J. Biochem. 2010;147(4):523–533 doi:10.1093/jb/mvp198

JB THE JOURNAL OF BIOCHEMISTRY

Identification and functional characterization of the NanH extracellular sialidase from *Corynebacterium diphtheriae*

Received September 24, 2009; accepted November 17, 2009; published online December 10, 2009

Seonghun Kim[1,*,†], Doo-Byoung Oh[1,*], Ohsuk Kwon[1,‡] and Hyun Ah Kang[2,§]

[1]Integrative Omics Research Center, Korea Research Institute of Bioscience and Biotechnology, 52 Eoeun-dong, Yuseong-gu, Daejeon, 305-333, Korea and [2]Department of Life Science, College of Natural Science, Chung-Ang University, Heukseok-dong, Dongjak-gu, Seoul 156-756, Korea

*These authors contributed equally to this work.

†Present address: Institut für of Mikrobiologie, Departement Biologie, Eidgenössische Technische Hochschule (ETH) Zürich, 8093 Zürich, Switzerland.

‡Ohsuk Kwon, Integrative Omics Research Center, Korea Research Institute of Bioscience and Biotechnology, 52 Eoeun-dong, Yuseong-gu, Daejeon, 305-333, Korea. Tel: +82 42 860 4459, Fax: +82 42 860 4594, E-mail: oskwon@kribb.re.kr

§Hyun Ah Kang, Department of Life Science, College of Natural Science, Chung-Ang University, Heukseok-dong, Dongjak-gu, Seoul 156-756, Korea, Tel: +82 2 820 5863, Fax: +82 2 825 5206, E-mail: hyunkang@cau.ac.kr

***Corynebacterium diphtheriae*, a pathogenic Gram-positive bacterium, contains sialic acids on its cell surface, but no genes related to sialic acid decoration or metabolism have been reported in *C. diphtheriae*. In the present study, we have identified a putative sialidase gene, *nanH*, from *C. diphtheriae* KCTC3075 and characterized its product for enzyme activity. Interestingly, the recombinant NanH protein was secreted as a catalytically active sialidase into the periplasmic space in *Escherichia coli*, while the short region at its C-terminus was truncated by proteolysis. We reconstructed a truncated NanH protein (His$_6$-NanH$_{\Delta N}$) devoid of its signal sequence as a mature enzyme fused with the 6xHis tag at the N-terminal region. The purified His$_6$-NanH$_{\Delta N}$ can cleave α-2,3- and α-2,6-linked sialic acid from sialic acid-containing substrates. In addition, even though the efficiency was low, the recombinant His$_6$-NanH$_{\Delta N}$ was able to catalyse the transfer of sialic acid using several sialoconjugates as donor, suggesting that the reversible nature of *C. diphtheriae* NanH can be used for the synthesis of sialyl oligosaccharides via transglycosylation reaction.**

Keywords: *Corynebacterium diphtheriae*/extracellular protein/sialic acid/sialidase/sialoglycoconjugate.

Abbreviations: TSB, trypticase soy broth; 3′SL, sialyl-α2,3-lactose; 6′SL, sialyl-α2,6-lactose; MU, 4-methylumbelliferone; Neu5Ac, *N*-acetylneuraminic acid; MU-Neu5Ac, 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid; MU-Gal, 4-methylumbelliferyl-α-D-galactopyranoside; His$_6$, hexahistidine; NanH$_{\Delta N}$, N-terminal truncated NanH; *p*NP-, paranitrophenyl-; Gal, galactose.

Sialidases, or neuraminidases (EC 3.2.1.18), belong to a class of glycosyl hydrolases that catalyse the removal of terminal sialic acid (*N*-acetylneuraminic acid, Neu5Ac) residues from a variety of glycoconjugates (*1*). Sialidases are found in diverse organisms including viruses, microorganisms (some fungi, protozoa, bacteria) and vertebrate animals (*2*, *3*), but not plants. For example, sialidase activity has been reported from over 70 different microorganisms in the orders Pseudomonadales and Eubacteriales. Interestingly, most of the sialidase-producing microorganisms have close contact with mammals as commensals or pathogens (*2*, *4*, *5*). Sialidases in many pathogenic bacteria are considered potential virulence factors, which can contribute to the recognition of sialic acids exposed on host cell surfaces (*5*). The biochemical properties of a number of sialidases have been characterized and some of their crystal structures have been solved (*2*, *3*, *6*). All sialidases share the same six-bladed β-propeller fold with conservation of key amino acids for catalytic activity (*3*). The non-viral sialidases usually contain four or five repeated Asp-boxes (Ser/Thr-x-Asp-x-Gly-x-Thr-Trp/Phe) in their amino acid sequences (*2*, *3*). Several sialidases are commercially available for glycan analysis, since their linkage-specific cleavages are useful for the terminal sialic acid analysis of glycans. Furthermore, it has been of interest to develop sialidases that have trans-sialylation activity to transfer sialic acids from sialo-donor substrates to asialo-acceptor substrates; this activity could be employed for the synthesis of sialoglycoconjugates (*7*, *8*). Because sialyltransferases display pronounced acceptor specificity and require expensive nucleotide sugar substrates, their uses are sometimes restricted. In contrast, the transglycosylation reaction based on the reversible nature of sialidases can be an alternative method for the synthesis of sialyl oligosaccharide because it is relatively simple, cheap and efficient (*8*).

Corynebacterium diphtheriae is a Gram-positive pathogenic bacterium that causes diphtheria. The bacterium colonizes the mucosal surface of the respiratory tract in humans, where it secretes the diphtheria toxin that causes necrotic injury to epithelial cells. The sialidase activity in *C. diphtheriae* was first identified in the extracellular crude diphtherial toxin (*9*), and the exo-sialidase was later characterized and shown to be induced in iron-enriched cultures (*10*). Recently, the iron ion concentration in a culture medium was shown to affect the sialidase production and the glycan differentiation on the cell surface of *C. diphtheriae* (*11*, *12*). Even though *C. diphtheriae* is known to have sialidase activities, no detailed studies of their identities or catalytic properties have been reported.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 28, 2012

© The Authors 2009. Published by Oxford University Press on behalf of the Japanese Biochemical Society. All rights reserved

In the present study, we purified an extracellular sialidase from *C. diphtheriae*, determined its identity by MS peptide-fingerprinting and cloned the corresponding *nanH* gene of the extracellular sialidase. Moreover, by biochemical characterization of recombinant NanH protein expressed in *Escherichia coli*, we investigated a potential activity of NanH protein to catalyze the sialidase-mediated transglycosylation reaction.

Materials and Methods

Bacterial strains, plasmids and culture condition

Corynebacterium diphtheriae KCTC3075 (ATCC11913) was obtained from Korean Collection for Type Culture (KCTC). *Corynebacterium diphtheriae* was cultivated in Trypticase Soy Broth (TSB; Difco Laboratories, Detroit, MI, USA). The cells were grown aerobically in a 200 ml flask at 37°C with shaking at 200 rpm. Growth was monitored spectrophotometrically at 600 nm. *Escherichia coli* strains, DH5α [λ^- *supE*44 Δ*lac*U169 (Φ80 *lac*Z ΔM15) *hsdR*17 *rec*A1 *end*A1 *gyrA*96 *thi*-1 *relA*1] and BL21(DE3)pLysS [F $^-$ *omp*T *hsd*S_B ($r_B^- m_B^-$) *gal dcm* (DE3) pLysS (*Cam*)^R], were used as hosts for the construction of recombinant plasmids and for the expression of the recombinant sialidase, respectively. *Escherichia coli* cells were grown in the LB medium supplied with an appropriate antibiotic at 37°C. The bacterial strains used in this study are summarized in supplementary Table S1.

Partial purification and identification of C. diphtheriae extracellular sialidase

Extracellular proteins in the cell-free culture broth were concentrated using Amicon® Ultra centrifugal filter device (Millipore, Billerica, MA, USA) equipped with YM-10 membrane, and then dialysed in 50 mM Tris–HCl (pH 7.6) overnight. The dialysed protein solution was loaded onto a Q-sepharose column (3.0 × 13 cm) equilibrated with 50 mM Tris–HCl (pH 7.6). The proteins were eluted with three bed volumes of the Tris–HCl buffer, followed by a linear gradient of 0–1.0 M NaCl. Fractions (5 ml each) were collected at a flow rate of 1 ml min^{-1}. Those containing sialidase activity were pooled, concentrated and dialysed in 50 mM Tris–HCl (pH 7.6). After addition of 1.0 M ammonium sulphate to the dialysed protein solution, the protein solution was loaded on a phenyl-sepharose column (1.0 × 15 cm) equilibrated with 50 mM Tris–HCl (pH 7.6) containing 1.0 M ammonium sulphate. After washing with the same buffer, the enzyme was eluted using a decreasing salt gradient (1.0–0 M ammonium sulphate) at 0.5 ml min^{-1}. Active fractions were concentrated by ultrafiltration and then analysed by SDS–PAGE.

