Chapter 13
Sialidases of Corynebacteria and their Biotechnological Applications

Seonghun Kim, Doo-Byoung Oh and Ohsuk Kwon

Abstract Sialidase (EC 3.2.1.18) is a glycosyl hydrolase which hydrolyzes terminal sialic acid residues from the glycans of glycoproteins, glycolipids, and polysaccharides. *Corynebacterium diphtheriae* harbors an extracellular exo-α-sialidase, NanH, which can cleave terminal sialic acids α(2,3)- or α(2,6)-linked to glycoconjugates. These catalytic activities of the sialidase can be used potentially for the enzymatical production of sialylated complex glycans using regioselective hydrolysis reactions. They can also be used for sialylation via transglycosylation. This chapter focuses on the biochemical properties and the structural features of *C. diphtheriae* NanH sialidase and its homologous proteins to synthesize sialyloligosaccharides through chemoenzymatic approaches. In addition, the chapter describes potential applications of NanH, including a putative vaccine candidate as a virulence factor and an exoglycosidase for analyses of the glycan structure.

Keywords *Corynebacterium diphtheriae* · *In vitro* trans-sialylation · Sialic acid · Sialidase · Sialoglycoconjugate

Abbreviations

CMP	cytidine monophosphate
Gal	galactose
Glc	glucose

O. Kwon (✉) · D.-B. Oh
Biochemicals and Synthetic Biology Research Center,
Korea Research Institute of Bioscience and Biotechnology (KRIBB),
125 Gwahak-ro, Yuseong-gu, Daejeon 305–806, Korea
e-mail: oskwon@kribb.re.kr

S. Kim
Jeonbuk Branch Institute, Korea Research Institute of Bioscience
and Biotechnology (KRIBB), 181 Ipsin-gil, Jeongeup 580–185, Korea
e-mail: seonghun@kribb.re.kr

D.-B. Oh
e-mail: dboh@kribb.re.kr

A. Burkovski (ed.), *Corynebacterium diphtheriae and Related Toxigenic Species*,
DOI 10.1007/978-94-007-7624-1_13,

MU-Gal	4-methylumbelliferyl-α-D-galactopyranoside
Neu5Ac	neuraminic acid (sialic acid)
*p*NP-	para-nitrophenyl-

13.1 Introduction

Sialic acids are a family of nine carbon α-keto aldonic acids, which are often occupied at the non-reducing end of oligosaccharide chains on glycoproteins, glycolipids, and polysaccharides. Sialic acid-containing structures naturally appear in diverse forms with different sialic acid linkages along with several functional group modifications. Four sialic acids, *N*-acetylneuraminic acid, *N*-glycolylneuraminic acid, deaminated neuraminic acid, and neuraminic acid, are major monosaccharide sugars. These major sialic acids are diversified by modification with additional substitutions at the hydroxyl group with *O*-acetyl, *O*-phosphate, *O*-sulfate, and *O*-methyl groups (Varki 1999; Chen and Varki 2010). More than 50 sialic acid structure derivatives have been detected and found to be widely distributed in various organisms, from bacteria to animals. The structural diversity of sialic acids reflects their involvement in the mediation and/or modulation of many biological processes, including intercellular interaction, cellular trafficking, intracellular adhesion, cell development, and microbial attachment (Varki 2007).

For microbial infections, the terminal sialic acids displayed on the surface of vertebrate cells, including erythrocytes and other blood cells and serum glycoproteins, mediate the adhesion of bacteria to host tissues as an initial and essential step in the infection process (Lehmann et al. 2006; Varki 2007). Several pathogenic bacteria have been shown to produce specific surface-adhesion proteins which harbor high affinity toward the sialic acids of oligosaccharide exposed on the host cell surface (Vimr et al. 2004; Lehmann et al. 2006). Some pathogenic bacteria, fungi, and protozoa can utilize the sialic acids of hosts as one type of carbon source for cell growth. In other cases, a form of pathogenic microorganism utilizes the sialic acids of host cell for the decoration of their cell surface to escape the host's immune system (Vimr et al. 2004). In the pathogenic machinery used to mediate specific interactions with sialic-acid-containing glycoconjugates on host cells, sialidases contribute to the recognition of sialic acids exposed on host cell surfaces.

Sialidase, or neuraminidase (E.C. 3.2.1.18), is an exo-α-glycosidase which hydrolyzes terminal sialic acids from a variety of sialoglycoconjugates. Sialidases are widely distributed in diverse organisms, including viruses, bacteria, fungi, protozoa, and vertebrate animals, but not plants. At least, 70 different microorganisms capable of sialidase activities have been reported (Kim et al. 2011). Several gram-positive and gram-negative bacteria-producing sialidases are commonly in close contact with mammals, acting as commensals or pathogens. Sialidases in several pathogenic microorganisms are considered to be a potential virulence factor.

Corynebacterium diphtheriae, a gram-positive pathogenic bacterium, causes diphtheria. The bacterium colonizes the mucosal surface of the respiratory tract in humans during the early stage of infection and then secretes the diphtheria toxin,

inducing necrosis and injury to epithelial cells. Sialidase activity in *C. diphtheriae* has been identified in a toxin preparation (Blumberg and Warren 1961), and exo-sialidase induced in an iron-enriched culture of the bacterium was later characterized (Warren and Spearing 1963). In addition, sialidase production and the cell surface-glycan contents of *C. diphtheriae* were found to be affected by the iron ion concentration in the culture (Mattos-Guaraldi et al. 1999; Moreira et al. 2003). However, the cellular regulatory mechanisms with three different effectors, the production of extracellular sialidase, the cell surface-glycan content and the iron ion concentration, in *C. diphtheriae* have yet to be clarified. Although no detailed studies of these cellular mechanisms in the bacterium have been reported, *C. diphtheriae* sialidase is a useful enzyme to apply to the chemoenzymatic synthesis of glycoconjugates. Recently, we identified an extracellular sialidase of *C. diphtheriae*, NanH, characterized its biochemical properties, and investigated its potential catalytic activity for sialidase-mediate transglycosylation reactions (Kim et al. 2010a; Kim et al. 2010b).

This chapter will focus on *C. diphtheriae* sialidase and its homologous proteins, with special emphasis placed on the biochemical features and structures of sialidases that are potentially useful for the chemoenzymatic synthesis of sialoglycoconjugates and/or other biotechnological applications.

