Sialic acid and *N*-acyl sialic acid analog production by fermentation of metabolically and genetically engineered *Escherichia coli*†

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Sialic acid is the terminal sugar found on most glycoproteins and is crucial in determining serum half-life and immunogenicity of glycoproteins. Sialic acid analogs are antiviral therapeutics as well as crucial tools in bacterial pathogenesis research, immunobiology and development of cancer diagnostic imaging. The scarce supply of sialic acid hinders production of these materials. We have developed an efficient, rapid and cost effective fermentation route to access sialic acid. Our approach uses low cost feedstock, produces an industrially relevant amount of sialic acid and is scalable to manufacturing levels. We have also shown that precursor directed biosynthesis can be used to produce a *N*-acyl sialic acid analog. This work demonstrates the feasibility of engineering manufacturing-friendly bacteria to produce complex, unavailable small molecules.

Introduction

Sialic acid encompasses a large family of acidic sugars found on the surfaces of eukaryotic cells.[1] The most common sialic acid is *N*-acetylneuraminic acid (**1**, Neu5Ac), a carboxylated nine-carbon monosaccharide. Sialic acid has key roles in glycoprotein stabilization, cell signaling, cell adhesion, cellular immunity and human diseases.[2] For example, excess sialylation on malignant cells has been shown to promote invasiveness[3] and reduce intercellular interactions,[4] which are features of tumor metastasis. Sialic acid analogs are currently being investigated for the non-invasive imaging of cancer tissue *in vivo*.[5] Analogs of sialic acid have found important therapeutic roles, especially as influenza neuraminidase inhibitors (*e.g.* Relenza®). The scarce supply of sialic acid and analogs has hindered advancement in basic research, diagnostic development and therapeutic production.

Because sialic acid is an unusual nine-carbon carbohydrate with numerous stereocenters, it is challenging to chemically synthesize. Sialic acid does not play a role in energy storage and is therefore difficult to produce *via* fermentation or cultivation. An *in vitro* enzymatic process is the current state of the art for sialic acid production.[6] This process is inherently costly and challenging to scale to the manufacturing level. To provide a low cost, scalable process for sialic acid production we have developed an *Escherichia coli* based fermentation route to produce sialic acid and *N*-acyl analogs.

In vivo production of sialic acid is an appealing alternative to *in vitro* production because it eliminates costly and difficult protein purification. Since cellular metabolism provides the building blocks for product formation, low cost carbon and nitrogen sources can be converted into product. This is particularly advantageous in sialic acid production because the *in vitro* process requires costly *N*-acetylglucosamine (GlcNAc) as the substrate. Industrial experience with manufacturing small molecules *via* fermentation (*e.g.* β-lactams) attests to the scalability and economic viability of large-scale metabolite production *via* fermentation. The main drawback of *in vivo* production is the increased difficulty associated with product purification. *In vivo* systems generate more complex mixtures of products, including excreted proteins, cellular debris and cellular waste products. Because sialic acid can be efficiently crystallized from aqueous solution at concentrations above 150 g L^{-1},[7] sufficiently high sialic acid titer will facilitate direct crystallization from the fermentation broth, simplifying purification.

In this study we demonstrate that sialic acid can be produced *in vivo via* fermentation of a metabolically and genetically engineered *E. coli*. By removing the sialic acid catabolism pathway and expressing sialic acid biosynthesis genes, multiple grams of sialic acid per liter of *E. coli* fermentation were produced. This *in vivo* system can be used to generate *N*-acyl sialic acid analogs. The ability to access analogs represents a major improvement in engineering fermentation-friendly organisms to produce complex molecules.

Results and discussion

An *E. coli* strain capable of producing sialic acid was generated by disabling the native sialic acid catabolic pathway and adding an exogenous synthetic pathway (Fig. 1). Native *E. coli* utilize sialic acid as a carbon source in cellular metabolism. Two key genes involved in sialic acid catabolism are *nanT* and *nanA*.[8] NanT is a major-facilitator transporter and is responsible for sialic acid uptake from the periplasmic space, and NanA is an aldolase that cleaves sialic acid **1** into pyruvate **3** and *N*-acetylmannosamine (**2**, ManNAc). A *nanT*$^-$ strain (FB27101) was obtained from the *E. coli* Genome Project (University of Wisconsin-Madison). This strain was generated *via* Tn5 insertion into *nanT* of *E. coli* K-12 MG1655 and therefore did not possess a T7 RNA polymerase gene, which enables high levels of recombinant protein expression. A λDE3 lysogen that encodes a T7 RNA polymerase under a *lac*-promoter was inserted into the chromosome of