To identify proteins, the protein bands were excised from the stained SDS–PAGE gel and then destained overnight. After the destaining solution was removed, 50 mM ammonium bicarbonate was added to the destained protein bands, and the mixture was agitated for 30 min. Finally, 100% acetonitrile was added to the rehydrated protein samples for the dehydration. The protein samples were then dried for 30 min in a Speed Vac and subsequently rehydrated in a digestion solution consisting of 50 mM ammonium bicarbonate and 0.1 μg μl^{-1} sequencing-grade modified trypsin (Promega, Madison, WI, USA). The mixtures were incubated overnight at 37°C. The supernatants containing the tryptic peptides were obtained from the mixtures and then dried. The dried samples were mixed with 20 μl of 0.1% trifluoroacetic acid (TFA), desalted with a ZipTip™ (Waters, Milford, MA, USA) and spotted on a MALDI-plate by elution with 2 μl 50% (v/v) acetonitrile containing 0.1% TFA and 7 mg ml^{-1} α-cyano-4-hydroxycinnamic acid. Peptide mass analysis was performed by the POSTECH Biotech Center (Pohang, Korea). The molecular masses of the peptides were determined with an Applied Biosystems 4700 Proteomics Analyzer MALDI-TOF-MS (Applied Biosystems, Foster City, CA, USA) or LC-MS-MS. A database search was carried out with ProFound (http://www.unb.br/cbsp/paginiciais/profound.htm) and MASCOT (http://www.matrixscience. com).

Vector construction for nanH and nanI expression in E. coli

The genomic DNA of *C. diphtheriae* was prepared with the AccuPrep™ Genomic DNA Extraction Kit (Bioneer, Daejeon, Korea). The *nanH* and *nanI* genes were amplified by PCR with Taq DNA polymerase (Bioneer, Daejeon, Korea) and appropriate primer pairs using *C. diphtheriae* genomic DNA as the template. The primers used in this study are summarized in supplementary Table SII. The amplified DNAs were cloned into pGEM-T easy vector (Promega) for DNA sequencing, yielding pGEM-nanH and pGEM-nanI, respectively.

The ORFs of *nanH* and *nanI* were amplified by PCR from pGEM-nanH and pGEM-nanI, and the amplified DNA products were subcloned into the pET-21a vector (Novagen, Madison, WI, USA), generating pET21-nanH and pET21-nanI, respectively. The in-frame cloning of these constructs enabled downstream fusion with the 6xHis tag coding sequence. Thus, pET21-nanH expresses the intact NanH protein tagged with 6xHis at its C-terminus (SS-NanH-His_6). The *nanH* gene fragment coding for a truncated NanH lacking a putative endogenous signal sequence ($NanH_{\Delta N}$) was amplified by PCR using pET21-nanH as a template with two appropriate primers, and then cloned into pET-21a vector, generating pET21-$nanH_{\Delta N}$ for the expression of the mature form of NanH protein tagged with 6×His at its N-terminus (His_6-$NanH_{\Delta N}$). All the constructed plasmids for sialidase expression were transformed into *E. coli* BL21(DE3)pLysS.

Plate assay for sialidase activity

Recombinant *E. coli* strains harbouring an empty pET21a vector, pET21-nanH or pET21-nanI, respectively, were streaked onto LB-agar plates containing ampicillin and grown overnight at 37°C. The plates were overlaid with 5 ml of 0.75% molten agar in 50 mM sodium acetate buffer (pH 5.5) containing 1 mg ml^{-1} 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MU-Neu5Ac), 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), 4 mM $CaCl_2$ and 150 mM NaCl. After the top agar on the plate was cooled and fixed, the plate was incubated at 37°C for 1 h. Sialidase-positive strains were detected by the bright blue halos around the colonies when excited with short wavelength UV light on the UV-illuminator.

Purification of recombinant NanH from E. coli

The *E. coli* strain harbouring pET21-$nanH_{\Delta N}$ was cultivated in the LB medium supplemented with 100 μg ml^{-1} of ampicillin at 37°C until the optical density at 600 nm reached 0.8–1.0, and then 0.5 mM IPTG was added to culture broth to induce gene expression. After induction for 6 h at 37°C with shaking, the cells were harvested, resuspended in 50 mM Tris–HCl (pH 7.6), and then disrupted by sonication. The crude extracts were clarified by centrifugation (40,000 × *g*, 30 min, 4°C). The clarified homogenate was loaded onto a Ni-NTA column (1.5 × 11 cm) pre-equilibrated with 50 mM Tris–HCl (pH 7.6), washed with three bed volumes of the buffer, and eluted by using a linear gradient of 0–0.2 M imidazole. Fractions of 3 ml were collected at a flow rate of 1.0 ml min^{-1}. The fractions containing the recombinant protein were pooled, dialysed and loaded onto a Q-sepharose column (3.0 × 11 cm) equilibrated with 50 mM Tris–HCl (pH 7.6). The enzyme was eluted using the linear gradient of 0–1.0 M NaCl. Fractions of 5 ml were collected at a flow rate of 1.0 ml min^{-1}. The fractions containing the recombinant protein were pooled, concentrated by ultrafiltration and then desalted by dialysis.

Molecular weight determination by MALDI-TOF MS analysis

A saturated solution of sinapinic acid was prepared in a mixture containing 25% acetonitrile and 0.1% TFA with a 1:2 volume ratio for the matrix solution. The sample for MALDI-TOF analysis was prepared by diluting 1.0 μl of protein (50 mg ml^{-1}) solution with 1.0 μl matrix solution and spotted onto a flat stainless steel target from Bruker Daltonik GmbH. The MALDI-TOF data were collected on a Microflex TOF instrument (Bruker Daltonik GmbH. Bremen, Germany) with delayed extraction in positive-ion reflector mode. Typical instrument operating settings were 25 kV total accelerating voltage, 91% grid voltage, 0.2% guide wire voltage, delay of 100–200 nanoseconds and 567.2 Da low mass gate. Data were analysed using FlexControl software (Brüker, Germany). The observed masses for one sialidase protein measured with sufficiently high intensity were used to internally calibrate each mass spectrum.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 28, 2012

Enzyme assays for sialidase and trans-sialidase activities

Sialidase activity was determined with 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MU-Neu5Ac) as follows. The reaction was performed in a total volume of 50 µl containing 50 mM Tris–HCl buffer (pH 7.5) and 1 mM MU-Neu5Ac. The reaction mixture was incubated at 37°C for 60 min. After the enzyme reaction, the fluorescence of 4-methylumbelliferone (MU) released from MU-Neu5Ac was measured by the Spectrofluorometer RF5301-PC (Shimadzu, Kyoto, Japan) with excitation and emission wavelengths of 365 nm and 450 nm, respectively. One unit was defined as the amount of enzyme required to release 1 µmol of MU from MU-Neu5Ac per min.

Trans-sialidase activity was determined using a method slightly modified from that described by Schrader *et al.* (*13*). Briefly, the reaction was performed at 37°C in a total volume of 50 µl containing 50 mM Tris–HCl buffer (pH 7.5), 1 mM sialyl-conjugate as a donor substrate, 0.5 mM 4-methylumbelliferyl-α-D-galactopyranoside (MU-Gal) as an acceptor substrate, and enzyme solution. After 60 min, the enzyme reaction was terminated by the addition of 450 µl of ice-cold water. The reaction mixture was applied to 0.2 ml Q-sepharose equilibrated with deionized water and then the unbound MU-Gal was washed from the column with deionized water (3×0.5 ml). 250 µl of 1 M HCl was then applied twice onto the column for elution of sialylated MU-Gal from the column. The acid hydrolysis of the eluted products was performed at 100°C for 45 min. The fluorescence of the released 4-MU was measured with excitation and emission wavelengths of 365 nm and 450 nm, respectively. All enzyme activities were determined in triplicate. One unit was defined as the amount of enzyme required to transfer 1 µmol of sialic acid from sialyl-conjugates (a donor) to MU-Gal (an acceptor) per min.

Biochemical characterizations of NanH activity

The substrate specificity of NanH hydrolysis activity was determined at 37°C for 1 h in 50 mM Tris–HCl buffer, pH 7.0, in a final volume of 50 µl, containing 1 mM sialosubstrate and 5 µl NanH protein (10 mU ml^{-1}). The sialylated glycans or glycoproteins used as sialidase substrates in this study were as follows: sialyl-α2,3-lactose, sialyl-α2,6-lactose, *p*NP-α-sialoside, MU-α-sialoside, fetuin, α_1-acid glycoprotein and transferrin. After enzyme reaction, free sialic acids released from sialylated glycans or glycoproteins were determined by the periodic acid/thiobarbituric acid-based method (*14*). The enzyme reaction mixture of 50 µl was oxidized using 125 µl of 25 mM periodic acid in 0.125 M H_2SO_4 at room temperature for 20 min. To terminate the oxidation, 250 µl of 2% (w/v) sodium arsenite dissolved in 0.5 M HCl was added. Subsequently, 1 ml of 0.3% thiobarbituric acid was added and the mixture was boiled at 100°C for 10 min. The amount of released sialic acid was calculated based on the extinction coefficient for *N*-acetylneuraminic acid, $\varepsilon_{549} = 6.8 \times 10^4\,M^{-1}cm^{-1}$. The effects of pH on the hydrolysis activity were determined at 37°C in 50 mM MES-buffer (pH 5.0–7.0), 50 mM MOPS-buffer (pH 6.5–8.0) or 50 mM Tris-HCl buffer (pH 7.5–9.0) using 1 mM sialyl-α2,3-lactose by the same methods described above. The effects of divalent ions (Mn^{2+}, Ba^{2+}, Mg^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Cd^{2+}, Ni^{2+} and Zn^{2+}) and EDTA on sialidase activities were determined by measuring the enzymatic activity after pre-incubation in 50 mM Tris–HCl (pH 7.6) at 37°C for 1 h in the presence of 1 mM of each of these divalent ions.

