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Sialyltransferases with enhanced legionaminic acid transferase activity for the preparation of analogs of sialoglycoconjugates

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Running title: STases with enhanced legionaminic acid transferase activity

The submission includes supplementary data (one table)

Abstract

Legionaminic acids (Leg) are bacterial analogs of neuraminic acid, with the same stereochemistry but different substituents at C5, C7 and C9. Hence they may be incorporated into useful analogs of sialoglycoconjugates, and we previously reported two sialyltransferases that could utilize CMP-Leg5Ac7Ac for preparation of Leg glycoconjugates, which were resistant to sialidases (Watson et al. Glycobiology vol 21 pp. 99-108 (2011)). These were the porcine ST3Gal1 and *Pasteurella multocida* sialyltransferases. We now report two additional sialyltransferases with superior Leg-transferase properties to the previous two. These are a) a truncated form of a *Photobacterium* α2,6-sialyltransferase with an Ala-Met mutation in its active site, and b) an α2,3-sialyltransferase from *Neisseria meningitidis* MC58 with a higher transferase activity than the *P. multocida* enzyme, with either CMP-Neu5Ac or CMP-Leg5Ac7Ac as the donor. These enzymes will enable the production of useful Leg5Ac7Ac glycoconjugate derivatives with either α2,6 or α2,3 linkages and unique biological properties.

Keywords: legionaminic acid; *Neisseria meningitidis*; *Neisseria gonorrhoeae;* *Photobacterium*; *Pasteurella multocida*; sialyltransferase

Introduction

Terminal sialic acid residues on glycoproteins and glycolipids can regulate their molecular and cellular interactions, often in either of two ways, which are opposite in nature (Schauer 2009). They can form recognition sites for proteins such as the Siglecs and for viruses or they can mask penultimate galactose residues, which would otherwise be recognised by proteins such as the asialoglycoprotein receptor. Natural and synthetic analogs of Neu5Ac, the most common sialic acid, are therefore of interest since they can alter these interactions either by impairing glycan binding to receptors or, if resistant to sialidases, prolonging the masking effects.

Leg5Ac7Ac, a legionaminic acid sugar, is a naturally-occurring analog of sialic acid (**Figure 1**), with the same stereochemistry as Neu5Ac but differing at C7 and C9, where it has acetamido and deoxy groups, respectively. We have previously found (Watson et al. 2011) two sialyltransferases that could efficiently transfer Leg5Ac7Ac from its CMP derivative to some glycoconjugates. Notably, the products were resistant to a set of sialidases which included bacterial, viral and human enzymes. Hence, Leg5Ac7Ac glycoconjugates are of interest as analogs of bioactive sialo-glycans. The two transferases were the porcine ST3Gal1 and the *Pasteurella multocida* PmST1 α2,3-sialyltransferases, and both were able to utilize CMP-Leg5Ac7Ac at ~60% of their activities with CMP-Neu5Ac. However, their acceptor specificities are restricted, with the porcine enzyme only modifying T-antigen on soluble saccharides or on *O*-linked glycoproteins, while the *P. multocida* STase is active only with relatively small substrates. Hence neither enzyme could be used to modify *N*-linked glycoproteins with

Leg5Ac7Ac. A third enzyme, the *Photobacterium* α2,6-sialyltransferase is capable of modifying large *N*-linked glycan substrates, but its activity with CMP-Leg5Ac7Ac was only around 5% of that with CMP-Neu5Ac.

We therefore investigated two routes to obtain better Leg-transferases, namely assaying further bacterial α2,3-sialyltransferases for Leg5Ac7Ac transferase activity and enhancing the Leg5Ac7Ac α2,6-transferase activity of the *Photobacterium* sialyltransferase by mutagenesis. We have previously reported the properties of *Neisseria meningitidis* and *N. gonorrhoeae* α2,3-sialyltransferases (Gilbert et al. 1996; 1997; Watson et al. 2011). However, recent work examining the substrate specificity of the *N. gonorrhoeae* α2,3-sialyltransferase (Gulati, S., Schoenhofen, I. et al., manuscript submitted) suggested that *Neisseria* enzymes may be good candidates for Leg5Ac7Ac transfer to LacNAc. *N. gonorrhoeae* was found to efficiently modify its lipooligosaccharide with several forms of sialic acid and Leg5Ac7Ac when the respective CMP-sugars were added to *N. gonorrhoeae* cell-based assays. Importantly, we have now found that both *N. meningitidis* and *N. gonorrhoeae* α2,3 sialyltransferases transfer Leg5Ac7Ac efficiently to LacNAc acceptors, exhibiting higher Leg5Ac7Ac transfer activity than that observed for the *P. multocida* α2,3 enzyme with T-antigen or Lac acceptors.

The *P. multocida* PmST1 and a truncated form of the *Photobacterium* sialyltransferase (Sun et al. 2008) have previously been utilized to add modified sialic acids to small molecule acceptors, with considerable success (Yu et al. 2005; Cheng et al. 2010; Khedri et al. 2014). Mutated forms of PmST1 with improved properties have been reported (Sugiarto et al. 2012; Choi et al. 2014), and interesting homologs have been

found in related species (Mine et al. 2010; Schmölzer et al. 2013). These sialyltransferases all belong to the same CAZy family, GT80, and the crystal structures of the *P. multocida* PmST1 and the *Photobacterium* sialyltransferase (Ni et al. 2007; Kakuta et al. 2008) are superimposable. A comparison of the donor binding site regions of the superimposed transferases (**Figure 2**) identified three amino acid differences that might influence their respective Leg5Ac7Ac-transferase activities. When these differences were introduced into the *Photobacterium* enzyme by site-directed mutagenesis, the Ala235Met mutant showed enhanced α2,6-Leg5Ac7Ac-transferase properties, and we therefore characterized its enzyme kinetics and investigated other mutations at this site. We show that the mutant enzymes can be used to produce α2,6-Leg5Ac7Ac glycoconjugates analogous to the previous α2,3-Leg5Ac7Ac ones (Watson et al. 2011).

