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Synthesis of oligosaccharides by bacterial enzymes

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Many human pathogens initiate disease by utilizing their microbial adhesin proteins to attach to glycoconjugates on host cell mucosal surfaces. Soluble oligosaccharides of identical or similar structure to these naturally occurring ligands can both prevent bacterial attachment as well as mediate the release of attached bacteria. Since it has not been possible to isolate large quantities of these compounds, we have developed enzyme-based technologies to synthesize several relevant human oligosaccharides. Using cloned bacterial glycosyltransferases, we can synthesize several hundred grams of these oligosaccharides at a time. The availability of these large quantities will allow these compounds to be tested as anti-adhesive pharmaceutical agents as well as lead to expanded practical applications.

Keywords: **oligosaccharide, bacterial, glycosyltransferase, enzymatic synthesis**

Introduction

Many human pathogens initiate disease by utilizing microbial adhesin proteins to bind to sugar chains attached to glycoconjugates on host cell mucosal surfaces. Unless bound to oligosaccharides anchored to the membranes of epithelial cells, the pathogens are cleared by physiological mechanisms characteristic of these mucosal surfaces. Both the prevention of bacterial attachment and the release of attached bacteria can be mediated by soluble oligosaccharides of identical or similar structure to the naturally occurring ligand. Babies are protected from many infectious agents by a large number of oligosaccharides present in breast milk [1]. Enzyme-based technologies utilizing cloned bacterial glycosyltransferases have allowed us to synthesize several human oligosaccharides on a lab scale of hundreds of grams with further scale-up possible in a production facility. The oligosaccharides can then be tested as anti-adhesive pharmaceutical agents, both *in vivo* and *in vitro.*

Pathogen/carbohydrate interactions

Numerous bacterial pathogens initiate specific binding to human cells by recognising oligosaccharide epitopes enriched on these cells [2]. These non-covalent interactions between adhesins and carbohydrates are used by bacterial toxins, viruses, yeasts and protozoans. Table 1 lists some of these pathogens. For instance, adherence of *Streptococcus pneumoniae* to respiratory epithelia cells is inhibited by oligosaccharides that terminate with the disaccharide *N*-acetylneuraminic acid linked α2→3 or α2→6 to galactose (NeuAcα2;→3/6Galβ1) [3]. The binding of many *Helicobacter pylori* strains to human duodenal cells is inhibited by NE0080, an oligosaccharide which contains the active non-reducing epitope, NeuAcα2→3 Galβ1 [4].

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Bacteria as a source of glycosyltransferases

Bacteria express a wide array of lipooligosaccharide (LOS) and lipopolysaccharide (LPS) structures on their surfaces, many of which mimic mammalian carbohydrate structures. A small representation of the diversity in LOS and LPS structures is shown in Table 2. This suggests that bacteria express a broad spectrum of glycosyltransferase activities.

The human gastric pathogen *Helicobacter pylori* can express the Type I and Type II Lewis blood group antigens that also are found in gastric epithelial cell surface glycoforms [5,6]. *Neisseria gonorrhoeae* and *Neisseria meningitidis* express a sialylated LOS [7,8], designated LST_D (see Table 2), which has the same structure as a those found in mammalian glycolipids. It has been postulated that this form of molecular mimicry allows the pathogen to evade the host immune response [5,6,9].

Glycosyltransferases generating lipooligosaccharides encoded in biosynthetic loci

The broad array of oligosaccharide structures found in mammals is mirrored in the diversity of carbohydrate

Table 1. Human pathogens with oligosaccharide targets

Gastrointestinal
Citrobacter freundii
Cryptosporidium parvum
Entamoeba histolytica
Escherichia coli
Helicobacter pylori
Salmonella typhimurium
Serratia marcescens
Shigella dysenteriae
Shigella flexneri
Vibrio cholerae
Genitourinary
Escherichia coli
Neisseria gonorrhoeae
Propionibacterium granulosum
Proteus mirabilis
Staphylococcus aureus
Staphylococcus saprophyticus
Respiratory
Bordetella pertussis
Haemophilus influenzae
Haemophilus parainfluenzae
Klebsiella pneumoniae
Mycobacterium tuberculosis
Mycoplasma pneumoniae
Pseudomonas aeruginosa
Pseudomonas cepacia
Pseudomonas maltophilia
Streptococcus pneumoniae
Streptococcus sanguis
Viruses
HIV
Influenza virus
Parvovirus
Rotavirus