13.2 *Corynebacterium diphtheriae* Sialidase and its Homologous Proteins

C. diphtheriae secrets an extracellular sialidase that can hydrolyze the sialic acid at the terminal position of glycans on glycoproteins and oligosaccharides (Warren and Spearing 1963; Kim et al. 2010a). Several microorganisms, including *Arthrobacter nicotianae*, *Arthrobacter ureafaciens*, *Bacteroides fragilis*, *Clostridium perfringens*, *Pasteurella multocida*, and *Streptococcus pneumoniae*, harbor more than one sialidase with different catalytic activities (Kim et al. 2011). Although the cellular functions of these isoenzymes remain unidentified, they vary in their hydrolysis activity towards various linkages of sialic acids as well as in their expression patterns (Corfield et al. 1983; Tanaka et al. 1994; Iwamori et al. 1997; Iwamori et al. 2005). These isoenzymes may play important roles in the interaction with other organisms or in the infection of a specific tissue by the reorganization of different sialic acid-linkages (King et al. 2006; Manco et al. 2006; Uchiyama et al. 2009).

C. diphtheriae NCTC13129 genome data also shows that this bacterium possesses two putative sialidases, NanH (DIP0543, protein accession no. NP_938919) and NanI (DIP0330 protein accession no. NP_938718) (Cerdeño-Tárraga et al. 2003). We partially purified NanH (protein accession no. ACS34893), a secreted protein of *C. diphtheriae* KCTC3075, and cloned the corresponding gene *nanH* together with the putative *nanI* gene encoding another sialidase (protein accession no. ACS34894) from its genomic DNA (Kim et al. 2010a). The amino acid sequence of *C. diphtheriae* KCTC3075 NanH and NanI demonstrates 75% and 100% identity levels with the DIP0543 and DIP0330 proteins of *C. diphtheriae* NCTC13129, respectively.

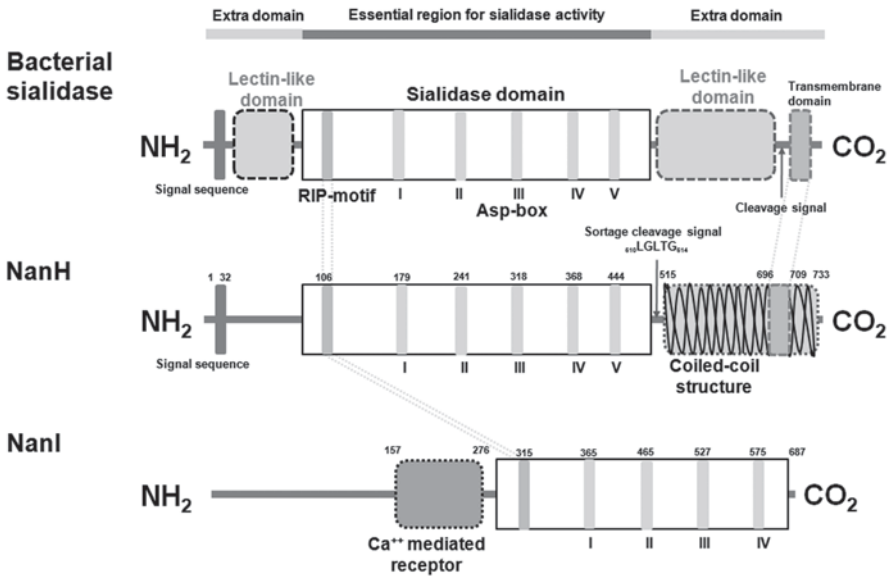

Fig. 13.1 Schematic representation and comparison of *C. diphtheriae* NanH and NanI sialidases with a typical bacterial sialidase structure. The location of the signal sequence, the RIP (Arg-Ile/Leu-Pro) motif, the Asp-boxes (I–V) lectin-like domain, and the transmembrane domain are indicated. The numbers indicate the position of each domain on the amino acid sequences of the *C. diphtheriae* NanH and NanI proteins. (Reprinted with minor adaptation from Kim et al. 2011. With permission)

Bioinformatics analysis reveals that the *C. diphtheriae* KCTC3075 NanH protein (733 amino acids) contains a putative signal sequence of 32 amino acids (Met_1-Ala_{32}) at its N-terminus and a hydrophobic transmembrane domain of 13 amino acids (Gly_{696}-Phe_{709}) at its C-terminus (Fig. 13.1). These predictions indicate that NanH is a membrane protein belonging to a typical type-Ia transmembrane protein and that its mature form would have the N_{out}-C_{in} orientation. On the other hand, *C. diphtheriae* KCTC3075 NanI does not have any signal sequence or membrane-anchored domain. It appears to be an intracellular protein in the cytoplasm. The amino acid sequences of both NanH and NanI of *C. diphtheriae* KCTC3075 possess the conserved motifs found in the bacterial sialidase family of a nonviral origin, i.e., four or five copies of an aspartate (Asp)-box (Ser/Thr-x-Asp-x-Gly-x-Thr-Trp/Phe; where x represents any amino acid) and the RIP (Arg-Ile/Leu-Phe)-motif observed upstream of the first Asp-box in the sialidase catalytic domain (Fig. 13.1). However, the NanH and NanI proteins do not contain any of the lectin-like domains occasionally observed at the N-terminal or C-terminal region of other bacterial sialidases as extra domains. Interestingly, the C-terminus of NanH contains a unique alanine-rich domain (Asp_{515}-Gln_{733}) homologous to a putative adhesion protein of *Haemophilus somnus* 129PT. This domain is predicted to be composed of α-helical coiled-coil structures, which may serve as a secreted virulence factor or as an

adhesion protein of pathogenic bacteria (Jedrzejas 2001; Delahay and Frankel 2002). In addition, the C-terminal region of NanH includes a putative sortase cleavage site, $L_{510}GLTG_{514}$, in front of a potential coiled-coil structure. These sequence analysis results indicate that the NanH protein can be localized on the cell surface or released extracellularly via sortase cleavage, playing a putative role as a virulence factor, like the sialidases of *S. pneumonia* and *Propionibacterium acnes* (Tai 2006; Nakatsuji et al. 2008).

On the other hand, although several features and functions of the *C. diphtheriae* KCTC3075 NanH protein are predictable, those of the NanI remain unclear. Although NanI includes sialidase motifs as well as potential active site residues, the recombinant protein did not show any activity toward sialoglycoconjugates when tested (Kim et al. 2010a). It may be one of the products encoding non-expressed pseudo-genes present in the cytoplasm. However, an analysis of its amino acid sequence showed that NanI may also include a putative calcium-ion-mediated receptor domain (a laminin-binding domain) containing a potential binding site for adhesion to laminin and the cell surface. This suggests that NanI is involved in adhesion, migration and differentiation through interaction with cell adhesion molecules, although further studies are required to understand its physiological roles (Magdesian et al. 2001; Tonelli et al. 2010).

