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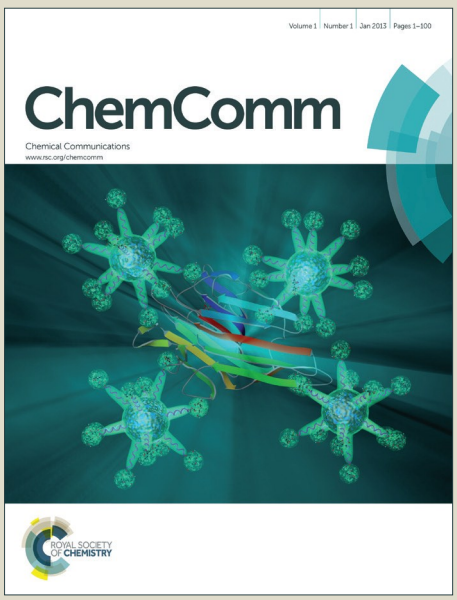

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Complete switch from α2,3- to α2,6-regioselectivity in *Pasteurella dagmatis* β-D-galactoside sialyltransferase by active-site redesign

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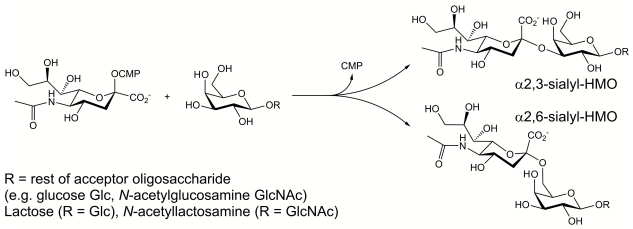
Structure-guided active-site redesign of a family GT-80 β-D-galactoside sialyltransferase (from *Pasteurella dagmatis*) to change enzyme regioselectivity from α2,3 in wild type to α2,6 in a P7H-M117A double mutant is reported. Biochemical data for sialylation of lactose together with protein crystal structures demonstrate highly precise enzyme engineering.

α2,3- and α2,6-sialic acid capped oligosaccharides are highly important in human glycobiology.[1] Sialylated human milk oligosaccharides (HMOs) are of interest due to their roles in the development and health protection of newborn infants.[2] Nearly 20% of the total HMOs comprise sialic acid,[2] and sialyllactose is one of their main components,[3] with both regioisomers being biologically active.[4] Sialylated HMOs contain *N*-acetylneuraminic acid (Neu5Ac) attached to D-galactosyl (Scheme 1) or *N*-acetyl-D-glucosaminyl residues through α2,3- or α2,6-linkage.[2-3] Since natural availability of sialylated HMOs is limited, synthetic sialyloligosaccharides (e.g. sialyllactose) are demanded as infant formula[2,5] and nutraceutical ingredients.[4]

Stereo- and regiocontrol are problems requiring special attention when installing a sialyl group on a nascent oligosaccharide.[6] Selective biocatalytic sialylation avoids use of protecting group chemistry and therefore presents a highly attractive route for sialylated HMO synthesis.[7] Sialyltransferases (STs; EC 2.4.99) catalyze transfer of a Neu5Ac residue from CMP-Neu5Ac to an acceptor oligosaccharide. Complementarily regioselective STs from bacterial,[8] mammalian[8i,8j,9] and viral[10] sources, were successfully applied for enzymatic α2,3- and α2,6-sialylation of various acceptor substrates. Sialyllactose and sialyl-(poly)-*N*-acetyllactosamine derivatives were produced in gram quantities.[8h-j,11] Engineered STs exhibiting tailored selectivities could be of interest for flexible sialoside synthesis.



Scheme 1 Enzymatic synthesis of α2,3- and α2,6-sialyl-HMOs by sialyltransferases with CMP-Neu5Ac and β-D-galactoside substrates.

Hitherto unprecedented switch in ST regioselectivity from α2,3 in wild type to α2,6 in a designed enzyme variant is reported. High-resolution protein structures show the active-site remodeling of the parent ST to have been precise at the atomic level. Results provide deepened insights into determinants of ST selectivity. They are also relevant for practical synthesis: a pair of "ST twins" is created that differ in regioselectivity, but otherwise have uniform synthesis conditions and substrate preferences. We show their application to alternative 3'- or 6'-sialylation of lactose and *N*-acetyllactosamine.

