus* cell enables the resulting *Bacillus* cell to internalize exogenously supplied lactose from the culture medium. The internalized lactose can then serve as an acceptor substrate for a sialic acid moiety to be transferred by a lactose-accepting sialyltransferase (see herein below), for example in the formation of 3'-SL or 6'-SL.

10 For the production of a sialylated oligosaccharide, the *Bacillus* has to be able to provide a donor substrate for transferring a NeuNAc moiety to an acceptor substrate. An exemplary donor substrate for a NeuNAc moiety is CMP-NeuNAc. Hence, the *Bacillus* cell has to be able to intracellularly produce CMP-NeuNAc for the production of sialylated oligosaccharides. For intracellular biosynthesis of CMP-NeuNAc, the *Bacillus* cell possesses a CMP-NeuNAc biosynthesis pathway (Fig. 1).
15 Thus, the *Bacillus* cell has been genetically engineered to possess a CMP-NeuNAc biosynthesis pathway.

The CMP-NeuNAc biosynthesis pathway comprises a NeuNAc salvage pathway or a sialic acid biosynthesis pathway for intracellular *de novo* biosynthesis of NeuNAc. Hence, the *Bacillus* cell possesses a CMP-NeuNAc biosynthesis pathway comprising a NeuNAc salvage pathway and/or a sialic acid biosynthesis pathway.
20

The NeuNAc salvage pathway comprise internalization of exogenous sialic acid by the *Bacillus* cell, and conversion of the internalized sialic acid to CMP-NeuNAc. For internalization of exogenous NeuNAc, the *Bacillus* cell possesses a sialic acid transporter. In some embodiments, the genetically engineered *Bacillus* cell has been
25 genetically engineered to possess a sialic acid transport for internalization of exogenous NeuNAc. A suitable sialic acid transporter is *E. coli* NanT (UniProtKB P41036). *E. coli* NanT catalyzes the proton-dependent symport of sialic acid. NanT can transport NeuNAc as well as the related sialic acids *N*-glycolylneuraminic acid (NeuNGc) and 3-keto-3-deoxy-D-glycero-D-galactononic acid (KDN). In an
30 embodiment, the genetically engineered *Bacillus* cell possesses *E. coli* NanT or a functional variant thereof.

In some embodiments, the *Bacillus* cell has been genetically engineered to contain and express a nucleotide sequence which encodes a sialic acid transporter for internalization of exogenous NeuNAc, preferably a nucleotide sequence that encodes *E. coli* NanT or a functional variant thereof.

- 5 The *E. coli* NanT is encoded by the protein -coding region of the *E. coli nanT* gene. Hence, in some embodiments, the *Bacillus* cell has been genetically engineered to contain and express a nucleotide sequence that encodes the *E. coli* sialic acid transporter NanT or a functional variant thereof.

- For expression of the sialic acid transporter, the *Bacillus* cell contains a recombinant
10 sialic acid transporter gene, wherein the nucleotide sequence that encodes the sialic acid transporter is operably linked to expression control sequences which mediate expression of the nucleotide sequence encoding the sialic acid transporter.

- The recombinant sialic acid transporter gene may be present as being integrated into the *Bacillus* chromosome, or present as an episomal version on an additional
15 plasmid in the *Bacillus* cell.

In some embodiment, the codon usage of the nucleotide sequence encoding the sialic acid transporter is adapted to the codon usage of *Bacillus*.

- In various embodiments, the CMP-NeuNAc biosynthesis pathway comprises a sialic acid biosynthesis pathway. Thus, the *Bacillus* cell may comprise a sialic acid
20 biosynthesis pathway for the intracellular biosynthesis of *N*-acetylneuraminic acid. The sialic acid biosynthesis pathway comprises the enzymatic activities of a glutamine:fructose-6-phosphate aminotransferase (Fig. 1: ①) and an *N*-acetylneuraminic acid synthase (= sialic acid synthase) (Fig. 1: ⑩). Hence, the *Bacillus* cell for the production of a sialylated oligosaccharide possesses a glutamine:fructose-6-phosphate aminotransferase and an *N*-acetylneuraminic acid synthase.
25

- The enzyme glutamine:fructose-6-phosphate aminotransferase (EC 2.6.1.16) catalyzes the conversion of fructose-6-phosphate (Frc-6P) to glucosamine-6-phosphate (GlcN-6P) using glutamine (Fig. 1: ①). This enzymatic reaction is typically considered to be the first step in the hexosamine biosynthesis pathway.
30 Alternative names of the glutamine:fructose-6-phosphate aminotransferase are D-

fructose-6-phosphate aminotransferase, GFAT, glucosamine-6-phosphate synthase, hexosephosphate aminotransferase, and L-glutamine–D-fructose-6-phosphate aminotransferase.

In an additional and/or alternative embodiment, the genetically engineered *Bacillus* cell possesses a glutamine:fructose-6-phosphate aminotransferase, preferably a heterologous a glutamine:fructose-6-phosphate aminotransferase. An example of a suitable heterologous glutamine:fructose-6-phosphate aminotransferase is derived from *E. coli*. The *E. coli* glutamine:fructose-6-phosphate aminotransferase (UniProtKB – P17169) is designated GlmS. Hence, the *Bacillus* cell possesses an *E. coli* GlmS or a functional variant of the *E. coli* GlmS. Preferably, the functional variant of the *E. coli* GlmS is a version which shows significantly reduced sensitivity to glucosamine-6-phosphate inhibition as compared to the wild-type enzyme. An example of a functional variant of the *E. coli* GlmS which shows significantly reduced sensitivity to glucosamine-6-phosphate inhibition. Examples of functional variants of the *E. coli* GlmS which shows significantly reduced sensitivity to glucosamine-6-phosphate inhibition are GlmS*54 and GlmS* as described in international application No. PCT/EP2019/063669 (incorporated herein by reference).

In an additional and/or alternative embodiment, the genetically engineered *Bacillus* cell contains a nucleic acid molecule comprising a nucleotide sequence which encodes a glutamine:fructose-6-phosphate aminotransferase, preferably the *E. coli* glutamine:fructose-6-phosphate aminotransferase GlmS, or a nucleotide sequence encoding a functional variant of the *E. coli* GlmS which shows significantly reduced sensitivity to glucosamine-6-phosphate inhibition as compared to the wild-type enzyme.

For expression of the glutamine:fructose-6-phosphate aminotransferase, the *Bacillus* cell contains a recombinant glutamine:fructose-6-phosphate aminotransferase gene, wherein the nucleotide sequence which encodes the glutamine:fructose-6-phosphate aminotransferase is operably linked to expression control sequences which mediate expression of the glutamine:fructose-6-phosphate aminotransferase open reading frame.

In an additional and/or alternative embodiment, the codon usage of the nucleotide sequence encoding the heterologous glutamine:fructose-6-phosphate aminotransferase is adapted to the codon usage of *Bacillus*.

The recombinant glutamine:fructose-6-phosphate aminotransferase gene may be
5 integrated into the *Bacillus* chromosome, or present as an episomal version on a plasmid within the *Bacillus* cell.

In an additional and/or alternative embodiment, the genetically engineered *Bacillus* cell comprises a sialic acid synthase. The sialic acid synthase catalyzes the condensation of *N*-acetylmannosamine (ManNAc) and phosphoenolpyruvate (PEP) to
10 *N*-acetylneuraminic acid (NeuNAc) (Fig. 1: ⑩). The enzymatic formation of NeuNAc is the last step of the sialic acid biosynthesis pathway.

In an additional and/or alternative embodiment, the genetically engineered *Bacillus* cell comprises a sialic acid synthase or a functional variant thereof, preferably a heterologous sialic acid synthase. Examples of sialic acid synthases are known from
15 a variety of bacterial species such as *Campylobacter jejuni*, *Streptococcus agalactiae*, *Butyrivibrio proteoclasticus*, *Methanobrevibacter ruminantium*, *Acetobacterium woodii*, *Desulfobacula toluolica*, *Escherichia coli*, *Prevotella nigescens*, *Halorhabdus tiamatea*, *Desulfotignum phosphitoxidans*, or *Candidatus Scalindua* sp., *Idomarina loihiensis*, *Fusobacterium nucleatum* or *Neisseria meningitidis*. An example of a
20 suitable sialic acid synthase is the *N*-acetylneuraminic acid synthase NeuB of *C. jejuni* as encoded by the *C. jejuni* *neuB* gene.

Thus, in an additional and/or alternative embodiment, the genetically engineered *Bacillus* cell contains a nucleic acid molecule comprising and expressing a nucleotide sequence which encodes a *N*-acetylneuraminic acid synthase. Hence, the
25 nucleotide sequence which encodes a *N*-acetylneuraminic acid synthase is operably linked to at least one nucleic acid expression control sequence effecting transcription and/or translation of said nucleotide sequence which encodes a *N*-acetylneuraminic acid synthase in the genetically engineered *Bacillus* cell to provide intracellular *N*-acetylneuraminic acid synthase activity.

In an additional and/or alternative embodiment, the codon usage of the nucleotide sequence encoding the heterologous *N*-acetylneuraminic acid synthase is adapted to the codon usage of *Bacillus*.

The recombinant *N*-acetylneuraminic acid synthase gene may be integrated into the
5 *Bacillus* chromosome, or present as an episomal version on a plasmid within the *Bacillus* cell.

In an embodiment, the sialic acid biosynthesis pathway comprises uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc) as an intermediate compound. The *Bacillus* cell comprising a sialic acid biosynthesis pathway which comprises UDP-
10 GlcNAc as intermediate compound further possesses a phosphoglucosamine mutase (Fig. 1: ②), a glucosamine-1-phosphate *N*-acetyltransferase (Fig. 1: ③), an *N*-acetylglucosamine-1-phosphate uridyltransferase (Fig. 1 ④), and a UDP-*N*-acetylglucosamine 2-epimerase (Fig. 1: ④).

The phosphoglucosamine mutase converts glucosamine-6-phosphate (GlcN-6P) to
15 glucosamine-1-phosphate (GlcN-1P).

In an additional and/or alternative embodiment, the *Bacillus* cell has been genetically engineered to possess a phosphoglucosamine mutase. A suitable phosphoglucosamine mutase is the *E. coli* phosphoglucosamine mutase GlmM or a functional variant thereof. Said *E. coli* GlmM is encoded by the *E. coli glmM* gene.

20 In an additional and/or alternative embodiment, the *Bacillus* cell has been genetically engineered to contain and express a nucleotide sequence which encodes a phosphoglucosamine mutase, preferably a nucleotide sequence that encodes the *E. coli* GlmM or a functional variant thereof.

The *E. coli* phosphoglucosamine mutase GlmM is encoded by the protein coding
25 region of the *E. coli glmM* gene. Hence, the *Bacillus* cell has been genetically engineered to contain and express a nucleotide sequence that encodes the *E. coli* GlmM.

Thus, in an additional and/or alternative embodiment, the genetically engineered *Bacillus* cell contains a nucleic acid molecule comprising and expressing a

nucleotide sequence which encodes a phosphoglucosamine mutase. Hence, the nucleotide sequence which encodes a phosphoglucosamine mutase is operably linked to at least one nucleic acid expression control sequence effecting transcription and/or translation of said nucleotide sequence which encodes a phosphoglucosamine mutase in the genetically engineered *Bacillus* cell to provide intracellular phosphoglucosamine mutase activity.

In an additional and/or alternative embodiment, the codon usage of the nucleotide sequence encoding the heterologous phosphoglucosamine mutase is adapted to the codon usage of *Bacillus*.

10 The recombinant phosphoglucosamine mutase gene may be integrated into the *Bacillus* chromosome, or present as an episomal version on a plasmid within the *Bacillus* cell.