Results

Structural comparison of P. multocida *and* Photobacterium *sialyltransferases*

On the basis of their sequences and an overlay of the tertiary structures of the two sialyltransferases (**Figure 2**), three positions in the binding site region were identified for mutation in the *Photobacterium* sialyltransferase. Two were single-residue mutations, His123Pro and Ala235Met, and one was an insertion, Val370a.

Construction of mutants and activity assays

The three mutations were introduced as single changes and in various combinations, by site-directed mutagenesis of a MalE fusion of a truncated form of the *Photobacterium* sp. JT-ISH-224 enzyme that lacks its first 109 residues (Kakuta et al. 2008, Chan et al. 2010). The residue numbering used here is according to PDB accession number 2Z4T. The combinations were His123Pro/Ala235Met, His123Pro/Val370a, Val370a/Asp473Glu and His123Pro/Ala235Met/Val370a. The activities of crude extracts and the purified Ala235Met mutant were tested with CMP-Leg5Ac7Ac or CMP-Neu5Ac as the donor, and LacNAc-FCHASE as the acceptor, in thin layer chromatography (TLC) and capillary electrophoresis (CE) assays (Watson et al. 2011).

Two mutants that included Ala235Met showed enhanced activity with CMP-Leg5Ac7Ac (**Figure 3,** lanes 4 and 8); the His123Pro mutants were inactive (lanes 3, 6 and 7), and the Val370a insertion was slightly more active than the wild-type (lanes 5 and 9). The Ala235Met mutant was therefore characterized in greater detail. The product from a larger-scale reaction was isolated by preparative TLC and the α2,6 linkage of the Leg to Gal was confirmed by NMR (Supplementary data).

Kinetic measurements for the Ala235Met mutant were performed with both CMP-Neu5Ac and CMP-Leg5Ac7Ac donors, and the results are reported in **Table 1**.

Further mutations at residue Ala235

Eleven more mutants were constructed in which Ala235 was replaced with residues of varying chemical character, *viz* Asp, Asn, Thr, Ile, Tyr, Leu, Val, Lys, His and Phe, and their activities with the two donors were compared with the same fluorescent acceptor as above. In TLC and CE analyses, several of the mutants showed higher activities with CMP-Neu5Ac compared to the wild-type or to the Ala235Met mutant. Two of the mutants, Leu and Ile, were close in activity to Ala235Met (**Figure 4A**) with CMP-Neu5Ac, and all three showed higher activities with CMP-Leg5Ac7Ac than the wildtype. Samples of the Ala235Ile and Ala235Leu mutants were therefore purified and the kinetic properties of the Ala235Leu mutant with the two donors are shown in **Table 1**. K_m values for the LacNAc-FCHASE acceptor were estimated to be >4 mM and therefore could not be determined accurately due to limitations in the availability and solubility of this compound. As previously reported (Watson et al. 2011), the activity of the wildtype *Photobacterium* enzyme with CMP-Leg5Ac7Ac donor was too low to be accurately determined.

Hydrolase activities of the mutants

Sialyltransferases that are used to make glycoconjugates *in vitro* often display hydrolase activity, breaking down the supply of donor, as well as glycosidase activity that can destroy the product. We therefore tested the three purified mutants with CMP-Neu5Ac

and CMP-Leg5Ac7Ac in the absence of acceptor, in order to determine if the increased α2,6 transferase activity was accompanied by undesirable hydrolysis of the donor. The results (**Figure 4B**) showed that the mutants had very high hydrolase activity with CMP-Neu5Ac, but considerably less with CMP-Leg5Ac7Ac. The hydrolase activity of the Ile mutant was lower than those of the Met and Leu mutants, and it may therefore be more economical to use for glycoconjugate modification, despite its slightly lower Leg-transferase activity.

Activity of Neisseria *sialyltransferases with CMP-Leg5Ac7Ac donor*

The lipooligosaccharide α2,3-sialyltransferases (LST) from three strains of *N. meningitidis* (LST-MC58, LST-126E and LST-406Y) and one strain of *N. gonorrhoeae* (LST-F62) (Gilbert et al. 1996; 1997) were cloned as full length enzymes without fusion tags and purified. When tested with CMP-Neu5Ac and CMP-Leg5Ac7Ac as donors, all showed significant activity with CMP-Leg5Ac7Ac (**Figure 5A**). Hence the enzymes from *Neisseria* species are naturally active with CMP-Leg5Ac7Ac. Their hydrolase activities were also measured with both donors. In each case, there was less hydrolysis of CMP-Leg5Ac7Ac than of CMP-Neu5Ac (**Figure 5B**), as was seen with the *Photobacterium* mutant enzymes. The overall hydrolysis rates were comparable to the spontaneous hydrolysis of each nonulosonic acid species (negative control), and below the rates seen with the *Photobacterium* mutant enzymes, indicating their superior utility for making Leg derivatives.

LST-MC58 was selected for further characterization. There were approximately three-fold differences in the k_{cat} and K_m values with the two donors (**Table 1**), resulting in very similar k_{cat}/K_m values overall.

Discussion

Substitution of residue 235 in the *Photobacterium* enzyme had a striking effect on its ability to transfer Leg from CMP-Leg5Ac7Ac to acceptors. This substitution site was chosen from a comparison of the two sialyltransferase structures in which the Ala residue was superimposable on the Met residue of the *P. multocida* enzyme (**Figure 2**). Of the three mutants with the greatest enhancements, the original Ala235Met one was more active than the corresponding Ile and Leu mutants. The k_{cat} values of the *Photobacterium* mutants with the CMP-Neu5Ac donor are about ten-fold higher than that of the wildtype, while their K_m values are similar. This suggests that an increase in overall rate is mostly responsible for the improved catalytic efficiency (k_{cat}/K_m) and probably explains the "acquisition" of activity with a non-natural donor, CMP-Leg5Ac7Ac. If so, then these mutants may well be useful for the preparation of glycoconjugates with other analogs of sialic acid.