The kinetic parameters of trans-sialylation were determined using 1 mM MU-Gal or MU-lactopyranoside (MU-Lac) as acceptors and various sialyl-conjugates (0.16–40 mM) as substrates. The apparent V_{max} and K_m values (means ± SD) were calculated by fitting the initial rate data to the Michaelis–Menten equation with the non-linear regression analysis program (Sigma Plot, ver. 9.0). The effect of pH on the transglycosylation activities was determined at 37°C in 50 mM MES-buffer (pH 5.0–7.0), 50 mM MOPS-buffer (pH 6.5–8.0) or 50 mM Tris–HCl buffer (pH 7.5–9.0) using the methods described above.

Sequence submission

The nucleotide sequences of *nanH* and *nanI* were deposited in the GenBank database under accession numbers GQ121278 and GQ121279, respectively.

Results

Identification of an extracellular sialidase from C. diphtheriae *KCTC3075*

It was reported that the exo-sialidase activity of *C. diphtheriae* was enhanced in iron-enriched cultures (*10*). Therefore, we measured sialidase activity of *C. diphtheriae* KCTC3075 cultivated in trypticase soy broth (TSB) supplemented with 1 mM $FeCl_3$ (iron-rich condition) or with 0.5 mM 2,2′-dipyridyl, a Fe-chelating agent (iron-free condition). The enzyme activity was determined in three separate fractions: the culture broth, the membrane and the cytoplasm fractions obtained from the cells in late stationary phase. Although the overall cell growth was increased, the extracellular protein production was decreased in the $FeCl_3$-supplemented cultivation (Table I). On the other hand, the addition of 2,2′-dipyridyl had a negative effect on cell growth and thus decreased the final extracellular protein yield. All the cell fractions prepared from *C. diphtheriae* grown in Fe-supplemented TSB had higher sialidase activities than those of cells cultivated under iron starvation condition and non-additive TSB, confirming the iron ion-induced pattern of sialidase activity in *C. diphtheria* KCTC3075 (Table I). In addition, some sialidases were secreted from the cells into the culture medium under iron-rich conditions.

Table I. Effect of iron ion on sialidase production in *C. diphtheriae*.

			Sialidase activity[b]		
Supplement to TSB medium	Cell growth[a] (OD_{600})	Extracellular protein (µg ml^{-1} culture broth)	Extracellular[c] (U ml^{-1})	Membrane[d] (U mg^{-1} protein)	Cytoplasm[d] (U mg^{-1} protein)
None	5.5 ± 0.5	100.9 ± 3.6	1.22 ± 0.03	203.8 ± 51.7	538.7 ± 39.3
$FeCl_3$ (1 mM)	6.6 ± 0.1	76.3 ± 1.8	4.71 ± 0.13	3252.0 ± 119.7	2122.2 ± 116.8
2,2′-Dipyridyl (0.5 mM)	1.4 ± 0.1	42.8 ± 2.3	0.45 ± 0.05	116.4 ± 23.4	472.2 ± 30.2

[a]The cell density of each sample was measured at late stationary phase.
[b]Sialidase activity was determined by measuring the amount of 4-methylumbelliferone (MU) hydrolysed from 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MU-Neu5Ac). One unit of enzyme activity was defined as the amount of enzyme producing 1 µmol of MU per min.
[c]The enzyme activity was determined from 100 µl of unconcentrated cell-free culture broth.
[d]*Corynebacterium diphtheriae* cells separated from the culture broth were washed twice with 50 mM Tris–HCl (pH 7.6), resuspended in 50 mM Tris–HCl (pH 7.6), and then disrupted by sonication. After ultracentrifugation ($100{,}000 \times g$, 60 min, 4°C), the clarified supernatant was harvested as the cytoplasmic fraction, and the pellet was resuspended in 50 mM Tris–HCl (pH 7.6) containing 3% Triton X-100 and 0.15 M NaCl. After ultracentrifugation of the resuspended pellet, the clarified supernatant was obtained as the membrane fraction.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 28, 2012

To further identify the sialidase secreted from *C. diphtheriae* KCTC3075, the enzyme was partially purified from the cell-free culture broth by two-step column chromatography using Q-sepharose and phenyl-sepharose columns. When the phenyl-sepharose column fractions containing sialidase activity were analysed on SDS–PAGE, eight polypeptides were clearly detected in the range of molecular weights from 37 to 80 kDa (data not shown). To identify each protein band, the respective bands on SDS–PAGE were digested by trypsin and subjected to MALDI-TOF-MS and LC-MS-MS analysis. The resulting spectrum was used to search for matching proteins in the NCBI database with the MASCOT search program. The search with the resulting spectrum from a major band around 80 kDa showed a top score of 182 for a putative sialidase precursor (accession number, gi|38233152) in *C. diphtheriae* NCTC13129 (a full-length protein of 77.5 kDa; protein scores > 78 are significant, $P < 0.05$). A search of the GenBank sequence database revealed that the 80 kDa protein corresponded to a putative exo-sialidase, the product of *nanH* (DIP0543, protein accession no. NP_938919) from *C. diphtheriae* NCTC13129. Moreover, our result was also in good agreement with the recent proteome analysis of the type strain *C. diphtheriae* $C7_s(-)^{tox-}$, which identified the product of DIP0543 as an extracellular and cell surface-associated protein (*15*).

Characterization of two putative sialidase genes, nanH *and* nanI*, isolated from* C. diphtheriae *KCTC3075*

Since the genome sequence of *C. diphtheriae* NCTC13129 (*16*) indicated the existence of another putative sialidase gene, *nanI* (encoding DIP0330, protein accession no. NP_938718), both *nanH* and *nanI* genes of *C. diphtheriae* KCTC3075 were amplified by PCR from the genomic DNA using primers (supplementary Table SII) designed based on genome information from *C. diphtheriae* NCTC13129. The amplified DNAs were cloned into pGEM-T easy vector (Promega), generating pGEM-nanH and pGEM-nanI, respectively, and subjected to DNA sequencing. The deduced amino acid sequence of *C. diphtheriae* KCTC3075 NanH shows 75% identity with DIP0543 protein (NP_938919) of *C. diphtheriae* NCTC13129. The sequence differences are mostly located in the regions putatively associated with the enzyme activity. In contrast, *C. diphtheriae* KCTC3075 NanI displays 100% sequence identity with the DIP0330 protein. The nucleotide sequences of *C. diphtheriae* KCTC3075 *nanH* and *nanI* were deposited in GenBank as accession numbers GQ121278 and GQ121279, respectively.

To predict the topologies of proteins encoded by *nanH* and *nanI,* their amino acid sequences were analysed using SignalP ver. 3.0 and TMHMM ver. 2.0 (http://www.cbs.dtu.dk/services). *Corynebacterium diphtheriae* KCTC3075 NanH protein (733 amino acids) was predicted to contain a signal sequence of 32 amino acids at its N-terminus (Met_1–Ala_{32}), as well as a putative transmembrane domain of 23 amino acids at its C-terminus (Gly_{696}–Phe_{709}), suggesting that *nanH* codes for an extracellular enzyme (Fig. 1A). The NanH protein seems to be a typical type Ia membrane protein that has a cleavable signal peptide at the N-terminus and a hydrophobic membrane-bound domain at the C-terminus (*17*). The orientation of the mature protein is predicted as N_{out}–C_{in}. On the other hand, there is no signal sequence and membrane-bound domain in the amino acid sequence of NanI (687 amino acids), indicating that NanI is an intracellular enzyme in the cytoplasm. Both *C. diphtheriae* KCTC3075 NanH and NanI contain the conserved catalytic residues found in the sialidase family, i.e. four repeated Asp-boxes, and the bacterial neuraminidase repeated signatures (Fig. 1A). NanH contains the $Arg_{106}Ile_{107}Phe_{108}$ (RIP) motif that is associated with the putative active site of bacterial sialidases (*3*, *18–20*). The protein also includes at least five copies of the Asp-box consensus motif, Ser/Thr-x-Asp-x-Gly-x-Thr-Trp/Phe, which is found in non-viral sialidases. NanI, which is slightly shorter than NanH, also contains the $Arg_{315}Ile_{316}Phe_{317}$ motif as well as at least four copies of the Asp-box motif (Fig. 1B). However, any lectin-like motifs that are usually found in bacterial sialidase protein structures could not be predictable in NanH and NanI proteins. Interestingly, the alanine-rich domain (Asp_{515}-Gln_{733}) in the C-terminus of NanH shows a significant homology (27% identity and 46% similarity) with a putative adhesion protein (NP_719270) of *Haemophilus somnus* 129PT. The domain was also predicted to compose α-helical coiled-coil structures, which are usually found in secreted virulence effector proteins and adhesion proteins of pathogenic bacteria (*21*, *22*). In addition, a putative consensus sortase cleavage signal site, $L_{510}GLTG_{514}$, is also found in front of a potential coiled-coil structure in the C-terminus of NanH (Fig. 1B). Although the typical consensus sequence of the sortase cleavage signal is LPxTG in several gram-positive bacteria (sortase substrate database: http://bamics3.cmbi.kun.nl/cgi-bin/sortase_substrates), the signal is L-[APG]-x-TG in the case of *C. diphtheriae*. The sequence analysis implied that the *C. diphtheriae* NanH protein could be localized on the cell surface or released extracellularly after cleavage by sortase.

