High-throughput screening methodology for the directed evolution of glycosyltransferases

Amir Aharoni[1], Karena Thieme[1], Cecilia P C Chiu[2], Sabrina Buchini[1], Luke L Lairson[1], Hongming Chen[1], Natalie C J Strynadka[2], Warren W Wakarchuk[3] & Stephen G Withers[1]

Engineering of glycosyltransferases (GTs) with desired substrate specificity for the synthesis of new oligosaccharides holds great potential for the development of the field of glycobiology. However, engineering of GTs by directed evolution methodologies is hampered by the lack of efficient screening systems for sugar-transfer activity. We report here the development of a new fluorescence-based high-throughput screening (HTS) methodology for the directed evolution of sialyltransferases (STs). Using this methodology, we detected the formation of sialosides in intact *Escherichia coli* cells by selectively trapping the fluorescently labeled transfer products in the cell and analyzing and sorting the resulting cell population using a fluorescence-activated cell sorter (FACS). We screened a library of $>10^6$ ST mutants using this methodology and found a variant with up to 400-fold higher catalytic efficiency for transfer to a variety of fluorescently labeled acceptor sugars, including a thiosugar, yielding a metabolically stable product.

Complex carbohydrates occur in a wide range of contexts in biology, including polysaccharides, proteoglycans, glycoproteins, glycolipids and antibodies. There they have important roles in a number of functions, including cell growth, cell-cell interactions[1], immune defense[2], inflammation[3] as well as viral and parasitic infections[4]. Assembly of these complex structures is orchestrated by a series of specific GTs, which sequentially transfer the monosaccharide moieties of their activated sugar donor to the required acceptor, with the correct positional and stereochemical outcome[5]. Consequently, there are many GTs with widely different specificities. Thus the prospects for engineering GTs to generate enzymes of desired specificity are very promising. This is important because, by contrast with the situation for peptides and oligonucleotides, the chemical synthesis of complex carbohydrates is an extremely challenging and labor-intensive process, and cannot generally be achieved in an automated fashion or on a large scale. New approaches to complex carbohydrate synthesis remain an urgent need in glycobiology in order to further our understanding as well as to facilitate the development of potential therapies.

In the past few years directed evolution approaches for protein engineering have proved to be highly useful in improving the stability of enzymes[6,7] and for altering their substrate specificities[8]. One of the crucial steps in any directed evolution experiment is the development of a screening assay to facilitate the screening of large libraries[9]. However, assaying for transfer activity and particularly GT activity is extremely challenging as no obvious change in fluorescence or absorbance is associated with glycosidic bond formation. As the screening for a desired phenotype, in most cases, is a random process, it is highly desirable to develop HTS methodologies to facilitate the screening of extremely large libraries[10,11]. These methodologies are particularly valuable for enrichment and isolation of rare mutants with beneficial activity from large mutant libraries[12].

Here we describe the development of a new fluorescence-based HTS methodology for the directed evolution of STs. STs represent a group of enzymes belonging to the GT superfamily, which transfer sialic acid (Neu5Ac) from CMP-Neu5Ac to the carbohydrate groups of various glycoproteins and glycolipids[13]. We have focused on the GTA family 42 ST CstII from the human pathogen *Campylobacter jejuni*, whose structure was recently solved[14] (see **Supplementary Fig. 1** online for the reaction scheme). We developed a fluorescence-based HTS methodology for the detection and sorting of ST activity in intact *E. coli* cells using FACS (**Fig. 1**). The development of a cell-based assay for GT activity using FACS is highly advantageous as it alleviates the need to lyse the cells and perform many other manipulations otherwise necessary for screening large mutant libraries. By using a carefully designed fluorescently labeled acceptor sugar and selectively trapping the sialylated fluorescent product in the cell, the formation of transfer product is directly correlated to the fluorescence of the cell. Here we describe the sensitivity and dynamic range of this screening system and its use to isolate a new CstII variant with up to 400-fold higher catalytic efficiency with fluorescent bodipy-labeled acceptor sugars. This large increase in catalytic efficiency was associated with a single mutation located 18 Å away from the donor-sugar binding site, likely resulting in exposure of a hydrophobic pocket to create a high-affinity aromatic aglycone binding site. Correspondingly,

