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MINI-REVIEW

Sialic acid metabolism and sialyltransferases: natural functions and applications

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Abstract Sialic acids are a family of negatively charged monosaccharides which are commonly presented as the terminal residues in glycans of the glycoconjugates on eukaryotic cell surface or as components of capsular polysaccharides or lipooligosaccharides of some pathogenic bacteria. Due to their important biological and pathological functions, the biosynthesis, activation, transfer, breaking down, and recycle of sialic acids are attracting increasing attention. The understanding of the sialic acid metabolism in eukaryotes and bacteria leads to the development of metabolic engineering approaches for elucidating the important functions of sialic acid in mammalian systems and for large-scale production of sialosides using engineered bacterial cells. As the key enzymes in biosynthesis of sialylated structures, sialyltransferases have been continuously identified from various sources and characterized. Protein crystal structures of seven sialyltransferases have been reported. Wild-type sialyltransferases and their mutants have been applied with or without other sialoside biosynthetic enzymes for producing complex sialic acid-containing oligosaccharides and glycoconjugates. This mini-review focuses on current understanding and applications of sialic acid metabolism and sialyltransferases.

Keywords Carbohydrate · Metabolism · Sialic acid · Sialoside · Sialyltransferase

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Introduction

Sialic acids are a family of α-keto acids with a nine-carbon backbone. More than 50 sialic acid forms have been found in nature including the most abundant *N*-acetylneuraminic acid (Neu5Ac), nonhuman *N*-glycolylneuraminic acid (Neu5Gc), 2-keto-3-deoxy-nonulosonic acid (or deaminoneuraminic acid) (Kdn), and their *O*-methyl, *O*-lactyl, *O*-sulfo, *O*-phospho-, and single or multiple *O*-acetyl derivatives (Angata and Varki 2002; Schauer 2000; Chen and Varki 2010).

Sialic acids are commonly found as the terminal monosaccharides of the glycans presented in glycoconjugates (glycoproteins and glycolipids) on cell surfaces of vertebrates and higher invertebrates (Varki et al. 2011; Chen and Varki 2010). They are also components of lipooligosaccharides or capsular polysaccharides of some pathogenic bacteria including well-studied pathogens *Escherichia coli* K1, *Haemophilus influenzae*, *Haemophilus ducreyi*, *Pasteurella multocida*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Campylobacter jejuni*, and *Streptococcus agalactiae* (Almagro-Moreno and Boyd 2009; Vimr et al. 2004; Severi et al. 2007). Sialic acids play pivotal roles in many physiologically and pathologically important processes, including nervous system embryogenesis, cancer metastasis, immunological regulation, bacterial and viral infection, etc. (Angata and Varki 2002; Chen and Varki 2010).

Although sialic acid metabolism pathways differ in eukaryotes and bacteria, both involve the coordinated action of several enzymes that catalyze the biosynthesis, activation, and transfer of sialic acids for the formation of sialyl glycoconjugates, as well as modifications and degradation of sialyl glycoconjugates and sialic acids. Abnormal metabolism of

sialic acid in human has been associated with various pathological conditions (Schwarzkopf et al. 2002). For example, mutation of human bifunctional enzyme GNE with both hydrolyzing uridine 5′-diphosphate-*N*-acetylglucosamine (UDP-GlcNAc) 2-epimerase and *N*-acetylmannosamine (ManNAc) kinase activities is related to two human disorders including sialuria (OMIM 269921) and hereditary inclusion body myopathy (HIBM, OMIM 600737) (Yardeni et al. 2011). Mutations of human lysosomal sialidase NEU1 have been related to the lysosomal storage disorder sialidosis (OMIM 256550) (Bonten et al. 1996; Pshezhetsky et al. 1997). In addition, normal metabolic incorporation of the nonhuman Neu5Gc from dietary sources (mainly red meat) to human tissues (mainly endothelia and epithelia) in the face of circulating anti-Neu5Gc antibodies led to chronic inflammation named xenosialitis (Varki et al. 2011).

Given the importance of sialic acids and the sequence and structural differences of human and bacterial enzymes involved in sialic acid metabolism, some enzymes are potential targets for drug development such as sialic acid synthases, CMP-sialic acid synthetases, sialyltransferases, sialidases, and sialic acid modification enzymes. In addition, although some human pathogenic bacteria (e.g., *Vibrio cholerae*, *Anthrobacter ureafaciens*, *Clostridium perfringens*, *Salmonella typhimurium*, *Streptococcus pneumoniae*, etc.) (Chokhawala et al. 2007b), commensals (e.g., *Bacteroides fragilis*) (Thompson et al. 2009), probiotics (e.g., *Bifidobacterium infantis*) (Sela et al. 2011), and viruses (e.g., Newcastle disease virus and influenza virus) (von Itzstein 2007; Paulson et al. 1982) do not biosynthesize sialic acid or sialylglycoconjugates, they produce sialidases or neuraminidases (sialic acid-cleaving enzymes) which have been shown to be virulence factors, to provide nutrient or to release the newly formed virons. Inhibitors against human influenza viruses generated by protein crystal structure-assisted rational drug design, such as Relenza (Zanamivir) and Tamiflu (Oseltamivir), have been commercialized and used as effective anti-influenza virus drugs although recent emerging drug-resistant strains demand new anti-flu therapeutics (von Itzstein and Thomson 2009; Mitrasinovic 2010). A recent study showed that sialidase substrate specificity-based inhibitor design was also an effective approach for identification of selective inhibitors against certain sialidases (Li et al. 2011). Pathogenic bacterial sialidases have been shown to disrupt the repressive immune-regulation of sialic acid-based interaction and cause server damage of host tissues during bacterial sepsis. A cocktail of two bacterial sialidase inhibitors has been used to protect mice dying from sepsis in a cecal ligation and puncture model (Chen et al. 2011). Besides sialidases, the crystal structures of many other sialic acid metabolic enzymes have been reported. Nevertheless, a clear understanding of the significance of nature's sialic acid structural diversity is still missing. This is mainly due to the analytical challenges in elucidating sialic acid-dependent interactions and synthetic difficulties in obtaining homogenous sialic acid-containing oligosaccharides and glycoconjugates, especially those contain diverse naturally occurring sialic acid modifications from natural sources (Yu and Chen 2007). Clearly, developing metabolic engineering approaches to characterize sialic acid-containing structures and sialic acid-binding proteins as well as establishing simple and efficient methods to synthesize sialylated structures in vitro are important to unravel the numerous biological roles of sialic acid and to assist drug sign.

This mini-review highlights current understanding of eukaryotic and bacterial sialic acid metabolic pathways and their applications in cell surface labeling and sialosides production via metabolic engineering. The natural functions of sialyltransferases and their applications in chemoenzymatic synthesis of various sialic acid-containing structures are also discussed.

Sialic acid metabolism

Among more than 50 different sialic acid forms that have been identified in nature, some are shared by bacteria, higher invertebrates, and vertebrates, while others have been identified exclusively in certain species. In addition, even for Neu5Ac, the most common sialic acid in nature, the metabolism pathways of bacteria and eukaryotes differ from each other, especially on the biosynthesis of sialic acids. In addition, the locations of enzymes involved in sialic acid activation and transfer as well as sialyl glycoconjugate degradation are different for bacteria and eukaryotes.

Sialic acid metabolism in eukaryotes

Sialic acid biosynthesis in vertebrates and higher invertebrates takes place in the cytosol involving three enzymes in a four-step process. The first two steps are catalyzed by a bifunctional enzyme called GNE with both hydrolyzing UDP-GlcNAc 2-epimerase and ManNAc kinase activities (Fig. 1). The epimerase function of the GNE converts UDP-GlcNAc to ManNAc with removal of the UDP moiety and epimerization of the GlcNAc. The kinase function of GNE then phosphorylates ManNAc to form ManNAc-6-P. Neu5Ac is then produced by the condensation and dephosphorylation reactions catalyzed by Neu5Ac 9-phosphate synthase (NANS) and Neu5Ac-9-phosphate phosphatase (NANP), respectively (Varki and Schauer 2008).