Since Met235 is important for enhanced sialic acid or legionaminic acid transfer, it is not surprising that Leu235 was also highly active as the two residues have similar size and hydrophobicity. The non-polar nature of the Met sidechain allows Met to participate in forming hydrophobic contacts between different protein segments, and substitution with Leu would be expected to preserve such contacts. These hydrophobic interactions may directly or indirectly position the acceptor/donor for optimal transfer.

It is interesting to compare the striking effects of the *Photobacterium* sialyltransferase Ala235 substitutions with work reported on a substitution at the corresponding position in the *P. multocida* sialyltransferase, Met144Asp (Sugiarto et al. 2012). This mutation reduced both the hydrolase and sialidase activities of the wildtype.

In the case of the *Photobacterium* enzyme, while the mutation to Met conferred the desired higher activity with CMP- Leg5Ac7Ac possessed by the *P. multocida* enzyme, the mutant Ala235Asp had much less activity (**Figure 4A**).

The activities of the *Neisseria* sialyltransferases with both the Leg5Ac7Ac and Neu5Ac donors were significantly higher than those of the *P. multocida* enzyme, though the latter were measured with Lac-FCHASE rather than LacNAc-FCHASE (Watson et al. 2011). The k_{cat}/K_m values with CMP-Neu5Ac and CMP-Leg5Ac7Ac for the *P. multocida* enzyme were 3.1 and 1.2 min^{-1} mM^{-1} respectively, while the activities of the *N. meningitidis* LST-MC58 enzyme were four to ten-fold higher, at 11.9 and 15.5 min^{-1} mM^{-1} (**Table 1**). Taken together with its low hydrolysis activity (Figure 5B) this enzyme (or one of its homologs) is therefore superior to the *Photobacterium* and the *P. multocida* enzymes for the preparation of legionaminic acid glycoconjugates. To note, the *Neisseria* LST activity with Leg5Ac7Ac measured here strongly suggests that the LST is responsible for the lipooligosaccharide modification with novel CMP-sugars observed in the *N. gonorrhoeae* cell-based assays (Gulati, S., Schoenhofen, I. et al., manuscript submitted) described earlier, given the partial similarity of the LacNAc-FCHASE and lipooligosaccharide acceptors from *in vitro* and *in vivo* assays, respectively.

There were ~3-fold differences in the activities of the four *Neisseria* enzymes with the two donors. In the crystal structure reported for LST-126E with co-crystallized CMP-3F-Neu5Ac (Lin et al. 2011; PDB entry 2YK7) the region in the enzyme closest to the O7 and O9 moieties of the glycerol sidechain of the Neu moiety (Figure 6) is around Ile^{168}. This region also shows notable sequence variation among the four enzymes. In the LST-126E the sequence is T^{167}INLIQSS, whereas in LST-MC58 and LST-406Y it is

TGNLIQSS and in the *N. gonorrhoeae* LST-F62 it is TNNLIRES. The activity differences probably arise from less favorable interactions around residue 168 with the bulkier C7 NAc group of Leg, or by changes in the Asp^{165} to Thr^{167} region which is close to the C9 atom. Interestingly, Ile^{168} has previously been found to be responsible for LST-126E showing some α2,6 sialyltransferase activity with a P^{k} acceptor (Galα1,4Galβ1,4Glcβ-), and mutation of Gly^{168} in LST-MC58 to Ile endowed it with the same activity (Wakarchuk et al. 2001).

In conclusion, the two enzymes described here offer advantages over the previously studied *P. multocida* and porcine ST3Gal1 sialyltransferases for making Leg analogs of sialylglycans with LacNAc acceptors, including access to α2,6 Leg compounds and greater overall activity. In addition to being useful in the development of sialoglycoconjugates with greater stability, i.e. to extend half-life of biotherapeutics, sialyltransferases with the ability to incorporate Leg5Ac7Ac within glycoconjugates will allow investigators to determine the impact this analog may have on interactions with sialic acid binding molecules such as Siglecs and viral receptors, with further potential applications.

Materials and methods

Reagents

CMP-Leg5Ac7Ac was prepared as previously described (Watson et al. 2011). The fluorescent acceptors LacNAc-FCHASE (Galβ1,4GlcNAcβ-FCHASE) and Lac-FCHASE (Galβ1,4Glcβ-FCHASE) were prepared as described by Wakarchuk and Cunningham (2003).

Capillary electrophoresis

CE analyses were performed using a P/ACE MDQ system equipped with either diode array detection or with a Laser module 488 (Beckman Coulter, Fullerton, CA, USA). Capillaries were bare silica 50 µm x 60.2 cm with a detector at 50 cm. For diode array detection, the running buffer was 25 mM sodium tetraborate, pH 9.4 and separations were performed at 27 kV for 20 min. For laser induced fluorescence (LIF) detection, the running buffer used was 90 mM Tris/89 mM borate/2 mM EDTA, pH 8.8 and samples were run at 30 kV for 15 min. Samples were introduced by pressure injection for 10 s and quantification performed by integration of trace peaks using the MDQ 32 Karat software.

Site-directed mutagenesis of the Photobacterium *sialyltransferase.*

The cloned *Photobacterium* sp. JT-ISH-224 sialyltransferase was mutagenized using a two-stage mutagenesis protocol. Forward and reverse PCR primers both containing the desired mutation were used in separate PCRs in conjunction with the 5' and 3' gene primers to generate two overlapping gene fragments. Both PCR products were then used as a template in a third PCR reaction with the primer pair designed to generate a full-

length *Photobacterium* sialyltransferase product. Full-length mutagenized PCR products were subsequently cloned in frame into a MalE containing expression vector (Chan et al. 2010) and verified by sequence analysis. In the case of the Val370a insertion, a second clone with a spontaneous mutation at a position remote from the binding site, Asp473Glu, was also characterised.

