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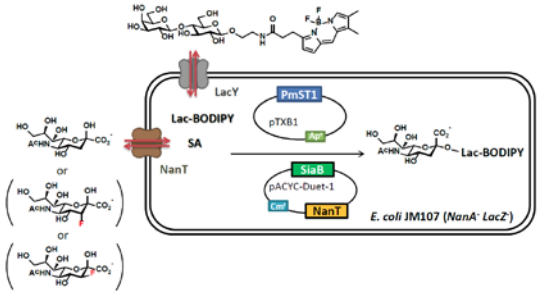
GRANT TITLE: Engineered sialyltransferases for sialidase-resistant therapeutic glycoproteins

a) **Abstract** Most therapeutic proteins in use are glycoproteins, and their circulatory half-lives are typically dependent on their constituent glycans, especially of terminal sialic acids. Desialylation of sialylated biopharmaceuticals occurs *in vivo*, exposing carbohydrates that are recognized by lectin-mediated endocytosis, promoting clearance and resulting in shortened circulatory half-life for the drug. We proposed that glycoproteins with longer circulatory half-lives could be made by replacing the terminal sialic acids with 3-fluorosialic acids as the 3-fluoro substituent dramatically slows rates of both enzymatic and spontaneous hydrolysis of sialosides due to inductive destabilisation of reaction transition states.

Introduction of these 3-fluorosialic acids must be done enzymatically. However the fluoro substituent also slows enzymatic sialyl transfer, making the preparation of these fluorosialylated glycoproteins impractically slow. We proposed to speed up enzymatic fluorosialic acid transfer by directed evolution using our high-throughput FACS entrapment assay (Figure). This assay depends upon the ability of lac permease (LacY) to transport fluorescent lactose derivatives in and out of *E. coli*, but cannot transport sialylated lactose derivatives out. Thus if *E coli* hosting an active sialyltransferase plus the enzyme that makes CMPsialic acid (SiaB) is incubated with BODIPYlactose plus sialic acid then BODIPYsialyl-lactose accumulates in the cells. After washing to remove unreacted BODIPYlactose fluorescent cells can be sorted by FACS and those bearing the most active sialyltransferases isolated. This approach should also work with 3-fluorosialic acid.



We synthesised both epimers of 3-fluorosialic acid as well as BODIPYlactose. The GT80 sialyltransferase from *Pasteurella multocida* (PmST1) was chosen for evolution and a random mutant library of PmST1 was generated by error-prone PCR and an appropriate mutation rate (2.3 per gene) confirmed by sequencing. Initial FACS screening was promising as we quickly enriched a population that could synthesise a sialylated BODIPYlactose. Unfortunately the sugar being transferred was not 3-fluorosialic acid but sialic acid present at low levels in LB growth medium. Switching to minimal media we saw no transfer in the presence or absence of 3-fluorosialic acid (but it worked with sialic acid). Increasing the expression level of PmST1 did not help, but if we lysed cells and added CMP3Fsialic acid we could see (some) transfer, suggesting that 3-fluorosialic acid was not getting into the cells. This could occur if 3-fluorosialic acid is not a substrate for NanT. Alternatively, since NanT synthesis requires induction, it may be that 3-fluorosialic acid is not an effective inducer. We therefore transformed *E coli* with a plasmid containing the gene for NanT, but still did not get uptake of 3-fluorosialic acid. Given these difficulties we explored whether other modified sialic acids could resist enzymatic and spontaneous hydrolysis. A good candidate was found and fortunately it can be transferred by the sialyltransferase without the need to engineer. Modified sialyl glycoproteins can be made in this way, and these are now being studied.