Despite the differences in the biosynthesis of sialic acids in bacteria and eukaryotes, the sialic acid activation and transfer processes are conserved from bacteria through humans although the locations of enzymes differ. In eukaryotic cells, The Neu5Ac synthesized in the cytosol is transferred to the nucleus and activated by

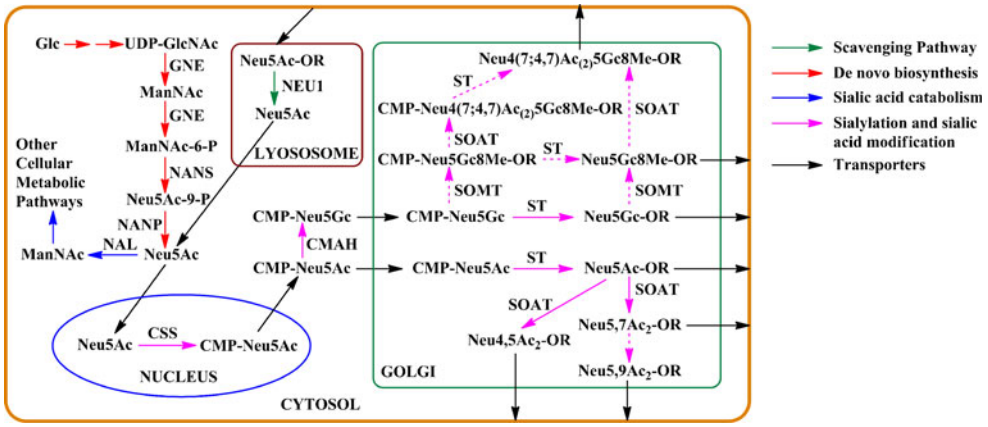

Fig. 1 Sialic acid metabolism in eukaryotes. Abbreviations: *Glc* glucose, *UDP-GlcNAc* uridine 5′-diphospho-*N*-acetylglucosamine, *ManNAc* *N*-acetylmannosamine, *ManNAc-6-P* *N*-acetylmannosamine 6-phosphate, *Neu5Ac-9-P* *N*-acetylneuraminic acid-9-phosphate, *Neu5Ac* *N*-acetylneuraminic acid, *CMP-Neu5Ac* cytidine 5′-monophospho-*N*-acetylneuraminic acid, *CMP-Neu5Gc* cytidine 5′-monophospho-*N*-glycolylneuraminic acid, *R* glycoprotein or glycolipid. Enzymes: *GNE* hydrolyzing UDP-GlcNAc 2-epimerase/ManNAc-6-kinase, *NANS* Neu5Ac-9-P synthetase, *NANP* Neu5Ac-9-P phosphatase, *NAL* *N*-acetylneuraminate lyase, *CSS* CMP-sialic acid synthetase, *CMAH* cytidine monophosphate-*N*-acetylneuraminic acid hydroxylase, *ST* sialyltransferase, *SOAT* sialate-*O*-acetyltransferase, *SOMT* sialate-*O*-methyltransferase, *NEU1* lysosomal sialidase

cytosine 5′-monophosphate *N*-acetylneuraminic acid (CMP-Neu5Ac) synthetase (EC 2.7.7.43) to form CMP-Neu5Ac which then goes to the Golgi to be used by sialyltransferases for the formation of glycoconjugates which are subsequently secreted or delivered to the cell surface (Kean et al. 2004; Altheide et al. 2006).

Sialic acids on glycolipids and glycoproteins are released in lysosome by sialidases (e.g., human NEU1) as part of the overall degradation of these glycoconjugates and are pumped back into the cytosol, where they can go through another cycle of sialyl glycoconjugate production or be broken down by Neu5Ac lyase (or sialic acid aldolase) to form ManNAc and pyruvate (Verheijen et al. 1999). Multiple sialidases have been found in human cells. Other than the lysosomal sialidase NEU1 (Pshezhetsky et al. 1997), three additional human sialidases including the cytosolic sialidase NEU2 (Monti et al. 1999), the plasma membrane-associated sialidase NEU3 (Wada et al. 1999), and the lysosomal or mitochondrial membrane-associated sialidase NEU4 (Monti et al. 2004) have been identified. These four human sialidases showed different substrate specificities and physiological functions (Miyagi 2008b). Aberrant sialylation is closely associated with the malignant phenotype of cancer cells including metastatic potential and invasiveness (Miyagi 2008a; Miyagi et al. 2004).

The de novo biosynthesis CMP-Kdn is believed to start from mannose and follow a similar route as CMP-Neu5Ac from ManNAc although it is not as well elucidated (Angata and Varki 2002; Terada et al. 1993; Angata et al. 1994, 1999).

The biosynthesis of sialyl glycoconjugates, however, is complicated by the additional modifications on sialic acid either before or after the formation of sialyl linkages in the Golgi. One such example is the nonhuman Neu5Gc. In animals including nonhuman hominids (previously great apes) such as chimpanzees, bonobos, gorillas, and orangutans, CMP-Neu5Ac is converted to CMP-Neu5Gc in the cytosol by Neu5Ac hydroxylase encoded by the *Cmah* gene. In contrast, humans do not synthesize CMP-Neu5Gc due to the inactivation of CMP-Neu5Ac hydroxylase by a frameshift mutation of the *CMAH* gene. Nevertheless, nonhuman Neu5Gc can be metabolically incorporated from dietary sources (mainly red meat) to human tissues (mainly endothelia and epithelia) in the face of circulating anti-Neu5Gc antibodies (Varki et al. 2011; Taylor et al. 2010; Pham et al. 2009; Padler-Karavani et al. 2008). Neu5Gc-containing glycoconjugates have also been found in the sera of cancer patients, human cancerous tissues, cultured human cell lines, and recombinant therapeutic glycoproteins (Inoue et al. 2010; Ghaderi et al. 2010). Human anti-Neu5Gc antibodies against Neu5Gc-sialyl Tn antigen have been identified as novel serum biomarkers and immunotherapeutics in human cancer based on studies using a novel sialoside glycan microarray (Padler-Karavani et al. 2011).

Another common sialic acid modification is *O*-acetylation. It is one of the most frequent modifications of sialic acids in eukaryotes cells, and evidence has shown that sialic acid *O*-acetylation on glycoconjugates in the Golgi involves both an acetyl-CoA transporter and an intraluminal *O*-acetyltransferase (Varki and Diaz 1985; Diaz et al. 1989; Higa et al. 1989). C7- and C9-*O*-acetylation have been shown in bovine submandibular gland (Vandamme-Feldhaus and Schauer 1998; Lrhorfi et al. 2007), and C4-*O*-acetylation has been observed in microsomes from equine submandibular glands (Tiralongo et al. 2000). Human colon mucosa is also a rich source of *O*-acetylated sialic acids, and the level of *O*-acetylation is

reduced significantly in colorectal cancer (Corfield et al. 1999). Modifications of Neu5Gc have been shown in salmonid fish egg glycoproteins (Sato et al. 1993). Sialate-*O*-acetyltransferases (SOATs) and sialate-*O*-acetylesterases (SOAEs) are responsible for the addition and removal of *O*-acetyl groups, respectively (Shen et al. 2004a; Srinivasan and Schauer 2009). In animals, the best characterized SOATs are sialate-4-*O*-acetyltransferase in guinea pig liver (Iwersen et al. 1998, 2003) and sialate-7(9)-*O*-acetyltransferase studied in rat liver (Higa et al. 1989), human colon (Shen et al. 2004a), and bovine submandibular gland (Lrhorfi et al. 2007). The C9-*O*-acetyl sialic acid is believed to be formed by the migration of the C7-*O*-acetyl group on glycosidically bound sialic acids, most likely also catalyzed by an enzyme (Vandamme-Feldhaus and Schauer 1998) (Fig. 1). The amount of *O*-acetylated sialic acid in a special tissue (e.g., human colon mucosa) or cell is believed to be dependent on the activities of both SOATs and SOAEs (Shen et al. 2004b). A positive correlation between the increased SOAT activity and the enhanced expression of Neu5,9Ac_2-glycoconjugates is seen in the microsomes of lymphoblasts (Mandal et al. 2009). In addition, SOAE activity is decreased in both lysosomal and cytosolic fractions of acute lymphoblastic leukemia cell lines (Mandal et al. 2012). Human *CasD1* gene, encoding a protein with a serine–glycine–asparagine–histidine hydrolase domain and a hydrophobic transmembrane domain, is believed to be involved in *O*-acetylation of α2–8-linked sialic acids (Arming et al. 2011).

Other than *O*-acetylation, *O*-methylation of sialic acids (mainly on lower invertebrates such as starfish) has also been well studied (Bergwerff et al. 1992; Zanetta et al. 2006; Kelm et al. 1998). In starfish *Asterias rubens* gonads, most Neu5Gc residues are 8-methylated in addition to C4- and/or C7-*O*-acetylation (Zanetta et al. 2006). Free Neu5Ac and Neu5Gc can be methylated, although those presented on oligosaccharides and glycoproteins are better substrates for enzymatic methylation (Kelm et al. 1998) (Fig. 1).