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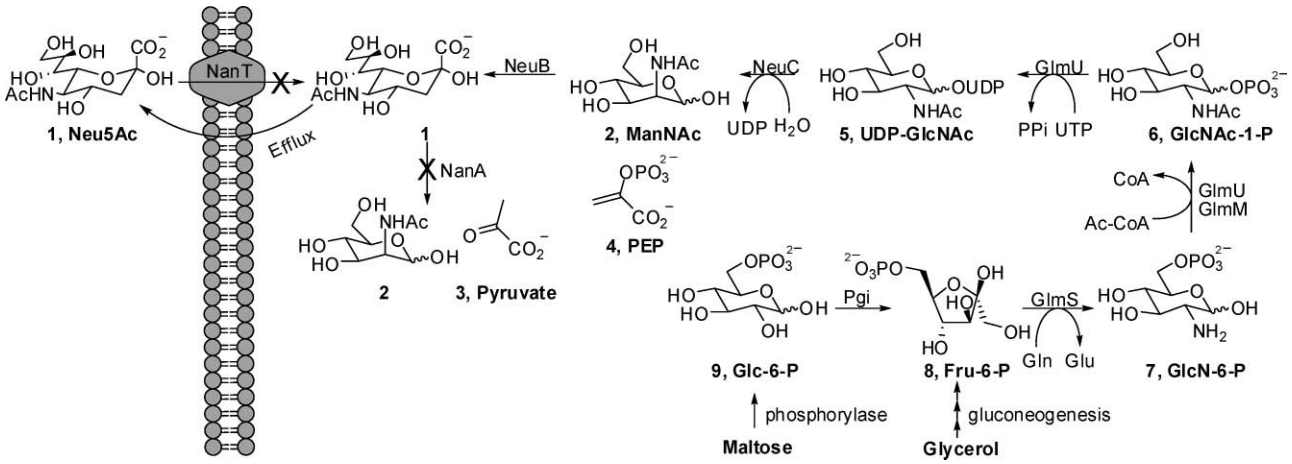

Fig. 1 Sialic acid production by metabolically engineered *E. coli* was achieved by removing the endogenous NanT and NanA proteins and introducing the NeuB and NeuC enzymes from *N. meningitidis* group B. The over-expression of *E. coli* GlmS was necessary for high sialic acid yields from inexpensive carbon sources including glucose, fructose and glycerol. Abbreviations: GlmM: glucosamine mutase, GlmS: glucosamine synthase, GlmU: GlcNAc-1-P uridyltransferase/GlcN-1-P acetyltransferase, Pgi: phosophoglucose isomerase.

nanT$^-$ strain FB27101 to generate strain BRL01. This enabled inducible expression of recombinant proteins in BRL01 using standard *E. coli* expression vectors. The *nanA* gene was inactivated using a gene-replacement strategy to generate a *nanT* $^-$ *nanA*$^-$ *E. coli* strain, BRL02.

The sialic acid biosynthetic genes, *neuC* and *neuB*, of *Neisseria meningitidis* group B were identified as suitable candidates for a sialic acid synthesis pathway. The NeuC and NeuB enzymes catalyze the *de novo* biosynthesis of intracellular sialic acid in *N. meningitidis* group B.[9] NeuC is a hydrolyzing epimerase that converts UDP-*N*-acetylglucosamine (**5**, UDP-GlcNAc), a common metabolite in bacteria, to ManNAc **2**. NeuB then catalyzes the condensation of ManNAc **2** with phosphoenolpyruvate (**4**, PEP) to generate sialic acid **1**. NeuC and NeuB were used as the primary synthetic enzymes because their *in vivo* and *in vitro* chemistry has been well established.[10–12] Both *neuC* and *neuB*, were amplified from genomic DNA of *N. meningitidis* group B by polymerase chain reaction (PCR) and subsequently cloned into an expression vector under the control of an inducible T7 promoter. Induced expression of the *neuBC* pathway in BRL02 yielded an *E. coli* strain capable of producing sialic acid from all carbon sources tested. Sialic acid titers ranged from 20–100 mg L^{-1} of *E. coli* fermentation broth. Without induction of the *neuBC* pathway, sialic acid production was at least ten fold lower.