structures found in bacterial LOS and LPS (Table 2). Using antibodies against human oligosaccharide structures in combination with the ease of generating LOS mutants in bacteria make these organisms a potentially rich source for isolating glycosyltransferase genes. Gotschlich [10] used this approach to characterize a genetic locus that was responsible for generating the lacto-*N*-neotetraose oligosaccharide present in *Neisseria gonorrhoeae.* Through the use of deletion mutants followed by characterization of the resultant LOS using anti-carbohydrate monoclonal antibodies, Gotschlich was able to assign a specific glycosyltransferase function to each of the five genes in this locus. He termed this locus lgt for *L*OS *g*lycosyl*t*ransferases. The LgtE gene encodes a UDP-Gal:glc (β1→4)galactosyltransferase (β1→4GalTase) that adds the first galactose to the inner core lipid to generate the structure Galβ1→4Glcβ1→4Hep→R (R is typically two keto-deoxy-octulosonic acid residues). Next *N*-acetylglucosamine is added in a β1→3 linkage to the terminal galactose by the product of the lgtA gene that encodes a UDP-GlcNAc:gal(β1→3)*N*-acetylglucosaminyltransferase (β1→3GlcNAcTase). The lacto-*N*-neotetraose structure is then completed by the action of the glycosyltransferase encoded by the lgtB gene, designated UDP-Gal: GlcNAc(β1→4)galactosyltransferase (β1→4GalTase), that adds galactose in a β1→4 linkage to the GlcNAc residue. The resultant lacto-*N*-neotetraose structure is a mimic of the human glycosphingolipid paragloboside (Table 2).

In gonococcal LOS the lacto-*N*-neotetraose frequently bears an additional *N*-acetylgalactosamine residue (GalNAcβ1→3Galβ1→GlcNAcβ1→3Galβ1→4Glcβ1→4→). The UDP-GalNAc:gal(β1→3)*N*-acetylgalactosaminyltransferase (β1→3GalNAcTase) encoded by the lgtD gene is responsible for generating this linkage. In some strains of gonococci, an alternative side chain is found that has the Galα1→4Galβ1→4Hep→R structure. This structure is a mimic of the oligosaccharide portion of the globoglycolipids and is generated by the UDP- Gal:gal(α1→4) galactosyltransferase (α1→4GalTase) encoded by the lgtC gene.

Jennings et al. [11] used the lic 2A gene, which is involved in LPS biosynthesis in *Haemophilus influenzae,* as a probe to isolate the locus responsible for generating lacto-*N*-neotetraose in *Neisseria meningitidis.* Interestingly, this locus only contained the lgtA, lgtB and lgtE genes. The activity of each glycosyltransferase was confirmed by analyzing LPS isolated from the various lgt mutants.

The above mentioned examples illustrate another advantage of isolating genes encoding glycosyltransferases from bacteria. The genes involved in generating LOS or LPS structures are often clustered in a locus. *Streptococcus pneumoniae* serotype 14 also express lacto-*N*-neotetraose in their capsule. The type 14 capsular polysaccharide locus, designated cps, contains 12 open reading frames (cps 14A-L) that encode all of the glycosyltransferases necessary to generate lacto-*N*-neotetraose [12,13]. The cps 14E gene encodes the UDP-Glc:lipid(β1→4)glucosyl-1-phosphate-transferase that adds glucose to the lipid carrier. Cps 14G encodes the UDP-gal:glc(β1→4) galactosyltransferase that adds the first galactose β1→4 to the glucose. The UDP-GlcNAc:gal(β1→3)*N*-acetylglucosaminyltransferase encoded by the cps14I gene catalyzes the addition of glcNAc in a β1→3 linkage to the terminal galactose residue. The cps14J gene encodes the UDP-Gal:glcNAc(β1→4)galactosyltransferase that transfers galactose to the terminal glcNAc residue to complete the lacto-*N*-neotetraose structure.