Expression and extraction of the Photobacterium *sialyltransferases*

MalE fusions of wild-type and mutant proteins were overexpressed in *E.coli* AD202 cells from 2YT cultures containing 0.2% glucose and lysed by mechanical disruption in 200 mM NaCl/20 mM Tris pH 7.5 buffer with an Avestin C5 Emulsiflex cell disrupter (Avestin, Ottawa, ON). Protease inhibitor cocktail (Roche Diagnostics, Laval, QC) was added to the protein extracts which were then clarified by centrifugation (27,000 X *g* for 30 min at 4^{o}C), and cell debris were discarded. Total membrane and soluble protein were obtained from clarified cell extracts by centrifugation (100,000 X *g* for 60 min at 4^{o}C).

Screening of sialyltransferases

Primary screening of the *Photobacterium* mutant enzymes was carried out with LacNAc-FCHASE as acceptor, and CMP-Leg5Ac7Ac and CMP-Neu5Ac as donors. The buffer conditions were 50 mM MES, pH 6.0. Assays were performed at 30^{o}C for 30 min using final concentrations of 200 µM LacNAc-FCHASE and 1 mM donor substrate with 3 µl of extract, in a total reaction volume of 10 µl. Reactions were stopped by the addition of 10 µl of 50% acetonitrile, 10 mM EDTA, 1% SDS. Reactivity was assessed by thin-layer chromatography on silica using a solvent system of ethyl acetate/methanol/water/acetic

acid 4:2:1:0.1. Those reactions that showed product formation were then quantified by CE-LIF as described previously (Wakarchuk and Cunningham 2003).

Preparation and assays of purified Photobacterium *sialyltransferases*

Wild-type and mutants proteins were purified from the soluble fractions by affinity chromatography on amylose resin according to the manufacturer's instructions (New England Biolabs, Beverley, MA). Kinetic analyses were carried out over a range of concentrations of donor (50 μM to 1 mM) and acceptor (50 μM to 3 mM) using a CE-LIF assay to quantify the formation of sialylated product as previously described (Gilbert et al. 1997).

The hydrolase activities of 2.5 μg aliquots of the enzymes were measured with 2 mM CMP-Neu5Ac or CMP-Leg5Ac7Ac as the substrate in 50 mM MES pH 6.0 buffer (10 μL), incubated at 30^{o} C for 30 min, and its conversion to free CMP was quantified by CE using diode array detection as described above.

Cloning, expression and purification of the Neisseria *sialyltransferases*

The four sialyltransferases that were studied were from *N. meningitidis* MC58 L3 immunotype (GenBank accession number U60660), 126E L1 immunotype (GenBank accession number U60662), 406Y L3 immunotype (GenBank accession number U60661) and from *N. gonorrhoeae* F62 (GenBank accession number U60664). The sialyltransferase gene (*lst*) from each strain was cloned using the NdeI and SalI cloning sites in pCWori+(-*lacZ*) giving constructs pNST-05 (LST-MC58), pNST-23 (LST-126E), pNST-25 (LST-406Y) and pNST-26 (LST-F62). The plasmids were electroporated into *E. coli* AD202. Cells were grown at 25^{o}C in 200 mL of 2YT medium supplemented with 150 μg/mL ampicillin. Over-expression of the transferases was induced by the addition of

0.5 mM isopropyl-β-D-thiogalactopyranoside at an A_{600nm} of 0.6, and growth was continued at 25°C for 16 h. Cells were harvested by centrifugation at 10,000 x *g* for 20 min, re-suspended in 10 mM HEPES buffer pH 7.5 and lysed by mechanical disruption with an Avestin C5 Emulsiflex cell disrupter (Avestin, Ottawa, ON). Protease inhibitor cocktail (Roche Diagnostics, Laval, QC) was added to the cell lysate which was then clarified by centrifugation at 27,000 x *g* for 30 min and cell debris were discarded. Cell membranes were obtained from clarified cell extracts by centrifugation at 100,000 x *g* for 60 min. Membrane pellets were re-suspended and washed in 10 mM HEPES buffer pH 7.5 and re-centrifuged at 100,000 x *g* for 1 h. Membrane pellets were re-suspended in 5 mL of 0.1M NaCl/10 mM HEPES pH 7.5/ 0.2% Triton X-100 and stirred for 4 h at 4°C followed by centrifugation at 100,000 x *g* for 1 h. Membrane extracts were stored at 4°C until use.

Transferase and hydrolase activities of the Neisseria *sialyltransferases*

Transferase reactions of the *Neisseria* LST's were performed using LacNAc-FCHASE as acceptor and CMP-Leg5Ac7Ac or CMP-Neu5Ac as donor. The buffer conditions were 50 mM MES pH 6.5 and 10 mM $MnCl_2$. Assays were performed in duplicates at 30°C for 10 min using CMP-Neu5Ac as donor or 30 min using CMP-Leg5Ac7Ac as donor. The final concentration of LacNAc-FCHASE acceptor was 100 mM and 1 mM of donor substrate with ~5 mU of enzyme in a total reaction volume of 10 µl. Reactions were stopped and reactivity assessed in the same manner as described for the *Photobacterium* mutants.

Hydrolase activities were measured using final concentrations of 2 mM CMP-Neu5Ac or CMP-Leg5Ac7Ac in 50 mM MES pH 6.5, 10mM $MnCl_2$ and ~5 mU of enzyme. Reactions were performed in duplicates at 30^{o}C for 30 min and quantitated by CE using diode array detection.

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Conflict of interest statement

None declared

Abbreviations

CE, capillary electrophoresis; FCHASE, 6-(fluorescein-5-carboxyamido)-hexanoic acid succinimidyl ester derivative of the *p*-aminophenyl glycoside; Lac, Galβ1,4Glc; LacNAc, Galβ1,4GlcNAc; Leg5Ac7Ac, diacetyllegionaminic acid or 5,7-diacetamido-3,5,7,9-tetradeoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid; LIF, laser induced fluorescence; TLC, thin layer chromatography.