To identify a feedstock for optimizing sialic acid production, we evaluated carbon sources that span the metabolic chemistry of *E. coli* (Fig. 2). Using GlcNAc as the feedstock generated the highest sialic acid titer of 104 mg L^{-1}. Extracellular GlcNAc is readily transported into *E. coli* and subsequently converted into UDP-GlcNAc **5** by a single enzymatic reaction.[13] In contrast, the disaccharide maltose produced the lowest sialic acid titer of 22 mg L^{-1}. Intracellular maltose must first be broken down into glucose by a maltose phosphorylase.[14] The liberated glucose can then be transformed into fructose-6-phosphate (**8**, Fru-6-P), which is the native substrate for the UDP-GlcNAc biosynthetic pathway in *E. coli*.[15] The necessity of the phosphorylase likely reduces sialic acid production by limiting the availability of intracellular glucose for UDP-GlcNAc biosynthesis, and thus

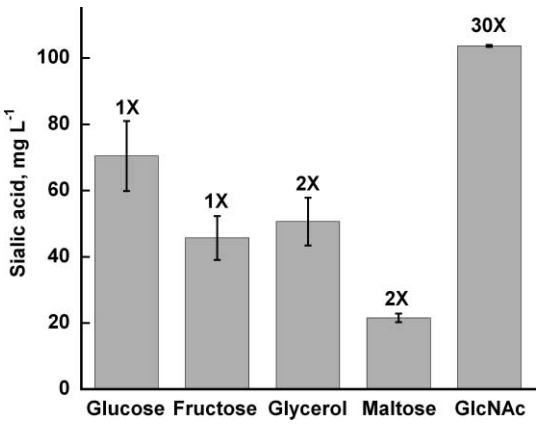

Fig. 2 Results of a feedstock comparison. Mean and standard deviation of sialic acid titers for triplicate 250 mL shake-flask cultures at 48 h are shown. The relative cost of the feedstock is indicated above each column. GlcNAc feeding generated the highest sialic acid titer from BRL02/*neuBC* fermentation. Due to its low cost and high titer glucose was identified as the best carbon source for further optimization.

making maltose a poor feedstock for sialic acid production. Glycerol, a common industrial carbon source, yielded a sialic acid titer of 51 mg L^{-1}. Intracellular glycerol is predominantly oxidized to dihydroxyacetone, which is then converted into Fru-6-P **8** *via* gluconeogenesis. Therefore, glycerol feeding promotes sugar biosynthesis including that of UDP-GlcNAc **5** and is expected to perform well.

Inexpensive glucose and fructose, which are excellent substrates for the aerobic growth of *E. coli*, were also evaluated as feedstocks for sialic acid production. Glucose and fructose feeding produced sialic acid at 70 and 46 mg L^{-1}, respectively. These non-amino sugars must first be committed into UDP-GlcNAc biosynthesis by the action of the glucosamine synthase, GlmS.[15] GlmS is the key regulatory enzyme in UDP-GlcNAc biosynthesis in *E. coli* where it transforms Fru-6-P **8** into glucosamine-6-phosphate (**7**, GlcN-6-P). GlcN-6-P **7** is then transformed into UDP-GlcNAc **5** by three enzymatic reactions. Upon intracellular transport, both

glucose and fructose are phosphorylated. Fructose-6-phosphate **8** readily enters UDP-GlcNAc biosynthesis whereas glucose-6-phosphate (**9**, Glc-6-P) has to be converted into Fru-6-P **8** by a phosphoglucose isomerase (Pgi). This isomerization occurs rapidly, as it is the first step in glycolysis. The comparable and relatively high titers for sialic acid production from glucose and fructose were consistent with rapid entry into the glucosamine biosynthetic pathway.

The level of sialic acid production from each carbon source varied inversely with the length of the metabolic pathways required to convert the feedstock into the key metabolite UDP-GlcNAc **5**. This metabolite is the substrate for the *neuBC* pathway and the rate of sialic acid production should be proportional to the intracellular concentration of **5**. Thus carbon sources that are rapidly converted into UDP-GlcNAc **5** *via* a short metabolic pathway provided the highest sialic acid titers. While GlcNAc was found to be the most productive feedstock in generating sialic acid, it was also the most expensive (Fig. 2). The high titers of glucose coupled to its low cost made it the feedstock of choice for optimization.