Expression of C. diphtheriae nanH *and* nanI *in* E. coli

To determine the enzyme activities of the *nanH* and *nanI* products, the whole open reading frames of these genes were amplified by PCR from pGEM-nanH and pGEM-nanI and then subcloned into the plasmid pET-21a (Novagen), yielding pET21-nanH and pET21-nanI, respectively. Recombinant *E. coli* BL21(DE3)pLysS harbouring pET21-nanH and pET21-nanI were streaked on LB-ampicillin plates. After the recombinant cells were incubated overnight, the plate was overlaid with soft agar containing 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MU-Neu5Ac) as a substrate to detect sialidase activity. If active sialidases were expressed, they could hydrolyse the substrate, MU-Neu5Ac, to release free 4-methylumbelliferone (MU), which generates a sky blue fluorescent halo under short wavelength

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 28, 2012

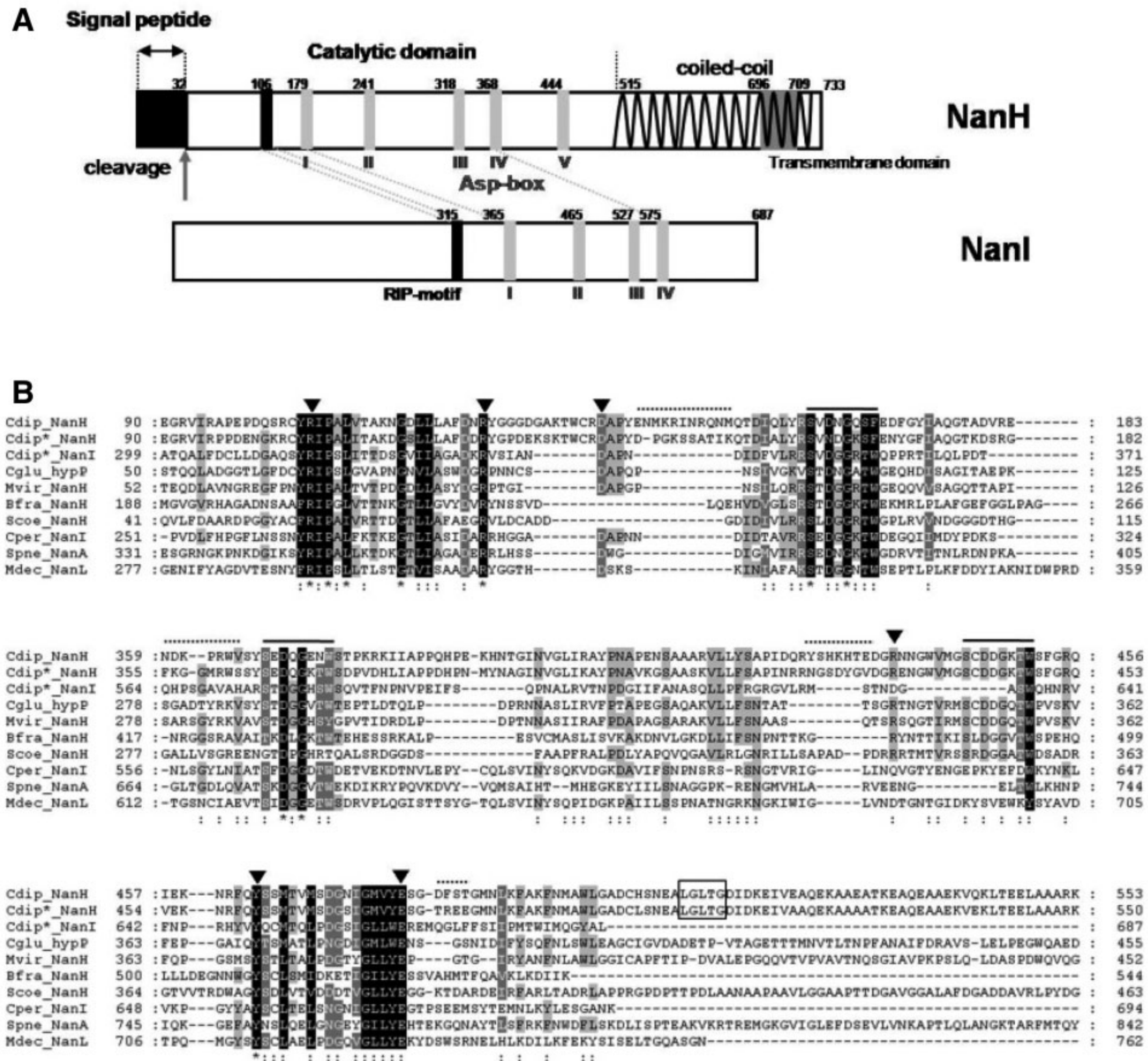

Fig. 1 Schematic representation of *C. diphtheriae* NanH and NanI. (A) Predicted structural features of NanH and NanI proteins. The topologies of proteins encoded by *nanH* and *nanI* were analyzed by using SignalP ver. 3.0 and TMHMM ver. 2.0 (http://www.cbs.dtu.dk/services). (**B**) Partial amino acids sequence alignment of *C. diphtheriae* KCTC3075 NanH and its homologues. NanH and NanI sequences from *C. diphtheriae* NCTC13129 genome sequence DB are designated as Cdip*_NanH (GenBank accession number, NP_938919) and Cdip*_NanI (NP_938718). *Corynebacterium glutamicum* R hypothetical protein (Cglu_hypP; YP_001138502), *Micromonospora viridifaciens* sialidase (Mvir_NanH; Q02834), *Bacteroides fragilis* YCH46 sialidase (Bfra_NanH; BAA05853), *Streptomyces coelicolor* A3(2) sialidase (Scoe_NanH; NP_630638), *Clostridium perfringens* NCTC 8239 sialidase I (Cper_NanI; ZP_02643014), *Streptococcus pneumoniae* R6 sialidase A (Spne_NanA; NP_359129), *Macrobdella decora* trans-sialidase (Mdec_NanL; AAC47263). The symbols '*' and ':' indicate conserved residues and conservative replacement, respectively. Putative active sites common among the bacterial sialidase family are indicated by inverted filled triangle. Dot and solid line indicate the different sequence region and Asp-box, respectively. The box marks a putative sortase cleavage signal.

UV light on the UV-illuminator. The sialidase activity was detected only in recombinant cells harbouring pET21-nanH (Fig. 2A). The fluorescence of this recombinant *E. coli* was much brighter than that of other clones harbouring an empty vector or pET21-nanI.

To further confirm the hydrolysis activity against MU-Neu5Ac, the sialidase activities in the cell lysates of the recombinant cells were also measured *in vitro*. After the enzyme reaction, the fluorescence intensity of MU released from the MU-Neu5Ac substrate was quantified by fluorospectrometer. As shown in the plate assay, the cell lysate from the *E. coli* strain containing pET21-nanH had about nine times higher activity than that from the strain harbouring pET21-nanI (Fig. 2A). Although the sialidase activity could not be detected in the plate assay, low hydrolytic enzyme activity against MU-Neu5Ac substrate was detected in the cell lysate from the recombinant *E. coli* containing pET21-nanI by *in vitro* enzyme assay. Based on the results from the indicator plate and the *in vitro* enzyme assays, NanH was chosen for further detailed biochemical study.