13.3 Structural Properties of *C. diphtheriae* NanH Sialidase for a Catalytic Activity

Generally, sialidases are categorized according to their origins and according to the similarities of their amino acid sequences in glycoside hydrolase families in the *C*arbohydrate-*A*ctive En*z*ymes database (CAZy): GH33 (bacterial and eukaryotic enzymes), GH34 (influenza virus derivative enzymes), and GH83 (other virus originated enzymes) for exo-α-sialidase and GH58 (bacteriophage endosialidase) for endosialidase families are grouped (http://www.cazy.org/Glycoside-Hydrolases.html). Although *C. diphtheriae* NanH and NanI sialidases, both belonging to the GH33 family, share less than 30% homology in their overall amino sequences, the topology of their sialidase catalytic domains described above are well conserved and share the same motifs and residues in their structures.

In a protein structure model predicted by PHYRE (Bennett-Lovsey et al. 2008) based on the homologous 3-D structure of bacterial sialidases, *C. diphtheriae* NanH displays a six-bladed β-propeller fold (Fig. 13.2a). The Asp-boxes of five copies are observed at topologically identified positions in the β-sheet folding. Although the precise NanH protein structure is still not available to elucidate the sialidase catalytic mechanism, we can predict the catalytic mechanism of *C. diphtheriae* NanH based on the structure models of other bacterial sialidases, as the motifs and special residues observed in the catalytic center are highly conserved in the bacterial enzymes (Luo et al. 1999; Newstead et al. 2008; Xu et al. 2008).

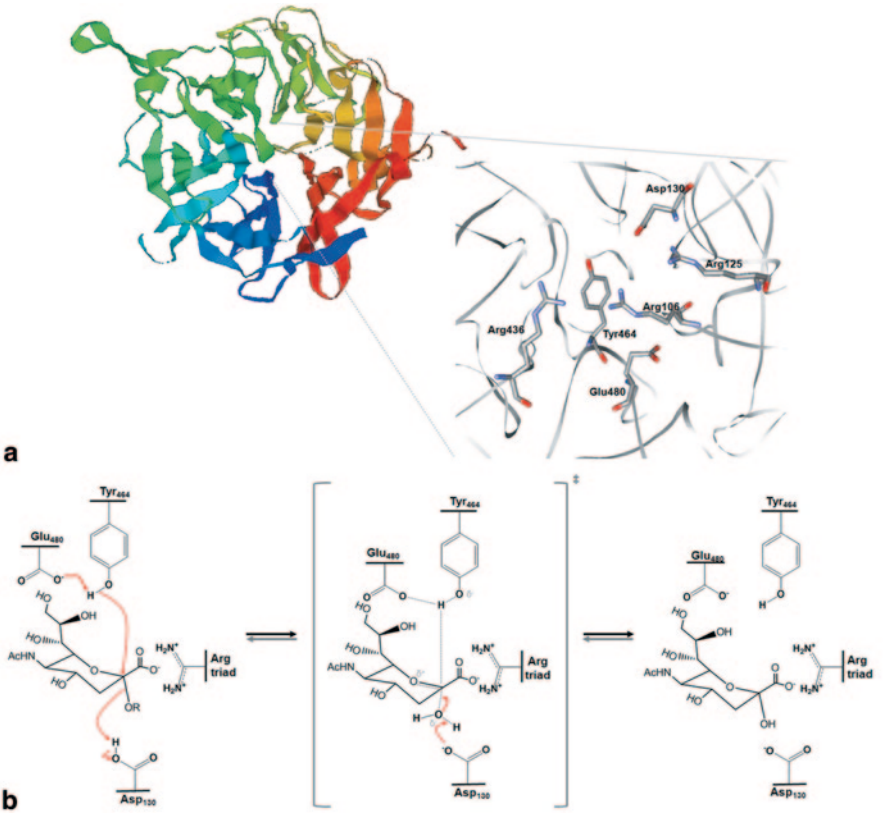

Fig.13.2 Structural model of *C. diphtheriae* NanH sialidase. **a** Overall structure of the NanH sialidase and zoomed-in view of its putative active site. The protein structure was generated by the PHYRE Protein fold recognition server (http://www.sbg.bio.ic.ac.uk/~phyre/) based on the homologous sialidase structures in the PDB database and via a PSI-blast search. **b** Proposed catalytic mechanism of the *C. diphtheriae* NanH sialidase based on bacterial sialidase structure models. (Reprinted with minor adaptation from Kim et al. 2011. With permission)

When comparing the active sites of the bacterial sialidases, several common features of *C. diphtheriae* NanH sialidase could be proposed. The highly conserved catalytic center of *C. diphtheriae* NanH consists of a tyrosine (Tyr_{464})/ glutamate (Glu_{480}) residue as a potential nucleophilic pair, an aspartic acid residue (Asp_{130}) as the acid/base catalyst, and an arginine triad (Arg_{106}-Arg_{125}-Arg_{436}) clustered with the Arg residue at the 106 position of the $R_{106}I_{107}P_{108}$ motif with two other Arg residues for stabilization through interaction with the carboxylate group of sialic acid. One of the conserved arginine residues in the active site can be stabilized by the glutamate residue (Glu_{480}). The hydroxyl group of a tyrosine residue (Tyr_{464}) is close to the C1 and C2 carbons of sialic acid (Fig. 13.2a). The catalytic mechanism for the hydrolysis reaction is initiated by the glutamate (Glu_{480}) residue, which facilitates the nucleophilic attack of the tyrosine (Tyr_{464}) residue. Once destabilized, the

bond-breaking energy is offset by the formation of a delocalized π orbital between the positively charged C2 and the electron-rich O6, and 2-carboxylate can attack the proton of the carboxyl group on the aspartate (Asp_{130}) residue in an electrophilic addition reaction (Fig. 13.2b). In the transient state of the enzyme-substrate-complex, the complex sialic acid (Neu5Ac) with NanH would result in an oxocarbenium ion intermediate, with the positive charge delocalized between the anomeric carbon and the endocyclic oxygen. The structure of the covalent complex with NanH shows that the intermediate can be stabilized through a covalent bond with the nucleophilic Tyr_{464}. Asp_{130} can then activate an incoming water molecule, a nucleophile, to attack the positive charge in the anomeric carbon, creating a protonated alcoholic intermediate. The loss of H^+ from this protonated alcohol back to Asp_{130} generates the hydrolyzed Neu5Ac.

Despite the fact that the catalytic residues involved in the enzyme activity are commonly shared with others bacterial sialidases, the preference for a specific linkage, the catalytic efficiency and the enzyme kinetics of *C. diphtheriae* NanH toward sialic acid-linked substrates represent different features. This implies that other amino acid residues around the active site or the substrate binding pocket in the protein influence these properties of *C. diphtheriae* NanH rather than any conserved residues.