The glucosamine-1-phosphate N-acetyltransferase catalyzes the transfer of an acetyl group from acetyl coenzyme A to glucosamine-1-phosphate (GlcN-1-P) to produce N-acetylglucosamine-1-phosphate (GlcNAc-1-P).

In an additional and/or alternative embodiment, the *Bacillus* cell has been genetically engineered to possess a glucosamine-1-phosphate N-acetyltransferase.

In an additional and/or alternative embodiment, the *Bacillus* cell has been genetically engineered to contain and express a nucleotide sequence which encodes a glucosamine-1-phosphate N-acetyltransferase.

Thus, in an additional and/or alternative embodiment, the genetically engineered *Bacillus* cell contains a nucleic acid molecule comprising and expressing a nucleotide sequence which encodes a glucosamine-1-phosphate N-acetyltransferase. Hence, the nucleotide sequence which encodes a glucosamine-1-phosphate N-acetyltransferase is operably linked to at least one nucleic acid expression control sequence effecting transcription and/or translation of said nucleotide sequence which encodes a glucosamine-1-phosphate N-acetyltransferase in the genetically engineered *Bacillus* cell to provide intracellular glucosamine-1-phosphate N-acetyltransferase activity.

In an additional and/or alternative embodiment, the codon usage of the nucleotide sequence encoding the heterologous glucosamine-1-phosphate *N*-acetyltransferase is adapted to the codon usage of *Bacillus*.

- 5 The recombinant glucosamine-1-phosphate *N*-acetyltransferase gene may be integrated into the *Bacillus* chromosome, or present as an episomal version on a plasmid within the *Bacillus* cell.

N-acetylglucosamine-1-phosphate uridyltransferase converts *N*-acetylglucosamine-1-phosphate (GlcNAc-1-P) into UDP-GlcNAc by the transfer of uridine 5-monophosphate (from uridine 5-triphosphate).

- 10 In an additional and/or alternative embodiment, the *Bacillus* cell has been genetically engineered to possess a *N*-acetylglucosamine-1-phosphate uridyltransferase.

In an additional and/or alternative embodiment, the *Bacillus* cell has been genetically engineered to contain and express a nucleotide sequence which encodes a *N*-acetylglucosamine-1-phosphate uridyltransferase.

- 15 Thus, in an additional and/or alternative embodiment, the genetically engineered *Bacillus* cell contains a nucleic acid molecule comprising and expressing a nucleotide sequence which encodes a *N*-acetylglucosamine-1-phosphate uridyltransferase. Hence, the nucleotide sequence which encodes a *N*-acetylglucosamine-1-phosphate uridyltransferase is operably linked to at least one nucleic acid expression
20 control sequence effecting transcription and/or translation of said nucleotide sequence which encodes a *N*-acetylglucosamine-1-phosphate uridyltransferase in the genetically engineered *Bacillus* cell to provide intracellular *N*-acetylglucosamine-1-phosphate uridyltransferase activity.

- 25 In an additional and/or alternative embodiment, the codon usage of the nucleotide sequence encoding the heterologous *N*-acetylglucosamine-1-phosphate uridyltransferase is adapted to the codon usage of *Bacillus*.

The recombinant *N*-acetylglucosamine-1-phosphate uridyltransferase gene may be integrated into the *Bacillus* chromosome, or present as an episomal version on a plasmid within the *Bacillus* cell.

In an additional and/or alternative embodiment, the glucosamine-1-phosphate *N*-acetyltransferase activity and the *N*-acetylglucosamine-1-phosphate uridyltransferase activity are provided by a bifunctional enzyme. An example of such bifunctional enzyme is *E. coli* GlmU. *E. coli* GlmU catalyzes the last two sequential
5 reactions which convert glucosamine-1-phosphate to UDP-GlcNAc (Fig. 1: ③). The C-terminal domain catalyzes the transfer of acetyl group from acetyl coenzyme A to glucosamine-1-phosphate (GlcN-1-P) to produce *N*-acetylglucosamine-1-phosphate (GlcNAc-1-P), which is converted into UDP-GlcNAc by the transfer of uridine 5-monophosphate (from uridine 5-triphosphate), a reaction catalyzed by the
10 N-terminal domain.

Such bifunctional enzyme is also known from *Bacillus subtilis* and *Haemophilus influenzae*.

In an additional and/or alternative embodiment, the *Bacillus* cell has been genetically engineered to possess a heterologous bifunctional glucosamine-1-phosphate
15 *N*-acetyltransferase / *N*-acetylglucosamine-1-phosphate uridyltransferase. A suitable example is *E. coli* GlmU or a functional variant thereof (including the *H. influenzae* GlmU). Said *E. coli* GlmU is encoded by the *E. coli glmU* gene.

In an additional and/or alternative embodiment, the *Bacillus* cell has been genetically engineered to contain and express or overexpress a nucleotide sequence
20 which encodes said bifunctional enzyme, preferably a nucleotide sequence that encodes the *E. coli* GlmU or a functional variant thereof.

Thus, in an additional and/or alternative embodiment, the genetically engineered *Bacillus* cell contains a nucleic acid molecule comprising and expressing a nucleotide sequence which encodes the bifunctional glucosamine-1-phosphate
25 *N*-acetyltransferase / *N*-acetylglucosamine-1-phosphate uridyltransferase. Hence, the nucleotide sequence which encodes a bifunctional glucosamine-1-phosphate *N*-acetyltransferase / *N*-acetylglucosamine-1-phosphate uridyltransferase is operably linked to at least one nucleic acid expression control sequence effecting transcription and/or translation of said nucleotide sequence which encodes a
30 bifunctional glucosamine-1-phosphate *N*-acetyltransferase / *N*-acetylglucosamine-1-phosphate uridyltransferase in the genetically engineered *Bacillus* cell to provide

intracellular glucosamine-1-phosphate *N*-acetyltransferase activity and
N-acetylglucosamine-1-phosphate uridylyltransferase activity.

In an additional and/or alternative embodiment, the codon usage of the nucleotide
sequence encoding the heterologous bifunctional glucosamine-1-phosphate
5 *N*-acetyltransferase / *N*-acetylglucosamine-1-phosphate uridylyltransferase is adapted
to the codon usage of *Bacillus*.

The recombinant bifunctional glucosamine-1-phosphate *N*-acetyltransferase / *N*-
acetylglucosamine-1-phosphate uridylyltransferase gene may be integrated into the
Bacillus chromosome, or present as an episomal version on a plasmid within the
10 *Bacillus* cell.

In an additional and/or alternative embodiment, the sialic acid biosynthesis pathway
comprises the conversion of UDP-GlcNAc to *N*-acetylmannosamine (ManNAc). This
conversion can be catalyzed by an UDP-*N*-acetylglucosamine 2-epimerase which
does not just convert UDP-*N*-acetylglucosamine to UDP-*N*-acetylmannosamine, but
15 releases UDP concomitantly (Fig. 1: ④).

Hence, the *Bacillus* cell comprises an UDP-*N*-acetylglucosamine 2-epimerase
epimerase which releases UDP concomitantly.

A suitable UDP-*N*-acetylglucosamine 2-epimerase epimerase with concomitant
release of UDP is the *Campylobacter jejuni* UDP-*N*-acetylglucosamine 2-epimerase
20 NeuC, encoded by the *C. jejuni neuC* gene.

In an additional and/or alternative embodiment, the *Bacillus* cell has been geneti-
cally engineered to possess an UDP-*N*-acetylglucosamine 2-epimerase with
concomitant release of UDP.

In an additional and/or alternative embodiment, the *Bacillus* cell has been
25 genetically engineered to contain and express a nucleotide sequence which
encodes a UDP-*N*-acetylglucosamine 2-epimerase which releases UDP
concomitantly, preferably to contain and express a nucleotide sequence that
encodes the *C. jejuni* NeuC or a functional variant thereof.

The *C. jejuni* UDP-*N*-acetylglucosamine 2-epimerase NeuC is encoded by the protein coding region of the *C. jejuni neuC* gene. Hence, the *Bacillus* cell has been genetically engineered to contain and express a nucleotide sequence that encodes the *C. jejuni* NeuC.

- 5 Thus, in an additional and/or alternative embodiment, the genetically engineered *Bacillus* cell contains a nucleic acid molecule comprising and expressing a nucleotide sequence which encodes the UDP-*N*-acetylglucosamine 2-epimerase with concomitant release of UDP during its enzymatic reaction to release ManNAc. Hence, the nucleotide sequence which encodes a UDP-*N*-acetylglucosamine 2-epi-
- 10 merase which releases UDP concomitantly is operably linked to at least one nucleic acid expression control sequence effecting transcription and/or translation of said nucleotide sequence which encodes said UDP-*N*-acetylglucosamine 2-epimerase in the genetically engineered *Bacillus* cell to provide intracellular UDP-*N*-acetylglucosamine 2-epimerase activity.
- 15 In an additional and/or alternative embodiment, the codon usage of the nucleotide sequence encoding the heterologous UDP-*N*-acetylglucosamine 2-epimerase with concomitant release of UDP is adapted to the codon usage of *Bacillus*.

The recombinant UDP-*N*-acetylglucosamine 2-epimerase gene may be integrated into the *Bacillus* chromosome, or present as an episomal version on a plasmid

20 within the *Bacillus* cell.

In an alternative embodiment, the sialic acid biosynthesis pathway utilizes *N*-acetylglucosamine-6-phosphate (GlcNAc-6-P) as an intermediate, but not utilize UDP-GlcNAc. The genetically engineered *Bacillus* cell comprises a sialic acid biosynthesis pathway for the intracellular biosynthesis of *N*-acetylneuraminic acid which

25 uses GlcNAc-6-P as intermediate comprises a glucosamine-6-phosphate *N*-acetyltransferase (Fig. 1: (5)). A sialic acid biosynthesis pathway using a glucosamine-6-phosphate *N*-acetyltransferase for the intracellular biosynthesis of *N*-acetylneuraminic acid does not utilize UDP-GlcNAc for the biosynthesis of sialic acid.

The sialic acid biosynthesis pathway using GlcNAc-6P comprises the enzymatic activities of a glutamine:fructose-6-phosphate aminotransferase (Fig. 1: ①) and an *N*-acetylneuraminic acid synthase (Fig. 1: ⑩). Said sialic acid biosynthesis pathway further comprises a) the enzymatic activities of a glucosamine-6-phosphate *N*-acetyltransferase (Fig. 1: ⑤), an *N*-acetylglucosamine-6-phosphate phosphatase (Fig. 1: ⑥) and an *N*-acetylglucosamine 2-epimerase (Fig. 1: ⑧) or b) the enzymatic activities of a glucosamine-6-phosphate *N*-acetyltransferase (Fig. 1: ⑤), an *N*-acetylglucosamine-6-phosphate epimerase (Fig. 1: ⑦) and an *N*-acetylmannosamine-6-phosphate phosphatase (Fig. 1: ⑨). Therefore, it is not necessary that the genetically engineered *Bacillus* cell comprises the enzymatic activities of a phosphoglucosamine mutase, an *N*-acetylglucosamine-1-phosphate uridyltransferase and an UDP *N*-acetylglucosamine 2-epimerase with concomitant release of UDP for intracellular sialic acid biosynthesis. Thus, in an additional and/or alternative embodiment, the genetically engineered *Bacillus* cell being capable of synthesizing sialic acid does not comprise one or more enzymatic activities selected from the group consisting of the enzymatic activities of a phosphoglucosamine mutase, an *N*-acetylglucosamine-1-phosphate uridyltransferase and an UDP *N*-acetylglucosamine 2-epimerase with concomitant release of UDP.

In an additional and/or alternative embodiment, the genetically engineered *Bacillus* cell possesses glucosamine-6-phosphate *N*-acetyltransferase activity. Said glucosamine-6-phosphate *N*-acetyltransferase activity converts GlcN-6P to *N*-acetylglucosamine-6-phosphate (GlcNAc-6P). An example of a glucosamine-6-phosphate *N*-acetyltransferase is the *Saccharomyces cerevisiae* Gna1 (UniProtKB – P43577).