Corynebacterium diphtheriae NanH includes a putative 32-amino-acid N-terminal signal peptide.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 28, 2012

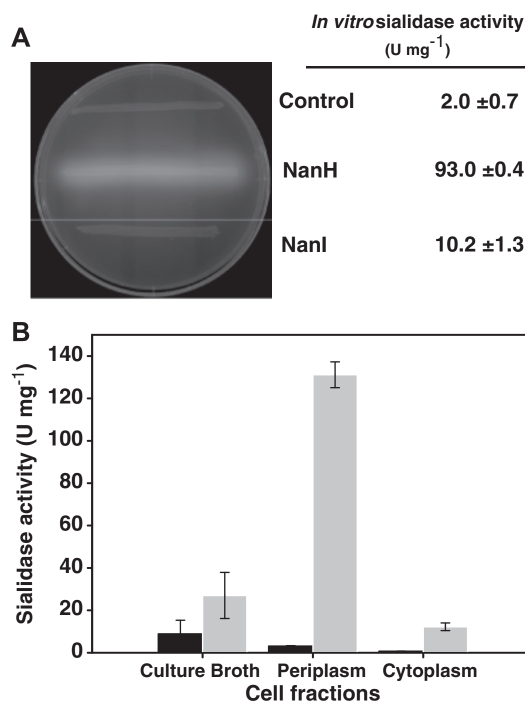

Fig. 2 Sialidase activity analysis to detect the expression of putative *C. diphtheriae* sialidase genes in *E. coli*. (A) Sialidase plate assay. Single colonies from each recombinant *E. coli* strain were streaked and cultivated overnight on LB plates containing ampicillin. The 0.75% soft agar in a buffer (50 mM sodium acetate, 150 mM NaCl, 4 mM $CaCl_2$) containing the inducer IPTG and the sialidase substrate MU-Neu5Ac was poured on the plate, and then the plate was further incubated at 37°C for 1 h. Control, recombinant *E. coli* BL21(DE3) harbouring pET21a as a negative control; NanH, recombinant *E. coli* BL21(DE3) harbouring pET21-nanH; NanI, recombinant *E. coli* BL21(DE3) harbouring pET21-nanI. The sialidase activities measured using total cell lysates are also displayed for recombinant strains harbouring pET21-nanH or pET21-nanI. (B) *In vitro* sialidase activity assay. The sialidase gene expression in the recombinant *E. coli* cells was induced for 6 h in the presence of 0.5 mM IPTG. The harvested *E. coli* cells were disrupted by sonication. After ultracentrifugation (100,000 × *g*, 60 min, 4°C) of the total cell lysates, the clarified supernatant was collected as the cytoplasmic fraction, and the pellets were resuspended in 50 mM Tris–HCl (pH 7.6) containing 3% Triton X-100 and 0.15 M NaCl. After ultracentrifugation of the resuspended pellets, the supernatant was obtained as the periplasm fraction. Grey bars, cell fractions from *E. coli* BL21(DE3) harbouring pET21-nanH; black bars, cell fractions from *E. coli* BL21(DE3) harbouring pET21a.

To determine whether the signal peptide can still function in the heterologous *E. coli* expression host, the sialidase activities were measured in the cell-free culture medium, periplasm and cytosol of the recombinant *E. coli*. Interestingly, among the tested fractions, the enzyme activity was highest in the periplasmic fraction (Fig. 2B). The cytosol fraction displayed 13 times less sialidase activity than the periplasmic fraction. The cell-free culture broth also displayed sialidase activity, although its activity was relatively low. The data indicate that the N-terminal signal sequence of NanH worked well in the heterologous *E. coli* host.

Purification of E. coli*-expressed recombinant NanH protein*

To facilitate the purification of recombinant NanH protein, a polyhistidine (6xHis) tag was first fused at the C-terminus of *C. diphtheriae* NanH to retain its own signal peptide sequence (NanH-His_6) (Fig. 3A, top). After IPTG induction, the expression level and the sialidase activity of recombinant NanH protein were measured by western blot analysis and hydrolysis activity of MU-Neu5Ac, respectively. Although the sialidase activity was detectable in the total cell lysate (Fig. 3B), the protein band corresponding to NanH-His_6 was not recognized by immunoblotting with the anti-His-tag antibody (Fig. 3C). It was speculated that the C-terminally tagged epitope of NanH-His_6 might be cleaved by endogenous proteases or masked in the structural protein so that it could not be detected in the western blot analysis. To detect the recombinant NanH protein expressed in *E. coli*, the 6xHis-tag was then fused at the N-terminus of NanH lacking the putative signal peptide sequence (His_6-$NanH_{\Delta N}$) (Fig. 3A, bottom). The total cell lysate of the recombinant *E. coli* expressing His_6-$NanH_{\Delta N}$ showed comparable sialidase activity to that of the *E. coli* strain expressing NanH-His_6 (Fig. 3B). Moreover, the recombinant His_6-$NanH_{\Delta N}$ protein

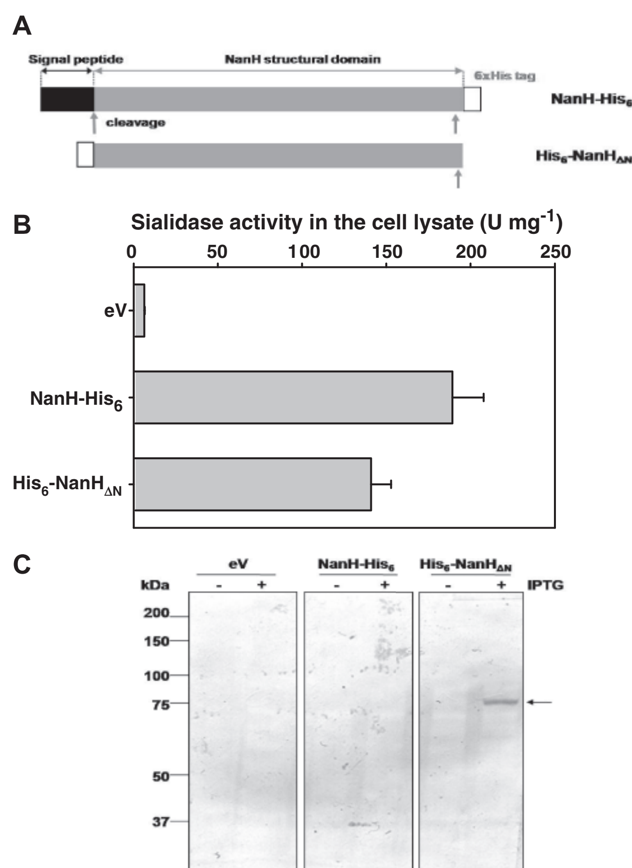

Fig. 3 Overexpression of His-tagged NanH proteins in *E. coli*. (A) Primary structure schemes of the recombinant NanH proteins tagged with six histidine residues to facilitate purification. (B) *In vitro* sialidase activity analysis using whole cell lysates from recombinant *E. coli* cells harbouring pET21a (eV), pET21-nanH (NanH-His_6) and pET21-$nanH_{\Delta N}$ (His_6-$NanH_{\Delta N}$), respectively. The data represent the mean ± SD from three independent measurements. (C) Western blot analysis of recombinant NanH-His_6 and His_6-$NanH_{\Delta N}$ proteins. The total cell lysates from recombinant *E. coli* cells were analysed by immunoblotting using the anti-His antibody (Santa Cruz Biotechnology).

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 28, 2012

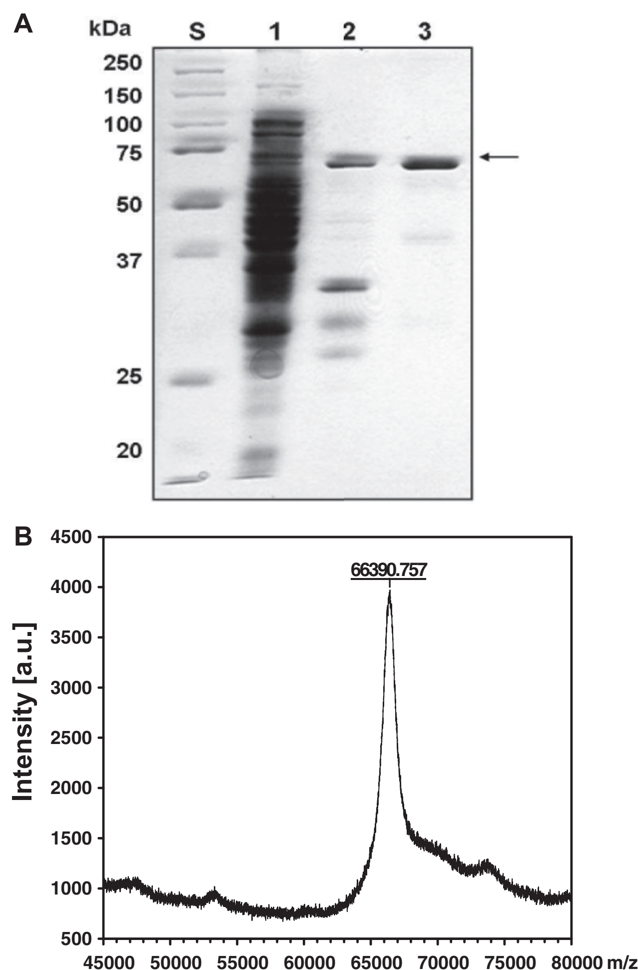

Fig. 4 Purification of recombinant NanH protein from *E. coli*. (A) SDS–polyacrylamide gel electrophoresis of recombinant His$_6$-NanH$_{\Delta N}$ protein during purification. Proteins were separated by SDS–PAGE (8%) and visualized by Coomassie staining: S, standard molecular markers; lane 1, crude extract; lane 2, Ni-NTA column eluate; lane 3, Q-sepharose column eluate. (B) Molecular weight determination of recombinant His$_6$-NanH$_{\Delta N}$ by MALDI-TOF MS.

was detected as a single band in the immunoblotting analysis using the anti-6xHis antibody (Fig. 3C).