Different from the de novo biosynthesis of sialic acid in most eukaryotes, some protozoa species, such as *Trypanosoma cruzi* (a causive agent of Chagas disease or American trypanosomiasis), use a surface α2–3-*trans*-sialidase (EC 2.4.1.-) to transfer α2–3-linked sialic acid residues directly from host sialyl glycoconjugates to the terminal β-galactose residues of the parasite mucins and form their own surface sialyl glycoconjugates. In comparison, a related American parasite *Trypanosoma rangeli* secretes a homologous sialidase but does not express *trans*-sialidase (Buschiazzo et al. 1997; Montagna et al. 2006). Two *trans*-sialidase (TS) forms (TS-1 and TS-2) have been purified from procyclic *Trypanosoma congolense* (a causive agent of animal African trypanosomiasis) cultures. The TS-1 form has higher TS activity and significantly less sialidase activity, whereas sialidase activity was predominately found in TS-2 form (Tiralongo et al. 2003). Two TS-1 variants have been cloned and showed activity in sialylating asialofetuin (Koliwer-Brandl et al. 2011). Interestingly, the procyclic stage of *Trypanosoma brucei* (a human African trypanosome that causes sleeping sickness) in the insect vector expresses a surface α2–3-*trans*-sialidase (TbTS) and an α2–3-sialidase separately (Montagna et al. 2006). More recently, a second catalytically active α2–3-*trans*-sialidase has also been identified from *T. brucei* (Nakatani et al. 2011). The activity of *trans*-sialidase is crucial for the pathogenesis of the parasites (Eugenia Giorgi and de Lederkremer 2011). Together with majority of eukaryotic and bacterial *exo*-sialidases, all *trans*-sialidases have been grouped into the glycosidase hydrolase family GH33 in the carbohydrate-active enzymes (CAZy) database (http://www.cazy.org) based on protein sequence homology (Henrissat 1991; Davies and Henrissat 1995). The most well-studied *trans*-sialidase is TcTS which is a glycosylphosphatidylinositol-anchored protein that is also shed into the milieu (Sartor et al. 2010). Its crystal structures have been reported (Buschiazzo et al. 2002; Amaya et al. 2004). Like *exo*-sialidases, TcTS follows a double displacement mechanism with the formation of an enzyme–substrate intermediate and an overall retention of the anomeric carbon stereoconfiguration of the sialic acid (Amaya et al. 2004; Damager et al. 2008; Watts et al. 2003). Tyr342 was identified as the nucleophile that attacks the anomeric carbon of the sialic acid and forms the enzyme–substrate intermediate (Watts et al. 2003). A Tyr342 to histidine mutation causes the inactivation of TcTSs and has been commonly observed for some *T. cruzi* strains (Cremona et al. 1996; Oppezzo et al. 2011). Due to its important roles in parasite infection and its functional difference from sialyltransferases or sialidases in human, TcTS is an attractive drug target (Sartor et al. 2010). Recently, a new generation of TcTS inhibitors have been designed (Buchini et al. 2008) and a highly specific high affinity (subnanomolar) TcTS neutralizing mouse monoclonal antibody (mAb 13 G9) has been identified (Buschiazzo et al. 2012). TbTS has also been suggested as a target for DNA vaccine development (Silva et al. 2009). An antibody (mAb 7/23) specifically against *T. congolense* TS-1 form is also available (Tiralongo et al. 2003).

Metabolic engineering of vertebrate cell surface sialic acids

The understanding of sialic acid metabolic pathways in eukaryotes leads to the development of metabolic engineering approaches for labeling and functional studies of sialic acid-containing glycoconjugates and sialic acid-binding proteins. Sialic acids modified with a small chemical handle (e.g., ketone, azide, alkyne, diazirine, or thiol) have been incorporated via metabolic engineering onto vertebrate cell surface in cell culture or in living animals and allow later

detections using a bioorthogonal ligand conjugated to a fluorophore, a biotin molecule, or an antigen that can be recognized by specific antibodies. In addition to in vitro and in vivo imaging and characterization of sialic acid-containing glycoconjugates using *N*-levulinoylmannosamine and *N*-azidoacetylmannosamine (ManNAz) as sialic acid precursors for metabolic engineering (Mahal et al. 1997; Prescher et al. 2004), a sialic acid analog with an *N*-butanoyl group has been used to inhibit expression of α2–8-linked polysialic acids on cell surface (Mahal et al. 2001). Neu5Ac analogs with *N*-propionyl, *N*-*iso*-butanoyl, and *N*-phenylacetyl derivatives of Neu5Ac have also been used to improve the immnuogenicity of glycan-based cancer vaccine (Krug et al. 2004; Chefalo et al. 2006; Wu and Guo 2006). Four processes have been used to metabolically incorporate sialic acid analogs onto cell surface. A less used approach is to start with GlcNAc analogs, which can form UDP-GlcNAc analogs to be used by sialic acid de novo synthetic pathway. It turns out that the strict substrate specificity of GNE limits the diversity of sialic acid analogs that can be generated using this approach (Yarema and Bertozzi 1998; Tanaka and Kohler 2008). A more frequently used approach is to start with ManNAc analogs, which are usually peracetylated to help their admission into cells. For this approach, the NANS is the bottleneck process and its substrate specificity excludes the use of long or branched *N*-acyl ManNAc (Jacobs et al. 2001; Viswanathan et al. 2003) or C6-modified ManNAc derivatives (Lawrence et al. 2000). Another frequently used approach is to start with peracetylated sialic acid analogs to bypass the substrate limitations of GNE and NANS. A minor drawback is the relatively higher cost or increased difficulties of synthesizing sialic acid analogs comparing to GlcNAc or ManNAc analogs. The least used approach is to start with CMP-sialic acid analogs as the synthesis of such compounds is more time-consuming and challenging. Besides the nonnatural sialic acids, analogs of other common monosaccharides found in eukaryotes have also been successfully incorporated into the glycoconjugates on the cell surface by metabolic glycoengineering methodologies. For more information on metabolic engineering of sialic acid and other sugars such as *N*-acetylgalactosamine (GalNAc), *N*-acetylglucosamine (GlcNAc), and fucose, readers are directed to two excellent reviews (Campbell et al. 2007; Du et al. 2009).

Bacterial sialic acid metabolism

Most bacteria are unable to synthesize sialic acid except for a limited number of pathogenic bacteria and commensals of which majority are related to human (Crocker and Varki 2001). Some pathogenic bacteria can coat themselves with sialic acid, protecting themselves from the detection of host immune system by regulating complement interaction as well as downregulate adaptive and innate immune responses (Carlin et al. 2009; Varki et al. 2011). Some bacteria use sialic acid as a nutrient (Severi et al. 2007).

Bacterial pathogens have evolved two ways to obtain sialic acid: de novo and scavenging pathways. Similar to that of eukaryotes, the de novo pathway of bacteria (e.g., *E. coli* K1, *N. meningitidis*, *C. jejuni*, and *S agalactiae*) begins with the conversion of UDP-GlcNAc to ManNAc by hydrolyzing UDP-GlcNAc 2-epimerase (NeuC) (Fig. 2). Different from eukaryotes, ManNAc produced by bacteria is directly used in the presence of phosphoenolpyruvate (PEP) by Neu5Ac synthase (NeuB) for the formation of Neu5Ac and inorganic phosphate without the involvement of a

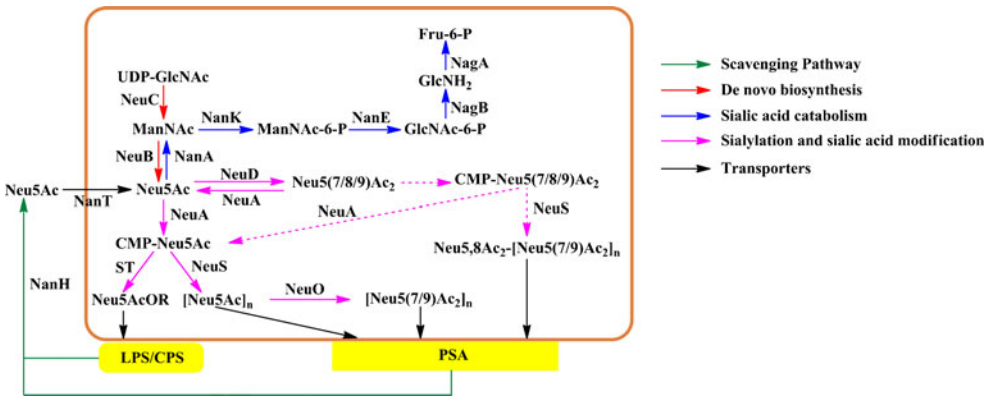

Fig. 2 Bacterial sialic acid metabolism using *E. coli* K1 as a model system. Abbreviations: *UDP-GlcNAc* uridine 5′-diphosphate-*N*-acetylglucosamine, *ManNAc* *N*-acetylmannosamine, *Neu5Ac* *N*-acetylneuraminic acid, *CMP-Neu5Ac* cytidine 5′-monophospho-*N*-acetylneuraminic acid, *GlcNAc* *N*-acetylglucosamine, *Fru* fructose, *LPS* lipopolysaccharide, *CPS* capsule polysaccharide, *PSA* polysialic acid, *R* LPS or CPS. Enzymes: *NeuC* hydrolyzing UDP-GlcNAc 2-epimerase, *NeuB* sialic acid synthase, *NanA* sialic acid aldolase, *NeuA* CMP-sialic acid synthetase (NeuA in *E. coli* K1 and *S. agalactiae* also possesses *O*-acetylesterase activity), *NeuD* sialic acid *O*-acetyltransferase, *ST* sialyltransferase, *NeuS* polysialyltransferase, *NeuO* polysialic acid *O*-acetyltransferase, *NanK* ManNAc kinase, *NanE* ManNAc-6-phosphate epimerase, *NagB* GlcNAc-6-phosphate deacetylase, *NagA* glucosamine-6-phosphate deaminase, *NanH* sialidase, *NanT* Neu5Ac transporter