Glycosyltransferase family GT-80 comprises α2,3-, α2,6- and also mixed α2,3/α2,6-selective STs.[8b,8d,8e,12] These STs are furthermore characterized by high specific activity and broad acceptor substrate scope, which typically includes lactose.[8b,8d,8e,12] Redesign of the naturally α2,3-selective β-D-galactoside ST from *Pasteurella dagmatis* (PdST)[12a] was developed from two family GT-80 protein structures that delineate distinct lactose binding modes in the α2,3/α2,6-selective ST from *Pasteurella multocida* (PmST1)[13] and the α2,6-selective ST from *Photobacterium sp.* JT-ISH-224[14] (Fig. 1). PdST is 70% identical in amino acid sequence to PmST1, and residues of their acceptor binding sites (Fig. 1a) are completely identical. Different orientations of the lactose's β-D-galactosyl

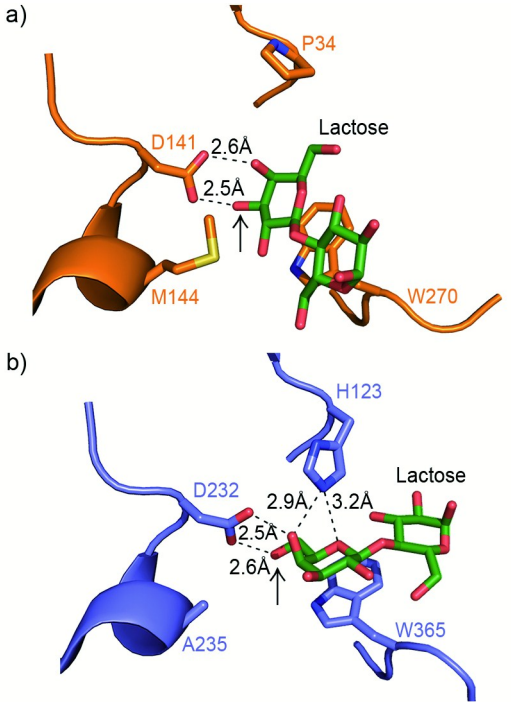


Fig. 1 Acceptor-binding site comparison of family GT-80 α2,3- and α2,6-sialytransferases. (a) α2,3/α2,6-sialyltransferase from *P. multocida* PmST1 (PDB code 2ILV)[13]. (b) α2,6-sialyltransferase from *Photobacterium* sp. JT-ISH-224 (PDB code 2Z4T)[14].

moiety relative to the proposed catalytic base of the enzyme (Asp^{141}, Asp^{232}),[13-15] such that either the 3-OH (Fig. 1a) or the 6-OH (Fig. 1b) is brought into a reactive position, appeared to have been evoked by a two amino acid residue substitution where Pro^{34} and Met^{144} in PmST1 are exchanged to, respectively, His^{123} and Ala^{235} in *Photobacterium* ST. Family-wide sequence comparison of GT-80 STs revealed clear sub-categorization of β-galactoside α2,3- and α2,6-STs according to a conserved Pro/Met or His/Ala(Ser) sequence pattern (see Table S1 in ESI†) that was therefore hypothesized to be decisive for α2,3 compared to α2,6 ST regioselectivity. Note, however, that a group of α2,3-selective STs within family GT-80 that are flexible in using α/β-galactosides for sialylation also possess an Ala instead of a Met (Table S1 in ESI†). To graft α2,6-selective ST activity on PdST, the relevant residues Pro^{7} and Met^{117} were replaced to generate P7H and P7H-M117A variants of the naturally α2,3-selective wild-type enzyme.