13.4 Application of *C. diphtheriae* NanH Sialidase for Sialoglycoconjugate Synthesis

Bacterial sialidases show different hydrolysis activities, kinetics, types of regioselectivity, and affinity toward various sialoglycoconjugates as a substrate. Many bacterial enzymes are capable of the hydrolysis of a broad range of sialoglycan substrates with either the α(2,3)-, α(2,6)-, or the α(2,8)-linked sialic acid. On the other hand, certain sialidase isoenzymes derived from gram-negative bacteria, *Salmonella typhimurium* LT2 and *Vibrio cholerae*, and gram-positive bacteria, *Clostridium chauvoei*, *Clostridium septicum*, *Clostridium sordellii*, and *Clostridium tertium*, have a relatively higher level of hydrolysis activity toward the α(2,3)-linked over the α(2,6)-linked sialic acid (Kim et al. 2011). These regioselective hydrolysis activities and stereoselective substrate specificities of the sialidases are useful characteristics for the chemoenzymatic synthesis of glycoconjugates using glycosidases and glycosyltransferases (Wang and Huang 2009; Chen and Varki 2010).

Generally, a chemical synthesis approach for glycoconjugates is known as challenging work. A synthetic procedure through several protection/de-protection steps for regio- and stereo-specific bond formations is very complicated. The purification and extraction steps occasionally require a considerable amount of time to recover intermediates. Moreover, the final products are usually obtained with low yields and low productivity levels. A chemical sialylation reaction for sialoglycoconjugates is more feasible than other types of syntheses of neutral glycans owing to the chemical structures of sialic acids, which include hindered and disfavored tertiary anomeric

centers among nine carbon α-keto aldonic acids, a carboxyl group linked to the anomeric carbon, acetyl group and other similar characteristics (Chen and Varki 2010).

In contrast, a chemoenzymatic approach for sialoglycoconjugates is a sophisticated tool involving enzymatic reactions by regio- and stereo-selective sialidases or sialyltransferases. Enzymatic reactions have been considered as a promising means of selectively creating regio- and stereo-specific bond formations of glycosides under mild conditions without the need for elaborate protection or de-protection processes. However the use of sialyltransferase requires an expensive nucleotide-sugar, CMP-sialic acid, as a sialic acid donor. In contrast, sialidase can synthesize sialoglycoconjugates using less expensive sialic acid-linked glycosides through condensation and trans-glycosylation reactions (Ajisaka et al. 1994; Crout and Vic 1998; Schmidt et al. 2000). In comparison with sialyltransferase-catalyzed reaction, sialidase-catalyzed trans-sialylation has several advantages, including the use of various ready-made or natural sialic acid-linked substrates as a donor substrate, relaxed substrate specificity for acceptors, easy access for many bacterial sialidases, and flexibility of the enzyme reaction conditions for enhanced productivity. This process is also feasible with the addition of other co-organic solvents as well as alternative reaction media (Wang and Huang 2009).

13.4.1 Trans-sialylation by NanH Sialidase for Sialyl-Linkage Formation

C. diphtheriae KCTC3075 NanH is a secreted sialidase which is able to transfer sialic acid to an asialoglycoconjugate and to hydrolyze α(2,3)- and α(2,6)-linked sialic acids (Kim et al. 2010a). Regarding the sialidase hydrolysis activity toward various substrates, NanH shows the capability to cleave α(2,3)- and α(2,6)-linked sialic acids (Table 13.1). The relative hydrolysis activity of the enzyme showed the highest value toward sialyl-α(2,6)-lactose [Neu5Ac-α(2,6)-Gal-β(1,4)-Glc], with more preferable cleavage for the α(2,6)-linkage, and comparable activity for the α(2,3)-linked sialyllactose [Neu5Ac-α(2,3)-Gal-β(1,4)-Glc]. NanH also revealed the higher affinity and hydrolysis activity toward natural sialic acid substrates as compared to synthetic substrates. It is also remarkable that the relative hydrolysis activity toward sialic acid linked to glycoproteins is much lower than that by sialo-oligosaccharides.

NanH sialidase is a hydrolase cleaving the α(2,3)- linkage and α(2,6)-linkage of sialic acid conjugated to oligosaccharides and glycoproteins. However, this enzyme can also catalyze the formation of sialic acid-linkage through trans-sialylation as a reverse reaction under appropriate conditions. *C. diphtheriae* KCTC3075 NanH is an exo-α-sialidase which catalyzes the transfer of the terminal sialic acid unit to an asialoglycoconjugate acceptor (Table 13.2). In a test of trans-sialylation activity using both α(2,3)- or α(2,6)-linked sialoglycoconjugates as a sialic acid donor and 4-methylumbelliferyl-α-D-galactopyranoside (MU-Gal) as a sialic acid acceptor, *C. diphtheriae* sialidase showed trans-sialylation activity toward all of the donor

Table 13.1 Hydrolysis activity of *C. diphtheriae* NanH sialidase toward sialoglycoconjugate substrates. (*Source*: From Kim et al. 2010a. With permission)

Sialidase substrates	Representative glycan structure	Relative enzyme activity[a](%)	K_m(mM)	V_{max}(U mg^{-1})
Natural sialic acid				
Sialyl-*α*2,3-lactose	Neu5Ac*α*2,3Gal*β*1,4Glc	89.8 ± 7.1	5.5 ± 1.7	41.0 ± 6.2
Sialyl-*α*2,6-lactose	Neu5Ac*α*2,6Gal*β*1,4Glc	100.0 ± 3.8	7.0 ± 2.3	57.0 ± 5.3
Unnatural sialic acid				
*p*NP-*α*-sialoside	–	65.5 ± 7.9	7.3 ± 2.0	11.8 ± 1.8
MU-*α*-sialoside	–	48.9 ± 5.0	7.9 ± 2.2	11.4 ± 1.8
Glycoprotein				
Fetuin	Neu5Ac*α*2,3Gal*β*1,4GlcNAc Neu5Ac*α*2,3Gal*β*1,3GalNAc Neu5Ac*α*2,3Gal*β*1,3(Neu5Ac*α*2,6) GalNAc	17.2 ± 2.4	N.D.	N.D.
α1-Acid glycoprotein	(Neu5Ac*α*2,3(6)) Gal*β*1,4GlcNAc	24.1 ± 1.3	N.D.	N.D.
Transferrin	Neu5Ac*α*2,6Gal*β*1,4GlcNAc	15.5 ± 1.7	N.D.	N.D.