In an additional and/or alternative embodiment, the genetically engineered *Bacillus* cell contains a glucosamine-6-phosphate *N*-acetyltransferase, preferably a heterologous glucosamine-6-phosphate *N*-acetyltransferase, more preferably Gna1 from *S. cerevisiae* or a functional variant thereof.

Thus, in an additional and/or alternative embodiment, the genetically engineered *Bacillus* cell contains a nucleic acid molecule comprising and expressing a nucleotide sequence encoding a glucosamine-6-phosphate *N*-acetyltransferase,

preferably *S. cerevisiae* Gna1, wherein said nucleotide sequence is operably linked to at least one nucleic acid expression control sequence effecting transcription and/or translation of said nucleotide sequence in the genetically engineered *Bacillus* cell to provide intracellular glucosamine-6-phosphate *N*-acetyltransferase activity.

- 5 In an additional and/or alternative embodiment, the codon usage of the nucleotide sequence encoding the heterologous glucosamine-6-phosphate *N*-acetyltransferase is adapted to the codon usage of *Bacillus*.

The recombinant glucosamine-6-phosphate *N*-acetyltransferase gene may be integrated into the *Bacillus* chromosome, or present as an episomal version on a
10 plasmid within the *Bacillus* cell.

In an additional and/or alternative embodiment, the genetically engineered *Bacillus* cell possesses a *N*-acetylglucosamine-6-phosphate phosphatase activity. Said *N*-acetylglucosamine-6-phosphate phosphatase activity converts GlcNAc-6P to *N*-acetylglucosamine (GlcNAc). Examples of an *N*-acetylglucosamine-6-phosphate
15 phosphatase are sugar phosphatases of the HAD-like superfamily which catalyze the conversion of GlcNAc6P to GlcNAc. The HAD-like superfamily of enzymes is named after the bacterial enzyme haloacid dehydrogenase and includes phosphatases. A suitable phosphatase of the HAD-like superfamily catalyzing the conversion of GlcNAc6P to GlcNAc may be selected from the group consisting of fructose-1-
20 phosphate phosphatase (YqaB, UniProtKB - P77475) and alpha-D-glucose 1-phosphate phosphatase (YihX, UniProtKB – P0A8Y3). The *E. coli* YqaB and *E. coli* YihX enzymes are considered to also act on GlcNAc6P (Lee, S.-W. and Oh, M.-K. (2015) Metabolic Engineering 28: 143-150).

In an additional and/or alternative embodiment, the sugar phosphatase of the HAD-
25 like superfamily catalyzing the conversion of GlcNAc-6P to GlcNAc is a heterologous enzyme in the genetically engineered *Bacillus* cell. In an additional and/or alternative embodiment, the sugar phosphatase of the HAD-like superfamily catalyzing the conversion of GlcNAc6P to GlcNAc is selected from the group consisting of *E. coli* YqaB, *E. coli* YihX, and functional variants thereof.

30 In an additional and/or alternative embodiment, the genetically engineered *Bacillus* cell contains a nucleic acid molecule which comprises and expresses a nucleotide

sequence encoding a sugar phosphatase of the HAD-like superfamily catalyzing the conversion of GlcNAc6P to GlcNAc. In an additional and/or alternative embodiment, the nucleotide sequence encoding the sugar phosphatase of the HAD-like superfamily catalyzing the conversion of GlcNAc6P to GlcNAc is a heterologous nucleotide sequence. In an additional and/or alternative embodiment, the nucleotide sequence encoding the sugar phosphatase of the HAD-like superfamily catalyzing the conversion of GlcNAc6P to GlcNAc encodes the *E. coli* YqaB or *E. coli* YihX or a functional variant of one of these two enzymes.

In an additional and/or alternative embodiment, the codon usage of the nucleotide sequence encoding the heterologous *N*-acetylglucosamine-6-phosphate phosphatase is adapted to the codon usage of *Bacillus*.

The recombinant *N*-acetylglucosamine-6-phosphate phosphatase gene may be integrated into the *Bacillus* chromosome, or present as an episomal version on a plasmid within the *Bacillus* cell.

In an additional and/or alternative embodiment, the genetically engineered *Bacillus* cell possesses *N*-acetylglucosamine 2-epimerase activity. *N*-acetylglucosamine 2-epimerase (EC 5.1.3.8) is an enzyme that catalyzes the conversion of *N*-acetylglucosamine (GlcNAc) to *N*-acetylmannosamine (ManNAc). The enzyme is a racemase acting on carbohydrates and their derivatives. The systematic name of this enzyme class is *N*-acyl-D-glucosamine 2-epimerase. This enzyme participates in amino-sugar metabolism and nucleotide-sugar metabolism, preferably a heterologous *N*-acetylglucosamine 2-epimerase.

In an additional and/or alternative embodiment, the genetically engineered *Bacillus* cell comprises an *N*-acetylglucosamine 2-epimerase, preferably a heterologous *N*-acetylglucosamine 2-epimerase. Examples of *N*-acetylglucosamine 2-epimerases were described from *Anabena variabilis*, *Acaryochloris* sp., *Nostoc* sp., *Nostoc punctiforme*, *Bacteroides ovatus* or *Synechocystis* sp. An example of a suitable *N*-acetylglucosamine 2-epimerase is the *N*-acetylglucosamine 2-epimerase of *B. ovatus* ATCC 8483 (UniProtKB - A7LVG6) as encoded by gene BACOVA_01816. Another example is the *N*-acetylglucosamine 2-epimerase of *Synechocystis* sp.

(strain PCC 6803) (UniProtKB - P74124) which is also known as renin-binding protein and is encoded by the *slr1975* gene.

In an additional and/or alternative embodiment, the genetically engineered *Bacillus* cell contains a nucleic acid molecule comprising a nucleotide sequence which
5 encodes an *N*-acetylglucosamine 2-epimerase, preferably the *N*-acetylglucosamine 2-epimerase of *B. ovatus* ATCC 8483 or *Synechocystis* sp. (strain PCC 6803) or a functional variant thereof.

Hence, the nucleotide sequence which encodes the *N*-acetylglucosamine 2-epimerase is operably linked to at least one nucleic acid expression control sequence
10 effecting transcription and/or translation of said nucleotide sequence which encodes a *N*-acetylglucosamine 2-epimerase in the genetically engineered *Bacillus* cell to provide intracellular *N*-acetylglucosamine 2-epimerase activity.

In an additional and/or alternative embodiment, the codon usage of the nucleotide sequence encoding the heterologous *N*-acetylglucosamine 2-epimerase is adapted
15 to the codon usage of *Bacillus*.

The recombinant *N*-acetylglucosamine 2-epimerase gene may be integrated into the *Bacillus* chromosome, or present as an episomal version on a plasmid within the *Bacillus* cell.

In an additional and/or alternative embodiment, the genetically engineered *Bacillus*
20 cell possesses *N*-acetylglucosamine-6-phosphate epimerase activity and *N*-acetylmannosamine-6-phosphate phosphatase activity. *N*-acetylglucosamine-6-phosphate epimerase converts *N*-acetylglucosamine-6-phosphate (GlcNAc-6P) to *N*-acetylmannosamine-6-phosphate (ManNAc-6P), whereas *N*-acetylmannosamine-6-phosphate phosphatase dephosphorylates ManNAc-6P to give *N*-acetylmanno-
25 samine (ManNAc). Possessing *N*-acetylglucosamine-6-phosphate epimerase activity and *N*-acetylmannosamine-6-phosphate phosphatase activity provides an additional or alternative way for providing ManNAc for Neu5Ac production.

In an additional and/or alternative embodiment, the genetically engineered *Bacillus* cell contains an *N*-acetylglucosamine-6-phosphate epimerase. An example of a

suitable *N*-acetylglucosamine-6-phosphate epimerase is *E. coli* NanE (UniprotKB P0A761) as encoded by the *E. coli nanE* gene.

Thus, in an additional and/or alternative embodiment, the genetically engineered *Bacillus* cell contains a nucleic acid molecule comprising and expressing a nucleotide sequence encoding an *N*-acetylglucosamine-6-phosphate epimerase, preferably
5 a nucleotide sequence encoding *E. coli* NanE or a functional variant thereof.

Thus, in an additional and/or alternative embodiment, the genetically engineered *Bacillus* cell contains a nucleic acid molecule comprising and expressing a nucleotide sequence encoding the *N*-acetylglucosamine-6-phosphate epimerase, wherein
10 said nucleotide sequence is operably linked to at least one nucleic acid expression control sequence effecting transcription and/or translation of said nucleotide sequence in the genetically engineered microbial cell to provide intracellular *N*-acetylglucosamine-6-phosphate epimerase activity.

In an additional and/or alternative embodiment, the codon usage of the nucleotide sequence encoding the heterologous *N*-acetylglucosamine-6-phosphate epimerase
15 is adapted to the codon usage of *Bacillus*.

In an additional and/or alternative embodiment, the genetically engineered *Bacillus* cell contains an *N*-acetylmannosamine-6-phosphate phosphatase which converts ManNAc-6P to ManNAc.

20 Thus, in additional and/or alternative embodiment, the genetically engineered *Bacillus* cell contains a nucleic acid molecule comprising and expressing a nucleotide sequence encoding an *N*-acetylmannosamine-6-phosphate phosphatase. Hence, the nucleotide sequence which encodes the *N*-acetylmannosamine-6-phosphate phosphatase is operably linked to at least one nucleic acid expression
25 control sequence effecting transcription and/or translation of said nucleotide sequence which encodes a *N*-acetylmannosamine-6-phosphate phosphatase in the genetically engineered *Bacillus* cell to provide intracellular *N*-acetylmannosamine-6-phosphate phosphatase activity.

In an additional and/or alternative embodiment, the codon usage of the nucleotide sequence encoding the heterologous *N*-acetylmannosamine-6-phosphate phosphatase is adapted to the codon usage of *Bacillus*.

5 The recombinant *N*-acetylmannosamine-6-phosphate phosphatase gene may be integrated into the *Bacillus* chromosome, or present as an episomal version on a plasmid within the *Bacillus* cell.

The genetically engineered *Bacillus* cell possesses cytidine 5'-monophospho- (CMP)-*N*-acetylneuraminic acid synthetase (Fig. 1: ⑪) activity for transferring cytidine 5'-monophosphate onto *N*-acetylneuraminic acid to generate a CMP-activated *N*-acetylneuraminic acid (CMP-NeuNAc). Several 5'-monophospho- (CMP)-sialic acid synthetases are known in the art and have been described, e.g. 5'-monophospho- (CMP)-sialic acid synthetases from *E. coli*, *Neisseria meningitidis*, *Campylobacter jejuni*, *Streptococcus sp.*, etc.

15 In an additional and/or alternative embodiment, the genetically engineered *Bacillus* cell contains a cytidine 5'-monophospho- (CMP)-*N*-acetylneuraminic acid synthetase, preferably a heterologous cytidine 5'-monophospho- (CMP)-*N*-acetylneuraminic acid synthetase, more preferably the *N*-acetylneuraminate cytidyltransferase NeuA from *C. jejuni*. The *C. jejuni* NeuA is encoded by the *C. jejuni neuA* gene.

20 Thus, in an additional and/or alternative embodiment, the genetically engineered *Bacillus* cell contains a nucleic acid molecule comprising and expressing a nucleotide sequence encoding a cytidine 5'-monophospho- (CMP)-*N*-acetylneuraminic acid synthetase, wherein said nucleotide sequence is operably linked to at least one nucleic acid expression control sequence effecting transcription and/or translation of said nucleotide sequence in the genetically engineered microbial cell to provide
25 *N*-acetylneuraminate cytidyltransferase activity.