To optimize our sialic acid titers from glucose and fructose, we modified the production strain. Over-expression of the key enzyme in the biosynthesis of UDP-GlcNAc[15] **5**, GlmS, was expected to direct more of the feedstock sugar into UDP-GlcNAc **5** biosynthesis. The increased levels of UDP-GlcNAc **5** should lead to greater flux through *neuC* and *neuB*, providing higher sialic acid titers. Simultaneous over-expression of *glmS* with the *neuBC* pathway in BRL02 increased sialic acid production 2- to 3-fold. The average sialic acid titer from fructose feedings increased from 46 to 130 mg L^{-1} with the addition of *glmS*. With glucose feedings, the average sialic acid titer increased from 70 to 170 mg L^{-1}.

Sialic acid production under shake-flask conditions was further optimized with multiple feedings of glucose. Multiple feedings are expected to sustain elevated UDP-GlcNAc **5** levels for longer periods of time, leading to a net increase in sialic acid titer. A titer of 1.7 g L^{-1} was obtained with 0.5% glucose feedings every 12 hours post induction for 3 d (Fig. 3). A maximum OD_{600} of 2 AU was obtained during the course of this experiment. Fed batch high-density *E. coli* fermentation is known to increase cell density upwards of 10 fold from shake-flask culture conditions with maximum OD_{600} of greater than 50 AU.[16] Application of this technology to our system should substantially increase sialic acid production to generate industrially relevant titers ($\geq$10 g L^{-1}).

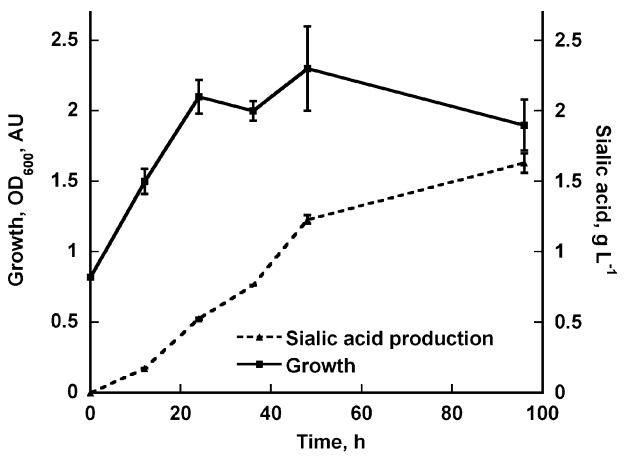

Fig. 3 Sialic acid production reached 1.7 g L^{-1} with multiple feedings of glucose in BRL02/*neuBCglmS* fermentation. Mean and standard deviation of triplicate 250 mL shake-flask results are shown.

A strength of our fermentation-based system is that sialic acid analogs can also be produced *via* precursor-directed biosynthesis. *N*-Acylglucosamine analogs can be transported into the cell and metabolized in place of GlcNAc. Conversion of these compounds into *N*-acyl analogs of *N*-acetylmannosamine **2** provides substrates for NeuB that can be converted into sialic acid analogs (Fig. 4). Multiple feeding of the BRL02/*neuBCglmS* strain with *N*-butanoylglucosamine led to production of *N*-butanoyl sialic acid at a titer of approximately 20 mg L^{-1} (Fig. 5).

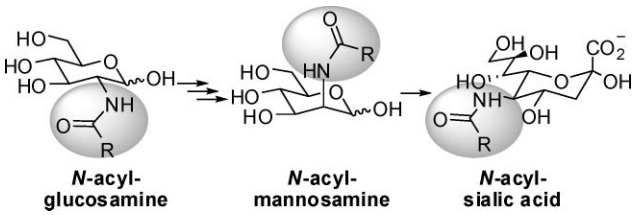

Fig. 4 Because the *N*-acyl group (shaded) from glucosamine is retained during the biosynthesis of sialic acid, feeding our *E. coli* system *N*-acylglucosamine analogs should lead to *N*-acyl sialic acid analog production.