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Figure legends

Fig. 1. Structures of neuraminic acid (Neu5Ac) and legionaminic acid (Leg5Ac7Ac)

Fig. 2. Comparisons of the two GT80 sialyltransferases. (A) superposition of their tertiary structures with *P. multocida* in green, and (B) comparison of their active-site regions. The *Photobacterium* numbering is shown, with the corresponding *P. multocida* numbers in brackets for some of them. Only the CMP portion of the *Photobacterium* donor is shown. The Val370a insertion was made into the *Photobacterium* loop at the bottom left (^{370}His-Glu-Arg-Glu^{374}); the corresponding loop in the *P. multocida* has been omitted for clarity. Two key active site residues, Trp and His (His405 and Trp365 in the *Photobacterium* enzyme), superimpose completely and only one of each is shown here. The PDB accession codes are 2Z4T for the *Photobacterium* enzyme (Kakuta et al. 2008) and 2IHJ for the *P. multocida* enzyme (Ni et al. 2007). Images were made with the PyMOL Molecular Graphics System, Version 1.7.0.3 Schrödinger, LLC.

Fig. 3. TLC analysis of the transferase activities of active-site mutants. Mixtures of the enzyme extracts with 1 mM donor and 0.3 mM LacNAc-FCHASE acceptor were incubated at pH 6.0 and 30°C for 1 h. Wildtype: lanes 1 and 2; single mutants: lane 3, His123Pro; lane 4, Ala235Met; lane 5, Val370a; double mutants: lane 6, His123Pro/Ala235Met; lane 7, His123Pro/Val370a; lane 8, Ala235Met/Val370a; lane 9, Val370a/Asp473Glu; triple mutant lane 10, His123Pro/Ala235Met/Val370a.

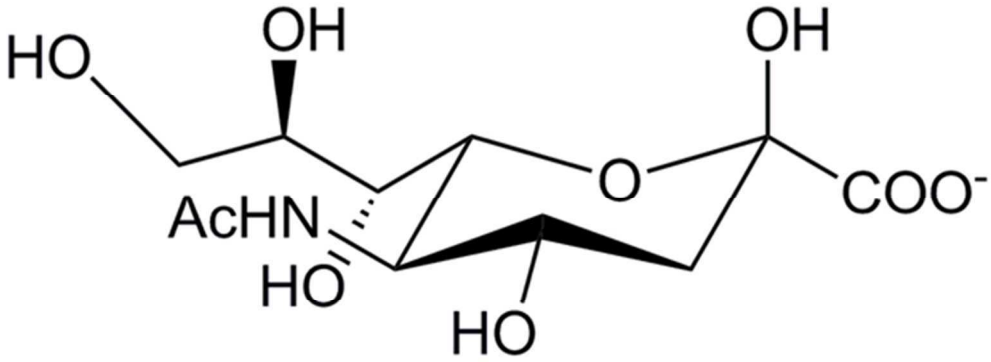
Fig. 4. Relative activities of Ala235 mutants of the *Photobacterium* STase. A. Relative transferase activities of crude enzyme extracts measured by CE-LIF and expressed as % conversion to the oligosaccharide product with LacNAc-FCHASE as acceptor and CMP-Neu5Ac (filled bars) or CMP-Leg5Ac7Ac (open bars) as donor. B. Hydrolase activities of the purified mutants with CMP-Neu5Ac (filled bars) and CMP-Leg5Ac7Ac (open bars), measured with CE with diode array detection. The negative control (without enzyme) was included because CMP-nonulosonates are naturally labile at the pH used.

Fig. 5. Relative activities of *Neisseria* STases. A. Relative transferase activities measured by CE-LIF and expressed as % conversion to the oligosaccharide product using LacNAc-FCHASE as acceptor and CMP-Neu5Ac (filled bars, 10 min reactions) or CMP-Leg5Ac7Ac (open bars, 30 min reactions) as donor. B. Relative hydrolase activities with CMP-Neu5Ac (filled bars) and CMP-Leg5Ac7Ac (open bars) measured with CE with diode array detection. The negative control (without enzyme) was included because CMP-nonulosonates are naturally labile at the pH used.

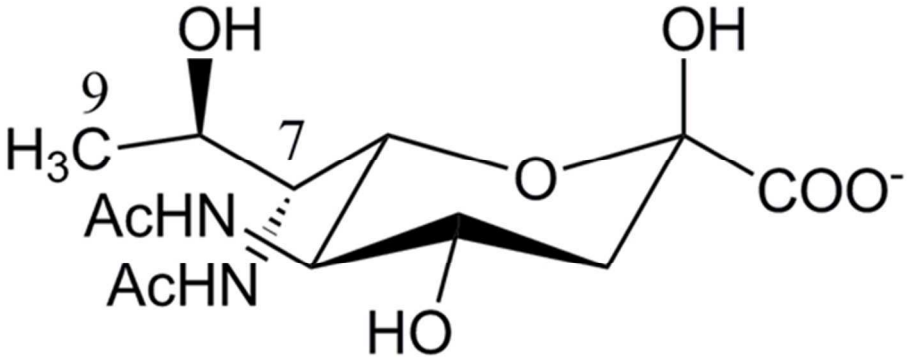
Fig. 6. Adjacency of residue Ile^{168} to the glycerol sidechain of CMP-3F-Neu5Ac. The distances between O9 and O7 of Leg5Ac7Ac and Thr167 and Ile168, respectively, are shown in Ångstroms. The stereo diagram is based on the crystal structure of *N. meningitidis* LST-126E (Lin et al. 2011; PDB entry 2YK7). Images were made with the PyMOL Molecular Graphics System, Version 1.7.0.3 Schrödinger, LLC.