In an additional and/or alternative embodiment, the codon usage of the nucleotide sequence encoding the heterologous cytidine 5'-monophospho- (CMP)-*N*-acetylneuraminic acid synthetase is adapted to the codon usage of *Bacillus*.

The recombinant cytidine 5'-monophospho- (CMP)-*N*-acetylneuraminic acid synthetase gene may be integrated into the *Bacillus* chromosome, or present as an episomal version on a plasmid within the *Bacillus* cell.

The genetically engineered *Bacillus* cell possesses a sialyltransferase, preferably a
5 heterologous sialyltransferase, and more preferably a sialyltransferase activity selected from the group consisting of α -2,3-sialyltransferase activity, α -2,6-sialyltransferase activity and/or α -2,8-sialyltransferase activity. The sialyltransferase activity is capable of transferring the *N*-acetylneuraminic acid moiety from the CMP-NeuNAc to an acceptor molecule, wherein said acceptor molecule is a saccharide
10 molecule, to provide a sialylated saccharide.

In an additional and/or alternative embodiment, the genetically engineered microbial cell contains at least one sialyltransferase, preferably at least one heterologous sialyltransferase, wherein said sialyltransferase is capable of possessing an α -2,3-sialyltransferase activity and/or an α -2,6-sialyltransferase activity and/or an α -2,8-sialyltransferase activity for transferring the NeuNAc moiety from CMP-NeuNAc as
15 donor substrate to the acceptor saccharide. Exemplary sialyltransferases and their genes are identified in Table 2.

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

The heterologous sialyltransferase is capable of transferring a sialic acid residue from a donor substrate to an acceptor molecule. The term “capable of” with respect to the heterologous sialyltransferase refers to the sialyltransferase activity of the heterologous sialyltransferase and the provision that suitable reaction conditions are required for the heterologous sialyltransferase to possess its enzymatic activity. In the absence of suitable reaction conditions, the heterologous sialyltransferase does not possess its enzymatic activity, but retains its enzymatic activity and possesses its enzymatic activity when suitable reaction conditions are restored. Suitable reaction conditions include the presence of a suitable donor substrate, the presence of suitable acceptor molecules, the presence of essential cofactors such as – for example – monovalent or divalent ions, a pH value in an appropriate range, a suitable temperature and the like. It is not necessary that the optimum values for each and every factor effecting the enzymatic reaction of the heterologous sialyltransferase is met, but the reaction conditions have to be such that the heterologous sialyltransferase performs its enzymatic activity. Accordingly, the term “capable of” excludes any conditions upon which the enzymatic activity of the heterologous sialyltransferase has been irreversibly impaired and also excluded exposure of the heterologous sialyltransferase to any such condition. Instead, “capable of” means that the sialyltransferase is enzymatically active, i.e. possesses its sialyltransferase activity, if permissive reactions conditions (where all requirements being necessary for the sialyltransferase to perform its enzymatic activity) are provided to the sialyltransferase.

Sialyltransferases can be distinguished on the type of sugar linkage they form. As used herein, the terms “ α -2,3-sialyltransferase” and “ α -2,3-sialyltransferase activity” refer to polypeptides and their enzymatic activity which add a sialic acid residue with an α -2,3 linkage to galactose, *N*-acetylgalactosamine or a galactose or *N*-acetylgalactosamine residue of the acceptor molecule. Likewise, the terms “ α -2,6-sialyltransferase” and “ α -2,6-sialyltransferase activity” refer to polypeptides and their enzymatic activity which add a sialic acid residue with an α -2,6 linkage to galactose, *N*-acetylgalactosamine or a galactose or *N*-acetylgalactosamine residue of the acceptor molecule. Likewise, the terms “ α -2,8-sialyltransferase” and “ α -2,8-sialyltransferase activity” refer to polypeptides and their enzymatic activity which add a

sialic acid residue with an α -2,8 linkage to galactose, *N*-acetylgalactosamine or a galactose or *N*-acetylgalactosamine residue of the acceptor molecule.

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

10 In another embodiment, the heterologous sialyltransferase is capable of possessing α -2,6-sialyltransferase activity.

In an additional and/or alternative embodiment, the genetically engineered *Bacillus* cell contains and expresses a nucleic acid molecule which comprises at least one nucleotide sequence encoding said heterologous sialyltransferase being capable of
15 possessing α -2,6-sialyltransferase activity.

In an additional and/or alternative embodiment, the heterologous sialyltransferase is capable of possessing α -2,8-sialyltransferase activity. An example of a heterologous sialyltransferase is capable of possessing α -2,8-sialyltransferase activity is the sialyltransferase CstII of *Campylobacter jejuni* OH4384.

20 The sialyltransferase is capable of transferring a sialic acid residue, e.g. a *N*-acetylneuraminic acid (Neu5Ac) residue, from a donor substrate, e.g. CMP-Neu5Ac, to an acceptor molecule. The acceptor molecule is a saccharide molecule, preferably a saccharide molecule set forth in Table 3.

In an additional and/or alternative embodiment, the genetically engineered microbial
25 cell contains a nucleic acid molecule which comprises and expresses at least one nucleotide sequence encoding said heterologous sialyltransferase being capable of possessing α -2,3-sialyltransferase activity.

Origin of the sialyltransferase gene	accession number of the sialyltransferase gene	Cloned as full length (FL) gene or without signal peptide (Δ)
<i>Neisseria meningitidis</i>	U60660	FL
<i>Campylobacter jejuni</i> strain OH4384	AF130466	FL
<i>Campylobacter jejuni</i> strain OH4384	AX934425	FL
<i>Helicobacter acinonychis</i>	NC_008229	FL
<i>Helicobacter acinonychis</i>	NC_008229	FL
<i>Photobacterium</i> sp. JT-ISH-224	BAF92026	Δ 17
<i>Pasteurella dagmatis</i> strain DSM 22969	AFY98851	FL
<i>Photobacterium</i> sp. JT-ISH-224	BAF92025	Δ 20
<i>Vibrio</i> sp. JT-FAJ-16	BAF91160	Δ 22
<i>Pasteurella multocida</i> PM70	AAK02272	Δ 25
<i>Photobacterium damsela</i> JT0160	BAA25316	FL
<i>Streptococcus agalactiae</i>	AB050723	FL
<i>Haemophilus somnus</i> -2336	ACA31578	FL
<i>Haemophilus ducreyi</i> 35000HP	AF101047	FL
<i>Haemophilus ducreyi</i> 35000HP	AAP95068	FL
<i>Photobacterium phosphoreum</i> JT-ISH-467	BAF63530	Δ 20
<i>Photobacterium leiognathi</i> JT-SHIZ-119	AB500947	Δ 15
<i>Photobacterium leiognathi</i> JT-SHIZ-145	BAF91416	Δ 15
<i>Campylobacter coli</i>	YP_008473374	FL
<i>Vibrio harveyi</i>	WP_017817635	Δ 24
<i>Streptococcus entericus</i>	WP_018369230	FL
<i>Avibacterium paragallinarum</i>	WP_021724759	FL
<i>Haemophilus parahaemolyticus</i> HK385	EIJ71207	FL
<i>Alistipes</i> sp. CAG:268	CDC95697	Δ 17
<i>Alistipes</i> sp. AL-1	WP_032134786	FL
<i>Pasteurella multocida</i> PM70	NC_002663	FL
<i>Campylobacter jejuni</i> strain 81-176	AAL09368	FL
<i>Alistipes shahii</i> WAL 8301	YP_007816735	Δ 21
<i>Actinobacillus suis</i> ATCC 33415	AIJ32009	FL
<i>Actinobacillus capsulatus</i> DSM 19761	WP_018652686	FL
<i>Bibersteinia trehalosi</i> USDA-ARS-USMARC-189	AHG84654	FL

Origin of the sialyltransferase gene	accession number of the sialyltransferase gene	Cloned as full length (FL) gene or without signal peptide (Δ)
<i>Photobacterium damsela</i> subsp. <i>damsela</i> CIP 102761	EEZ40509	FL
<i>Haemophilus somnus</i> 2336	ACA31170	FL
<i>Streptococcus suis</i>	AGL48117	FL
<i>Streptococcus suis</i>	EEF64118	FL
<i>Photobacterium phosphoreum</i>	CDN97322	
<i>Alistipes timonensis</i>	WP_026020701	
<i>Haemophilus parasuis</i>	KEZ22922	
<i>Acinetobacter pittii</i>	EOQ74854	
<i>Photobacterium leiognathi</i>	BAI49484	
<i>Porphyromonas</i> sp.	KGO0080	
<i>Photobacterium damsela</i>	WP_005298232	
<i>Pasteurella multocida</i>	EGP04633	
<i>Actinobacillus ureae</i>	WP_005624339	
<i>Actinobacillus equuli</i>	WP_039196193	
<i>Acinetobacter calcoaceticus</i>	WP_017391462	
<i>Acinetobacter baumannii</i>	WP_032039596	
<i>Acinetobacter oleivorans</i>	WP_042898369	
<i>Acinetobacter baumannii</i>	ELX04805	
<i>Acinetobacter gyllenbergii</i>	WP_032865812	
<i>Shigella boydii</i>	ACD37071	
<i>Kingella kingae</i>	WP_026036248	
<i>Alsiella crassa</i>	WP_034294366	
<i>Alistipes</i> sp.	EFR56292	
<i>Streptococcus suis</i>	WP_024397787	
<i>Fusobacterium mortiferum</i>	EEO36694	
<i>Porphyromonas catoniae</i>	WP_005467220	

Table 2: List of sialyltransferase-encoding nucleotide sequences. The sialyltransferase-encoding nucleotide sequences were either cloned as full length constructs (FL) or without a predicted signal peptide (Δ) as

compared to their wild-type protein coding regions. The number behind the Δ indicates the N-terminal amino acids deleted from the corresponding sequence.

- In an additional and/or alternative embodiment, the acceptor molecule is a mono-saccharide, preferably a monosaccharide selected from the group consisting of
- 5 *N*-acetylglucosamine, galactose and *N*-acetylgalactosamine.

In an additional and/or alternative embodiment, the acceptor molecule is a disaccharide, preferably a disaccharide selected from the group consisting lactose, lactulose, *N*-acetyllactosamine, lacto-*N*-biose, lactulose and melibiose.

- 10 In an additional and/or alternative embodiment, the acceptor molecule is a trisaccharide, preferably a trisaccharide selected from the group consisting of raffinose, lacto-*N*-triose II, 2'-fucosyllactose, 3-fucosyllactose, 3'-sialyllactose, 6'-sialyllactose, 3'-sialyl-*N*-acetyllactosamine, 6'-sialyl-*N*-acetyllactosamine, 3'-galactosyllactose and 6'-galactosyllactose.