[a] The relative hydrolysis activities were determined for *C. diphtheriae* NanH with 1 mM each of sialosubstrate. The value for sialyl-α2,6-lactose obtained after 1 h incubation at 37°C was set at 100%.
ND not determined

Table 13.2 Trans-sialylation activity of *C. diphtheriae* NanH sialidase toward various sialoglycoconjugate donor substrates. (*Source:* From Kim et al. 2010a. With permission)

Donor substrates[a]	Relative enzyme activity (%)	K_m (mM)	V_{max}(U mg^{-1})
Natural sialic acid			
Sialyl-α2,3-lactose; Neu5Acα2,3Galβ1,4Glc	100.0 ± 5.6	12.2 ± 1.9	1.6 ± 0.2
Sialyl-α2,6-lactose; Neu5Acα2,6Galβ1,4Glc	99.2 ± 8.5	15.4 ± 3.8	1.7 ± 0.2
Unnatural sialic acid			
pNP-α-sialoside	90.0 ± 7.0	N.D	N.D
MU-α-sialoside	68.2 ± 2.8	N.D	N.D
Glycoprotein			
Fetuin	61.2 ± 5.3	N.D	N.D
Transferrin	47.5 ± 12.2	N.D	N.D

[a] MU-α-D-galactopyranoside (MU-Gal) was used for the sialic acid acceptor sugar.
ND not determined

substrates tested (Table 13.2). Interestingly, the enzyme displays relatively equivalent activity levels toward both sialyl-α(2,3)-lactose [Neu5Ac-α(2,3)-Gal-β(1,4)-Glc] and sialyl-α(2,6)-lactose [Neu5Ac-α(2,6)-Gal-β(1,4)-Glc], at 1.6 ± 0.2 U mg^{-1} and 1.7 ± 0.2 U mg^{-1} towards the α(2,3)- and α(2,6)-isomers, respectively. In comparison with the level of hydrolysis activity toward various sialoglycoconjugates,

C. diphtheriae NanH sialidase can also transfer sialic acids of unnatural substrates and glycoproteins to MU-Gal. Interestingly, the enzyme showed relatively high levels of activity toward two unnatural sialic acid donors, *p*NP-α-sialoside and MU-α-sialoside, whereas it showed relatively low detectable levels of activity towards the glycoprotein substrates fetuin and transferrin compared to other sialoglycoconjugate donor substrates when tested.

13.4.2 Synthesis of Sialylated Glycoproteins by Trans-sialylation

Sialic acid in the *N*-linked glycan of glycoproteins is observed at the terminal position. The terminal sialic acid is an important factor which determines the quality of a therapeutic glycoprotein. It influences the *in vivo* half-life of the glycoprotein by protecting the protein from clearance by the hepatic asialoglycoprotein receptor (Bork et al. 2009; Kim et al. 2011). To enhance the sialylation of recombinant therapeutic proteins, *in vivo* and *in vitro* modification of the glycans have been extensively developed over the last few decades (Bork et al. 2009). For example, as *in vivo* approaches, several strategies have been developed to increase the metabolic flux in the biosynthetic pathway for an activated sialic acid, CMP-Neu5Ac, through the introduction of genes encoding the CMP-sialic acid transporter, control of the UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (GNE) activity to reduce feedback inhibition, and the deletion of genes encoding endogenous sialidases. Other efforts have sought to synthesize α(2,3)- or α(2,6)-conjugated sialic acid formation by the heterologous expression of a gene encoding a linkage specific-sialyltransferase (Bork et al. 2009). The *in vitro* alternative method for an enhancement of sialylation in the glycoprotein is enzymatic sialylation using a sialyltransferase or a sialidase (Raju et al. 2001; Bork et al. 2009; Kim et al. 2010b).

One good example for *in vitro* sialylation using sialyltransferase and CMP-Neu5Ac is Etanercept®, a tetra *N*-glycosylated recombinant fusion glycoprotein, which is the homodimeric human type-2 tumor necrosis factor receptor fused to the hinge and Fc regions of the human IgG1 heavy chain (Raju et al. 2001; Bork et al. 2009). The terminal sialylation content of the protein within the heterologous glycan produced in a mammalian cell was increased to approximately 20-23% through a two-step enzyme reaction using β(1,4)-galactosyltransferase and α(2,3)-sialyltransferase (Raju et al. 2001).

Trans-sialylation of a therapeutic glycoprotein using sialidase or trans-sialidase is one type of reaction that mimics the behavior of pathogenic microorganisms. Certain pathogenic strains of clinically isolated *C. diphtheriae* or *Trypanosoma* species are unable to synthesize sialic acid by themselves. Thus, these pathogens take out the sugar at the sialoglycoconjugates of infectious hosts *via* their surface-localized sialidase or trans-sialidase to decorate their cell surface-glycoconjugates to escape the host's immune system and to interact with the host cells (Scudder et al. 1993; Mattos-Guaraldi et al. 1998; Vimr et al. 2004). As a type of functional mimicry of these enzymes of pathogenic microorganisms, *Trypanosoma cruzi* trans-sialidase displayed on a yeast cell surface was applied to synthesize an α(2,3)-sialylated

glycoconjugate using sialyl-α(2,3)-lactose as a sialic acid donor and a biantennary bigalactosylated complex *N*-glycan as an acceptor (Ryckaert et al. 2005).