Purified preparations of wild type and variant PdST were obtained from *Escherichia coli* overexpression culture producing target protein equipped with a C-terminal His_6-tag for purification by metal chelate chromatography. Specific activity for sialyltransfer to lactose (1 mM) from CMP-Neu5Ac (1 mM) was determined at pH 8.0, measuring the consumption of CMP-Neu5Ac and the release of CMP by HPLC, and the formation of sialyllactose by HPAEC-PAD (high-performance anion exchange chromatography with pulsed amperometric detection). The specific activity of P7H mutant was identical (5.8 U mg^{-1}) to that of the wild-type enzyme. The specific activity of the P7H-M117A double mutant was lowered somewhat in comparison (2.2 U mg^{-1}). Sialyllactose regioisomers formed in the different enzymatic reactions were identified from their elution in HPAEC-PAD referenced against authentic standards of 3'- and 6'-sialyllactose, as shown in Fig. 2. The wild-type enzyme produced 3'-

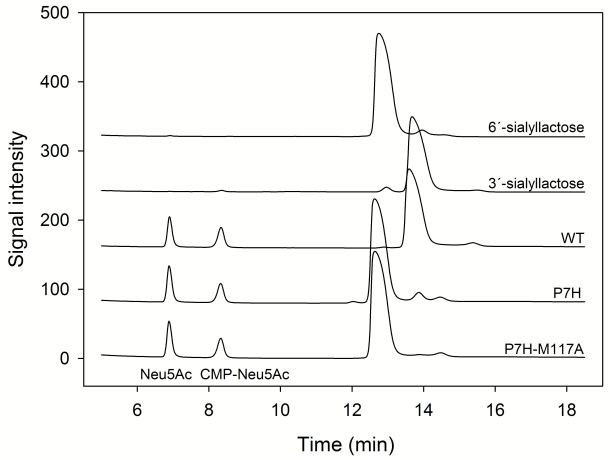


Fig 2 HPAEC-PAD analysis showing gradual change from α2,3- to α2,6-regioselective sialyltransfer to lactose, resulting from substitution of Pro^{7} by His and from additional substitution of Met^{117} by Ala in PdST. First 5 min are omitted for clarity (see Fig. S1 in ESI†). Note that the identity of the sialyllactose products formed was additionally confirmed by NMR.

sialyllactose exclusively. Single mutation of Pro^{7} to His resulted in drastic change of enzyme regioselectivity, so that 6'-sialylation of lactose was now strongly favored by the enzyme. However, 3'-sialyllactose was still present to ~4% of total transfer product (Fig. 2), showing that α2,3-ST activity had not been completely abolished in the P7H mutant. The double mutant P7H-M117A, by contrast, featured complete α2,3 to α2,6 switch in ST regioselectivity. No 3'-sialyllactose was detectable (≤ 1%) next to 6'-sialyllactose as product of the enzymatic sialyltransfer (Fig. 2). Therefore, these results suggested distinct and divergingly important roles for His and Ala in conferring α2,6-regioselectivity to STs of family GT-80. While the His was clearly essential, the Ala seemed to fulfil an auxiliary function in fine-tuning of enzyme selectivity. Consistent with this notion, the M117A mutant of PdST did not exhibit significant change in regioselectivity as compared to the wild-type enzyme (Table 1). Evidence from enzyme kinetic characterization (Table 1) supports the idea that in terms of catalytic efficiency (k_{cat}/K_m) and also turnover frequency (k_{cat}), the replacement Pro→His is tolerated much better by the enzyme than the Met→Ala replacement. Interestingly, Pro→His replacement in M117A variant resulted in clear recovery of activity parameters that had been decreased in the single-site variant as compared to wild-type PdST.

Table 1 Activity and selectivity parameters of wild-type PdST and site-directed variants thereof.

	WT	M117A	P7H	P7H-M117A
Regioselectivity	α2,3	α2,3[a]	α2,6[b]	α2,6
Specific activity [U mg^{-1}]	5.7	0.70	5.8	2.2
K_m [mM]	1.5	15	3.8	6.4
k_{cat} [s^{-1}]	24	11	16	18
k_{cat}/K_m [s^{-1} mM^{-1}]	16	0.74[c]	4.3	2.8

[a] 4% of 6'-sialyllactose and [b] 4% of 3'-sialyllactose were detected. Apparent kinetic parameters for lactose as sialyltransferase acceptor substrate. Kinetic parameters have S.D. of ≤10%, except [c] 20%. Measurements were done using CMP-Neu5Ac (10 mM) and a varied concentration of lactose.