Name	Abbreviation	Structure
<i>N</i> -acetyl-glucosamine	GlcNAc	GlcNAc
Galactose	Gal	Gal
<i>N</i> -acetylgalactosamine	GalNAc	GalNAc
Lactose	Lac	Gal(β1,4)Glc
<i>N</i> -acetyllactosamine	LacNAc	Gal(β1,4)GlcNAc
Lacto- <i>N</i> -biose	LNB	Gal(β1,3)GlcNAc
Lactulose	LacU	Gal(β1,4)Frc
Melibiose	Mel	Gal(α1,6)Glc
Raffinose	Raf	Gal(α1,6)Glc(α1,2)Frc
2'-Fucosyllactose	2'-FL	Fuc(α1,2)Gal(β1,4)Glc
3-Fucosyllactose	3-FL	Gal(β1,4)[Fuc(α1,3)]Glc
2',3-Difucosyllactose	DFL	Fuc(α1,2)Gal(β1,4)[Fuc(α1,3)]Glc
6'-Galactosyllactose	6'-GL	Gal(β1,6)Gal(β1,4)Glc
3'-Galactosyllactose	3'-GL	Gal(β1,3)Gal(β1,4)Glc
Lacto- <i>N</i> -triose II	LNT II	GlcNAc(β1,3)Gal(β1,4)Glc
Lacto- <i>N</i> -tetraose	LNT	Gal(β1,3)GlcNAc(β1,3)Gal(β1,4)Glc
Lacto- <i>N</i> -neotetraose	LNnT	Gal(β1,4)GlcNAc(β1,3)Gal(β1,4)Glc
Lacto- <i>N</i> -fucopentaose I	LNFP I	Fuc(α1,2)Gal(β1,3)GlcNAc(β1,3)Gal(β1,4)Glc
Lacto- <i>N</i> -neofucopentaose I	LNnFP I	Fuc(α1,2)Gal(β1,4)GlcNAc(β1,3)Gal(β1,4)Glc

Name	Abbreviation	Structure
Lacto- <i>N</i> -fucopentaose II	LNFP II	Gal(β1,3)[Fuc(α1,4)]GlcNAc(β1,3)Gal(β1,4)Glc
Lacto- <i>N</i> -fucopentaose III	LNFP III	Gal(β1,4)[Fuc(α1,3)]GlcNAc(β1,3)Gal(β1,4)Glc
Lacto- <i>N</i> -fucopentaose V	LNFP V	Gal(β1,3)GlcNAc(β1,3)Gal(β1,4)[Fuc(α1,3)]Glc
Lacto- <i>N</i> -neofucopentaose V	LNnFP V	Gal(β1,4)GlcNAc(β1,3)Gal(β1,4)[Fuc(α1,3)]Glc
Lacto- <i>N</i> -difucohexaose I	LNDH I	Fuc(α1,2)Gal(β1,3)[Fuc(α1,4)]GlcNAc(β1,3)Gal(β1,4)Glc
Lacto- <i>N</i> -difucohexaose II	LND	Gal(β1,3)[Fuc(α1,4)]GlcNAc(β1,3)Gal(β1,4)[Fuc(α1,3)]Glc
Lacto- <i>N</i> -neodifucohexaose I	LNnDFH I	Gal(β1,4)[Fuc(α1,3)]GlcNAc(β1,3)Gal(β1,4)[Fuc(α1,3)]Glc
Lacto- <i>N</i> -hexaose	LNH	Gal(β1,4)GlcNAc(β1,6)[Gal(β1,3)GlcNAc(β1,3)]Gal(β1,4)Glc
Lacto- <i>N</i> -neohexaose	LNnH	Gal(β1,4)GlcNAc(β1,6)[Gal(β1,4)GlcNAc(β1,3)]Gal(β1,4)Glc
<i>para</i> -Lacto- <i>N</i> -hexaose	<i>para</i> LNT	Gal(β1,3)GlcNAc(β1,3)Gal(β1,4)GlcNAc(β1,3)Gal(β1,4)Glc
<i>para</i> -Lacto- <i>N</i> -neohexaose	<i>para</i> LNnH	Gal(β1,4)GlcNAc(β1,3)Gal(β1,4)GlcNAc(β1,3)Gal(β1,4)Glc
3'-Sialyllactose	3'-SL	Neu5Ac(α2,3)Gal(β1,4)Glc
6'-Sialyllactose	6'-SL	Neu5Ac(α2,6)Gal(β1,4)Glc
Sialyllacto- <i>N</i> -tetraose a	LSTa	Neu5Ac(α2,3)Gal(β1,3)GlcNAc(β1,3)Gal(β1,4)Glc
Sialyllacto- <i>N</i> -tetraose b	LSTb	Gal(β1,3)[Neu5Ac(α2,6)]GlcNAc(β1,3)Gal(β1,4)Glc
Sialyllacto- <i>N</i> -tetraose c	LSTc	Neu5Ac(α2,6)Gal(β1,4)GlcNAc(β1,3)Gal(β1,4)Glc
Fucosyllacto- <i>N</i> -sialylpentaose a	F-LST-a	Neu5Ac(α2,3)Gal(β1,3)[Fuc(α1,4)]GlcNAc(β1,3)Gal(β1,4)Glc
Fucosyllacto- <i>N</i> -sialylpentaose b	F-LST-b	Fuc(α1,2)Gal(β1,3)[Neu5Ac(α2,6)]GlcNAc(β1,3)Gal(β1,4)Glc
Fucosyllacto- <i>N</i> -sialylpentaose c	F-LST-c	Neu5Ac(α2,3)Gal(β1,3)GlcNAc(β1,3)Gal(β1,4)[Fuc(α1,3)]Glc
Disialyllacto- <i>N</i> -tetraose	DS-LNT	Neu5Ac(α2,3)Gal(β1,3)[Neu5Ac(α2,6)]GlcNAc(β1,3)Gal(β1,4)Glc
3-Fucosyl-3'-sialyllactose	3F-3'-SL	Neu5Ac(α2,3)Gal(β1,4)[Fuc(α1,3)]Glc
3-Fucosyl-6'-sialyllactose	3F-6'-SL	Neu5Ac(α2,6)Gal(β1,4)[Fuc(α1,3)]Glc
3'-sialyl- <i>N</i> -acetyllactosamine	3'-SLN	Neu5Ac(α2,3)Gal(β1,4)GlcNAc
6'-sialyl- <i>N</i> -acetyllactosamine	6'-SLN	Neu5Ac(α2,6)Gal(β1,4)GlcNAc

Table 3: List of saccharides that may be used as acceptor substrate for the production of a sialylated saccharide. The sialylated oligosaccharide itself may also be used as acceptor substrate for the production of a further sialylated oligosaccharide.

In an additional and/or alternative embodiment, the acceptor molecule is a tetrasaccharide, preferably a tetrasaccharide selected from the group consisting of lacto-*N*-tetraose, lacto-*N*-neotetraose, 2'3-difucosyllactose, 3-fucosyl-3'-sialyllactose and 3-fucosyl-6'-sialyllactose.

- 5 In an additional and/or alternative embodiment, the acceptor molecule is a pentasaccharide, preferably a pentasaccharide selected from the group consisting of sialyllacto-*N*-tetraose a, sialyllacto-*N*-tetraose b, sialyllacto-*N*-tetraose c, lacto-*N*-fucopentaose I, lacto-*N*-fucopentaose II, lacto-*N*-fucopentaose III, lacto-*N*-fucopentaose V, lacto-*N*-neofucopentaose I and lacto-*N*-neofucopentaose V.
- 10 It is to be understood that a *Bacillus* cell already carrying one or more genes encoding said enzymes, and expressing said genes in a manner sufficient to produce NeuNAc, CMP-NeuNAc and/or the sialylated saccharide does not need to be genetically engineered to complete sialic acid biosynthesis and to transfer a sialic acid moiety to a saccharide acceptor, but may nevertheless be genetically engi-
- 15 neered to alter the expression level of one or more of said genes to increase the intracellular level of said one or more gene products such as – for example the quantity of glutamine:fructose-6-phosphate aminotransferase, glucosamine-6-phosphate *N*-acetyltransferase, *N*-acetylglucosamine-6-phosphate phosphatase, *N*-acetylglucosamine 2-epimerase and/or *N*-acetylneuraminic acid synthase, thus
- 20 increasing the rate of Neu5Ac biosynthesis and, as a consequence, of the sialylated saccharide, in the genetically engineered cell.

In some embodiments, the *Bacillus* cell is for use in the production of 3'-SL and has been genetically engineered to possess a lactose permease, a CMP-NeuNAc biosynthesis pathway that uses GlcN-1P as intermediate, and a α -2,3- sialyltrans-

25 ferase.

In various embodiments, the *Bacillus* cell is for use in the production of 6'-SL and has been genetically engineered to possess a lactose permease, a CMP-NeuNAc biosynthesis pathway that uses GlcN-1P as intermediate, and a α -2,6- sialyltrans-

ferase.

- 30 In an additional and/or alternative embodiment, the genetically engineered *Bacillus* cell synthesizes more PEP than the wildtype of the cell. In an additional and/or

alternative embodiment, the genetically engineered microbial cell has been genetically engineered to possess an enhanced PEP biosynthesis pathway. Preferably, the genetically engineered microbial cell has been genetically engineered to possess an increased phosphoenolpyruvate synthase activity, for example in that the *ppsA* gene encoding phosphoenolpyruvate synthase gene is overexpressed and/or in that the non-naturally-occurring microorganisms contains at least one additional copy of a nucleotide sequence allowing the expression of a phosphoenolpyruvate synthase or a functional variant thereof. Overexpression of *ppsA* enhances intracellular PEP synthesis such that more PEP is available for the production of sialic acid. For example, a suitable phosphoenolpyruvate synthase is PpsA of *E. coli*.

In an additional and/or alternative embodiment, the genetically engineered *Bacillus* cell contains a nucleic acid molecule comprising a nucleotide sequence encoding *E. coli* PpsA or a functional variant thereof. Said nucleotide sequence encoding *E. coli* PpsA or a functional variant thereof has a sequence identity of at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% to the *E. coli ppsA* gene.

In an additional and/or alternative embodiment, the genetically engineered *Bacillus* cell is further modified to be capable to transfer said sole carbon source into the cell via a mechanism, that is not consuming PEP.

In an additional and/or alternative embodiment, the *Bacillus* cell for producing a sialylated oligosaccharide lacks any β -galactosidase activity or possesses reduced β -galactosidase activity as compared to a wild type *Bacillus* cell of the same species.

The intracellular biosynthesis of sialylated oligosaccharides requires import of lactose as acceptor substrate for the lactose-accepting sialyltransferase. Any intracellular enzyme activity that hydrolyzes internalized lactose would affect the efficacy of sialyllactose formation as the pool of intracellular lactose would be diminished. Therefore, it would be advantageous if the *Bacillus* cell for producing a sialylated oligosaccharide would lack or at least possess – as compared to the wild-type *Bacillus* cell – reduced beta-galactosidase activity.

In an additional and/or alternative embodiment, the *Bacillus* cell has been genetically engineered to abolish or at least reduce the cell's β -galactosidase activity.

In an additional and/or alternative embodiment, the *Bacillus* cell has been
5 genetically engineered by deletion or functional inactivation of the *ganA* gene. In another embodiment, the *Bacillus* cell has been genetically engineered to reduce the expression level of the *ganA* gene – as compared to the wild-type *Bacillus* cell.

The *Bacillus ganA* gene is also called *yvñ* or *lacA*. It is a gene of the GanR regulon which contains genes encoding enzymes involved in the utilization of galactan. The
10 *ganA* gene encodes a beta-galactosidase that is involved in the galactan utilization of *Bacillus*.

Deletion or functional inactivation of the *ganA* gene abolishes the GanA-mediated β -galactosidase activity in the *Bacillus* cell, whereas reduction of the *ganA* expression lowers the amount of GanA in the *Bacillus* cell and hence the β -galactosidase
15 activity which could interfere with the biosynthesis of a sialylated oligosaccharide.

In an additional and/or alternative embodiment, the *Bacillus* cell has been genetically engineered by deletion or functional inactivation of the *yesZ* gene. The *Bacillus yesZ* gene encodes the beta-galactosidase YesZ which plays a role in the degradation of rhamnogalacturonan being derived from plant cell walls. The
20 expression of *Bacillus yesZ* gene is induced by rhamnogalacturonan I. In another embodiment, the *Bacillus* cell has been genetically engineered to reduce the expression level of the *yesZ* gene – as compared to the wild-type *Bacillus* cell.

Deletion or functional inactivation of the *yesZ* gene abolishes the YesZ-mediated β -galactosidase activity in the *Bacillus* cell, whereas reduction of the *yesZ* expression
25 lowers the amount of YesZ in the *Bacillus* cell and hence the β -galactosidase activity which could interfere with the biosynthesis of a sialylated oligosaccharide.