kinase or a phosphatase (Bravo et al. 2004; Angata and Varki 2002). The scavenging pathway involves two scenarios: donor scavenging (only found in *Neisseria gonorrheae*) in which CMP-sialic acid is scavenged from the hosts (Parsons et al. 1994), and precursor scavenging (e.g., *H. influenzae*, *H. ducreyi*, *Haemophilus somnus*, and *P. multocida* belonging to the *Haemophilus–Actinobacillus–Pasteurella* or HAP group) in which free sialic acid is obtained directly from the host (Steenbergen et al. 2005; Schilling et al. 2001).

Similar to eukaryotes, sialylation in bacteria is mainly catalyzed by sialyltransferases (STs) and sialic acid catabolism is carried out by sialidases and Neu5Ac lyase (or sialic acid aldolases, NanA in *E. coli* K1 and K12) which catalyzes the breakdown of Neu5Ac to form ManNAc and pyruvate (Vimr et al. 2004).

In some bacteria (e.g., *C. jejuni*, *E. coli* K1, and *S. agalactiae*), sialic acid can be further modified, such as by *O*-acetylation. For example, the terminal sialic acid on the disialylated LPS in *C. jejuni* can be modified by an *O*-acetyltransferase identified recently (Houliston et al. 2006). Both *E. coli* K1 and *S. agalactiae* (or group B *Streptococcus*, GBS) have sialic acid *O*-acetyltransferases, and the C-terminal sequence of their CMP-sialic acid synthetases has sialic acid *O*-acetylesterase activity (Steenbergen et al. 2006; Lewis et al. 2007). In vitro studies showed that GBS CMP-sialic acid synthetase (NeuA) de-*O*-acetylated sialic acid by two alternate pathways: de-*O*-acetylation of Neu5,9Ac_2 followed by CMP activation of Neu5Ac and activation of Neu5,9Ac_2 followed by de-*O*-acetylation of CMP-Neu5,9Ac_2 (Lewis et al. 2007). In comparison, *N. meningitidis* has a shorter CMP-sialic acid synthetase lacking the C-terminal sialic acid *O*-acetylesterase activity in *E. coli* K1 and GBS enzymes. Different from *O*-acetylation of sialic acids on oligosaccharide moieties of glycoconjugates, separate pathways are proposed for sialic acid-containing polymers. For example, *O*-acetylation at C-7 and C-9 of the sialic acid in polysialic acid capsules is catalyzed by NeuO in *E. coli* K1 and Oat C in *N. meningitidis* serogroup B. A minor NeuO-independent, NeuD-dependent, *O*-acetylation pathway with the involvement of NeuA and NeuS is also proposed (Steenbergen et al. 2006). For more information about sialic acid metabolism and function in bacterial pathogens, readers are referred to two excellent reviews (Vimr et al. 2004; Severi et al. 2007).

Production of sialosides by engineering bacterial sialic acid metabolic pathways

The understanding of sialic acid metabolic pathways in sialic acid-producing bacteria has helped to develop metabolic engineering approaches for large-scale production of sialosides using whole cell catalysts or by fermenting living cells (so called the living factory approach) (Chen and Varki 2010). Large-scale production of 3′-sialyllactose (Endo et al. 2000) and the carbohydrate portion of the sialyl-Tn epitope, Neu5Acα2–6GalNAc (Endo et al. 2001), has been achieved using the whole cell catalysts strategy. This strategy uses a UTP/CDP-producing *Corynebacterium ammoniagenes* strain and three *E. coli* strains harboring plasmids encoding a CTP synthetase, a CMP-Neu5Ac synthetase, and a suitable sialyltransferase, respectively. All cells were grown and collected separately. They were permeablized and combined in one pot for sialoside production. In comparison, fermenting living cells engineered by adding suitable sialic acid biosynthetic genes and eliminating genes involved in divergent pathways seems to be more convenient and cost effective. An earlier version of this approach was reported in 2002 for large-scale production of 3′-sialyllactose using a *lacZ*$^-$ *E. coli* strain engineered by inactivating the endogenous sialic acid aldolase gene (*nanA*$^-$) and adding plasmids containing *N. meningitidis* CMP-Neu5Ac synthase gene (*neuA*) and *N. meningitidis* α2–3-sialyltransferase gene, respectively. In this system, relatively expensive sialic acid was added exogenously and transported into the cells by endogenous permease NanT for the production of the target sialoside (Priem et al. 2002). A similar system with additional glycosyltransferase genes has been generated for large-scale synthesis of GM1 and GM2 oligosaccharides (Antoine et al. 2003; Fort et al. 2005). An improved more economic version of 3′-sialyllactose-production strain eliminates the need of adding exogenous sialic acid by knocking out the endogenous ManNAc kinase (*nanK*$^-$) gene in *lacZ*$^-$ *E. coli* K12 strains devoid of sialic acid aldolase (*nanA*$^-$) and introducing additional genes including *neuC* and *neuB* from *C. jejuni* in addition to the previously introduced *neuA* and α2–3-sialyltransferase genes. This strategy allows the bacterium to generate sialic acid from endogenous UDP-GlcNAc produced through its own metabolism (Fierfort and Samain 2008). Recently, this improved strategy has been used to produce 6′-sialyllactose, 6,6′-disialyllactose, and 6′-Kdo-lactose with metabolically engineered *E. coli* harboring a sialyltransferase from *Photobacterium* sp. JT-ISH-224 (Drouillard et al. 2010).

Sialyltransferases

As described above, STs (EC 2.4.99.X) are key enzymes in the biosynthesis of sialic acid-containing oligosaccharides and glycoconjugates (Harduin-Lepers et al. 2005; Harduin-Lepers et al. 1995). They catalyze the reaction that transfers a sialic acid residue from its activated sugar nucleotide donor cytidine 5′-monophosphate sialic acid to a variety of acceptor molecules, usually a structure terminated with a galactose (Gal), an GalNAc, or another sialic acid (Sia) residue (Chen and Varki 2010).

Classification of sialyltransferases

Based on the linkages that they form, common sialyltransferases have been classified to α2–3-, α2–6-, α2–8-sialyltransferases, and polysialyltransferases. In human and animals, α2–3- and α2–6-sialyltransferases can modify a numbers of core glycan structures on different proteins, while polysialyltransferases that catalyze the formation of α2–8-linked sialic acid homopolymers appear to be highly selective for their protein carriers. Other than autopolysialylation of ST8Sia IV and ST8Sia II, only four other protein carriers have been identified for ST8Sia IV and ST8Sia II: the neural cell adhesion molecule (also called CD56), the α-subunit of voltage-gated sodium channel, CD36, and neuropilin (Drake et al. 2008). In bacteria, polysialyltransferases that catalyze the formation of α2–8- and/or α2–9-linked capsular polysaccharides of *N. meningitidis* serogroups B and C and *E. coli* K1 and K92 strains have been identified (Steenbergen and Vimr 2003). Examples of the products formed by different sialyltransferases are shown in Fig. 3. In addition, capsular polysaccharide polymerases that contain both sialyltransferase and hexosyltransferase activities for the construction of sialic acid-containing heteropolymeric capsular polysaccharides of *N. meningitidis* serogroups W-135 and Y are also presented in nature (Claus et al. 2009).

Based on their protein sequence homology, all known sialyltransferase have been classified into six glycosyltransferase (GT) families (Thon et al. 2011) in the CAZy database (http://www.cazy.org) (Campbell et al. 1997; Coutinho et al. 2003). All sialyltransferases and polysialyltransferases from eukaryotes and viruses are grouped into glycosyltransferase family 29 (GT 29). Bacterial STs have been grouped into GT4, GT38, GT42, GT52, and GT80 five CAZy GT families. More details are discussed below for eukaryotic and bacterial sialyltransferases, respectively.