[1]Department of Chemistry, University of British Columbia, 2036 Main Mall, Vancouver, British Columbia V6T 1Z1, Canada. [2]Department of Biochemistry, Life Sciences Center, 2350 Health Sciences Mall, Vancouver, British Columbia V6T 1Z3, Canada. [3]Institute of Biological Sciences, National Research Council, Room 3157, 100 Sussex Drive, Ottawa, Ontario K1A OR6, Canada. Correspondence should be addressed to S.G.W. (withers@chem.ubc.ca).

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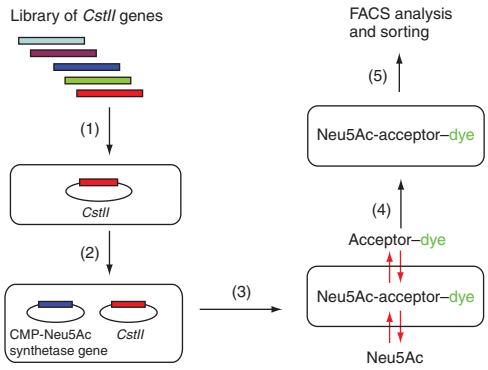

Figure 1 | Cell-based assay for ST activity. A gene library is transformed and cloned in *E. coli* (1). The encoded ST protein is expressed in the cytoplasm of the engineered JM107 *NanA*$^-$ strain together with CMP-Neu5Ac synthetase (2). Cells are incubated with Neu5Ac donor sugar and fluorescently labeled acceptor sugars (3). After incubation, cells are extensively washed to remove unreacted fluorescent acceptor sugar (4). Cells are directly analyzed and sorted by FACS (5).

appendage of this aromatic aglycone to a range of otherwise incompetent acceptors endowed them with efficient acceptor activity with this specific mutant. This fluorescence-based HTS method should therefore allow for the evolution of enzymes with new catalytic activities, providing new synthetic routes for complex carbohydrates and other conjugates such as phosphates or sulfates.

RESULTS

Detection and sorting of ST activity in intact *E. coli* cells

To develop a fluorescence cell based assay for CstII activity, we used an engineered mutant *E. coli* cell strain. This strain (JM107 *NanA*$^-$), previously used for the production of sialylated oligosaccharides[15], efficiently transports the Neu5Ac donor and lactose acceptor sugars to the cytoplasm through specific transporters. To prevent catabolism of lactose and Neu5Ac, the mutant strain lacks both β-galactosidase (*lacZ*) and Neu5Ac aldolase activities (*NanA*). To allow cell-based synthesis of sialosides, the cells express both CMP-Neu5Ac synthetase and CstII. CMP-Neu5Ac synthetase activates the Neu5Ac *in situ* to CMP-Neu5Ac, and the CstII uses the latter as its donor sugar for sialyltransfer to β-galactoside acceptors[14].

The second component required for this screen was a fluorescently labeled galactose-containing acceptor that is freely transported into and out of the cell. To this end, we synthesized a series of fluorescently labeled acceptor sugars (**Fig. 2**). The general scheme for the detection and sorting of ST activity in the cells is shown in **Figure 1**. We incubated the engineered cells with fluorescently labeled acceptor sugars and Neu5Ac. After an incubation time of 30–60 min, we subjected the cells to three rounds of centrifugation and resuspension to wash out any unreacted fluorescent lactoside. At this point, fluorescent sialylated lactoside product remains trapped in the cells because of its size and charge, but the unreacted fluorescent lactose is washed away. This wash step is extremely important to reduce background fluorescence and to facilitate detection of even weak ST activity. Finally the cells are subjected to FACS analysis and sorting to assess the amount of fluorescently labeled sialylated product trapped in the cell.