In a consequent next step, we applied the two regio-complementary STs (wild type; P7H-M117A) and also the P7H mutant to the synthesis of 3'- and 6'-sialyllactose from CMP-Neu5Ac and lactose (each 1 mM) (Table 2). Full reaction time courses were

Table 2 Synthesis of sialyllactose and sialyl-*N*-acetyllactosamine using wild-type PdST and mutants thereof.

	WT	P7H	P7H-M117A
Lactose[a]			
Yield SL [%]	75	75	72
$R_{6'/3'SL}$[b]	≤ 0.006	18	≥ 240
N-acetyllactosamine[a]			
Yield SLN [%]	77	83	71
$R_{6'/3'SLN}$[b]	≤ 0.006	8	≥ 180

[a] 1 mM of acceptor substrate was used. [b] Ratio of 6'- to 3'-sialoside, whereby product identity was verified by NMR. SL, sialyllactose; SLN, sialyl-*N*-acetyllactosamine.

determined (see Fig. S2 in ESI†), and the reported sample composition at each point was carefully verified by a closed mass balance. Using wild-type PdST (see Fig. S2a in ESI†), 3'-sialylation product was obtained in about 75% yield (0.75 mM). The concentration of 6'-sialyllactose never exceeded 0.005 mM. The enzyme's inherent hydrolase activity caused partly non-productive utilization of the CMP-Neu5Ac donor substrate, thus restricting the 3'-sialyllactose yield under the conditions used. Hydrolytic competition was however strongly reduced at elevated lactose concentration (10 mM) where ≥ 95% of the initial CMP-Neu5Ac (1 mM) was utilized for sialyltransfer to acceptor substrate (see Fig. S3 in ESI†). Using the P7H-M117A double mutant, 6'-sialyllactose was synthesized in about 72% yield (0.72 mM) whereas 3'-sialyllactose was present at only ≤ 0.003 mM (see Fig. S2b in ESI†). At the equivalent protein concentration used (0.1 µM), the space-time yield of sialyllactose product was 2.5-fold lower for the reaction of the double mutant as compared to the reaction of wild-type PdST, explained by the different specific ST activities of the two enzymes. Like in wild-type PdST, non-productive (i.e. hydrolytic) utilization of donor substrate by the double mutant was prevented almost completely when lactose was employed in 10-fold molar excess over CMP-Neu5Ac (1 mM) (see Fig. S3 in ESI†). The sialoside product was fully stable up to extended incubation times (180 min) in both enzymatic reactions, demonstrating that minor sialyllactose hydrolase activity of both enzymes (0.1 U mg^{-1}) did not interfere with synthesis under the conditions used. The P7H mutant produced a mixture of regioisomers (Table 2).

Similar results were obtained in synthesis experiments of sialyl-*N*-acetyllactosamine (Table 2; see Fig. S4 –S5 in ESI†), which is an important building block towards sialyl-lacto-*N*-neotetraose that is also highly abundant in human milk.[3,16] Moreover, *N*-acetyllactosamine (LacNAc) sialylation is of high importance as most asialo-HMOs bear LacNAc on their non-reducing end. Product identity of 3'- or 6'- sialyllactose and 3'- or 6'-sialyl-*N*-acetyllactosamine, obtained through wild-type or double mutant catalyzed synthesis, was verified by several methods, including NMR spectroscopy. Overlay of NMR spectra of enzymatic conversions and respective commercial standards (Carbosynth, UK) (HSQC, ^{1}H; see Fig. S6–S13 in ESI†) unequivocally demonstrated strict α2,3-selectivity of wild-type PdST and strict α2,6-selectivity of the P7H-M117A variant.

To obtain molecular interpretation of the regioselectivity switch achieved in PdST (Tables 1 and 2), we determined the structures of wild-type enzyme, P7H and P7H-M117A in their respective apo-form (X-ray data collection and refinement statistics is shown in the ESI†, Table S2). For a detailed description of crystallization and structure determination see the ESI†. All structures show an open conformation with the same positions of mutated (Pro→His, Met→Ala) and catalytic (Asp[114], His[284])[13,15] residues (Fig. 3). The two PdST variants were also co-crystallized in the presence of CMP-Neu5Ac and lactose or 2-nitrophenyl-β-D-galactopyranoside. Additionally, the crystals were soaked with lactose or 2-nitrophenyl-β-D-galactopyranoside.