Eukaryotic sialyltransferases

Together with some viral sialyltransferases, all identified eukaryotic sialyltransferases share sequence homology and belong to CAZy GT29 family (Harduin-Lepers et al. 2001, 2005; Harduin-Lepers 2010). Due to the diversities of the sialyl linkages that they form and the acceptor substrates that they recognize, multiple sialyltransferases are produced by single species of eukaryotes. For example, 20 human sialyltransferases and polysialyltransferases have been identified and are classified into four groups according to the type of linkage formed and the nature of the sugar acceptor. These include six beta-galactoside α2–3-sialyltransferases (ST3Gal I–VI), two beta-galactoside α2–6-sialyltransferases (ST6Gal-I–II), six GalNAc α2–6-sialyltransferases (ST6GalNAc-I–

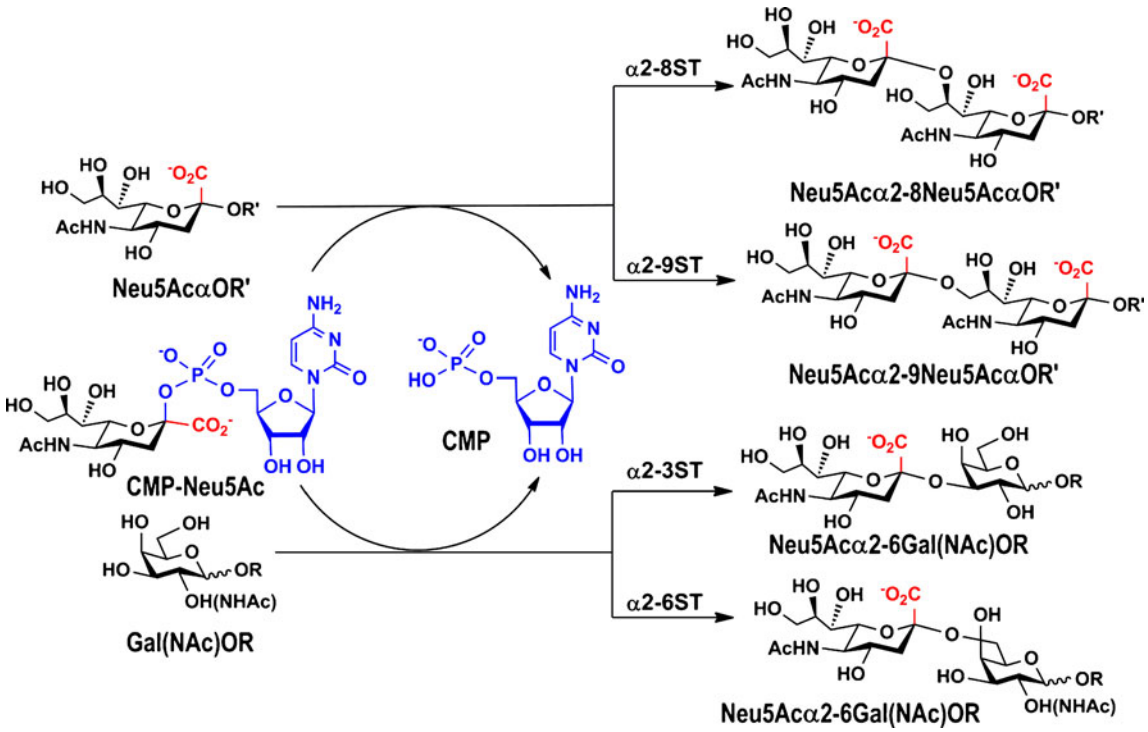

Fig. 3 Sialyltransferases catalyze the transfer of sialic acid from CMP-sialic acid to a suitable acceptor, commonly a Gal, GalNAc, or Sia terminated glycan or glycoconjugates. The most common sialic acid form, *Neu5Ac*, is shown as an example. *R* glycans or glycoconjugates, *R′* Neu5Ac- or Gal-terminated glycans or glycoconjugates

VI), and six α2–8-sialyltransferases (ST8Sia-I–VI, among which ST8Sia-II and ST8Sia-IV are polysialyltransferases). The sialyltransferases in the same group share some overlapping but not identical acceptor substrate specificities. The presence of such a large number of sialyltransferases in human and other animals is another indication of the important roles of sialic acid-containing structures. Mammalian STs have quite strict acceptor substrate specificity but relatively more relaxed donor substrate specificity (Harduin-Lepers 2010; Datta 2009), although their donor substrate specificities have been less explored. CMP-Neu5Gc, CMP-Neu5,9Ac_2 (Higa and Paulson 1985), and CMP-Kdn (Angata et al. 1998) have shown to be acceptable donor substrates by some mammalian sialyltransferases.

Like other vertebrate glycosyltransferases, mammalian sialyltransferases are type II membrane proteins. They are localized in the Golgi and have an N-terminal cytoplasmic domain, a single transmembrane domain of 16–20 amino acid residues, a size variable (20–300 amino acid residues) stem region, and a soluble relatively well-conserved C-terminal catalytic domain of 300±20 amino acid residues in the Golgi lumen (Audry et al. 2011; Chen and Varki 2010). The catalytic domains of mammalian sialyltransferases and a viral sialyltransferase vST3Gal-I that can use fucose-containing Lewis x antigens as acceptors (Sugiarto et al. 2011b) have four conserved sialyl motifs including long (L), short (S), very small (VS) motifs, and motif 3 (Datta and Paulson 1995; Datta et al. 1998; Jeanneau et al. 2004). A disulfide bond stabilizing the L- and the S-motifs is also conserved among these sialyltransferases (Datta et al. 2001). Site-directed mutagenesis and structures of porcine ST3Gal-I show that the L-motif is involved in the donor binding, motifs 3 and VS contribute to the binding of the acceptor, and the S-motif participates in the binding of both donor and acceptor substrates (Datta 2009; Audry et al. 2011; Rao et al. 2009; Paulson and Rademacher 2009). A conserved histidine residue located in the VS-motif has been identified as the catalytic base from the X-ray crystal structures of porcine ST3Gal-I which have the GT-A fold with a single Rossmann domain (Rao et al. 2009).

Bacterial sialyltransferases

Bacterial sialyltransferases have been mainly identified and characterized from several pathogenic bacteria including *N. meningitidis*, *N. gonorrheae*, *C. jejuni*, *H. influenzae* and *H. ducreyi*, *P. multocida*, *S. agalactiae*, and some marine bacteria. Some of these bacteria including *N. meningitidis*, *H. influenzae*, *H. ducreyi*, *P. multocida*, and *Photobacterium* species JT-ISH-224 have multiple sialyltransferase genes. Different from mammalian sialyltransferases which are commonly monofunctional sialyltransferases, many bacterial sialyltransferases have multiple functions including sialyltransferase activities responsible for forming different sialyl linkages with or without additional sialidase and *trans*-sialidase activities (Chen and Varki 2010).