To test the feasibility of this approach, we separately incubated cells expressing the target CstII enzyme and cells expressing empty pUC18 plasmid with Neu5Ac and either bodipy-lactose acceptor or the less efficient acceptor bodipy-galactose (**Fig. 2**). After extensive washing, the fluorescence intensity of cells expressing CstII was substantially higher than that of control cells as visualized under an ultraviolet light lamp (**Fig. 3a**). To quantify the difference in fluorescence and test the dynamic range of the cell-based ST assay, we subjected the samples to FACS analysis. The mean fluorescence intensity of the cells expressing the CstII enzyme and incubated with bodipy-lactose was about 80-fold higher than that of the control cells (**Fig. 3b**). This demonstrates the high dynamic range and potential to detect even slow transfer reactions.

Next we used the cell-based assay to simultaneously detect CstII transfer activity to two different acceptor sugars.

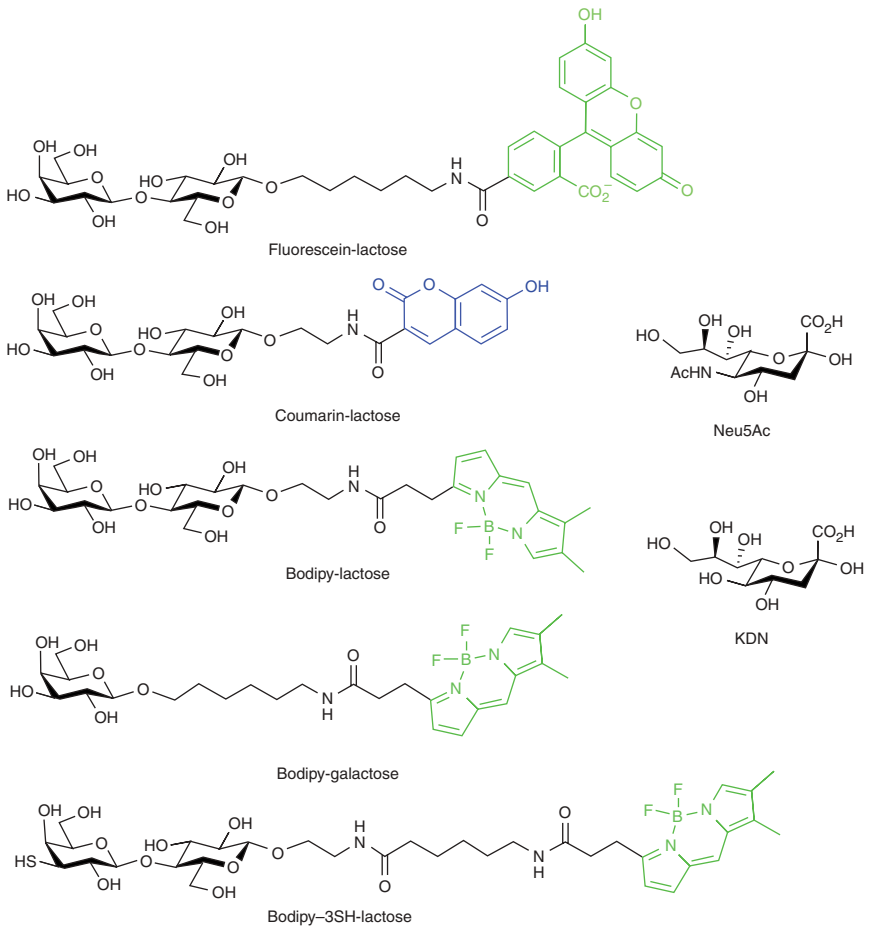

Figure 2 | Donor sugars and fluorescent acceptor sugars used in this study. KDN, 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid.