Table I. Kinetic properties of wildtype, Met and Leu substitutions of the *Photobacterium* enzyme and the *N. meningitidis* enzyme LST-MC58, with LacNAc-FCHASE as the acceptor, and *P. multocida* data from Watson et al. (2011) with Lac-FCHASE as the acceptor. *Photobacterium* WT (Ala235) activity with CMP-Leg5Ac7Ac was too low for accurate kinetic measurements (Watson et al. 2011).

Enzyme	Donor	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (min^{-1} mM^{-1})
Ala235	CMP-Neu5Ac	33.5 ± 1.0	0.7 ± 0.1	48
Ala235Met	CMP-Neu5Ac	336 ± 10	0.7 ± 0.1	480
Ala235Met	CMP-Leg5Ac7Ac	4.2 ± 0.2	0.3 ± 0.1	14
Ala235Leu	CMP-Neu5Ac	243 ± 11	0.9 ± 0.1	270
Ala235Leu	CMP-Leg5Ac7Ac	9.5 ± 0.7	1.7 ± 0.3	5.6
LST-MC58	CMP-Neu5Ac	6.7 ± 0.2	0.6 ± 0.1	11.2
LST-MC58	CMP-Leg5Ac7Ac	2.5 ± 0.1	0.16 ± 0.01	15.6
P. multocida	CMP-Neu5Ac	0.78	0.25 ± 0.03	3.1
P. multocida	CMP-Leg5Ac7Ac	0.56	0.46 ± 0.04	1.2



Sialic acid
(Neu5Ac)



N,N'-diacetyllegionaminic acid
(Leg5Ac7Ac)

Figure 1
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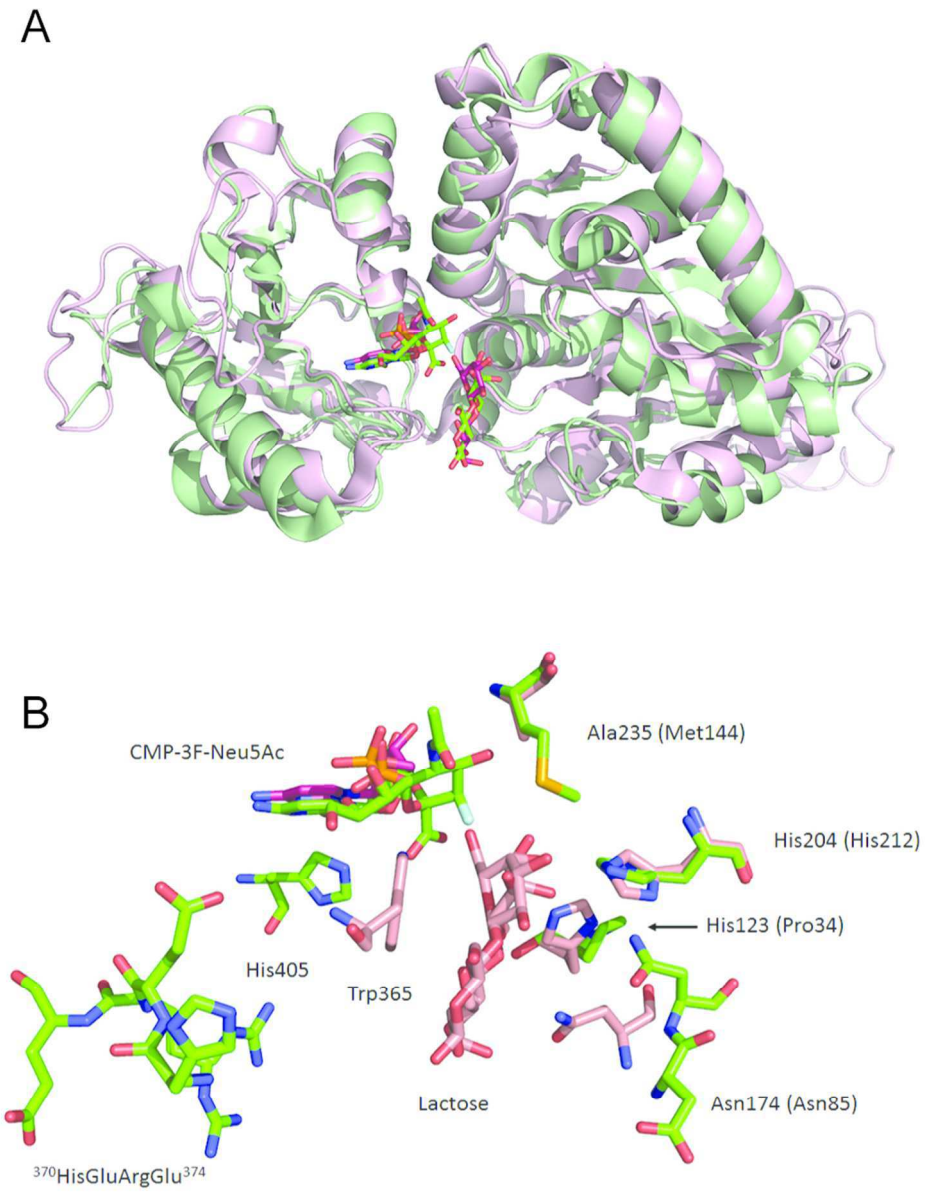