Unlike vertebrate and viral sialyltransferases which share protein sequence homology and all belong to CAZy GT29 family, bacterial sialyltransferases have less conserved protein sequences and are distributed into five CAZy GT families. The highly homologous capsular polysaccharide (CPS) polymerases (SiaDs) of *N. meningitidis* serogroups W135 and Y which have both hexosyltransferase (α1–4-galactosyltransferase activity for $SiaD_{W135}$ and α1–4-glucosyltransferase activity for $SiaD_Y$) and sialyltransferase activities responsible for the synthesis of sialic acid-containing heteropolymeric CPSs [-6Gal/Glcα1–4Neu5Acα2-]$_n$ belong to GT4 along with other glycosyltransferases. In comparison, *E. coli* K1 α2–8-polysialyltransferase (NeuS), *E. coli* K92 alternating α2–8/9-polysialyltransferase (Vimr et al. 1992; Steenbergen et al. 1992; Shen et al. 1999), *N. meningitidis* serogroup B α2–8-polysialyltransferase (SiaD) encoded by *siaD*/*synD*, and *N. meningitidis* serogroup C α2–9-polysialyltransferase encoded by *synE* are grouped into GT38 (Peterson et al. 2011). GT42 includes α2–3-sialyltransferases (Cst-I and Cst-III) and a multifunctional α2–3/8-sialyltransferase (Cst-II) which also has α2–8-sialidase and α2–8-*trans*-sialidase activities (Cheng et al. 2008) from *C. jejuni* (Gilbert et al. 2000, 2002), a *P. multocida* α2–3-sialyltransferase (PmST3) encoded by *Pm1174* gene (Thon et al. 2012), as well as an α2–3-sialyltransferase encoded by *lic3A* gene (Harrison et al. 2005) and a multifunctional α2–3/8-sialyltransferase (Lic3B) from *H. influenzae* (Fox et al. 2006). GT52 family contains characterized α2–3/6-sialyltransferases (Lsts) from *N. meningitidis* and *N. gonorrhoeae* (Gilbert et al. 1996), an *H. influenzae* α2–3-sialyltransferase (LsgB) (Jones et al. 2002), a *H. ducreyi* α2–3-sialyltransferase (Lst encoded by *Hd0686* gene) (Bozue et al. 1999), and a recently reported *P. multocida* glycolipid α2–3-sialyltransferase (PmST2) encoded by *Pm0508* gene (Thon et al. 2011). CpsK, another member of GT52 family and a homolog to the Lst of *H. ducreyi*, has also been identified as a putative α2–3-sialyltransferase for the synthesis of sialic acid-terminated capsular polysaccharide of *S. agalactiae* (group B *Streptococcus*) (Chaffin et al. 2002). GT80 family contains bacterial α2–3- and/or α2–6-sialyltransferases including a multifunctional *P. multocida* α2–3/6-sialyltransferase (PmST1) encoded by *Pm0188* gene which also has α2–3-sialidase and α2–3-*trans*-sialidase activities (Yu et al. 2005), an *H. ducreyi* α2–3-sialyltransferase (Hd2,3ST encoded by *Hd0053* gene) (Li et al. 2007), and several marine bacterial sialyltransferases such as a *Photobacterium damselae* α2–6-sialyltransferase (Pd2,6ST or JT0160 Bst) (Yamamoto et al. 1998; Sun et al. 2008) which also has α2–6-sialidase and α2–6-*trans*-sialidase activity

(Cheng et al. 2010), *Photobacterium leiognathi* α2–6-sialyltransferases with (Mine et al. 2010) or without additional α2–6-sialidase activity (Yamamoto et al. 2007), *Photobacterium phosphoreum* α2–3-sialyltransferase (Tsukamoto et al. 2007), an α2–3-sialyltransferase from *Vibrio* species (Takakura et al. 2007), as well as α2–3- and α2–6-sialyltransferases from *Photobacterium* species JT-ISH-224 (Tsukamoto et al. 2008).

Crystal structures of sialyltransferases

Despite the diversity of their primary protein sequences (grouped into 94 classified and one non-classified CAZy GT families), the tertiary structures of all glycosyltransferases characterized so far fall into only two structural folds: GT-A and GT-B folds. The GT-A fold consists of a single nucleotide-binding Rossmann domain with a typical α/β/α sandwich topology and a smaller fold. Although GT-A enzymes are commonly metal-ion-dependent and contain a conserved Asp-x-Asp (DxD) or equivalent motif that is crucial for catalysis, sialyltransferases usually do not require metal ion for catalysis, and GT-A fold sialyltransferases have been identified as two variants that do not have the metal-binding DxD conserved motif (Chiu et al. 2004, 2007). The GT-B fold consists of two separate Rossmann domains with a connecting linker region and a substrate binding site located in the cleft between the two domains. Although divalent cations may be required for full activity of some non-sialyltransferase GT-B enzymes, a bound metal ion associated with catalysis has not been seen in the GT-B fold glycosyltransferase structures characterized so far (Audry et al. 2011; Buschiazzo and Alzari 2008).

The crystal structures of seven sialyltransferases become available since the first report of the crystal structures of a bacterial multifunctional sialyltransferase Cst-II (a GT42 sialyltransferase) in 2004, 10 years after the first report of the crystal structures of a glycosyltransferase (Vrielink et al. 1994). These sialyltransferases span four sialyltransferase GT families including GT29, GT42, GT52, and GT80. The crystal structures of CPS polymerases (SiaDs) of *N. meningitidis* serogroups W135 and Y in GT4 family and any of the polysialyltransferases in GT38 family are still unknown.

The first crystal structure of the ST reported in 2004 for bacterial Cst-II from *C. jejuni* (GT42) belongs to the GT-A family (variant 1) (Chiu et al. 2004). Another *C. jejuni* sialyltransferase Cst-I (GT42) adopts a similar GT-A (variant 1) fold (Chiu et al. 2007). Both Cst-I and Cst-II are tetrameric. The recently reported crystal structures of porcine ST3Gal-I (GT29) adopts a second distinct GT-A variant (variant 2) (Rao et al. 2009). The multifunctional bacterial ST, PmST1 (GT80) from *P. multocida* was the second structure of an ST to be reported which belongs to the GT-B structural superfamily (Ni et al. 2006). In the same GT80 family, the crystal structures of two other STs sharing the GT-B fold, an α2–6ST from *Photobacterium sp.* JT-ISH-224 in complex with CMP and lactose (Kakuta et al. 2008) and an α2–3ST from *P. phosphoreum* in complex with CMP (Iwatani et al. 2009), have also been reported. Very recently, the first sialyltransferase structure of GT52 family for a membrane-associated α2–3/6 lipooligosaccharide sialyltransferase from *N. meningitidis* serotype L1 (NST) adopting a GT-B fold has been solved (Lin et al. 2011).

Despite their sequence and structural differences, all sialyltransferases characterized to date are inverting glycosyltransferases which catalyze the formation of α-sialyl linkage in the product from β-linked sialic acid in CMP-sialic acid donor substrate. The sialyltransferase is believed to follow a single displacement mechanism that involves nucleophilic attack of the acceptor hydroxyl (activated by a catalytic base in the enzyme) to the C2 anomeric center of the sialic acid moiety in the donor substrate with inversion of stereochemistry. In STs of GT29 and GT42, a histidine residue [H319 in pST3Gal-I (Rao et al. 2009), H202 in Cst-I (Chiu et al. 2007), and H188 in Cst-II (Chan et al. 2009)] serves as a catalytic base to deprotonate the reactive oxygen of the acceptor, while the most likely candidate for the catalytic base is an aspartic acid residue in the STs of GT80 [D141 in Δ24PmST1 (Ni et al. 2007)] and GT52 [D258 in NST (Lin et al. 2011)].

The inverting reaction of glycosyltransferases follows a S_N2-like mechanism (Lairson et al. 2008) and involves the formation of an oxocarbenium-like transition state with the concomitant departure of the nucleotide leaving group. In Cst-I and Cst-II, two conserved tyrosine residues [Y171 and Y177 in Cst-I (Chiu et al. 2007) and Y156 and Y162 in Cst-II (Chiu et al. 2004)] are involved in the departure of the CMP group of CMP-Neu5Ac, while in GT29, GT52, and GT80 STs, a conserved histidine residue [H302 in pST3GalI (Rao et al. 2009), H280 in NST (Lin et al. 2011), and H311 in Δ24PmST1 (Ni et al. 2007)] is involved in stabilizing the phosphate of the departing CMP.

Sialyltransferase mutants by crystal structure-based design or directed evolution

The structures of PmST1, a multifunctional highly active α2–3-sialyltransferase (pH 6.0–10.0) with α2–3-sialidase (pH=5.0–5.5), α2–3-*trans*-sialidase (pH=5.5–6.5), and α2–6-sialyltransferase (pH=4.5–7.0) activities, in the presence or the absence of CMP, CMP-3F(*axial*)Neu5Ac, CMP-3F(*equatorial*)Neu5Ac, and/or lactose provide a good understanding of the mechanism and the key residues involved in the sialyltransferase and the sialidase activities of the enzyme and allow the rational design of a PmST1 double mutant E271F/R313Y with significantly decreased α2–3-sialidase

activity without affecting its α2–3-sialyltransferase activity (Sugiarto et al. 2011a). Besides using the site-directed mutagenesis methodology to engineer sialyltransferases for desired properties, a fluorescence-based high-throughput screening method was developed for screening sialyltransferase mutants generated via error-prone polymerase chain reactions (PCRs). Using this directed evolution approach, a library of $>10^5$ sialyltransferase mutants were screened, and a variant with up to 400-fold higher catalytic efficiency using a fluorescently labeled acceptor was obtained (Aharoni et al. 2006). Recently, this method was improved by the introduction of a two-color screening protocol to minimize the possibility of false-positive mutants (Yang et al. 2010).

Sialyltransferase-catalyzed enzymatic and chemoenzymatic syntheses of sialosides

Chemical sialylation has been considered one of the most challenging glycosylation reactions due to the hindered tertiary anomeric center and the lack of a neighboring participating group in sialic acids (Boons and Demchenko 2000). Sialyltransferase-catalyzed reaction has been used as a preferred alternative approach (Izumi et al. 2001).