When *B. subtilis* enters the post-exponential growth phase, they (start to) produce large quantities of extracellular proteases. Foreign proteins are often protease sensitive. Therefore, an exoprotease-free strain is desirable to increase the stability
30 of heterologous proteins and to allow accumulation of high levels of foreign proteins.

The genome of *Bacillus* encodes for at least eight extracellular proteases, namely *nprE*, *aprE*, *epr*, *bpr*, *mpr*, *nprB*, *vpr* and *wprA*. Thus, in an additional and/or alternative embodiment, the *Bacillus* cell has been genetically engineered in that at least one gene encoding for an extracellular protease has been deleted or

5 functionally inactivated, preferably at least one of the genes selected from the group consisting of *nprE*, *aprE*, *epr*, *bpr*, *mpr*, *nprB*, *vpr* and *wprA*. Preferably, two, three, four, five six, seven or eight of the genes selected from the group consisting of *nprE*, *aprE*, *epr*, *bpr*, *mpr*, *nprB*, *vpr* and *wprA* have been deleted or functionally inactivated.

10 *B. subtilis* synthesizes pulcherriminic acid when growing in media containing a carbohydrate such as glucose or lactose. Excreted pulcherriminic acid forms the red pigment pulcherrimin, the salt of pulcherriminic acid (a ferric chelate), when iron is present in the growth medium. The formation of this undesired by-product during fermentation processes can be avoided/abolished by deletion or disruption of the

15 genes *yvmC* and/or *cypX*. The gene *yvmC* encodes a cyclodipeptide synthase and the gene *cypX* encodes a cytochrome P450 cyclo-l-leucyl-l-leucyl dipeptide oxidase.

Thus, in an additional and/or alternative embodiment, the *Bacillus* cell has been genetically engineered in that at least one of the genes *yvmC* and *cypX* has been deleted or functionally inactivated.

20 The rhizobacterium *B. subtilis* possesses genes for the synthesis of more than 20 antibiotics. Among them are peptide antibiotics like *Bacillus subtilis* lantibiotics and lantibiotic-like peptides (subtilin, ericin S, mersacidin, sublancin 168, subtilosin A) and non-ribosomally synthesized (peptide) antibiotics (surfactin, iturin, bacillomycin, mycosubtilin, corynebactin/ bacillibactin, fengycin plipastatin, mycobacillin, TL-119,

25 bacilysin, bacilysocin, amicoumacin, 3,3'-neotrehalosdiamine, difficidin, rhizocticin).

For producing a sialylated oligosaccharide, it is preferred to use a *Bacillus* cell that does not produce an antibiotic. Thus, in an additional and/or alternative embodiment, the *Bacillus* cell does not synthesize one or more of the antibiotics selected from the group consisting of lantibiotics and lantibiotic-like peptides such

30 as subtilin, ericin S, mersacidin, sublancin 168, subtilosin A; non-ribosomally synthesized (peptide) antibiotics such as surfactin, iturin, bacillomycin, mycosubtilin,

corynebactin/ bacillibactin, fengycin plipastatin, mycobacillin, TL-119, bacilysin, bacilysoicin, amicoumacin, 3,3'-neotrehalosadamine, difficidin and rhizocticin. The *Bacillus* cell may have been genetically engineered to impair or abolish their ability to synthesize one or more of said antibiotics.

- 5 The genetically engineered *Bacillus* cell that can produce sialylated oligosaccharides may – optionally – include additional features, and may be genetically engineered to possess these additional features. These additional features are considered to improve the productivity of the non-naturally-occurring microorganism leading to higher sialylated oligosaccharide yields.
- 10 The *Bacillus* cell of the invention is able to produce a sialylated oligosaccharide when cultivated in the presence of lactose in a medium and under conditions that are permissive for the *Bacillus* cell to produce the sialylated oligosaccharide. Thus, provided is also the use of a genetically engineered *Bacillus* cell as described herein before for the production of a sialylated oligosaccharide.
- 15 In an aspect, provided is a method for the production of a sialylated oligosaccharide. The method comprises (i) providing a *Bacillus* cell as describe herein before, (ii) cultivating said *Bacillus* cell in a culture medium or fermentation broth and under conditions that are permissive for the production of the sialylated oligosaccharide. The method may further include retrieving the sialylated oligosaccharide from the
- 20 culture medium/fermentation broth and/or from the *Bacillus* cell.

The fermentation broth contains at least one carbon source for the *Bacillus* cell's metabolism. In some embodiments, the at least one carbon source is selected from the group consisting of glucose, fructose, sucrose, glycerol and combinations thereof. The carbon source can be internalized by the *Bacillus* cell and may be used

25 by the *Bacillus* cell's metabolism to generate biomass and/or energy in form of energy rich triphosphates.

- In some embodiments, the fermentation broth contains lactose, in particular if the *Bacillus* cell is not able to synthesize lactose by its own. The lactose may be supplied exogenously to the fermentation broth. Lactose may serve as acceptor
- 30 substrate for a NeuNAc moiety in the production of some sialylated oligosaccharides, in particular in the production of sialyllactoses.

In various embodiments, the fermentation broth contains sialic acid, in particular if a *Bacillus* cell is used for the production of a sialylated oligosaccharide which does not possess a sialic acid biosynthesis pathway, but a sialic acid salvage pathway.

In some embodiments, the production of a sialylated oligosaccharide does not
5 require addition of *N*-acetylglucosamine, *N*-acetylmannosamine and/or *N*-acetylneuraminic acid to the fermentation broth and/or cultivating the genetically engineered microbial cell in the presence of *N*-acetylglucosamine, *N*-acetylmannosamine and/or *N*-acetylneuraminic acid for the intracellular biosynthesis of the sialylated oligosaccharide, because a *Bacillus* cell is used for the production of
10 the sialylated oligosaccharide which possesses a *de novo* sialic acid biosynthesis pathway for intracellular biosynthesis of NeuNAC.

In the method, the at least one genetically engineered *Bacillus* cell is cultivated in a fermentation broth and under conditions which are permissive for the production of the saccharide comprising at least one *N*-acetylneuraminic acid moiety.

15 While the process employs a carbon source in the fermentation broth for the genetically engineered *Bacillus* cell, it is not necessary to add glucosamine and/or *N*-acetylneuraminic acid and/or *N*-acetylglucosamine and/or *N*-acetylmannosamine to the fermentation broth when the *Bacillus* cell is able to intracellularly produce *N*-acetylneuraminic acid. Thus, in various embodiments for the production of a
20 sialylated oligosaccharide, the genetically engineered *Bacillus* cell is cultivated in the absence of and/or without addition of one or more selected from the group consisting of glucosamine, *N*-acetylglucosamine, *N*-acetylmannosamine and *N*-acetylneuraminic acid. The genetically engineered *Bacillus* cell may be cultivated in the absence and/or without addition of galactose, as far as galactose is not
25 supplied as an acceptor substrate for the sialyltransferase reaction.

In additional and/or alternatives embodiments, the genetically engineered *Bacillus* cell is cultivated in the presence of one or more monosaccharides (e.g. galactose), disaccharides (e.g. lactose), trisaccharides (e.g. lacto-*N*-triose II), tetrasaccharides (e.g. lacto-*N*-tetraose) and/or pentasaccharides (e.g. sialyllacto-*N*-tetraose a).

30 According to an additional and/or alternative embodiment, the genetically engineered *Bacillus* cell is cultivated in the presence of at least one acceptor

substrate selected from the group consisting of galactose, *N*-acetylgalactosamine, *N*-acetylglucosamine, lactose, lactulose, *N*-acetyllactosamine, lacto-*N*-biose, lacto-*N*-triose, 2'-fucosyllactose, 3-fucosyllactose, 3'-sialyllactose, 6'-sialyllactose, 3'-sialyl-*N*-acetyllactosamine, 6'-sialyl-*N*-acetyllactosamine, 3'-galactosyllactose, 6'-galactosyllactose, lacto-*N*-triose II, lacto-*N*-tetraose, lacto-*N*-neotetraose, 2'3-difucosyllactose, 3-fucosyl-3'-sialyllactose and 3-fucosyl-6'-sialyllactose. These substrates are imported into the cell and used as acceptor molecules in the cell.

The method comprises the optional step of recovering the sialylated oligosaccharide that has been produced by the genetically engineered *Bacillus* cell during its cultivation and proliferation in the fermentation broth. The sialylated oligosaccharide can be recovered from the fermentation broth after the genetically engineered *Bacillus* cells have been removed, for example by centrifugation or filtration, and/or can be recovered from the cells, for example in that the cells are harvested from the fermentation broth by centrifugation and are subjected to a cell lysis step. Subsequently, the sialylated oligosaccharides can be further purified from the fermentation broth and/or cell lysates by suitable techniques known to the skilled artisan. Suitable techniques include microfiltration, ultrafiltration, diafiltration, simulated moving bed type chromatography, electrodialysis, reverse osmosis, gel filtration, anion exchange chromatography, cation exchange chromatography, and the like.

The method and the genetically engineered microbial cell that is employed in the method are used for the production of a sialylated oligosaccharide. The term "sialylated oligosaccharide" refers to an oligosaccharide molecule comprising at least one *N*-acetylneuraminic acid moiety.

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

moieties. The term "sialylated oligosaccharide" as used herein refers to oligosaccharides comprising one or more *N*-acetylneuraminic acid moieties.

According to additional and/or alternative embodiment, the sialylated oligosaccharide is selected from the group consisting of 3'-sialyllactose, 6'-sialyllactose, sialyl-lacto-*N*-tetraose a, sialyllacto-*N*-tetraose b, sialyllacto-*N*-tetraose c, fucosyl-sialyl-lacto-*N*-tetraose a, fucosyl-sialyllacto-*N*-tetraose b, fucosyl-sialyllacto-*N*-tetraose c, disialyllacto-*N*-tetraose, fucosyldisialyllacto-*N*-tetraose I, fucosyldisialyllacto-*N*-tetraose II, 3'-sialylgalactose, 6'-sialylgalactose, 3'-sialyl-*N*-acetyllactosamine and 6'-sialyl-*N*-acetyllactosamine.

10 In another aspect of the invention, the use of a genetically engineered *Bacillus* cell as described herein before for the production of a sialylated oligosaccharide in a whole cell fermentation process is provided, i.e. the sialylated oligosaccharide is synthesized by and in the genetically engineered *Bacillus* cell.

15 In another aspect of the invention, a sialylated oligosaccharide is provided that has been produced by the method and/or by using the genetically engineered *Bacillus* cell as described herein before.

In another aspect of the invention, provided is the use of a sialylated oligosaccharide that has been produced by a method as described herein before and/or by using the genetically engineered *Bacillus* cell as described herein before for the manufacture
20 of a nutritional composition.

Thus, provided is a nutritional composition containing at least one sialylated oligosaccharide which has been produced by the method and/or by the genetically engineered *Bacillus* cell as described herein before.

In an additional and/or alternative embodiment, the sialylated oligosaccharide is
25 selected from the group consisting of 3'-sialyllactose, 6'-sialyllactose, sialyllacto-*N*-tetraose a, sialyllacto-*N*-tetraose b, sialyllacto-*N*-tetraose c, fucosyl-sialyllacto-*N*-tetraose a, fucosyl-sialyllacto-*N*-tetraose b, fucosyl-sialyllacto-*N*-tetraose c, disialyllacto-*N*-tetraose, fucosyldisialyllacto-*N*-tetraose I, fucosyldisialyllacto-*N*-tetraose II, 3'-sialylgalactose, 6'-sialylgalactose, 3'-sialyl-*N*-acetyllactosamine and 6'-sialyl-*N*-
30 acetyllactosamine.