Bacterial and vertebrate glycosyltransferases share regions of homology

Five members of the human GDP-fucose:glcNAc (α1→3)fucosyltransferase (α1→3FucTase) gene family have been cloned [14–23]. Homologs of some of these

Table 2. Carbohydrate structures common to bacteria and mammals*

Name	*Structure*	*Organism*
Lacto-*N*-neotetraose (paragloboside)	Galβ1→4GlcNAcβ1→3Galβ1→4Glc→R	*N. gonorrhoeae* *N. meningitidis*
LST_D	Galβ1→4GlcNAcβ1→3Galβ1→4Glc→R ↑2,3 NeuAcα	*N. gonorrhoeae* *N. meningitidis*
Globotriose	Galα1→4Galβ1→4Glc→R	*N. gonorrhoeae* *H. influenzae*
Lewis X	Galβ1→4GlcNAcβ1→3Galβ1→R ↑1,3 (GlcNAc) Fucα	*H. pylori*
Lewis A	Galβ1→3GlcNAcβ1→3Galβ1→R ↑1,4 (GlcNAc) Fucα	*H. pylori*
Lewis Y	Galβ1→4GlcNAcβ1→3Galβ1→R ↑1,2 (Gal) ↑1,3 (GlcNAc) Fucα Fucα	*H. pylori*

*Refer to reference[54].

genes have also been cloned from mouse [24–26], rat [27] and chicken [28]. The high degree of sequence similarity in the catalytic domains of the cloned mammalian and chicken [29] α1→3FucTases were used to design degenerate oligonucleotide primers that were then used in the polymerase chain reaction to probe *Helicobacter pylori* genomic DNA. Two groups used this approach to identify a putative α1→3FucTase in this pathogen [30,31]. *E. coli* transformed with this *H. pylori* gene expressed a recombinant α1→3FucTase activity that was capable of generating the Lewis X structure using GDP-fucose and galβ1→4glcNAc. This approach may find widespread utility as more bacterial genomes are sequenced and similarities to known mammalian glycosyltransferases are found.

Bacteria make sialylated carbohydrates

Mammals synthesize oligosaccharide structures containing *N*-acetylneuraminic acid (NeuAc) in α2→3gal, α2→6gal, α2→6galNAc, α2→6glcNAc and α2→8NeuAc linkages [32]. Each of these linkages also exist in the bacterial world. The bacterial enzyme generating the polyα2→8NeuAc (PSA) capsule in *Escherichia coli* K1 has been described previously [33]. The same enzyme adds the first and all subsequent sialic acid residues [34–36], whereas mammals have one enzyme that initiates PSA formation and another enzyme extends the chain [37]. In addition mammals possess other α2→8STases, e.g., UDP-Gal:GM_3(α2→8)sialyltransferase that is involved in generating the gangliosides GD_3 and GD_2 [38–40].

Gilbert et al. [41] have cloned the gene encoding CMP-NeuAc:gal(α2→3)sialyltransferase (α2→3STase) from *Neisseria meningitidis* and *Neisseria gonorrhoeae* and expressed this gene in *E. coli.* Unlike mammalian α2→3STases [42], the *Neisseria* enzymes can use α-terminal and β-terminal galactose residues, including β1→4-linked and β1→3-linked galactosyl residues as acceptors [43].

The occurrence of α2→6NeuAc residues in bacterial LOS has been a point of much debate. Recently, a gene encoding a CMP-NeuAc:gal(α2→6)sialyltransferase (α2→6STase) has been cloned from the marine bacterium *Photobacterium damsella* [44]. Expression of this gene in *E. coli* yielded a recombinant protein that catalyzed the transfer of NeuAc from CMP-NeuAc to galactose in an α2→6 linkage. In contrast to mammals that have separate αg2→6STases for adding NeuAc to terminal galactose or *N*-acetylgalactosamine residues [45,46], the *P. damsella* α2→6STase adds NeuAc either to terminal galactose or *N*-acetylgalactosamine. The *P. damsella* α2→6STase can also catalyze the addition of NeuAc α2→6 to the galactose residues in Fucα1→2Galβ1→4Glc and Neu5Acβ2→3Galβ1→4Glc [47]. There is no known mammalian α2→6STase with these acceptor specificities.