The low titer of sialic acid analog production is likely due to high substrate specificity of GlmM or GlmU. GlmM converts GlcN-6-P **7** into the activated 1-phosphate sugar and GlmU generates the UDP-activated sugar **5** from the 1-phosphate sugar. This is the principle route for UDP-GlcNAc **5** formation. *In vitro* studies suggests that these enzymes are substrate specific and may not be able to efficiently process *N*-acyl analogs of glucosamine,[17,18] leading to low levels of UDP-activated *N*-acylglucosamine. Low levels of UDP-activated *N*-acylglucosamine decreases the flux through our sialic acid biosynthetic pathway as well as inhibiting cell growth and slowing cell wall biosynthesis. Because NeuB and NeuC orthologs are known to be broadly substrate tolerant, NeuB and NeuC are expected to process *N*-acyl analogs of their native substrates.[19,20]

Broadening the substrate tolerance of GlmM and GlmU should dramatically increase analog product titer. This should facilitate large scale production of analogs such as *N*-azidoacetyl- and *N*-levulinoyl sialic acid, which have been used in *in vivo* tumor imaging and modulation of cell–cell interactions, respectively.[21]

Conclusions

We have successfully engineered *E. coli* to produce sialic acid and *N*-acyl sialic acid analogs, complex, biologically important molecules that have significant applications in both medicine and biotechnology. The cost of sialic acid production from metabolically engineered *E. coli* (<$1 per gram) is substantially lower than that of chemical synthesis (retail value >$400 per gram[22]), and unlike enzymatic synthesis, metabolically engineered *E. coli* are more readily scalable for industrial production. From low-cell density shake-flask cultures, sialic acid production reached a maximum of 1.7 g L^{-1}. High-cell density fermentation coupled with strain optimization should substantially increase sialic acid production.

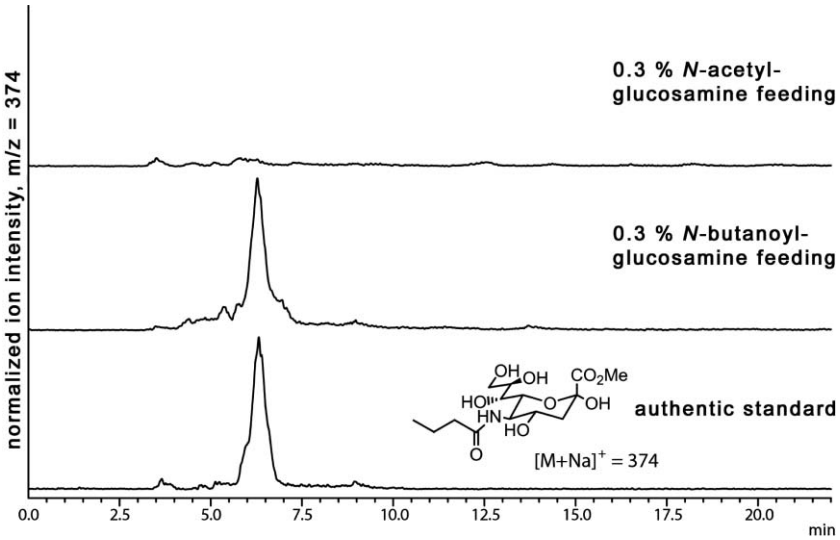

Fig. 5 Sialic acid analogs could be produced when cultures were fed with modified glucosamine derivatives. LCMS (ESI positive) analyses of fermentation broths from shake flask feeding experiments are shown. All experiments were performed in duplicate. Acid catalyzed esterification was used to generate the methyl esters of the products for improved HPLC separation. HPLC conditions: Alltima C_{18} LC-MS, 3 u, 150 mm × 2.1 mm; Mobile phase A, 5.0% acetonitrile, 0.05% formic acid in H_2O; Mobile phase B, 5.0% H_2O 0.05% formic acid in acetonitrile: Flow rate, 0.100 mL min^{-1}; gradient, 10 min at 0% B then a linear gradient from 0% B to 95% B over 10 min.