The recombinant His$_6$-NanH$_{\Delta N}$ protein was expressed at about 50 mg l^{-1} and the sialidase activity, measured using MU-Neu5Ac as a substrate, was found exclusively in the cytosol. The His$_6$-NanH$_{\Delta N}$ protein was purified more than 25-fold from the cytosol fraction of the recombinant *E. coli* by two-step column chromatography using a Ni-NTA affinity column and a Q-sepharose anionic exchange column (Fig. 4A). The molecular weight of the denatured His$_6$-NanH$_{\Delta N,}$ separated on SDS–PAGE, appeared to be ~70 kDa. The more precise molecular weight of the purified recombinant His$_6$-NanH$_{\Delta N}$ was measured by MALDI-TOF MS analysis. The mass spectrum of the purified His$_6$-NanH$_{\Delta N}$ showed one peak corresponding to a protein with a molecular mass of 66,390.75 Da (Fig. 4B). The obtained molecular weight was 9,790.26 Da lower than the theoretical molecular mass (76,181.01 Da) of the His$_6$-NanH$_{\Delta N}$ protein containing the N-terminal 6xHis-tag without the signal peptide. This data strongly supported the truncation of the C-terminal region of NanH by endogenous proteases in the recombinant *E. coli*.

Biochemical characterization of NanH sialidase

The biochemical assays for the sialidase activity of the recombinant *C. diphtheriae* NanH were carried out using various sialoconjugates and sialoglycoprotein as sialidase substrates. The amount of free sialic acid released by the enzyme reaction was measured by periodic acid/thiobarbituric acid-based method (*14*). The relative hydrolysis activities for sialosubstrates tested are summarized in Table II. *C. diphtheria* NanH showed the highest cleavage rate for the α-2,6-linked sialic acids of sialyllactose, with comparable activity for the α-2,3-linked sialic acids. *Corynebacterium diphtheriae* NanH showed the higher substrate affinities and hydrolysis activities towards the natural sialic acid substrates than the synthetic substrates.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 28, 2012

Table II. Hydrolysis activity of recombinant NanH protein toward sialylated substrates.

Sialidase substrates	Representative glycan structure	Relative enzyme activity[a] (%)	K_m (mM)	V_{max} (U mg^{-1})
Natural sialic acid				
Sialyl-α2,3-lactose	Neu5Acα2,3Galβ1,4Glc	89.8 ± 7.1	5.5 ± 1.7	41.0 ± 6.2
Sialyl-α2,6-lactose	Neu5Acα2,6Galβ1,4Glc	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	Neu5Acα2,3Galβ1,4GlcNAc Neu5Acα2,3Galβ1,3GalNAc Neu5Acα2,3Galβ1,3(Neu5Acα2,6)GalNAc	17.2 ± 2.4	ND	ND
α1-Acid glycoprotein	(Neu5Acα2,3(6)) Galβ1,4GlcNAc	24.1 ± 1.3	ND	ND
Transferrin	Neu5Acα2,6Galβ1,4GlcNAc	15.5 ± 1.7	ND	ND

ND: not determined.
[a]Free sialic acids hydrolysed from sialylated substrates were measured by the periodic acid/thiobarbituric acid assay (*14*). 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%.

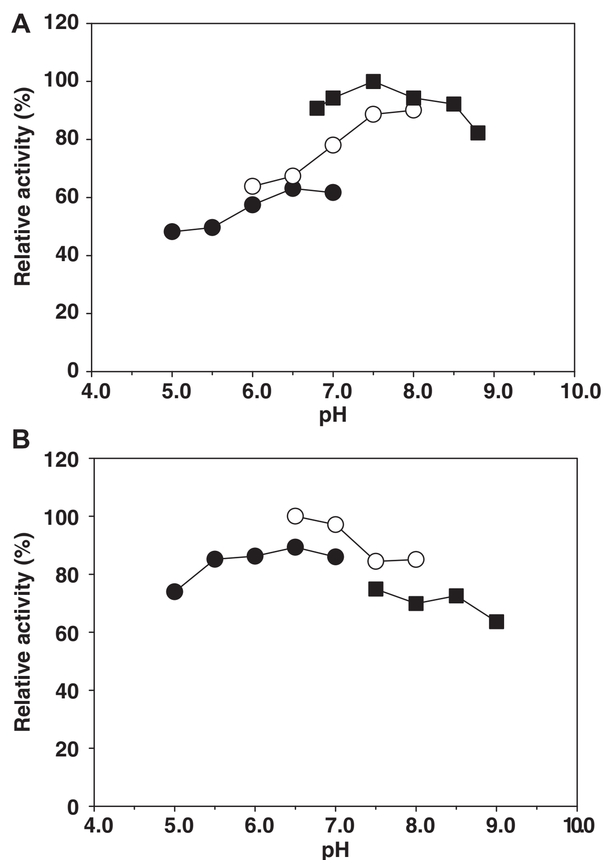

Fig. 5 Effect of pH on the activity of recombinant NanH protein. (A) Effect of pH on the hydrolysis activity of the Nan H protein. The assays were carried out with 50 mM MES-buffer (filled circle), 50 mM MOPS-buffer (open circle) and 50 mM Tris–HCl buffer (filled square), respectively. The hydrolysis activity was determined using sialyl-α2,3-lactose as a substrate. (B) Effect of pH on the transglycosylation activity of the NanH protein. The assays were performed with the same buffers described above. The transglycosylation activity of the purified recombinant NanH was determined using α-2,3-linked sialyllactose as a donor sugar and MU-Gal as an acceptor sugar.

It is also notable that the hydrolysis rates of sialic acid conjugated to glycoproteins were apparently much lower than those of the free glycans.

It was suggested that a single protein could express both neuraminidase and trans-sialidase activity in *C. diphtheriae* (*23*). To examine the transglycosylation activity of purified NanH, six sialoglycoconjugates containing α-2,3- or α-2,6-linkage were tested as sialic acid donors using 4-methylumbelliferyl-α-D-galactopyranoside (MU-Gal) as a sialic acid acceptor substrate. Sialic acids transferred from sialic acid donor substrates to MU-Gal were measured by the method of Schrader *et al.* (*13*). *Corynebacterium diphtheriae* NanH showed trans-sialylation activity in the reactions using sialyl-α2,3-lactose [Neu5Ac α2,3 Gal β1,4Glc] (1.6 ± 0.2 U mg^{-1}) and sialyl-α2,6-lactose [Neu5Ac α2,6 Gal β1,4Glc] (1.7 ± 0.2 U mg^{-1}) (mean ± SD, $n = 3$) as donors. The k_{cat}/K_m values towards α2,3- and α2,6-isomers were 19.5 $mM^{-1} min^{-1}$ and 16.4 $mM^{-1} min^{-1}$, respectively. In addition, although the rates were relatively lower, *C. diphtheriae* NanH could also catalyse the transfer of sialic acid from several other sialoconjugates to MU-Gal. The comparative rates of sialic acid transfer from several sialoglycoconjugate-donor substrates were shown in Table II. Recombinant *C. diphtheriae* NanH showed detectable transglycosylation activities using sialic acid-conjugated free oligosaccharides and glycoproteins as donor substrates. Interestingly, this enzyme displayed similar donor preference for α-2,3- or α-2,6-linked siayllactose substrates containing β1,4-linked galactose with similar kinetic parameters. Also, the enzyme showed relatively high activity towards two unnatural sialic acid donors, *p*NP-α-sialoside and MU-α-sialoside. Although the relative activities towards fetuin and transferrin were lower than those towards other sialoconjugates, these sialoglycoproteins could still serve as donors since the relative activities were more than 50% of those towards sialyl-α-2,3- or sialyl-α-2,6-lactose. When analysed on TLC, we did not detect any oligo-sialyl structures from the reaction products (data not shown), indicating that all the sialylated products of the NanH reaction appeared to be mono-sialylated.

The effect of pH on the sialidase and transglycosylation activities of NanH was examined at 37°C in 50 mM MES-buffer (pH 5.0–7.0), 50 mM MOPS-buffer (pH 6.5–8.0) or 50 mM Tris–HCl buffer (pH 7.5–9.0). The sialidase activity was measured using sialyl-α-2,3-lactose as substrate for hydrolysis, whereas the transglycosylation activity was assayed using sialyl-α-2,3-lactose as donor substrate and MU-Gal as acceptor substrate. It was shown that *C. diphtheriae* NanH displayed the optimal hydrolysis activity in the neutral pH range (Fig. 5A), whereas the optimal transglycosylation activity in the range between pH 5.5 and 7.5 (Fig. 5B).