Bacterial glycosyltransferases can be expressed as soluble recombinant proteins

Mammalian glycosyltransferases are often type II membrane glycoproteins which are localized to the Golgi apparatus. Mammalian glycosyltransferases require solubilization with detergents to retain their activity *in vitro* and are difficult to purify. The gonococcal lgtA-lgtE genes, cps 14I and cps14J have been expressed in *E. coli* as active soluble fusion proteins (unpublished results [13]). Gilbert

et al. [41] expressed in *E. coli* the *Neisseria meningitidis* and *Neisseria gonorrhoeae* α2→3STases as soluble proteins. In addition the *H. pylori* α1→3FucTase [30,31] and the *P. damsella* α2→6STase [44] have all been expressed as soluble proteins in *E. coli.* Each of these recombinant glycoyltransferases was soluble in the absence of detergent.

Bacterial glycosyltransferases are best expressed in bacterial expression systems

Unlike mammalian glycosyltransferases, bacterial glycosyltransferases are not glycoproteins. Most mammalian glycosyltransferases are inactive in bacterial expression systems, if the proteins are synthesized at all (unpublished results). This phenomenon may be due to the absence of post-translational modifications in bacterial expression systems are needed for glycosyltransferase activity. One notable exception is the expression of the bovine UDP-Gal:gal(α-1→3)galactosyltransferase in *E. coli* [48]. For purposes of generating enzymes on a production scale, bacterial expression systems offer significant cost advantages over mammalian expression systems such as Sf9 or Chinese hamster ovary cells.

Bacterial glycosyltransferases use a broader range of acceptors

Mammalian glycosyltransferases tend to have narrowly defined acceptor specificities. In contrast, the gonococcal β1→3GlcNAcTase (lgtA) can use octyl-β-D-lactoside, lactose and even lacto-*N*-neotetraose as acceptors (Table 3). The gonococcal β1→4GalTase (lgtB) can use octyl-β-D-glucopyranoside, glucose, *N*-acetylglucosamine and GlcNAcβ1→3Galβ1→Glc (LNT-2) as acceptors in the absence of any additional regulatory proteins (Table 3). The bovine milk UDP-Gal:glcNAc(β1→4)galactosyltransferase normally transfers galactose to GlcNAc. However, in the presence of the regulatory protein α-lactalbumin the bovine milk β1→4GalTase can catalyze the transfer of galactose to glucose [49–51]. In the presence of α-lactalbumin the enzyme can also catalyze the transfer of GalNAc from UDP-GalNAc to free GlcNAc [52]. In general, mammalian glycosyltransferases have more clearly defined acceptor specificities.

Lab-scale production of globotriose and globotetraose

The trisaccharide portion of globotriose (Gb_3) was synthesized from lactose and UDP-galactose using an enzyme encoded by the *Neisseria gonorrhoeae* lgtC gene. The product of the lgtC gene was expressed as a soluble glutathione-S-transferase fusion protein in *E. coli.* A 2 L reaction containing lactose, UDP-galactose and 12 U α1→4GalTase (Unit is defined as μmol product made per minute) produced, after purification, 5 grams of globotriose oligosaccharide with an overall yield of 75% based on starting lactose.

The tetrasaccharide portion of globotetraose (Gb_4) was synthesized from the globotriose oligosaccharide and UDP-*N*-acetylgalactosamine (UDP-GalNAc) using an enzyme encoded by the *Neisseria gonorrhoeae* lgtD gene. The product of the lgtD gene was expressed as a soluble glutathione-S-transferase fusion protein in *E. coli.* Approximately 1.5 grams of the tetrasaccharide was obtained in a 60% overall yield based on the input globotriose from a 200 mL reaction containing the trisaccharide, UDP-GalNAc and 6 U of β1→3GalNAcTase. Both of these reactions can be scaled up to yield several hundred grams of oligosaccharide.