In a parallel approach, *C. diphtheriae* NanH was engineered to display on a yeast cell surface (Kim et al. 2010b). In a trans-sialylation reaction with pyridylamino (PA)-labeled asialo-*N*-glycan as an acceptor and *p*NP-α-Neu5Ac as a sialic acid donor, *C. diphtheriae* NanH sialidase immobilized on the yeast surface transferred sialic acid to human-type asialo-*N*-glycans with a yield of less than 15% (Fig. 13.3a): Asialo-biantennary *N*-glycan (PA-001), asialo-triantennary *N*-glycan (PA-002), and fucosylated asialo-triantennary *N*-glycan (PA-010) were sialylated at yields of 8.7%, 13.7%, and 9.0%, respectively. Interestingly, the sialylation efficiency of triantennary *N*-glycan was slightly higher than that of the other biantennary *N*-glycans, with a conversion ratio approximately 13% higher. For a further evaluation of the trans-sialylation activity of the immobilized *C. diphtheriae* NanH sialidase toward an asialoglycoprotein, the enzyme activity was tested using asialofetuin as a model acceptor glycoprotein and *p*NP-α-Neu5Ac as a sialic acid donor. After an enzyme reaction, the syntheses of the sialic acid linkages in the sialylated products were detected by lectin blot analyses using two sialic acid-specific lectins, *Maackia amurensis* (MAA) and *Sambucus nigra* (SNA-1), recognizing the terminal α(2,3)- and α(2,6)-sialic acids linked to galactose (Gal), respectively. The lectin blot analysis clearly revealed that the enzyme is able to transfer sialic acid to the glycan of asialofetuin with the α(2,6)- as well as the α(2,3)-linkage, although not all of the protein was completely sialylated (Fig. 13.3b). In a negative control, no protein bands of unreacted asialofetuin were observed on the lectin blot analysis. However, the sialylated fetuin, detected as a single band, implied that not all of the asialofetuin or not all of asialoglycans on the protein in the reaction mixture could be completely sialylated. Thus, only a small portion of the sialylated protein was detected in the assay. The glycoproteins sialylated by *C. diphtheriae* NanH sialidase could be also confirmed by a mobility shifting assay using an isoelectric focusing (IEF) gel and a lectin blot analysis (Fig. 13.3c). The sialylated fetuin was found to have a ladder pattern (lane 2) in the lectin blot, in which its mobility was shifted to a negative charge due to the change in the pI value of proteins harboring the sialic acid. To improve the sialylation efficiency in the enzyme reaction, 30% (v/v) of dimethyl sulfoxide as an organic co-solvent was added to the reaction mixture. However, the protein mobility was not changed (lane 1). The sialylation efficiency of an asialoglycoprotein by the immobilized *C. diphtheriae* NanH sialidase may be similar to that of the free asialo-*N*-glycans, with a conversion ratio of less than 15% (Fig. 13.3a).

For an *in vitro* trans-sialylation reaction to synthesize sialoglycoconjugates, the NanH sialidase has huddles of a low production yield as well as hydrolysis of sialylated products by itself. This can be overcome by protein engineering through the mutagenesis of *C. diphtheriae* NanH sialidase for protection of the newly formed sialyl linkage against hydrolysis. Moreover, optimization of the trans-sialylation reaction conditions, such as the enzyme reaction temperature, acceptor/donor ratio, reaction time, and appropriate co-solvents for the enhancement of the transglycosylation process will lead to an improvement of the productivity and the yields for sialoglycoconjugate synthesis.

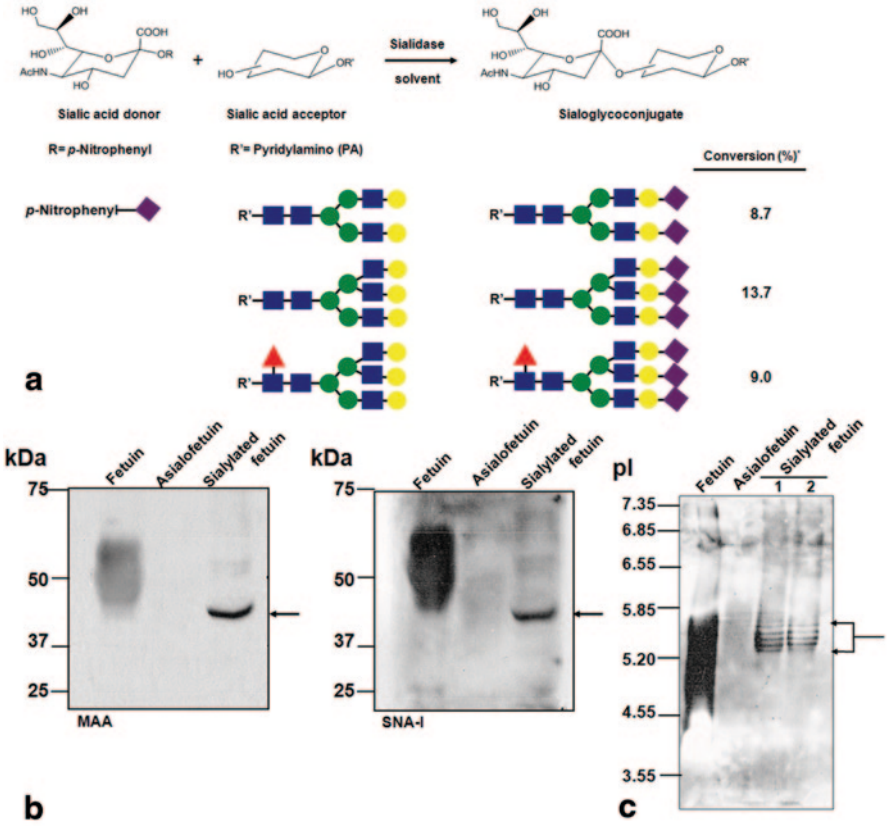

Fig. 13.3 Trans-sialylation activities of *C. diphtheriae* NanH sialidase immobilized on the surface of a yeast cell. **a** Sialylation of pyridylamino (PA)-labeled asialoglycans as a sialic acid acceptor using *p*NP-α-Neu5Ac as a sialic acid donor. *The conversion ratio (percent) was calculated by the following formula: Conversion (%) = 100 × $[\text{product}]_t$ / ($[\text{substrate}]_t$ + $[\text{product}]_t$). **b** Lectin blot analysis of the sialylation of asialofetuin separated on 8% SDS-PAGE. The sialylated products were detected by *M. amurensis* (MAA) and *S. nigra* (SNA-1) lectins. Fetuin and asialofetuin were used as a positive and a negative control, respectively. **c** Mobility shifting test of the sialylated fetuin by electrophoresis using IEF-gel (pH gradient 3–7) and a lectin blot analysis with MAA lectin. Lane 1, sialylated products reacted with 30% (v/v) dimethyl sulfoxide (DMSO) as a co-solvent; lane 2, sialylated fetuin reacted without a co-solvent. The arrows indicate the sialylated products. (Reprinted with minor adaptation from Kim et al. 2010b; Kim et al. 2011. With permission)

13.4.3 Other Potential Applications

The identification of glycan structures which enhance the functional diversity and influence the biological activity in therapeutic glycoproteins or in clinical samples is an important research area (Bork et al. 2009; Mariño et al. 2010). Because naturally occurring glycoconjugates harbor regio- and stereo-specific bond formations, analy-

ses of the glycan structures containing these complex linkages are worthy but difficult tasks. Nevertheless, the development of new analytical technologies (instrumentation-based chemical analyses) will lead to more precise, sensitive, reproducible, and robust analyses of these complex glycan structures (Mariño et al. 2010). Moreover, the glycan-linkage-specific exo-/endo-hydrolases in many bacterial enzymes, including α-sialidase, α-fucosidase, β-galactosidase, β-hexosaminidase, α-mannosidase and amidase (peptide N-glycosidase F, PNGase F), have been highlighted as useful tools for releasing a specific glycan or a glycan moiety from a glycoconjugate. Currently, sialidases derived from *S. pneumoniae*, *S. typhimurium,* and *A. ureafaciens* are mainly used in analyses of terminal sialic acids (Mariño et al. 2010). In addition to the applications of *C. diphtheriae* NanH sialidase for sialoglycoconjugate synthesis, as described previously, *vice versa,* the enzyme can be also used as a potential enzyme in an analysis of the glycan structure. This sialidase contains broad hydrolysis activities toward various sialoglyconjugates and is able to hydrolyze both α(2,3)- and α(2,6)-linked sialic acids (Table 13.1).