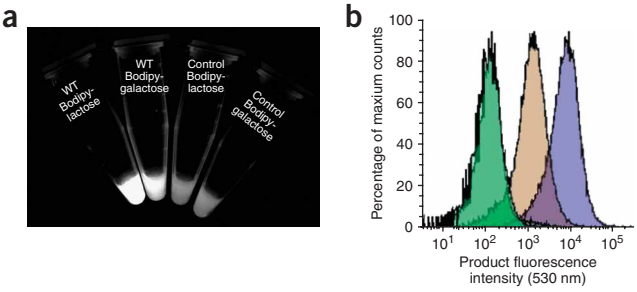

Figure 3 | Cell-based ST assay for cells expressing wild-type CstII (WT) and containing the pUC18 plasmid (control). The different cell samples were incubated with Neu5Ac and bodipy-lactose or bodipy-galactose. After extensive washing the cells were analyzed either visually or by FACS. (**a**) Eppendorfs containing the four different cell samples as visualized under an ultraviolet light lamp; the cell fluorescence intensity is correlated to the CstII activity. (**b**) FACS histogram analysis of the four different cell samples. Overlapping light blue and light green, cells expressing empty pUC18 plasmid incubated with bodipy-galactose and bodipy-lactose respectively; brown, cells expressing wild-type CstII incubated with bodipy-galactose; purple, cells expressing wild-type CstII incubated with bodipy-lactose.

We incubated cells expressing CstII with Neu5Ac together with bodipy-galactose and coumarin-lactose (**Fig. 2**), two acceptor sugars of different transfer efficiency, and compared the transfer activity to that of control cells. We performed FACS detection and analysis of both dyes in the cells through separate excitation and emission channels. The fluorescence intensity of cells expressing the CstII was much higher for both fluorescently labeled acceptor sugars (**Supplementary Fig. 2** online). Finally, to verify that cells containing active CstII enzyme can be sorted from a large heterogeneous cell population using FACS, we performed a model selection[16]. After sorting, we calculated an enrichment factor of 80 fold for a cell population in which cells expressing wt CstII were mixed with a large excess (200-fold) of cells expressing a control plasmid (for a detailed experimental description, see **Supplementary Note** online).

Selection of CstII library for increase in ST activity

With all of these controls in place and indicating the establishment of an effective screen, we used this screen to probe libraries of CstII mutants. We constructed a large CstII gene library by inserting random mutations along the full-length CstII gene. We cloned this library into a pUC18 vector and propagated it in *E. coli* cells to yield $>10^6$ different colonies. We extracted the plasmid DNA library, transformed it into JM107 *NanA*$^-$ cells carrying the CMP synthetase expression plasmid, grew them and induced for protein expression. We incubated the cells with Neu5Ac and the bodipy-lactose, washed them, analyzed and sorted more than 10^7 cells by FACS (**Fig. 1**). In each round we performed three iterative rounds of enrichment, sorting multiple 'positive' events ($3–5 \times 10^4$) within the top 1–2% of the green fluorescence intensity, collected these cells into growth medium and plated them on agar for a new round of enrichment. After each round of sorting we observed an increase in ST activity of the crude cell lysates, as judged by thin layer chromatography (TLC) analysis (**Fig. 4a**). Accordingly, the mean fluorescence of the library after three rounds of sorting was substantially higher than that of the wild-type cells (**Fig. 4b**).

To identify and isolate single clones with improved transfer activity, we transformed the plasmid DNA extracted after the third round of sorting into fresh JM107 *NanA*$^-$ cells, and selected 20 random clones, which we grew individually and tested for CstII activity. We analyzed product formation by TLC at different time points and compared it to the wild-type CstII activity. Approximately 20% of the clones had much higher activity than the wild-type clone using bodipy-lactose and Neu5Ac (**Supplementary Fig. 3** online). We sequenced four of the improved clones and found that one mutation, F91Y, was repeated in two of the four most active clones (see **Supplementary Fig. 3** for a complete list of mutations). We subcoloned the gene encoding the CstII variant containing only the F91Y mutation, showing the highest transfer activity, into a pET28 vector, overexpressed it and purified it for further characterization.