Discussion

According to its genome sequence, *C. diphtheriae* NCTC13129 is expected to possess two genes encoding putative sialidases, NanH (DIP0543) and NanI (DIP0330) (*16*). In the present study, we partially purified and identified the NanH protein of *C. diphtheriae* KCTC3075 as a secreted sialidase using MALDI-TOF MS analysis. Bioinformatic analysis also revealed that NanH has a putative signal sequence of 32 amino acids at its N-terminus and a membrane-spanning α-helix structure at its C-terminus. Thus, we speculate that NanH is a membrane-bound protein with a putative function as a virulence factor, like the sialidases of *Streptococcus pneumoniae* and *Propionibacterium acnes* (*24*, *25*). Sialidases localized on cell surfaces have been investigated as a vaccine target for several bacterial pathogen-mediated diseases (*3*, *23*, *24*). The variation in the *nanH* gene between *C. diphtheriae* NCTC13129 and *C. diphtheriae* KCTC3075 might reflect the genome interrogation and population diversity often found in the pathogenic genes of *Corynebacterium* (*26*). Virulence factor genes might be evolutionarily diverse in order to escape the immune response of host cells. In contrast to NanH, the NanI sequence of *C. diphtheriae* KCTC3075 shows 100% identity with that of *C. diphtheriae* NCTC13129

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 28, 2012

Table III. Donor substrate specificity of recombinant NanH protein for transglycosylation reaction.

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			
*p*NP-α-sialoside	90.0 ± 7.0	ND	ND
MU-α-sialoside	68.2 ± 2.8	ND	ND
Glycoprotein			
Fetuin	61.2 ± 5.3	ND	ND
Transferrin	47.5 ± 12.2	ND	ND

ND: not determined.
[a]MU-α-D-galactopyranoside (MU-Gal) was used for the sialic acid acceptor sugar and sialic acids transferred from donor substrates to MU-Gal were measured by the method of Schrader *et al.* (*13*).

(DIP0330). NanI might reside in the cytoplasm or be a non-expressed pseudo-gene.

It has been known for several decades that *C. diphtheriae* has both sialidase and trans-sialidase activities, although direct evidence for corresponding genes or proteins was lacking (*10*, *23*, *27*). No genes coding for proteins belonging to *Trypanosoma* trans-sialidase family have been found in the *C. diphtheriae* NCTC13129 genome database. Thus, we investigated the possibility that *C. diphtheriae* NanH can catalyse both sialidation and transglycosylation as reported previously in other bacterial sialidases. Sialidases from *Clostridium perfringens*, *Arthrobacter ureafaciens* or *Vibrio cholerae* were shown to transfer sialic acid to *N*-acetyl-lactosamine by a transglycosylation reaction (*7*). These enzymes preferentially catalyse the formation of sialyl-α-2,6-linkages, whereas *Salmonella typhimurium* and Newcastle disease virus sialidases show a distinct preference for α2,3-linkage sialylation (*8*). It has been reported that the regioselectivity for the sialoconjugate products from the transglycosylation by these enzymes were correlated with the substrate-linkage specificity in the hydrolysis activity (*7*, *8*).

Sequence alignments of NanH revealed the conserved residues commonly found in the active site of sialidase superfamily members for hydrolysis and transglycosylation activity. The NanH amino acid sequence contains Arg_{106}, Arg_{125} and Arg_{436}, a tri-arginine cluster (Fig. 1B) that could interact with the carboxyl group of Neu5Ac. The position of Arg_{106} located within $R_{106}I_{107}P_{108}$-motif may be stabilized by a conserved glutamic acid, Glu_{480} (*18*, *19*). A tyrosine (Tyr_{464}) and a glutamic acid (Glu_{480}) might be involved in the catalytic reaction to stabilize the positive charge at the sialic acid (*17*, *18*). The Asp_{138} residue found in the sialidase active sites, close to the Tyr_{464} residue, might function as a putative acid/base during catalysis (*18*–*20*). Most sialidase active sites have a hydrophobic pocket to accept the *N*-acetyl group of the Neu5Ac substrate, but the amino acid residues comprising this substrate-binding pocket are not usually conserved.

One peculiar feature of the *C. diphtheriae* NanH protein is the relatively large size for a sialidase. The canonical sialidase is an ~42 kDa polypeptide with diagnostic Asp-boxes and catalytic site residues (*3*, *18*, *19*). Therefore, the 70 kDa size of the NanH sialidase suggests the presence of at least one additional domain, which might be a lectin-like carbohydrate-binding domain as observed in some sialidases and trans-sialidases of *Trypanosoma* species (*3*, *18*–*20*, *28*–*30*). However, *C. diphtheriae* NanH protein has a putative coiled-coil structure, which is usually involved in the secretion mechanism of proteins associated with virulence and adhesion in pathogenic bacteria, at its C-terminus instead of lectin-like domains (*21*, *22*). The function of the coiled-coil structure in the *C. diphtheriae* NanH protein is equivocal: whether it might be involved in interaction with sialic acid-containing glycans or it would have a function for protein secretion. It would be intriguing to elucidate the physiological significance of the additional domain of *C. diphtheriae* NanH.

Comparative analysis of kinetic values for hydrolysis and transglycosylation by the *C. diphtheriae* NanH protein implies that NanH thermodynamically prefers the hydrolysis reaction over the transglycosylation (Tables II and III). The hydrolysis reaction towards natural sialic acid substrates was faster than the transglycosylation reaction. The higher V_{max} values for natural substrates indicate that the hydrolysis reaction is more preferable than transglycosylation for the synthesis of sialoconjugates. Moreover the V_{max}/K_m values (sialyl-α-2,3-lactose, 7.5; sialyl-α-2,6-lactose, 8.1) in NanH hydrolysis reaction are 70 times higher compared to those in transglycosylation reaction. Further intensive analysis on the glycan structure should be required to elucidate the linkage specificity of the trans-sialylated products by NanH sialidase.

The K_m value of *C. diphtheriae* NanH for sialyl-α-2,3-lactose is higher than those of the enzymes of three *Trypanosoma* species catalysing trans-sialylation reactions, and the V_{max} value of NanH for sialyl-α-2,3-lactose is much lower than those of the *Trypanosoma* trans-sialidases (*29*–*31*). Moreover, the optimum pH of NanH (pH 5.5–7.5) is lower than those of *Trypanosoma* species (pH 7.0–7.9). However, the substrate specificity of *C. diphtheriae* NanH against sialic acid donors appears to be broader than those of the trans-sialidases of *Trypanosoma* species. Interestingly, *C. diphtheriae* NanH can convert both α-2,3- and α-2,6-linked sialic acid donors, although

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 28, 2012

the previously reported *Trypanosoma* enzymes did not use α-2,6-linked sialic acid as their substrate (*28–30*).

Some sialidases such as *V. cholera* sialidase need divalent ions such as Ca^{2+} for catalytic activity (*31*). However, we observed that *C. diphtheriae* NanH does not require divalent metal ions for hydrolysis or transglycosylation reaction, and that the addition of EDTA did not affect its enzyme activity. Thus, it seems that divalent metal ions are not involved in the *C. diphtheriae* NanH-mediated reactions for sialic acid hydrolysis and transglycosylation. Rather, several divalent ions, such as Hg^{2+}, Cu^{2+} and Fe^{2+}, inhibited the hydrolysis activity (data not shown). Inhibition of sialidase activity by some cations was also previously reported with several other bacterial sialidases (*32*, *33*). It is speculated that these cations might be bound to Cys residues containing SH groups important for the sialidase activity or protein stabilization (*33*).

Previous experiments detected sialic acids on the cell surfaces of both sucrose fermentation and non-sucrose fermentation strains of *C. diphtheriae* (*11*, *12*). If *C. diphtheriae* does not synthesize sialic acid, it must acquire the sugar from a host during infection or from culture medium when grown *in vitro*. The comparison of those genes involved in biosynthesis and catabolism of sialic acid derivatives from the genomes of pathogenic bacteria strongly suggests that *C. diphtheriae* cannot produce or metabolize sialic acid *in vivo*. However, the NanH sialidase located on the cell surface could be used for decoration of galactose moiety acceptors with sialic acid for invasion of hosts under certain conditions. On the other hand, the sialic acids generated by NanH from host cells might be transported into *C. diphtheriae*, although no sialic acid transporters have been reported in *C. diphtheriae*. A sialic acid transporter was recently identified in *Haemophilus ducreyi* (*34*), which cannot produce sialic acid by itself, and thus should obtain the sugar from its host. By homology analysis of *C. diphtheriae* genome with the *H. ducreyi* sialic acid transporter protein (NanT) as a query sequence, we found that the gene cluster (Hd1669-Hd1672) of *H. ducreyi* corresponded to an operon-like cluster (DIP0512-0515) in the *C. diphtheriae* genome, and that the *H. ducreyi* NanT protein showed high amino acid sequence homology with a putative membrane protein (46% identity and 60% positive) of *C. diphtheriae*. Thus, *C. diphtheriae* may transport sialic acids hydrolysed by extracellular sialidases for the synthesis of sialic acid derivatives *in vivo*. However, further studies are needed to elucidate whether NanH is really involved in sialic acid decoration on the cell surface and how NanH and NanT play roles in sialic acid metabolism.