Figure 2
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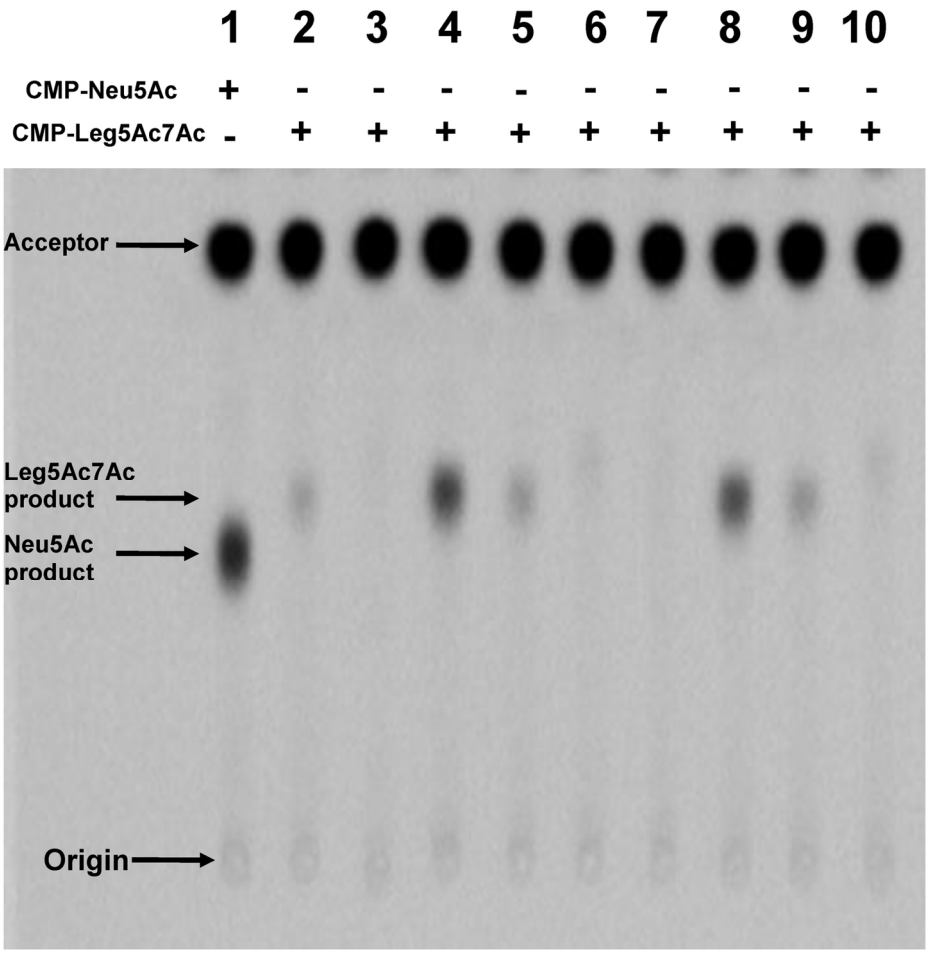

Figure 3
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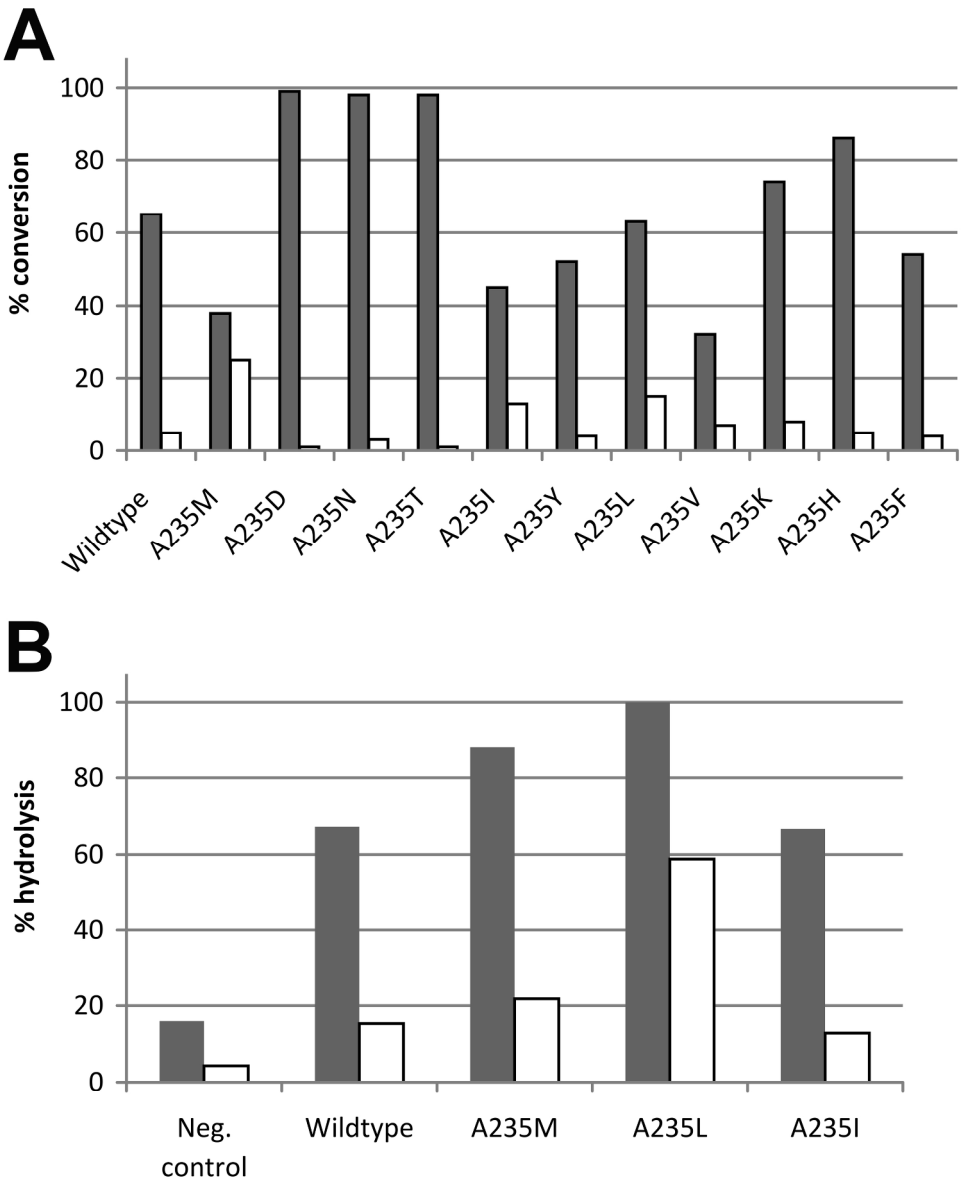