Characterization of the CstII F91Y mutant

We confirmed the ability of the F91Y mutant to specifically form an α-2,3 glycosidic linkage by TLC detection of bodipy lactose formation after incubation of the bodipy-labeled sialyl lactose product with the specific recombinant α-2,3-neuraminidase from *Salmonella typhimurium* (data not shown), and per kinetic analysis of the pure F91Y mutant using a spectrophotometric continuous coupled assay[17], and measured the F91Y mutant transferase activity with a variety of acceptor sugars including bodipy-lactose, bodipy-galactose and bodipy-3SH-lactose (**Fig. 2** and **Table 1**). We assessed the contribution of the dye to the transfer efficiency of the F91Y mutation using unmodified lactose and galactose as acceptors (**Table 1**), and observed a dramatic difference in catalytic efficiency of 153- and 367-fold for bodipy-lactose and bodipy-galactose, respectively, relative to the natural lactose and galactose sugars. Observation of this dramatic rate improvement with dye-tagged acceptor sugars raised the question of whether lactose analogs that do not function with the wild-type enzyme could be turned into useful acceptors for F91Y by dye-tagging. Of particular interest in this regard was an ability to form glycosidase-resistant

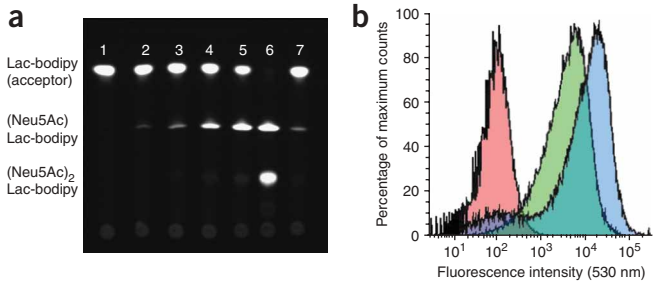

Figure 4 | Library selection through three iterative rounds of sorting by FACS. (**a**) Activity analysis of the various rounds of FACS enrichment. CstII transfer activity was measured on crude cell lysates prepared from the pool of cells obtained after each round of enrichment and analyzed by TLC. Activity was measured and compared to cells expressing pUC18 plasmid (control), wild-type CstII and the evolved CstII F91Y mutant. Lane 1, pUC18 plasmid; lane 2, naive CstII library; lanes 3–5, library after one, two and three rounds of enrichment, respectively; lane 6, evolved F91Y CstII mutant; and lane 7, wild-type CstII. (**b**) FACS histogram analysis of cells expressing pUC18 (pink), wild-type CstII (green) and library following three rounds of enrichments (blue) following a 1-h incubation with 1 mM Neu5Ac and 0.5 mM bodipy-lactose.

Table 1 | The evolved CstII F91Y mutant: catalytic efficiency for the transfer of CMP-Neu5Ac to different acceptors

Substrate	k_{cat}/K_M (min^{-1} M^{-1})[a]	Fold increase[b]
Bodipy-lactose	35.1	153
Lactose	0.23	
Bodipy-galactose	11	367
Galactose	0.03	
Bodipy–3SH-lactose	6.5	>>407[c]

[a]The k_{cat}/K_M was calculated from a linear fit of plots of initial transfer rates from CMP-Neu5Ac (0.5 mM) to the bodipy acceptors at a range of concentrations from 0.05–0.2 mM. Measurements of transfer to lactose and galactose were performed at higher acceptor concentrations (up to 150 mM). [b]Fold increase in catalytic efficiency of bodipy acceptor relative to the unmodified acceptor. [c]Minimum estimate of the fold increase in rate for bodipy–3SH-lactose relative to 3SH-lactose.

thioglycosidic linkages, which would be metabolically stable. Neither 3SH-lactose nor bodipy–3SH-lactose acts as an acceptor for the wild-type CstII. By contrast bodipy–3SH-lactose acted as a good acceptor for the CstII F91Y mutant, with a k_{cat}/K_M value only fivefold lower than its parent bodipy-lactose (**Table 1**). Product analysis by mass spectrometry confirmed the formation of a sialylated 3-SH lactose derivative, as did TLC analysis (**Supplementary Fig. 4** online). We also detected an increase in transfer activity for the alternative donor sugar CMP-2-keto-3-deoxy-D-glycero-D-galacto-nononic acid (CMP-KDN) both in cell lysates (**Fig. 2** and **Supplementary Fig. 4**) and using the cell-based assay (data not shown). Finally, we determined the effects of the CstII F91Y mutation on reaction rates with untagged substrates, as well as the enzyme catalyzed hydrolysis of CMP-NeuAc. The mutation actually decreases these inherent rates by three- to fivefold, highlighting the importance of the bodipy dye binding for the acceleration of the transfer reaction (**Supplementary Table 1** online).