In conclusion, the present study identified NanH from *C. diphtheriae* KCTC3075 as an active extracellular sialidase that could transfer a sialic acid from sialylconjugates to asialoglycans via transglycosylation. Our combined data from biochemical studies, genetic information and bioinformatics prediction strongly suggest that the NanH protein might play important roles in infection, although further studies are required to understand its physiological roles.

Supplementary data

Supplementary Data are available at *JB* Online.

Acknowledgements

The authors would like to thank J.W. Byun in POSTECH for MS protein identification and J.M. Lee in KRIBB for technical assistance with MALDI-TOF MS analysis to determine the molecular weight of the recombinant sialidase.

Funding

Korean Ministry of Knowledge and Economy (Next Generation New Technology Development Program) grant and the Korea Science and Engineering Foundations grant (F01-2006-001-10080-0); Postdoctoral fellowship grant to S.K. from Korea Research Foundation (KRF-2006-D00071).

Conflict of interest None declared.

References

1. Vimr, E.R. (1992) Microbial sialidases: does bigger always mean better? *Trends Microbiol.* **2**, 271–277
2. Roggentin, P., Schauer, R., Hoyer, L.L., and Vimr, E.R. (1993) The sialidase superfamily and its spread by horizontal gene transfer. *Mol. Microbiol.* **9**, 915–921
3. Taylor, G. (1996) Sialidases: structures, biological significance and therapeutic potential. *Curr. Opin. Struct. Biol.* **6**, 830–837
4. Vimr, E. and Lichtensteiger, C. (2002) To sialylate, or not to sialylate: that is the question. *Trends Microbiol.* **10**, 254–257
5. Vimr, E.R., Kalivoda, K.A., Deszo, E.L., and Steenbergen, S.M. (2004) Diversity of microbial sialic acid metabolism. *Microbiol. Mol. Biol. Rev.* **68**, 132–153
6. Monti, E., Preti, A., Venerando, B., and Borsani, G. (2002) Recent development in mammalian sialidase molecular biology. *Neurochem. Res.* **27**, 649–663
7. Ajisaka, K., Fujimoto, H., and Isomura, M. (1994) Regioselective transglycosylation in the synthesis of oligosaccharides: comparison of β-galactosidases and sialidases of various origins. *Carbohydr. Res.* **259**, 103–115
8. Schmidt, D., Sauerbrei, B., and Thiem, J. (2000) Chemoenzymatic synthesis of sialyl oligosaccharides with sialidases employing transglycosylation methodology. *J. Org. Chem.* **65**, 8518–8526
9. Blumberg, B.S. and Warren, L. (1961) The effect of sialidase on transferrins and other serum proteins. *Biochim. Biophys. Acta* **50**, 90–101
10. Warren, L. and Spearing, C.W. (1963) Sialidase (Neuraminidase) of *Corynebacterium diphtheriae*. *J. Bacteriol.* **86**, 950–955
11. Mattos-Guaraldi, A.L., Cappelli, E.A., Previato, J.O., Formiga, L.C., and Andrade, A.F. (1999) Characterization of surface saccharides in two *Corynebacterium diphtheriae* strains. *FEMS Microbiol. Lett.* **170**, 159–166
12. Moreira, L. de O., Andrade, A.F., Vale, M.D., Souza, S.M., Hirata, R. Jr, Asad, L.M., Asad, N.R., Monteiro-Leal, L.H., Previato, J.O., and Mattos-Guaraldi, A.L. (2003) Effects of iron limitation on adherence and cell surface carbohydrates of *Corynebacterium diphtheriae* strains. *Appl. Environ. Microbiol.* **69**, 5907–5913
13. Schrader, S., Tiralongo, E., Paris, G., Yoshino, T., and Schauer, R. (2003) A nonradioactive 96-well plate assay for screening of trans-sialidase activity. *Anal. Biochem.* **322**, 139–147

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 28, 2012

14. Warren, L. (1959) The thiobarbituric acid assay of sialic acids. *J. Biol. Chem.* **234**, 1971–1975
15. Hansmeier, N., Chao, T.C., Kalinowski, J., Puhler, A., and Tauch, A. (2006) Mapping and comprehensive analysis of the extracellular and cell surface proteome of the human pathogen. *Corynebacterium diphtheriae*. *Proteomics* **6**, 2465–2476
16. Cerdeño-Tárraga, A.M., Efstratiou, A., Dover, L.G., Holden, M.T., Pallen, M., Bentley, S.D., Besra, G.S., Churcher, C., James, K.D., De Zoysa, A., Chillingworth, T., Cronin, A., Dowd, L., Feltwell, T., Hamlin, N., Holroyd, S., Jagels, K., Moule, S., Quail, M.A., Rabbinowitsch, E., Rutherford, K.M., Thomson, N.R., Unwin, L., Whitehead, S., Barrell, B.G., and Parkhill, J. (2003) The complete genome sequence and analysis of *Corynebacterium diphtheriae* NCTC13129. *Nucleic Acids Res.* **31**, 6516–6523
17. Von Heijne, G. (1994) Decoding the signals of membrane protein sequence. In *Membrane Protein Structure* (White, S.H., eds), pp. 27–40, Oxford University Press, NY, USA
18. Newstead, S.L., Potter, J.A., Wilson, J.C., Xu, G., Chien, C.H., Watts, A.G., Withers, S.G., and Taylor, G.L. (2008) The structure of *Clostridium perfringens* NanI sialidase and its catalytic intermediates. *J. Biol. Chem.* **283**, 9080–9088
19. Xu, G., Li, X., Andrew, P.W., and Taylor, G.L. (2008) Structure of the catalytic domain of *Streptococcus pneumoniae* sialidase NanA. *Acta Crystallogr. Sect F Struct. Biol. Cryst. Commun.* **64**, 772–775
20. Xu, G., Potter, J.A., Russell, R.J., Oggioni, M.R., Andrew, P.W., and Taylor, G.L. (2008) Crystal structure of the NanB sialidase from *Streptococcus pneumoniae*. *J. Mol. Biol.* **384**, 436–449
21. Delahay, R.M. and Frankel, G. (2002) Coiled-coil proteins associated with type III secretion systems: a versatile domain revisited. *Mol. Microbiol.* **45**, 905–916
22. Jedrzejas, M.J. (2001) Pneumococcal virulence factors: structure and function. *Microbiol. Mol. Biol. Rev.* **65**, 187–207
23. Mattos-Guaraldi, A.L., Formiga, L.C., and Andrade, A.F. (1998) Trans-sialidase activity for sialic acid incorporation on *Corynebacterium diphtheriae*. *FEMS Microbiol. Lett.* **168**, 167–172
24. Tai, S.S. (2006) *Streptococcus pneumoniae* protein vaccine candidates: properties, activities and animal studies. *Crit. Rev. Microbiol.* **32**, 139–153
25. Nakatsuji, T., Liu, Y.-T., Huang, C.-P., Gallo, R.L., and Huang, C.-M. (2008) Vaccination targeting a surface sialidase of *P. acnes*: implication for new treatment of acne vulgaris. *PLoS one* **3**, e1551
26. Mokrousov, I. (2009) *Corynebacterium diphtheriae*: genome diversity, population structure and genotyping perspectives. *Infect. Genet. Evol.* **9**, 1–15
27. Moriyama, T. and Barksdale, L. (1967) Neuraminidase of *Corynebacterium diphtheriae*. *J. Bacteriol.* **94**, 1565–1581
28. Scudder, P., Doom, J.P., Chuenkova, M., Manger, I.D., and Pereira, M.E. (1993) Enzymatic characterization of β-D-galactoside α2,3-trans-sialidase from *Trypanosoma cruzi*. *J. Biol. Chem.* **268**, 9886–9891
29. Montagna, G., Cremona, M.L., Paris, G., Amaya, M.F., Buschiazzo, A., Alzari, P.M., and Frasch, A.C. (2002) The trans-sialidase from the African trypanosome *Trypanosoma brucei*. *Eur. J. Biochem.* **269**, 2941–2950
30. Tiralongo, E., Schrader, S., Lange, H., Lemke, H., Tiralongo, J., and Schauer, R. (2003) Two trans-sialidase forms with different sialic acid transfer and sialidase activities from *Trypanosoma congolense*. *J. Biol. Chem.* **278**, 23301–23310
31. Vimr, E.R., Lawrisuk, L., Galen, J., and Kaper, J.B. (1988) Cloning and Expression of the *Vibrio cholerae* neuraminidase gene *nanH* in *Escherichia coli*. *J. Bacteriol.* **170**, 1495–1504
32. Abrashev, I. and Dulguerova, G. (2000) Neuraminidases (Sialidases) from bacterial origin. *Exp. Pathol. Parasitol.* **4**, 35–40
33. Zenz, K.I., Roggentin, P., and Schauer, R. (1993) Isolation and properties of the natural and the recombinant sialidase from *Clostridium septicum* NC 0054714. *Glycoconj. J.* **10**, 50–56
34. Post, D.M.B., Mungur, R., Gibson, B.W., and Munson, R.S. (2005) Identification of a novel sialic acid transporter in *Haemophilis ducreyi*. *Infect. Immun.* **73**, 6727–6735

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 28, 2012