Another potential application of *C. diphtheriae* NanH sialidase and the homologous protein NanI may be as a target antigen for vaccinations. As bacterial sialidases are considered to be a virulence factor which recognizes sialic acid for host infection or adhesion, *C. diphtheriae* enzymes would be putative target proteins (Jedrzejas 2001; King et al. 2006 Lehmann et al. 2006; Li et al. 2011). Sialidase has been used as a vaccine target for virus influenza, bacterial pneumonia, and pathogenic protozoa (Tai 2006; Johansson and Brett 2007; Silva et al. 2009). Recently, an acne vaccine target with a surface sialidase of *Propionibacterium acnes*, a gram-positive bacterium associated with acne vulgaris, showed a successful immune response *in vivo* and *in vitro* model systems (Nakatsuji et al. 2008). Interestingly, *P. acnes* sialidase is also a cell-wall-anchored protein containing the sortase-cleavage signal LPXTG at its C-terminus, like *C. diphtheriae* NanH sialidase. Iron-mediated inhibition of diphtheria toxin production may be related to the expression of a surface-localized and secreted NanH sialidase, although the mechanism of these factors and how they influence each other remain vague (Warren and Spearing 1963; Mattos-Guaraldi et al. 1999; Moreira et al. 2003). Thus, this suggests the possibility of NanH and its homologous protein as alternative antigenic targets against the pathogenic bacterium *C. diphtheriae*. Moreover, a yeast system for the NanH sialidase immobilized on the cell surface, which was used for a trans-sialylation reaction, can be applied to the development of a novel sialidase vaccine as a natural adjuvant to improve immune responses and to increase the level of protein stability (Kim et al. 2010b; Jahns and Rehm 2012).

13.5 Conclusion

Bacterial sialidases are potential enzymes for trimming terminal α(2,3)-, α(2,6)-, and α(2,8)-sialic acids linked to glycoconjugates. These catalytic activities of sialidases can be applied to the synthesis of sialylated glycoconjugates and glycoproteins with

a regio- and stereo-selective sialic acid-linkage *via* trans-sialylation. This chapter concentrates on the biochemical properties and the structural features of *C. diphtheriae* NanH sialidase and its homologous protein to synthesize sialoglycoconjugates through chemoenzymatic approaches. Although the cellular mechanisms and the biological functions of *C. diphtheriae* sialidases as a putative virulence factor are unidentified, the NanH enzyme displayed potential catalytic activities for hydrolysis and trans-sialylation toward natural and unnatural sialic acids conjugated to oligosaccharides as well as glycoproteins. In addition, the trans-sialylation activity for asialoglycoprotein harboring complex *N*-glycans showed that the sialidase can be utilized for the *in vitro* engineering of the *N*-glycan of glycoproteins. However, an improvement of the synthetic yield for sialoglycoconjugates through transglycosylation requires further investigation to increase the enzymatic activity of trans-sialylation via protein engineering, to optimize the reaction condition, and to develop a novel enzymatic process.

References

Ajisaka H, Fujimoto H, Isomura M (1994) Regioselective transglycosylation in the synthesis of oligosaccharide: comparison of β-galactosidases and sialidases of various origin. Carbohydr Res 259:103–115

Bennett-Lovsey RM, Herbert AD, Sternberg MJ, Kelley LA (2008) Exploring the extremes of sequence/structure space with ensemble fold recognition in the program Phyre. Proteins 70:611–625

Blumberg BS, Warren L (1961) The effect of sialidase on transferrins and other serum proteins. Biochim Biophys Acta 50:90–101

Bork K, Horstkorte R, Weidemann W (2009) Increasing the sialylation of therapeutic glycoproteins: the potential of the sialic acid biosynthetic pathway. J Pharm Sci 98:3499–3508

Cerdeño-Tárraga AM, Efstratiou A, Dover LG, Holden MT, Pallen M, Bentley SD, Besra GS, Churcher C, James KD, De Zoysa A, Chillingworth T, Cronin A, Dowd L, Feltwell T, Hamlin N, Holroyd S, Jagels K, Moule S, Quail MA, Rabbinowitsch E, Rutherford KM, Thomson NR, Unwin L, Whitehead S, Barrell BG, Parkhill J (2003) The complete genome sequence and analysis of *Corynebacterium diphtheriae* NCTC13129. Nucleic Acids Res 31:6516–6523

Chen X, Varki A (2010) Advances in the biology and chemistry of sialic acids. ACS Chem Biol 5:163–176

Corfield AP, Higa H, Paulson JC, Schauer R (1983) The specificity of viral and bacterial sialidases for α(2–3)- and α(2–6)-linked sialic acids in glycoproteins. Biochim Biophys Acta 744:121–126

Crout DHG, Vic G (1998) Glycosidases and glycosyl transferases in glycoside and oligosaccharide synthesis. Curr Opin Chem Biol 2:98–111

Delahay RM, Frankel G (2002) Coiled-coil proteins associated with type III secretion systems: a versatile domain revisited. Mol Microbiol 45:905–916

Iwamori M, Ohta Y, Uchida Y, Tsukada Y (1997) *Arthrobacter ureafaciens* sialidase isoenzymes, L, M1 and M2, cleave fucosyl GM1. Glycoconj J 14:67–73

Iwamori M, Kaido T, Iwamori Y, Ohta Y, Tsukamoto K, Kozaki S (2005) Involvement of the C-terminal tail of *Arthrobacter ureafaciens* sialidase isoenzyme M in cleavage of the internal sialic acid of ganglioside GM1. J Biochem 1(38):327–334

Jahns AC, Rehm BH (2012) Relevant uses surface proteins-display on self-organized biological structures. Microb Biotechnol 5:188–202

Jedrzejas MJ (2001) Pneumococcal virulence factors: structure and function. Microbiol Mol Biol Rev 65:187–207

Johansson BE, Brett IC (2007) Changing perspective on immunization against influenza. Vaccine 25:3062–3065