To study the effect of the F91Y mutation on the structure and catalytic activity of CstII, we solved the crystal structure of the CstII F91Y mutant in complex with CMP-3FNeuAc (see **Supplementary Table 2** online for X-ray refinement statistics) and compared it to the recently solved structure of the wild-type CstII together with CMP-3FNeuAc[14]. In the wild-type CstII, the phenyl side chain of Phe91 protrudes into the enzyme core where it is tightly packed by several surrounding hydrophobic residues. Substitution by the larger and more hydrophilic tyrosine apparently disrupts that tight hydrophobic packing and results in a dramatic flip of the side chain to a completely solvent-exposed orientation (**Fig. 5a**). This movement creates a hydrophobic pocket, which is fortuitously complementary to the fused aromatic ring system of the bodipy dye structure (**Fig. 2**). Analysis of a model of bodipy-lactose bound to CstII (**Fig. 5b**) suggests that with the bodipy dye specifically bound in the newly formed hydrophobic cavity of the CstII F91Y mutant, the lactose would be appropriately positioned in the vicinity of the donor sugar to facilitate the formation of the sialyl-lactose product. This model suggests two adjacent but distinct binding sites for the sugar and bodipy dye, which result in an overall dramatic increase in catalytic proficiency. The formation of an additional binding site may explain why tagging of the bodipy dye to otherwise incompetent acceptors (for example, 3-SH-lactose) results in a dramatic increase of the transfer activity and suggests a general way to increase the transfer activity of GTs by generating a specific aglycone binding site without compromising the transfer activity and regioselectivity.

In accordance with the notion that improved activities of the CstII F91Y mutant are due mostly to improved binding of bodipy-lactose, we observed a saturation in transfer rates, as judged by TLC, between 0.6 mM and 0.8 mM of bodipy-lactose whereas we observed no sign of saturation with the wild-type enzyme up to 5 mM (data not shown). Additionally, we probed the specificity of this newly created dye binding site by monitoring reactions with both coumarin lactose and fluorescein lactose. In neither case was any substantial improvement in transfer rate seen for the F91Y mutant relative to WT CstII, indicating that the site formed was indeed specific for bodipy.

DISCUSSION

We have developed a fluorescence based HTS methodology for the detection of ST activity in intact *E. coli* cells. Coupling the