Vertebrate sialyltransferases are usually monofunctional although they have certain tolerance towards acceptors with some variations and are more promiscuous towards donor substrates (Harduin-Lepers 2010; Datta 2009). Before the identification and cloning of sialyltransferases from bacterial sources, mammalian sialyltransferases have been used in enzymatic and chemoenzymatic syntheses but only a few reactions were carried out in preparative scales mainly due to the limited access of a large amount of sialyltransferases and the high cost of sugar nucleotide donor CMP-sialic acid (Blixt et al. 2002). Several sialyltransferases such as rat recombinant α2–3-(N)-sialyltransferase, rat recombinant α2–3-(O)-sialyltransferase, and human recombinant α2–6-(N)-sialyltransferase were commercially available from CalBioChem (now EMD). Due to their limited expression levels in insect cells such as *Spodoptera frugiperda*, challenges in adapting to *E. coli* expression systems, and less flexible substrate tolerance, vertebrate sialyltransferases have found less application in synthesis, especially in preparative and large-scale preparation, of sialosides. In comparison, bacterial sialyltransferases have been increasingly used in enzymatic synthesis of sialosides since the reports of cloning α2–3-sialyltransferases from *N. meningitidis* and *N. gonorrhoeae* in 1996 (Gilbert et al. 1996) and an α2–6-sialyltransferase from *P. damselae* (Yamamoto et al. 1998).

To avoid the use of expensive CMP-Neu5Ac for sialyltransferase-catalyzed synthesis of sialosides, an in situ CMP-Neu5Ac regeneration system was developed by the Wong group (Ichikawa et al. 1991a, 1991b, 1992). In this system, The CMP formed from sialyltransferase-catalyzed reaction was reacted with ATP to form CDP and ADP by nucleoside monophosphate kinase. A pyruvate kinase in the presence of PEP was used to regenerate CTP and ATP from CDP and ADP, respectively. The CTP formed reacted with Neu5Ac to form CMP-Neu5Ac and pyrophosphate by CMP-Neu5Ac synthetase. An inorganic phosphorylase was used to breakdown the pyrophosphate to drive the reaction towards completion. The inputs of this system are metal cofactor(s), stoichiometric amounts of a sialyltransferase acceptor and Neu5Ac, at least two equivalents of PEP, and catalytic amount of ATP and CMP. Neu5Ac can also be replaced by excess ManNAc and pyruvate using a sialic acid aldolase-catalyzed reaction (Ichikawa et al. 1991a).

Earlier applications of mammalian or bacterial sialyltransferases and other sialic acid biosynthetic enzymes in preparative-scale or large-scale synthesis of sialosides with or without in situ cofactor regeneration have been mainly focused on Neu5Ac-containing sialosides (Ichikawa et al. 1991a, 1991b, 1992; Ito 1993; Gilbert et al. 1998; Johnson 1999). More recently, systematic synthesis of a large library of sialosides containing diverse sialic acid forms linked to various underlying glycans with α2–3-, α2–6-, or α2–8-sialyl linkages in preparative scale has been achieved efficiently from ManNAc, mannose, and their derivative as precursors for naturally existing and nonnatural sialic acid forms using highly reactive and substrate promiscuous recombinant bacterial sialyltransferases in the presence of a sialic acid aldolase and a CMP-sialic acid synthetase in a one-pot three-enzyme system (Fig. 4) (Yu et al. 2006a).

Sialic acid aldolase (*N*-acetylneuraminate lyase, NAL; EC 4.1.3.3) is a class I aldolase that catalyzes the cleavage of sialic acid to pyruvate and ManNAc with an equilibrium that favors Neu5Ac cleavage. It has been found in pathogenic as well as non-pathogenic bacteria (Aisaka et al. 1991) and in mammalian tissues. Both C2- and C5-modified ManNAc or mannose derivatives can be used as substrates by *E. coli* K-12 sialic acid aldolase (EcNanA) and *P. multocida* P-1059 sialic acid aldolase (PmNanA) (Li et al. 2008; Cao et al. 2009b). Nevertheless, PmNanA is a more efficient enzyme than EcNanA for synthesizing C8-modified sialic acids, especially for 5-*O*-methyl ManNAc, 5-*O*-methyl *N*-glycolylmannosamine (ManNGc5OMe), 5-*O*-methyl mannose, and 5-deoxy-mannose (Yu et al. 2011; Li et al. 2008). For activating sialic acid to form CMP-sialic acid, the donor of sialyltransferase, CMP-sialic acid synthetase (CSS or sialic acid cytidylyltransferase, EC 2.7.7.43) from *N. meningitidis* (NmCSS) was the first bacterial CSS that was characterized in detail (Warren and Blacklow 1962; Yu et al. 2004). The crystal structures of NmCSS (Horsfall et al. 2010) and N-terminal catalytically active domain of murine CSS (Krapp et al. 2003) have been reported. The enzyme has also been cloned from *E. coli* K1 (Yu et al. 2004), *S.*

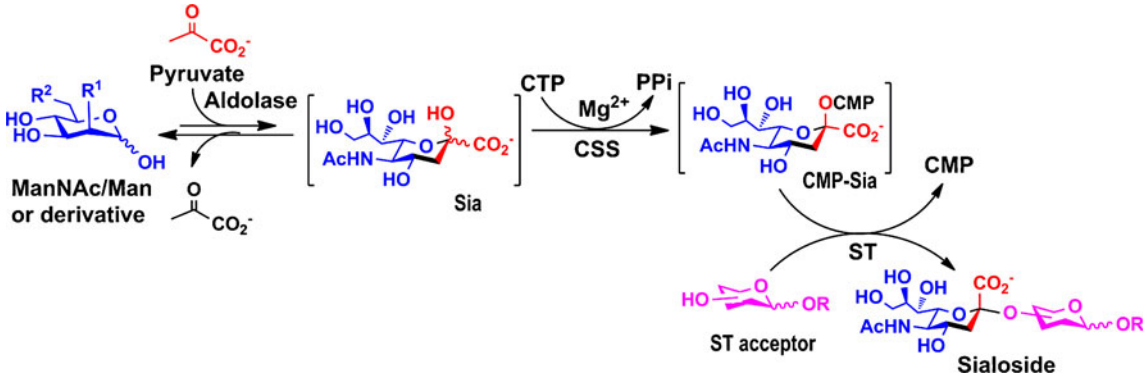

Fig. 4 One-pot three-enzyme synthesis of sialosides. *R* glycans or glycoconjugates, R^1 [–NHAc, –OH, –NHC(O)CH_2OH, –OAc, –OMe, –NHC(O)CH2OAc, –NHC(O)CH_2OMe, –NHC(O)CH_2NHCbz, –N_3, –NHC(O)CH_2N_3, –NHC(O)$OCH_2C \equiv CH$, –NHC(O)CH_2F, –H, –F], and R^2 [–OH, –OAc, –OC(O)CH(CH_3)OH, –N_3], various substituents

agalactiae or GBS (Yu et al. 2006c), *H. ducreyi* (Tullius et al. 1996), *Pasteurella haemolytica* A2 (Bravo et al. 2001), *Clostridium thermocellum* (Mizanur and Pohl 2007) as summarized in a recent review (Mizanur and Pohl 2008), and more recently from *P. multocida* (Li et al. 2012). The substrate promiscuity of NmCSS enables its application in catalyzing the formation of diverse CMP-sialic acids (Morley and Withers 2010; Yu et al. 2004; Rauvolfova et al. 2008; Hartlieb et al. 2008; Gilbert et al. 1998). A recent study has shown that the substrate promiscuity of NmCSS can be further improved by site-directed mutagenesis (e.g. NmCSS_S81R and NmCSS_Q163A) (Li et al. 2012).

Using the one-pot three-enzyme sialylation system containing a multifunctional *P. multocida* sialyltransferase PmST1 encoded by *Pm0188* gene, an *E. coli* or a *P. multocida* sialic acid aldolase (EcNanA or PmNanA) and NmCSS, α2–3-linked structurally diverse sialosides containing C5-, C8-, C9-, and other modified sialic acids and/or various acceptors including mono- and oligosaccharides, were synthesized in preparative scale at 37 °C, pH 8.5 (for sialosides that do not have a base labile *O*-acetyl or *O*-lactyl group) or pH 7.5 (for sialosides with an *O*-acetyl or *O*-lactyl group) (Yu et al. 2011,2005; Cao et al. 2008; Lau et al. 2011; Yu et al. 2006a). Typical yields for preparative-scale (>20 mg) synthesis were higher than 60 %, many reactions were achieved with more than 90 % yields. Sialosides containing an azido group on the sialic acids were readily obtained, and the azido group can be easily converted to an amino group and used as a handle for chemical acylation to generate a series of sialosides containing various sialic acid forms (Cao et al. 2009b). The tumor-associated sialyl T-antigens and derivatives were also synthesized using PmST1 in an efficient sequential two-step multienzyme approach (Lau et al. 2011). Recently, both PmST1 and Cst-I, an α2–3-sialyltransferase from *C. jejuni*, have been used synthesizing sialosides containing C8-modified sialic acid (Yu et al. 2011; Morley and Withers 2010).