Kim S, Oh DB, Kwon O, Kang HA (2010a) Identification and functional characterization of the NanH extracellular sialidase from *Corynebacterium diphtheriae*. J Biochem 147:523–533

Kim S, Oh DB, Kwon O, Kang HA (2010b) Construction of an *in vitro* trans-sialylation system: surface display of *Corynebacterium diphtheriae* sialidase on *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 88:893–903

Kim S, Oh DB, Kang HA, Kwon O (2011) Features and applications of bacterial sialidases. Appl Microbiol Biotechnol 91:1–15

King SJ, Hippe KR, Weiser JN (2006) Deglycosylation of human glycoconjugates by the sequential activities of exoglycosidases expressed by *Streptococcus pneumoniae*. Mol Microbiol 59:961–974

Lehmann F, Tiralongo E, Tiralongo J (2006) Sialic acid-specific lectins: occurrence, specificity and function. Cell Mol Life Sci 63:1331–1354

Li J, Sayeed S, Robertson S, Chen J, McClane BA (2011) Sialidases affect the host cell adherence and epsilon toxin-induced cytotoxicity of *Clostridium perfringens* type D strain CN3718. PLoS Pathog 7:e1002429

Luo Y, Li SC, Li YT, Luo M (1999) The 1.8 Å structures of Leech intramolecular trans-sialidase complexes: evidence of its enzymatic mechanism. J Mol Biol 285:323–332

Magdesian MH, Giordano R, Ulrich H, Juliano MA, Juliano L, Schumacher RI, Colli W, Alves MJ (2001) Infection by *Trypanosoma cruzi*. Identification of a parasite ligand and its host cell receptor. J Biol Chem 276:19382–19389

Manco S, Hernon F, Yesilkaya H, Paton JC, Andrew PW, Kadioglu A (2006) Pneumococcal neuraminidases A and B both have essential roles during infection of the respiratory tract and sepsis. Infect Immun 74:4014–4020

Mariño K, Bones J, Kattla JJ, Rudd PM (2010) A systematic approach to protein glycosylation analysis: a path through the maze. Nat Chem Biol 6:713–723

Mattos-Guaraldi AL, Formiga LC, Andrade AF (1998) Trans-sialidase activity for sialic acid incorporation on *Corynebacterium diphtheriae*. FEMS Microbiol Lett 168:167–172

Mattos-Guaraldi AL, Cappelli EA, Previato JO, Formiga LC, Andrade AF (1999) Characterization of surface saccharides in two *Corynebacterium diphtheriae* strains. FEMS Microbiol Lett 170:159–166

Moreira L de O, Andrade AF, Vale MD, Souza SM, Hirata R Jr, Asad LM, Asad NR, Monteiro-Leal LH, Previato JO, Mattos-Guaraldi AL (2003) Effects of iron limitation on adherence and cell surface carbohydrates of *Corynebacterium diphtheriae* strains. Appl Environ Microbiol 69:5907–5913

Nakatsuji T, Liu YT, Huang CP, Gallo RL, Huang CM (2008) Vaccination targeting a surface sialidase of *P. acnes*: Implication for new treatment of acne vulgaris. PLoS one 3:e1551

Newstead SL, Potter JA, Wilson JC, Xu G, Chien CH, Watts AG, Withers SG, Taylor GL (2008) The structure of *Clostridium perfringens* NanI sialidase and its catalytic intermediates. J Biol Chem 283:9080–9088

Raju TS, Briggs JB, Chamow SM, Winkler ME, Jones AJ (2001) Glycoengineering of therapeutic glycoproteins: *in vitro* galactosylation and sialylation of glycoproteins with terminal *N*-acetylglucosamine and galactose residues. BioChemistry 40:8868–8876

Ryckaert S, Martens V, De Vusser K, Contreras R (2005) Development of a *S. cerevisiae* whole cell biocatalyst for *in vitro* sialylation of oligosaccharides. J Biotechnol 119:379–388

Schmidt D, Sauerbrei B, Thiem J (2000) Chemoenzymatic synthesis of sialyl oligosaccharides with sialidases employing transglycosylation methodology. J Org Chem 65:8518–8526

Scudder P, Doom JP, Chuenkova M, Manger ID, Pereira ME (1993) Enzymatic characterization of β-D-galactoside α2,3-trans-sialidase from *Trypanosoma cruzi*. J Biol Chem 268:9886–9891

Silva MS, Prazeres DM, Lança A, Atouguia J, Monteiro GA (2009) Trans-sialidase from *Trypanosoma brucei* as a potential target for DNA vaccine development against African trypanosomiasis. Parasitol Res 105:1223–1229

Tai SS (2006) *Streptococcus pneumoniae* protein vaccine candidates: properties, activities and animal studies. Crit Rev Microbiol 32:139–153

Tanaka H, Ito F, Iwasaki T (1994) Two sialidases which preferentially hydrolyze sialyl α2–8 linkage from *Bacteroides fragilis* SBT3182. J Biochem 115:318–321

Tonelli RR, Giordano RJ, Barbu EM, Torrecilhas AC, Kobayashi GS, Langley RR, Arap W, Pasqualini R, Colli W, Alves MJ (2010) Role of the gp85/trans-sialidases in *Trypanosoma cruzi* tissue tropism: preferential binding of a conserved peptide motif to the vasculature *in vivo*. PLoS Negl Trop Dis 4:e864

Uchiyama S, Carlin AF, Khosravi A, Weiman S, Banerjee A, Quach D, Hightower G, Mitchell TJ, Doran KS, Nizet V (2009) The surface-anchored NanA protein promotes pneumococcal brain endothelial cell invasion. J Exp Med 206:1845–1852

Varki A (1999) Sialic acids. In: Varki A, Cummings R, Esko J, Freeze H, Hart G, Marth J (eds) Essential of glycobiology. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp 195–209

Varki A (2007) Glycan-based interactions involving vertebrate sialic-acid-recognizing proteins. Nature 446:1023–1029

Vimr ER, Kathryn AK, Deszo EL, Steenbergen SM (2004) Diversity of microbial sialic acid metabolism. Microbiol Mol Biol Rev 68:132–153

Wang LX, Huang W (2009) Enzymatic transglycosylation for glycoconjugate synthesis. Curr Opin Chem Biol 13:592–600

Warren L, Spearing CW (1963) Sialidase (Neuraminidase) of *Corynebacterium diphtheriae*. J Bacteriol 86:950–955

Xu G, Potter JA, Russell RJM, Oggioni MR, Andrew PW, Taylor GL (2008) Crystal structure of the NanB sialidase from *Streptococcus pneumoniae*. J Mol Biol 384:436–449