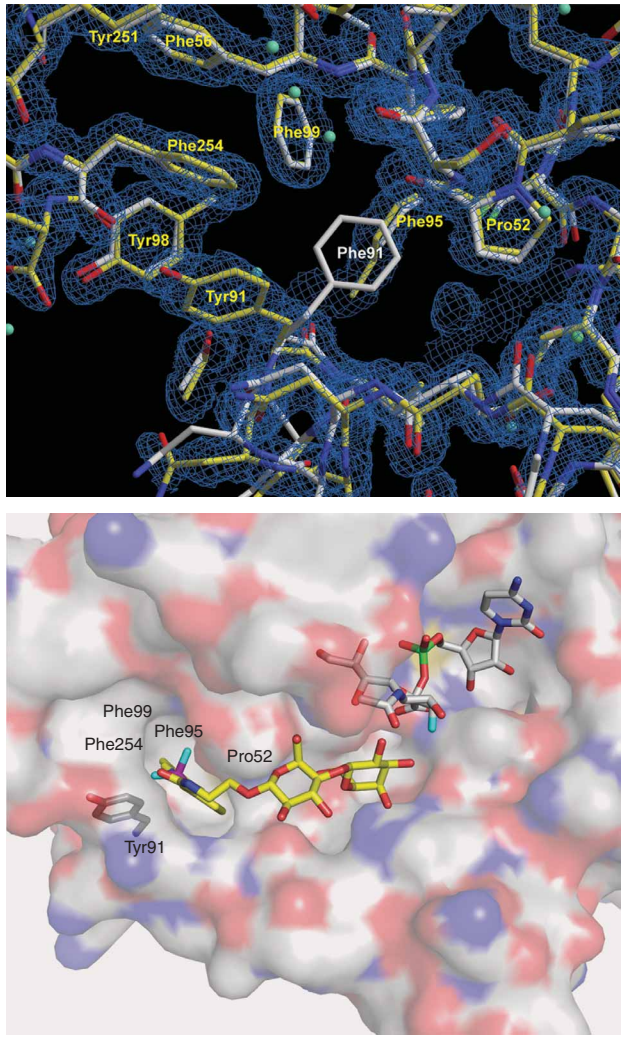

Figure 5 | Structure of the CstII F91Y mutant. (**a**) Overlay of the wild-type (white) and F91Y (yellow) mutant forms of CstII. Corresponding $2F_o–F_c$ electron density at 1 σ for the F91Y mutant is also shown. The Tyr91 residue in the mutant (yellow) is flipped out exposing a hydrophobic pocket that is complementary to the bodipy dye ring structure. (**b**) Molecular surface representation of the active site cleft and the Tyr91 region in the CstII F91Y mutant (red, negative potential; blue, positive potential). The CMP-3FNeuAc donor sugar (white) is depicted in a stick representation. The bodipy-lactose (yellow) is modeled so that the bodipy is positioned in the exposed hydrophobic pocket, and the lactose directly adjacent to the CMP-3FNeuAc.

fluorescence cell based assay to FACS allowed us to screen a library of $>10^6$ different variants in less than two hours. Unlike other bulk selection methodologies such as panning on immobilized ligands[18], FACS allows fine-tuning of the selection threshold, enrichment and recovery. We have also demonstrated how parallel selection can be performed on two different acceptor sugars by using a different dye for each acceptor sugar and monitoring the transfer reaction simultaneously in the cell (**Supplementary Fig. 2**). This approach could be possibly used to exert positive and negative selective pressures for the isolation of highly selective enzyme variants[19]. Additionally, monitoring of the transfer reaction simultaneously in the cell using acceptor sugar labeled with two different dyes could be used to reduce cross-reactivity toward the fluorophore.

Other methodologies that have been developed to screen large enzyme libraries[10,11] are based on different display technologies (for example, phage display[18] or bacterial display[19]). Recently an HTS method was developed in which the diffusion of substrate and product is restricted by using double water-in-oil-in-water emulsion[16,20]. No such technologies, however, have been developed previously for glycosyl transfer reactions. Indeed, the only screening systems are those developed for glycosynthases. This included the use of an agar plate–based coupled enzyme assay for the *Agrobacterium* sp. β-glucosidase (Abg) glycosynthase in which an endocellulase was used to release a fluorescent dye only from the reaction product[21]. Recently a selection assay for the glycosynthase activity was developed for the E197A mutant of the Cel7B from *Humicola insolens*[22]. Using the yeast three-hybrid system, product formation was directly coupled to yeast growth, but the approach was only applied to very small libraries.

The outcome of our selection of $>10^6$ different CstII mutants for increase in transfer activity of Neu5Ac to bodipy-lactose is a CstII variant containing a single F91Y mutation. Although this mutation substantially increased the transfer activity with a variety of bodipy-labeled acceptor sugars, the activity of this mutant with unlabeled acceptor sugars or acceptor sugars labeled with different dyes (for example, coumarin, fluorescein) is barely affected. This suggests that our methodology could be used for the parallel directed evolution of other desirable properties using the F91Y mutant as a starting point. Generation of a new binding site for the bodipy dye in the CstII F91Y mutant, which we isolated in our selection, provides a general strategy to increase the transfer efficiency to poor acceptor sugars by temporarily tagging them with a hydrophobic moiety. Indeed, using this approach a sugar that does not function as an acceptor for wild-type CstII, 3-SH-lactose, is converted into an acceptor for the F91Y mutant with a catalytic proficiency (k_{cat}/K_M) for the transfer of Neu5Ac to bodipy-3SH-lactose that is only ~5 times lower than that for the transfer to bodipy lactose using the CstII F91Y mutant (**Table 1**). This result suggests that the low transfer activity to 3SH lactose is mainly due to inefficient binding of the acceptor rather than any intrinsic difference in the catalytic transfer mechanism between the 3-thio and 3-hydroxy analogs. The thiosialylated product is of particular interest as thiooligosaccharides are metabolically stable mimics of their naturally occurring counterparts[23]. While a very limited set of glycosyltransferases has been shown to catalyze the synthesis of thiooligosaccharides[24], no thioglycoside product was reported for any ST previously.