Figure 4
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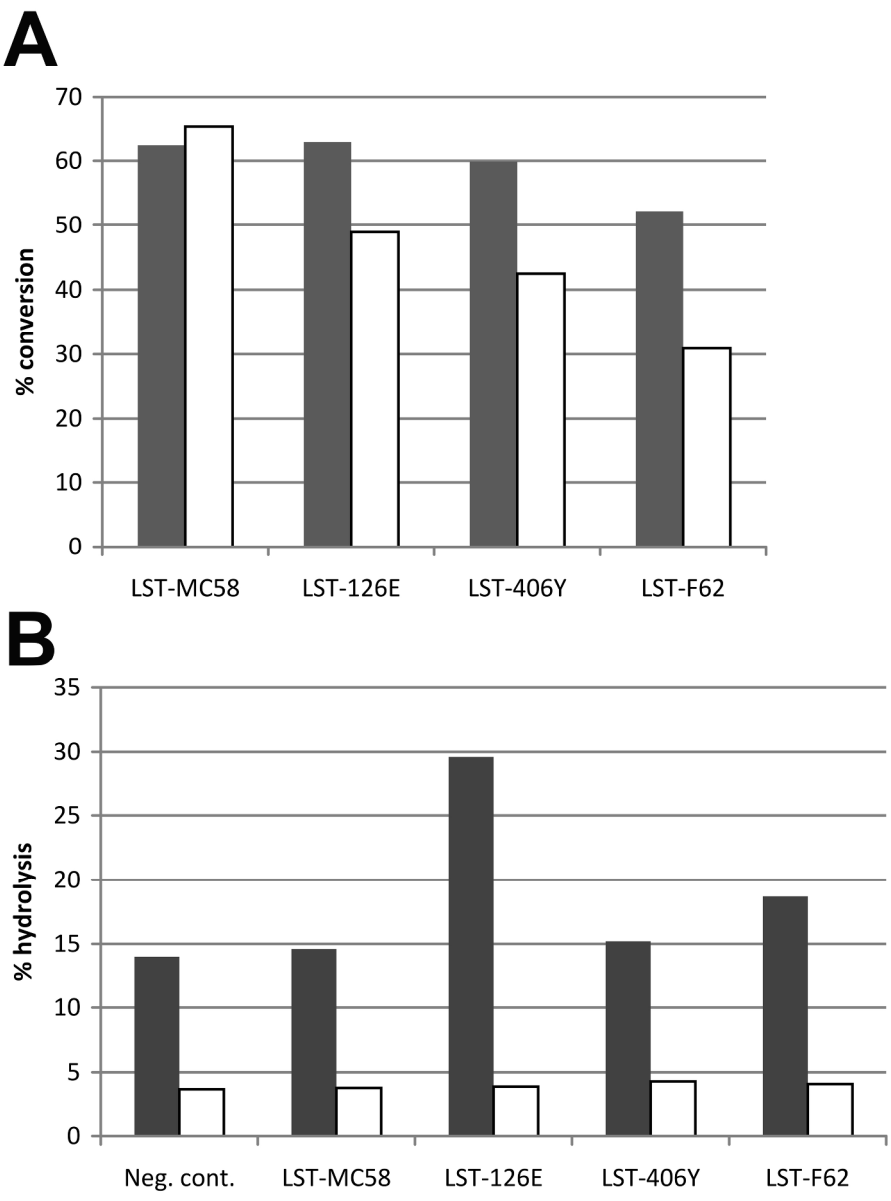

Figure 5
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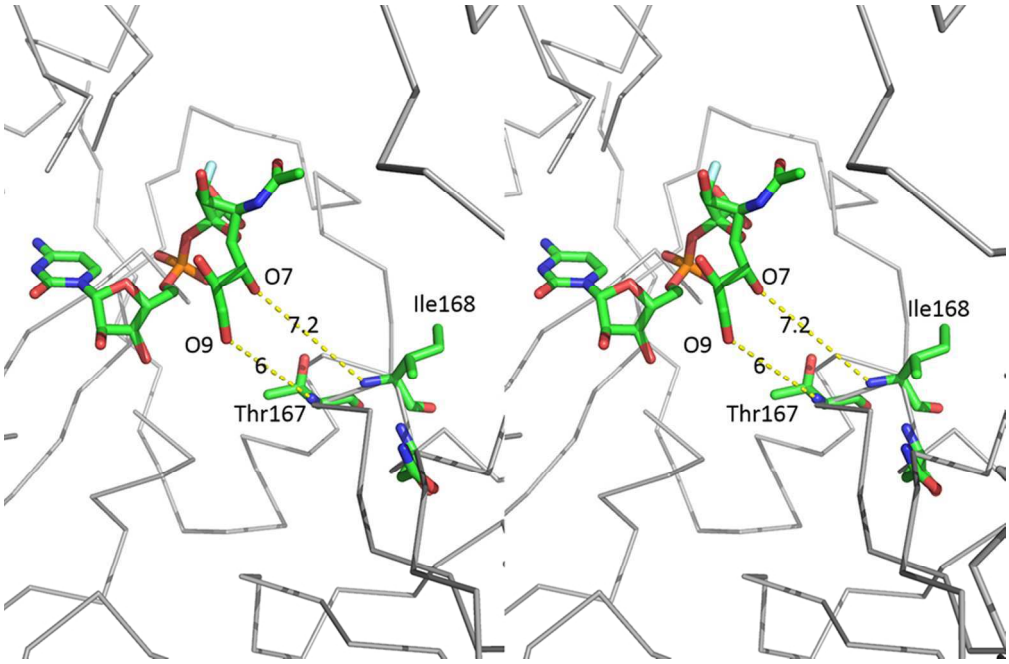

Figure 6
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