In some embodiments, the nutritional composition further contains at least one neutral HMO, preferably at least one neutral HMO that has been produced by using a genetically engineered *Bacillus* cell. The at least one neutral HMO is selected from the group consisting of 2'-fucosyllactose, 3-fucosyllactose, 2',3-difucosyllactose, 5 lacto-*N*-triose II, lacto-*N*-tetraose, lacto-*N*-neotetraose, lacto-*N*-fucopentaose I, lacto-*N*-neofucopentaose I, lacto-*N*-fucopentaose II, lacto-*N*-fucopentaose III, lacto-*N*-fucopentaose V, lacto-*N*-neofucopentaose V, lacto-*N*-difucohexaose I, lacto-*N*-difucohexaose II, lacto-*N*-neodifucohexaose I, lacto-*N*-hexaose, lacto-*N*-neohexaose, *para*-lacto-*N*-hexaose, and *para*-lacto-*N*-neohexaose.

- 10 In an additional embodiment, the nutritional composition is selected from the group consisting of medicinal compositions, pharmaceutical compositions, nutraceutical formulations, infant formula and dietary supplements.

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

- 15 The present invention will be described with respect to particular embodiments, but the invention is not limited thereto but only by the claims. Furthermore, the terms first, second and the like in the description and in the claims, are used for distinguishing between similar elements and not necessarily for describing a sequence, either temporally, spatially, in ranking or in any other manner. It is to be understood 20 that the terms so used are interchangeable under appropriate circumstances and that the embodiments of the invention described herein are capable of operation in other sequences than described or illustrated herein.

- It is to be noticed that the term "comprising", used in the claims, should not be interpreted as being restricted to the means listed thereafter; it does not exclude 25 other elements or steps. It is thus to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not preclude the presence or addition of one or more other features, integers, steps or components, or groups thereof. Thus, the scope of the expression "a device comprising means A and B" should not be limited to devices consisting only of components A 30 and B. It means that with respect to the present invention, the only relevant components of the device are A and B.

- Reference throughout this specification to “one embodiment” or “an embodiment” means that a particular feature, structure or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, appearances of the phrases “in one embodiment” or “in an
- 5 embodiment” in various places throughout this specification are not necessarily all referring to the same embodiment, but may. Furthermore, the particular features, structures or characteristics may be combined in any suitable manner, as would be apparent to one of ordinary skill in the art from this disclosure, in one or more embodiments.
- 10 Similarly, it should be appreciated that in the description of exemplary embodiments of the invention, various features of the invention are sometimes grouped together in a single embodiment, figure, or description thereof for the purpose of streamlining the disclosure and aiding in the understanding of one or more of the various inventive aspects. This method of disclosure, however, is not to be interpreted as
- 15 reflecting an intention that the claimed invention requires more features than are expressly recited in each claim. Rather, as the following claims reflect, inventive aspects lie in less than all features of a single foregoing disclosed embodiment. Thus, the claims following the detailed description are hereby expressly incorporated into this detailed description, with each claim standing on its own as a
- 20 separate embodiment of this invention.

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

25 following claims, any of the claimed embodiments can be used in any combination.

Furthermore, some of the embodiments are described herein as a method or combination of elements of a method that can be implemented by a processor of a computer system or by other means of carrying out the function. Thus, a processor with the necessary instructions for carrying out such a method or element of a

30 method forms a means for carrying out the method or element of a method. Furthermore, an element described herein of an apparatus embodiment is an

example of a means for carrying out the function performed by the element for the purpose of carrying out the invention.

In the description and drawings provided herein, numerous specific details are set forth. However, it is understood that embodiments of the invention may be practiced
5 without these specific details. In other instances, well-known methods, structures and techniques have not been shown in detail in order not to obscure an understanding. The invention will now be described by a detailed description of several embodiments of the invention. It is clear that other embodiments of the invention can be configured according to the knowledge of persons skilled in the art
10 without departing from the true spirit or technical teaching of the invention, the invention being limited only by the terms of the appended claims.

Examples

Example 1: Transformation of *Bacillus subtilis*

Bacillus subtilis can be genetically manipulated by various techniques. For transformation of *B. subtilis*, competent cells were prepared by a modified protocol of the
15 two-step method (Anagnostopoulos, C. and Spizizen, J. (1961) J Bacteriol 81 (5): 741–746). An overnight culture was inoculated in MG1 medium and shaken at 37 °C. MG1 medium is Spizizen's minimal medium, which is supplemented with 0.5 % glucose, 5 mM MgSO₄ and 0.02 % casamino acids (optionally additionally
20 supplemented with biotin and/or L-tryptophan). At the next morning, this culture was diluted 1:20 in fresh MG1 medium and incubated at 37 °C for approximately 6 h. 1 ml of the culture was diluted in 8 ml MG2 medium which differs from MG1 medium in the concentration of casamino acids (0.01 % instead of 0.02 %). In a shortened protocol the overnight culture is directly diluted in MG2 medium. After incubation for
25 another 90 min, a 1-ml portion of the culture was mixed with 1-3 µg multimeric plasmid DNA or linear DNA and incubated at 37 °C for 30-60 min with shaking. Multimeric plasmid DNA was obtained either by using *E. coli* strain NM538 for propagation of plasmid DNA or by linearization of the plasmid by digestion with a single-cutter restriction enzyme, which cleaves within the backbone, followed by re-ligation
30 with T4 DNA ligase.

Afterwards, cells were spread on 2x YT agar plates containing the appropriate antibiotic. Antibiotics were added in the following concentrations: 5 $\mu\text{g}\cdot\text{mL}^{-1}$ erythromycin, 5 $\mu\text{g}\cdot\text{mL}^{-1}$ chloramphenicol, 10 $\mu\text{g}\cdot\text{mL}^{-1}$ kanamycin, 100 $\mu\text{g}\cdot\text{mL}^{-1}$ spectinomycin.

- 5 Alternatively, for protoplast transformation (Romero, D. et al. (2006) Journal of Microbiological Methods 66:556-559), cells were grown in 20 ml of Penassay broth (PAB) at 37 °C until the onset of the stationary phase of growth ($\text{OD}_{600}=1.7-2$). Cells were then pelleted and resuspended in 10 ml of SMPP medium (0.3% bovine serum albumin, 5% 2 M sucrose, 25% 4x PAB, 50% 2x SMM), composition of 2x SMM
- 10 being 1 M sucrose, 0.04 M maleic acid disodium salt hydrate and 0.04 M MgCl_2 (pH 6.5). After the addition of lysozyme (10 mg mL^{-1}) and mutanolysin (75 U mL^{-1}) the mixture was incubated at 37 °C with shaking to generate protoplasts. Protoplast formation was checked microscopically. Protoplasts were then carefully harvested by centrifugation at 5200 $\times g$ and 4 °C for 5 min, washed twice with ice cold washing
- 15 electrotransformation buffer (1x SMM) and finally suspended in this solution. Plasmid DNA (1-3 μg) was added to 120 μl of protoplast suspension and the mixture was kept on ice for at least 5 min. The transformation mixture was transferred to a 0.2-cm cuvette and a single electroporation pulse was applied at 25 μF , 400 Ω and 0.7 kV. Immediately after the electroporation shock 1 ml of recovering medium
- 20 (equal volumes of 4x PAB and 2x SMM, prepared freshly before use) was added to the cuvette. The transformation reaction was then transferred to a 2 ml tube and incubated at 37 °C with shaking for 12 h. For regeneration, the cell suspension was spread on DM3 agar plates (Chang, S. and Cohen, S. (1979) MGG 168(1):111-115) and incubated at 37 °C for 48 h. DM3 regeneration medium contained the following
- 25 sterile solutions per liter: 200 ml 4% agar, 100 ml 5% casamino acids, 50 ml 10% yeast extract, 100 ml 3.5% K_2HPO_4 and 1.5% KH_2PO_4 , 25 ml 20% glucose, 20 ml 1 M MgCl_2 , 500 ml 0.5 M sorbitol and 5 ml filter sterilized 2% bovine serum albumin (added to the mixture when the temperature is below 55 °C) and was supplemented with the appropriate antibiotic.
- 30 Electroporation of *B. subtilis* was done according to a modified protocol from Zhang et al. (2011) provided by MoBiTec GmbH (Zhang, G., Bao, P., Zhang, Y., Deng, A., Chen, N. and Wen, T. (2011) Anal. Biochem., 409:130-137). A 2x YT overnight

culture was diluted 100-fold with fresh 2x YT medium and the culture was grown to an OD₆₀₀ of 0.2 at 37 °C on a rotary shaker. Then the culture was supplemented with 1% DL-threonine, 2% glycine, 0.1% tryptophan and 0.03% Tween 80. After cultivation for another 60 min, the cell suspension was cooled on ice for 20 min, centrifuged at 5000 x g for 10 min at 4 °C and washed twice with electroporation buffer (0.5 M trehalose, 0.5 M sorbitol, 0.5 M mannitol, 0.5 mM MgCl₂, 0.5 mM K₂HPO₄, 0.5 mM KH₂PO₄, pH 7.4, filter-sterilized and stored frozen). Finally, cells were resuspended in electroporation buffer at 1/100 of the original culture volume and 100 µl of cell suspension was mixed with DNA. The transformation mixture was transferred to a 0.1-cm cuvette and electroporation was performed at 1.8 kV with a single pulse delivered with a MicroPulser™ device (Bio-Rad). Immediately after pulse delivery, 1 ml 2x YT broth containing 0.5 M sorbitol and 0.38 M mannitol was added to the cuvette. The transformation suspension was transferred to a 2 ml tube and incubated at 37 °C for 3 h on a rotary shaker. The cells were spread on a selective 2x YT agar plate and incubated at 37 °C overnight.

Using an alternative electroporation protocol (Xue, G. P., J. S. Johnson, and B. P. Dalrymple: 1999; Journal of Microbiological Methods 34:183-191), 5 ml of LB medium containing 0.5 M glucitol was inoculated with *B. subtilis* and incubated overnight at 37 °C. Subsequently, the overnight culture was diluted (1:16) by 75 ml of LB containing 0.5 M glucitol and incubated until an OD₆₀₀ of 0.85 – 0.95 was obtained. The cells were then pelleted by centrifugation for 10 min at 4 °C at 5.000xg and washed four times by ice-cooled electroporation buffer (10 % glycerol, 0.5 M glucitol, 0.5 M mannitol). Finally, the cells were resuspended in 1-2 ml electroporation buffer. Electroporation was performed using 60 µl of competent cells with DNA in a cooled electroporation cuvette (1-mm electrode gap). The cell-DNA mixture was subjected to a single electrical pulse at 25 µF, 200 Ω and 21 kV/cm. Finally, 1 ml recovery broth (LB containing 0.5 M glucitol and 0.38 mannitol) was added to the electroporated cells and the bacterial culture was incubated for 3 h at 37 °C followed by plating on LB agar supplemented by an antibiotic.

Two different rich media were used, namely Luria Broth (LB) and 2x YT.

Luria Broth (LB) medium consisted of 1% tryptone, 0.5% yeast extract and 0.5% NaCl (pH 7.2).

2x YT medium consisted of 1.6% tryptone, 1% yeast extract and 0.5% NaCl (pH 7.5).

To prepare rich-medium agar plates 15 g L⁻¹ agar were added.

For shaking flask experiments, Spizizen's minimal medium (Spizizen, J. 1958 Proc. Natl. Acad. Sci. U. S. A. 44(10):1072-1078) was used.

Spizizen's minimal medium contains the following salts: 2 g/L (NH₄)₂SO₄, 14 g/L K₂HPO₄, 6 g/L KH₂PO₄, 1 g/L Na₃ citrate × 2·H₂O and 0.2 g/L MgSO₄ × 7·H₂O.

The preculture medium consisted of Spizizen's minimal salts supplemented with 2 % D-glucose, 0.05 % casamino acids and MgSO₄ to a final concentration of 2 mM (optionally additionally supplemented with biotin and/or L-tryptophan).