To the best of our knowledge, we describe the first directed evolution experiment for GTs that is based on a genuinely HTS methodology. This work opens up new avenues for directed evolution of GTs for the glycosylation of a variety of acceptors. In the case of GTs that form neutral sugar products the CstII could serve as a coupling enzyme to trap the reaction product in the cell, thus extending the methodology to other GTs that transfer galactose to fluorescently labeled acceptor sugars. We believe that our methodology, based on selectively trapping the transfer product, can be extended to detect other transfer reactions (for example, phosphorylation or sulfation) in which a charged moiety is transferred to a variety of acceptors. Indeed, preliminary experiments with cells expressing cytosolic sulfotransferases have indicated that the sulfated fluorescent transfer product is trapped in the cells, and the unreacted fluorescent substrate is washed away (data not shown). However, each substrate for the cell-based assay must be carefully designed and examined for penetration into the cells and entrapment after the transfer reaction. This methodology is also applicable to the detection of transfer reactions by fluorescence resonance energy transfer by using acceptors and donors that are both fluorescently labeled.

METHODS

DNA manipulation. We PCR-amplified the gene encoding the soluble form of CstII (a C-terminal 32-amino-acid truncation)[14] from the pET28-CstII plasmid and subcloned it into the pUC18 plasmid. We PCR-amplified the CMP sialic acid synthetase gene from pNSY-05 (ref. 25) and subcloned it into a low-copy-number plasmid, pACKC18. We generated *CstII* libraries by error-prone PCR according to established protocols[26] (a detailed description of library preparation is available in **Supplementary Methods** online).

Screening CstII libraries, isolation and characterization of CstII F91Y mutant. We transformed *E. coli* JM107 *NanA*$^-$ cells (derivative of *E. coli* K12, which contains a chromosomal deletion of the Neu5Ac aldolase gene)[15] with pACKC18 plasmid encoding for CMP-synthetase. We prepared electrocompetent cells of a subsequent clone. We transformed plasmid DNA (pUC18-CstII) containing the gene encoding CstII variants and library into these cells and grew the cells, induced and screened them for transfer activity using FACS (detailed description of the screening process and isolation of F91Y mutant is available in **Supplementary Methods**). We subcloned the CstII F91Y mutant into the pET28 plasmid, over expressed it and purified it as previously described[14]. We performed the kinetic analysis of the CstII F91Y mutant essentially as described[14,17].

Crystallization, data collection and structure determination. We concentrated pure CstII F91Y protein to ~10 mg/ml at room temperature (22 °C) and subjected it to cocrystallization screens together with the inert donor sugar analog CMP-3FNeu5Ac using the vapor diffusion method. The structure of the mutant was solved by molecular replacement using a monomer of wild-type CstII as the starting model (Protein Data Bank accession code 1RO7; a detailed description of the crystallization process and structural determination is available in **Supplementary Methods**).

Accession codes. Protein Data Bank: coordinates for the CstII F91Y structure have been deposited with accession code 2DRJ.

Note: Supplementary information is available on the Nature Methods website.

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AUTHOR CONTRIBUTIONS

A.A. conceived the strategy and carried out the majority of the work. K.T., S.B., L.L.L. and H.C. carried out the synthesis. C.P.C.C. and N.C.J.S. carried out the crystallography. W.W.W. and S.G.W. helped in development of the strategy. A.A. and S.G.W. wrote the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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