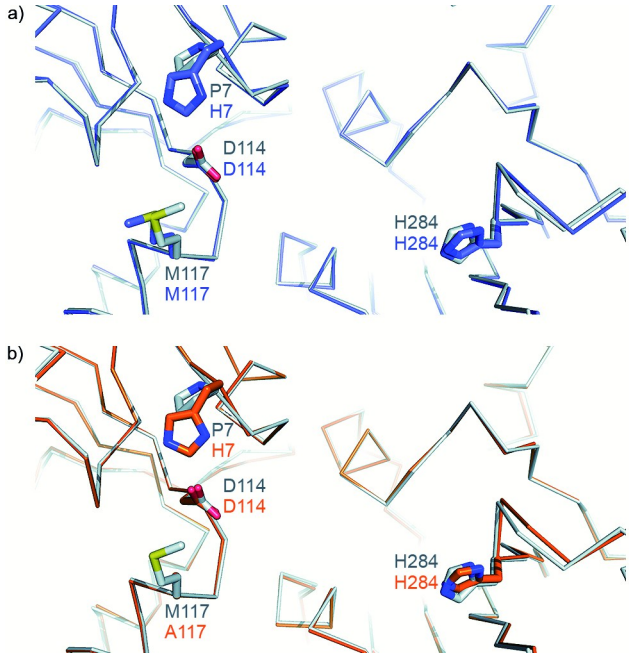


Fig 3 Close-up views of experimental apo PdST structures. Shown is an overlay of wild-type (grey, PDB code 4V2U) and single P7H mutant (blue, PDB code 4V38) (a) and wild-type (grey, PDB code 4V2U) and double P7H-M117A mutant (orange, PDB code 4V39) (b). Key active site residues (Asp[114], His[284])[13,15] and mutation sites (Pro[7], Pro→His; Met[117], Met→Ala) are drawn in sticks.

Unfortunately, only CMP could be clearly placed in the electron density (X-ray data collection and refinement statistics see the ESI†, Table S2, Fig. S14). Note that co-crystallization of *Photobacterium* sp. α2,6-ST (PDB code 2Z4T) with CMP and lactose resulted in a ternary complex.[14] However, overlay of the apo structure of P7H-M117A double mutant (open conformation) with the N- and C-terminal domains of *Photobacterium* sp. α2,6-ST (PDB code 2Z4T, closed conformation)[14] shows almost perfect superimposition of important active site residues for catalytic function (Asp[114]/Asp[232], His[284]/His[405]) and regioselectivity (His[7]/His[123], Ala[117]/Ala[235]) as well as the Phe[34]-Lys[38] loop (Arg[36]/Arg[153]) region (see Fig. S15 in ESI†). Our structural observations, taken together with the biochemical characterization, therefore clearly support a highly successful mutational strategy.

Alternative enzymatic α2,3- and α2,6-sialylation of various acceptor substrates, including lactose and sialyl-(poly)-*N*-acetyllactosamine derivatives, was demonstrated before.[8-9] What is new here is the use of a *designed pair* of regio-complementary STs

instead of two individual enzymes. The approach of ST engineering might offer a convenient exchange of enzymatic regioselectivity for synthesis of sialyllactose and sialyl-*N*-acetyllactosamine (Table 2).

In conclusion, sequence motifs determining α2,3- and α2,6-regioselectivity in β-D-galactoside STs of family GT-80 were identified and exploited through protein engineering of PdST to create a unique pair of regio-complementary STs. Relationships between PdST atomic structure and enzyme selectivity were established. The two STs were applied for α2,3/α2,6-sialylation of lactose and LacNAc from CMP-Neu5Ac.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Experimental procedures, sequence alignment, HPAEC-PAD analysis, time-courses of synthesis experiments, ^{1}H and HSQC NMR spectra, crystallization and structure determination. See DOI: 10.1039/c000000x/

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