The main culture medium consisted of Spizizen's minimal salts supplemented with 2 % D-glucose, 0.05 % casamino acids, MgSO₄ to a final concentration of 2 mM and 0.5 mL·L⁻¹ 1000x trace element solution (optionally additionally supplemented with biotin and/or L-tryptophan).

Trace element solution (1000x) consisted of 100.6 g/L C₆H₅NO₆, 56.4 g/L ammonium ferric citrate, 9.8 g/L MnCl₂ × 4·H₂O, 1.6 g/L CoCl₂ × 6·H₂O, 1 g/L CuCl₂ × 2·H₂O, 1.9 g/L H₃BO₃, 9 g/L ZnSO₄ × 7·H₂O, 1.1 g/L Na₂MoO₄ × 2·H₂O, 1.5 g/L Na₂SeO₃, 1.5 g/L NiSO₄ × 6·H₂O.

If necessary, the appropriate antibiotic(s) were added to the medium to make it selective.

B. subtilis strains were initially grown on rich-media agar plates to obtain single colonies. These plates were grown for 1 day at 30-37°C. For shaking flask experiments, a 20-ml preculture was inoculated with a single colony and grown overnight at 30-37 °C on a rotary shaker. Subsequent 20-ml main cultures were inoculated with this preculture to a starting OD₆₀₀ of about 0.1 and incubated at 30-37 °C on a rotary shaker. If induction was required, a 40-60 ml main culture was splitted into 20-ml proportions at the timepoint of induction. The culture volume did not exceed 20 % of the shaking flask capacity.

Example 2: Construction of a *Bacillus subtilis* production strain for 3'-sialyllactose

For the synthesis of 3'-sialyllactose from the metabolic intermediate UDP-*N*-acetylglucosamine using the *neuCBA* pathway, a *B. subtilis* expression plasmid (SEQ ID NO: 1) was constructed. Firstly, the genes *neuA*, *neuB*, *neuC* as well as a α -2,3-sialyltransferase were codon-optimized for expression in *B. subtilis* and prepared synthetically by GenScript Corp. The *Campylobacter jejuni* gene *neuA* (UniProtKB accession number: Q93MP7) encodes a CMP-Neu5Ac synthetase. The *Campylobacter jejuni* gene *neuB* (UniProtKB accession number: Q93MP9) encodes a sialic acid synthase. The *Campylobacter jejuni* gene *neuC* (UniProtKB accession number: Q93MP8) encodes a *N*-acetylglucosamine-6-phosphate 2-epimerase. The *Pasteurella multocida* gene *siaT* (UniProtKB accession number: Q9CLP3) encodes an α -2,3-sialyltransferase. The open reading frame of the *E. coli* gene *lacY* (Gen Bank accession number: NP_414877.1), encoding a lactose permease, was amplified by PCR from chromosomal DNA. Afterwards, the expression cassette <P_{grac100}-*neuBCA-siaT-lacY-terminator*> was constructed, that comprises all necessary genes under the control of the inducible promoter P_{grac100}. For this, the *B. subtilis* expression vector pHT253 (MoBiTec GmbH, Göttingen, Germany) was used as backbone. Each gene within the expression cassette was linked to a *B. subtilis* RBS sequence. Additionally, a suitable *B. subtilis* terminator sequence from the iGem part repository (sequence ID: BBa_B0015) was placed downstream of the expression cassette. The resulting plasmid <pHT253-P_{grac100}-*neuBCA-2,3siaT-lacY-terminator*> (SEQ ID NO: 1) was used to transform a sporulating *B. subtilis* strain (table 1) by exploiting its natural competence.

Gene expression was confirmed by targeted proteomics and/or by real-time PCR.

Transformants were cultivated under conditions that are permissive for *B. subtilis* to produce 3'-sialyllactose in the presence of exogenous lactose.

Example 3: Construction of a *Bacillus subtilis* production strain for 3'-sialyllactose

For the synthesis of 3'-sialyllactose from the metabolic intermediate UDP-*N*-acetylglucosamine using the *neuCBA* pathway, a constitutive *B. subtilis* expression plasmid (SEQ ID NO: 2) was constructed. Firstly, the genes *neuA*, *neuB*, *neuC* and
 5 *siaT* were codon-optimized for expression in *B. subtilis* and prepared synthetically by GenScript Corp. The *Campylobacter jejuni* gene *neuA* (UniProtKB accession number: Q93MP7) encodes a CMP-Neu5Ac synthetase. The *Campylobacter jejuni* gene *neuB* (UniProtKB accession number: Q93MP9) encodes a sialic acid
 10 synthase.

The *Campylobacter jejuni* gene *neuC* (UniProtKB accession number: Q93MP8) encodes a *N*-acetylglucosamine-6-phosphate 2-epimerase. The *Pasteurella multocida* gene *siaT* (UniProtKB accession number: Q9CLP3) encodes an α -2,3-sialyltransferase. The open reading frame of the *E. coli* gene *lacY* (Gen Bank
 15 accession number: NP_414877.1), encoding a lactose permease, was amplified by PCR from chromosomal DNA. Afterwards, the expression cassette <P43-*neuBCA*-*P_{lepA}*-*siaT*-*lacY*-terminator> was constructed, that comprises all necessary genes operably linked to the two strong constitutive *B. subtilis* promoters *P_{lepA}* and/or P43. The *B. subtilis* expression vector pHT253 (MoBiTec GmbH, Göttingen, Germany)
 20 was used as plasmid backbone. Each gene within the expression cassette was linked to a *B. subtilis* RBS sequence. Additionally, a suitable *B. subtilis* terminator sequence from the iGem part repository (sequence ID: BBa_B0015) was placed downstream of the expression cassette.

The resulting plasmid <pHT253-P43-*neuBCA*-*P_{lepA}*-*siaT*-*lacY*-terminator> (SEQ ID
 25 NO: 2) was used to transform spore-forming and non-sporulating *B. subtilis* strains (Table 1) by exploiting their natural competence.

Gene expression was confirmed by targeted proteomics and/or by real-time PCR.

Transformants were cultivated under conditions that are permissive for *B. subtilis* to produce 3'-sialyllactose in the presence of exogenous lactose.

Example 4: Construction of a *Bacillus subtilis* production strain for 6'-sialyllactose

For production of 6'-sialyllactose in *B. subtilis* the α -2,6-sialyltransferase from *Photobacterium leiognathi* was used. The open reading frame of the gene *siaT* (UniProtKB accession number: D0VYB7) was codon-optimized for expression in *B. subtilis* and prepared synthetically by GenScript Corp.

The expression plasmid <pHT253-P_{grac100}-*neuBCA-2,6siaT-lacY-terminator*> (SEQ ID NO: 3) was constructed as described in example 2. The resulting plasmid (SEQ ID NO: 3) was used to transform spore-forming and non-sporulating *B. subtilis* strains (Table 1) by exploiting their natural competence. Gene expression was confirmed by targeted proteomics and/or by real-time PCR. Transformants were cultivated under conditions that are permissive for *B. subtilis* to produce 6'-sialyllactose in the presence of exogenous lactose.

Example 5: Construction of a *Bacillus subtilis* strain for production of 3'-sialyllactose

Metabolic engineering of *B. subtilis* (Table 1) was achieved by integration of the heterologous genes *Campylobacter jejuni neuA*, *E. coli nanT* and *Haemophilus parahaemolyticus siaT* and simultaneous deletion of the endogenous gene *ganA* by homologous recombination. The *B. subtilis* gene *ganA* (*yvfN*, *lacA*), which is positioned within the galactan operon, encodes a beta-galactosidase.

For the production of 3'-sialyllactose from exogenous sialic acid/N-acetylneuraminic acid and lactose, the open reading frames of *neuA*, *nanT* and *siaT* were operably linked to the *B. subtilis* constitutive promoter P43 (iGem part repository: sequence ID: BBa_K143013) as an operon. For this purpose, firstly, the genes *neuA*, *nanT* and *siaT* were codon-optimized for expression in *B. subtilis* and prepared synthetically by GenScript Corp. The *Campylobacter jejuni* gene *neuA* (UniProtKB accession number: Q93MP7) encodes a CMP-Neu5Ac synthetase, the *E. coli* gene *nanT* (GenBank accession number: NP_417691.4) encodes a sialic acid transporter and the *H. parahaemolyticus* gene *siaT* (UniProtKB accession number: I3DHL4)

encodes an alpha-*N*-acetylneuraminyl-2,3-beta-galactosyl-1,3-*N*-acetylgalactosaminide 6-alpha-sialyltransferase (alpha-2,3-sialyltransferase).

Each gene within the expression cassette was linked to a *B. subtilis* RBS sequence. Additionally, a suitable *B. subtilis* terminator sequence from the iGem part repository
5 (sequence ID: BBa_B0015) was placed downstream of the expression cassette.

The cloning vector pBR322 (New England Biolabs GmbH, Frankfurt, Germany) was used as plasmid backbone. The complete integration cassette was assembled to create the suicide plasmid <pBR322 flank *ganA* up-P43-*siaT-neuA-nanT-terminator-erm*-flank *ganA* down> (SEQ ID NO: 4). Afterwards, *B. subtilis* was transformed with
10 this plasmid by its natural competence. Cells were spread on 2x YT agar plates containing the appropriate antibiotic (5 µg mL⁻¹ erythromycin). Integration of the expression cassette <P43-*siaT-neuA-nanT-terminator*> into the *ganA* locus of the *B. subtilis* genome, yielding strain A, was verified by colony PCR. Gene expression was confirmed by targeted proteomics and/or by real-time PCR.

15 For import of exogenous lactose into the *Bacillus* cell, the *E. coli* gene *lacY* was integrated into the endogenous *amyE* (*amyA*) locus of the *B. subtilis* genome (encoding for an alpha-amylase). For this purpose, the open reading frame of the *E. coli* *lacY* gene (Gen Bank accession number: NP_414877.1), encoding a lactose permease, was amplified by PCR from chromosomal DNA. The integration cassette
20 <flank *amyE* up-*lox71-aad9-lox66*-P43-*lacY*-flank *amyE* down> (SEQ ID NO: 5) was constructed and cloned into pBR322 (New England Biolabs GmbH, Frankfurt, Germany). Strain A was transformed with the resulting suicide plasmid by natural competence. Integration of the expression cassette <P43-*lacY*> into the *amyE* locus of strain A, yielding the final strain B, was verified by colony PCR.

25 Gene expression was confirmed by targeted proteomics and/or by real-time PCR. Strain B was cultivated under conditions that are permissive for *B. subtilis* to produce 3'-sialyllactose in the presence of exogenous lactose and sialic acid/*N*-acetylneuraminic acid.

**Example 6: Production of sialyllactose using metabolically engineered
Bacillus subtilis strains**

A preculture was inoculated with a metabolically engineered *Bacillus subtilis* strain suitable for the biosynthesis of sialyllactose (as described in examples 2-5).

- 5 The preculture was incubated at 30-37 °C over night and then diluted to a starting OD₆₀₀ of about 0.1 in fresh main culture medium. When the main culture reached an OD₆₀₀ of approximately 0.5, 2 mM lactose was added to the growth medium. When the inducible promoter P_{grac100} was used for gene expression, induction was carried out with lactose (2 mM) or with both lactose (2 mM) and IPTG (1 mM). For the
- 10 salvage biosynthesis of CMP-*N*-acetylneuraminic acid, additionally, 2 mM sialic acid/*N*-acetylneuraminic acid were added. Cultivation was discontinued after about 24 h / 48 h after induction and intracellular and extracellular sialyllactose was analyzed by thin layer chromatography and/or HPLC and/or mass spectrometry (as described in WO 2017/042382 A or WO 2019/008133 A). Biosynthesis of substantial
- 15 amounts of sialyllactose (3'-sialyllactose/6'-sialyllactose) was detectable.