Table 3. Activities and acceptor specificities of lgt glycosyltransferases

Enzyme	*Acceptor*	*Activity pmol/μl.min*
β1→3*N*-acetylglucosaminyltransferase (lgtA)	Galβ1→4Glc	3000
	octyl-β-D-lactopyranoside	2000
	lacto-*N*-neotetraose	100
β1→4-galactosyltransferase (lgtB)	Glc	1200
	octyl-β-D-glucopyranoside	1000
	GlcNAc	1100
	GlcNAcβ1→3Galβ1→4Glc	1500
	Galβ1→4Glc	1100
α1→4galactosyltransferase (lgtC)	Galβ1→4Glc	500
β1→3*N*-acetylgalactosaminyltransferase (lgtD)	Galα1→4Galβ1→4Glc	1200

Enzymatic synthesis of LST_D

The synthesis of GlcNAcβ1→3Galβ1→4-Glc (LNT-2) has been successfully scaled up from several hundred milligrams to several hundred grams with no loss in conversion rate. A 100 L reactor containing lactose, UDP-GlcNAc and 1000 U of *E. coli* expressed β1→3GlcNAcTase (lgtA) at 25°C produced 250 grams of LNT-2 in 16 hours. The overall yield based on the input amount of sugar nucleotide was greater than 85%. A second enzymatic step was also performed in a 100 L reactor. LNT-2, UDP-galactose and 1000 U of *E. coli* expressed β1→4GalTase (lgtB) were incubated at 25°C for 16 hours. The reaction produced more than 300 grams of LNnT with an overall yield based on the input LNT-2 of greater than 85%. LST_D was generated from LNnT and NeuAcα2→3galβ1→4glc using the recombinant transglycosidase produced by *E. coli* transformed with *Trypanosoma cruzi* α2→3transialidase gene [53]. A 5 L reactor produced 50 grams of LST_D, after purification, with an overall conversion of 90% relative to the input amount of LNnT. The production of LST_D can be easily scaled up to the kilogram scale.

Conclusions

Bacteria are an excellent source of highly active glycosyltransferases. A number of oligosaccharide structures have been synthesized on scales ranging from grams to several hundred grams using the glycosyltransferases isolated from *Neisseria.* The production of LST_D and the globotriose and globotetraose oligosaccharides is efficient and cost effective. All of these reactions are easily scaleable to the multikilogram level in a production facility.

Another advantage of using bacterial glycosyltransferases is the relative ease with which these enzymes can be cloned and expressed in *E. coli.* The diversity of known bacterial LOS and LPS structures is an indication of the variety of glycosyltransferases present in the procaryotic world. The combination of mutation and characterization of the resultant mutant LOS using anti-human oligosaccharide monoclonal antibodies is a powerful approach to identify glycosyltransferases [10]. The tendency of bacteria to organize the enzymes necessary for generating their LOS and LPS structures in a defined locus allows for the isolation of several glycosyltransferases in the DNA surrounding a characterized mutant.

The bacterial glycosyltransferases themselves have some useful properties that their mammalian counterparts do not. These enzymes are soluble whereas the mammalian glycosyltransferases commonly need detergents to be solubilized. Unlike most mammalian glycosyltransferases, the genes encoding bacterial glycosyltransferases are efficiently translated and expressed as active proteins in *E. coli.* Fermentation costs for bacterial expression systems are less than for those systems that are needed to express mammalian glycosyltransferases, e.g., Sf9 and Chinese hamster ovary cells. The bacterial glycosyltransferases tested have all been expressed in an active form in *E. coli* while the only mammalian glycosyltransferase shown to be expressed in an active form in bacteria is the bovine α-1,3-galactosyltransferase [48]. The bacterial enzymes have broad substrate specificities. In addition they either lack entirely or possess higher K_I values for inhibition by the sugar nucleotide donor and its metabolites. This minimizes the need to use sugar nucleotide cycling reactions to keep the concentration of the sugar donor and its metabolites low. We have just begun to tap the vast bacterial gene pool for glycosyltransferases involved in oligosaccharide synthesis. As more bacterial genomes are sequenced and glycosyltransferase sequences are identified, the full potential of this relatively untapped resource will become apparent.

Large scale production and availability of oligosaccharides will bring down the cost and stimulate research in the glycobiology field, ultimately leading to new scientific discoveries and expanded practical applications of oligosaccharides.

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