Quite interesting, unlike PmST1 encoded by *Pm0188* gene homolog in Pm strain P-1059 which is a multifunctional α2–3-sialyltransferase that prefers Gal-terminated oligosaccharides as acceptors, PmST2 encoded by *Pm0508* gene in the same *P. multocida* strain is a monofunctional α2–3-sialyltransferase that prefers β1-4-linked galactosyl glycolipids as acceptors (Thon et al. 2011). In comparison, PmST3 encoded by *Pm1174* gene in Pm70 strain is absent from Pm strains P-1059 and P-934. It is a monofunctional α2–3-sialyltransferase that can use both β1–4-linked galactosyl oligosaccharides and glycolipids as acceptors (Thon et al. 2012).

The one-pot three-enzyme system (Fig. 4) has also been used for synthesizing α2–6-linked sialosides. The extremely flexible substrate specificity of the *P. damselae* α2–6 sialyltransferase (Pd2,6ST) enables its application in highly efficient chemoenzymatic synthesis of naturally occurring and non-naturally α2–6-linked sialosides (Yu et al. 2006b, 2006a, 2011; Muthana et al. 2007). Size-defined polysaccharide analogs were also chemoenzymatically synthesis by Pd2,6ST-catalyzed block transfer of di- or tetraoligosaccharides from their CMP-activated forms (Muthana et al. 2007). These polysaccharides were used to produce novel macrocyclic structures (Muthana et al. 2009). For synthesizing sialyl Tn antigens (Siaα2–6GalNAcα1-*O*-Ser/Thr) and derivatives, the recombinant marine bacterial α2,6-sialyltransferase cloned from *Photobacterium* sp. JT-ISH-224 (Psp2,6ST) (Tsukamoto et al. 2008) was shown to be a more efficient catalyst than Pd2,6ST (Yu et al. 2006b, 2007).

CMP-sialic acids and α2–3- and α2–6-linked sialosides containing 3F(*axial*)-sialic acid or 3F(*equatorial*)-sialic acid residue have also been obtained using a one-pot two-enzyme system containing a sialyltransferase and NmCSS from purified 3F(*axial*)-sialic acid or 3F(*equatorial*)-sialic acid

synthesized by an *E. coli* sialic acid-catalyzed reaction (Chokhawala et al. 2007a).

In addition to α2–3- and α2–6-linked sialosides, a recombinant Cst-II mutant, Cst-IIΔ32^{I53S}, in which the predicted C-terminal membrane-associated domain of 32 amino acids was removed and an I53S mutation was introduced to enhance its stability and α2–8-sialyltransferase activity (Gilbert et al. 2002), was used in an efficient chemoenzymatic approach for the synthesis of a series of ganglioside oligosaccharides including GM3 (Neu5Acα2–3Lac), GD3 (Neu5Acα2–8Neu5Acα2–3Lac), GT3 (Neu5Acα2–8Neu5Acα2–8Neu5Acα2–3Lac), and other disialyl glycans containing a terminal Siaα2–8Sia component with different natural and nonnatural sialic acids (Yu et al. 2009; Blixt et al. 2005).

Three types of bacterial polysialyltransferases (polySTs) that catalyze the sialic acid polymerization have been cloned and characterized. The polySTs encoded by *neuS* gene in *E. coli* K1 (Vimr et al. 1992; Steenbergen et al. 1992) and *synD* gene in *N. meningitidis* serogroup B (Steenbergen and Vimr 2003) catalyze the synthesis of α2–8-linked polysialic acid homopolymers. The polyST encoded by *synE* gene in *N. meningitidis* serogroup C catalyzes the formation α2–9-linked polysialic acid (Steenbergen and Vimr 2003). The polyST encoded by the *E. coli* K92 *neuS* gene can synthesize polysialic acid capsules with alternating sialyl α2–8- and α2–9 linkages (Vimr et al. 1992; Steenbergen et al. 1992; McGowen et al. 2001). Neither *E. coli* nor *N. meningitidis* can initiate polysialic acid synthesis de novo. They require oligosialic acids or endogenous acceptors (Ferrero and Aparicio 2010). All polysialyltransferases are cytoplasmic membrane-associated enzymes. Therefore, earlier attempts of purification of polySTs failed and resulted in the inactivation of the enzymes. Success in expressing soluble *N. meningitidis* serogroup B polyST was achieved by introducing a maltose-binding protein as a fusion protein partner (Freiberger et al. 2007; Willis et al. 2008). Recently, the characterization of several soluble chimeras of the *N. meningitidis* serogroup C polySTs clearly demonstrated that only a single protein is required for elongation of polysialic acid acceptors (Peterson et al. 2011). Several vaccines based on the *N. meningococcal* capsular polysialic acids have been licensed (Tan et al. 2010). The polysialyltransferase genes have been used for the diagnosis of *N. meningitidis* infection by PCR-based assays (Lewis et al. 2003).

Sialosides synthesized by the one-pot multienzyme system have been used to probe the interactions of sialic acid-binding proteins. Many sialosides synthesized have an alkyl azido aglycon which can be conveniently reduced to an amido group for efficient conjugation to proteins (Yu et al. 2007) for ELISA-based studies. It has also been used to produce biotinylated sialosides for surface plasmon resonance imaging (Linman et al. 2008, 2009, 2012) and glycan array studies (Fei et al. 2011) to probe the interaction of sialosides and sialic acid-binding proteins. Some have a *para*-nitrophenyl aglycon for high-throughput substrate specificity studies of sialidases (Chokhawala et al. 2007b; Cao et al. 2009a; Li et al. 2011). The propyl azide aglycon on a library of α2–3/6/8-linked sialosides has also been reduced to propyl amine for sialyl glycan array studies (Padler-Karavani et al. 2011). The one-pot three-enzyme system has also been used to sialylate fluorophore-derivatized complex glycans such as 2-amino-*N*-(2-aminoethyl)-benzamide-derivatized lactose, lacto-*N*-tetraose, lacto-*N*-neotetraose, and complex-type biantennary N-glycan with diverse natural occurring and nonnatural sialic acid forms for sialyl glycan microarray studies (Song et al. 2011; Bradley et al. 2011).

Although the one-pot multienzyme chemoenzymatic synthesis is an efficient approach to obtain structurally defined sialosides, the product purification steps are still tedious and time-consuming for generating a large library of sialosides. To avoid the tedious product purification process, a combinatorial chemoenzymatic sialylation approach has been developed. In this strategy, sialyltransferase acceptors were linked to a biotin molecule through a hexaethylene glycol linker to minimize nonspecific binding in the protein binding assays. One-pot multienzyme sialylation using the biotinylated acceptors followed by the ELISA-type protein binding analysis using NeutrAvidin-coated plates allows high-throughput screening of sialic acid-binding proteins without product purification (Chokhawala et al. 2008).

Prospective

Despite a good understanding of general sialic acid metabolic processes for eukaryotes and bacteria, the details and significance of diverse sialic acid post-glycosylational modifications are still not fully elucidated and required further investigation. Due to the importance of sialyl glycoconjugates in cellular recognition, cell signaling, immune regulation, as well as bacterial and viral infection, key enzymes in the metabolism of sialyl glycoconjugates, such as sialyltransferases and sialidases, are attractive targets for drug design. In spite of the characterization of an increasing number of sialyltransferases, CMP-sialic acid synthetases, and sialic acid aldolase for chemoenzymatic synthesis of diverse sialyl oligosaccharides and glycoconjugates, the substrate specificity of the sialoside biosynthetic enzymes limits the diversity of sialoside products. Cloning, characterizing, and exploring substrate specificity of additional wild-type bacterial enzymes, protein crystal structure-based tailor design of enzyme mutants with altered substrate specificity, and directed evolution-based screening of enzyme mutagenesis with broader substrate promiscuity will

contribute greatly to increase the size of obtainable sialic acid-containing structures. Obtaining structurally diverse sialosides including those with modified underlying sugars (e.g., *O*-sulfated Gal or GlcNAc in *O*-sulfated sialyl Lewis x structures) and various sialic acid forms such as Neu5Ac, Neu5Gc, Kdn, and their natural existing *O*-acetylated and other post-glycosylationally modified forms is critical for unraveling the important functions of sialic acid-containing molecules.

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