There are a limited number of examples of rationally engineered fermentation friendly organisms (*E. coli* and *Saccharomyces cerevisiae*) that produce substantial amounts of non-native complex molecules.[23] A herculean effort enabled production of the antimalarial isoprenoid artemisinin (C_{14}, 7 stereocenters) in *S. cerevisiea* with a titer of 0.10 g L^{-1}.[24] High-cell density fed-batch *E. coli* fermentation has been used to produce the polyketide 6-deoxyerythronolide B[25] (C_{21}, 10 stereocenters) at a titer of 0.70 g L^{-1} and the primary metabolite shikimic acid[26] (C_7, 3 stereocenters) at a titer of 52 g L^{-1}. Our system compares favorably with these studies. While sialic acid (C_9, 6 stereocenters) is less complex than artemisinin and 6-deoxyerythronolide B, it is more complex than shikimic acid. Our multigram per liter titers from low density shake-flask cultures are superior to artemisinin and 6-deoxyerythronolide B titers and with high-density fermentation techniques may be comparable to the shikimic acid titers. This work thus represents a major contribution to the field by demonstrating that fermentation friendly organisms can be rationally engineered to produce complex, non-native carbohydrates.

Our ability to access an analog as well as the natural product is a major advance in the engineering of fermentation-friendly organisms to produce complex molecules. We have demonstrated in this study that precursor-directed biosynthesis[27] can be used to introduce limited diversity into the *N*-acyl group of sialic acid. This is an important site for diversity in sialic acid since *N*-acyl sialic acid analogs have been shown to differentially modulate cell–cell interactions.[28,29] Future studies will determine if this method is a general approach for introducing diversity into the *N*-acyl group of sialic acid. Additionally our fermentation strategy should be sufficiently plastic to permit the introduction of product tailoring enzymes. Because many sialic acid tailoring enzymes have been characterized,[30–32] we anticipate being able to generate even greater sialic acid diversity through this approach. Because of the high demand and limited availability of sialic acid analogs, access to these compounds will have a large, positive impact on sialic acid biology.

Experimental

Reagents were purchased from Fisher Scientific/Aldrich and were used without further purification. NMR spectra were recorded on a Bruker DPX-300 spectrometer calibrated using residual undeuterated solvent as an internal standard. LC-MS was performed on a Shimadzu LCMS 2010 A single quadrupole mass spectrometer using electrospray ionization.

Construction of metabolically engineered *Escherichia coli* strain BRL02

Genomic DNA from *Neisseria meningitidis* ATCC® BAA-335D and XL1 Blue *Escherichia coli* (Stratagene, La Jolla, California) were used for PCR. Pfu Turbo® DNA polymerase (Stratagene, La Jolla, California) and the thermocylcer Mastercycler® personal (Eppendorf, Hamburg, Germany) were used for all PCR reactions. The *neuB* and *neuC* genes were amplified from *N. meningitidis* MC58 group B genomic DNA by Polymerase Chain Reaction (PCR). The *glmS* gene was amplified from *E. coli* genomic DNA by PCR. The *neuB*, *neuC* and *glmS* primers used for PCR are listed in the electronic supplementary information†. Touchdown and hotstart PCR were performed for all amplifications, and the thermocycler conditions were as follows: one cycle of 95 °C for 5 min, 15 cycles of 30 s at 95 °C, 30 s at 72–57 °C (−1 °C per cycle), and 90 s at 72 °C, 15 cycles of 30 s at 95 °C, 30 s at 57 °C, and 90 s at 72 °C, and one cycle of 10 min at 72 °C. PCR products for *neuB*, *neuC* and *glmS* were cloned into pCR-Blunt (Invitrogen, Carlsbad, California) following the manufacturer's instructions. Cloned genes were sequenced (DNA Core Facility, Upstate Medical University, Syracuse, New York). An internal

EcoRI site in *neuB* was removed through the Quikchange® site-directed mutagenesis kit (Qiagen, Valencia, California). Genes were subsequently subcloned under the T7-promoter of the vector pKH22 to generate the plasmids pBRL22 (pKH22-*neuBneuC*) and pBRL47 (pKH22-*neuBneuCglmS*) using standard molecular cloning techniques.[33] Plasmids have ampicillin resistance markers.

A *nanT* knockout *E. coli* K-12 strain MG1655 named FB21071 was requested from the Blattner laboratory (*E. coli* Genome Project) at the University of Wisconsin, Madison, WI. This strain has a Tn5 insertion into the *nanT* gene that confers kanamycin resistance. To allow for gene expression from T7-promoter plasmids, a T7 RNA polymerase gene under a lac-promoter was inserted into the chromosome of FB21071 using the λDE3 lysogenization kit (Novagen, San Diego, California) to generate the strain BRL01. The lambda Red system[34] was used to knockout the *nanA* gene of BRL01 to generate the *nanT*$^-$ *nanA*$^-$ *E. coli* strain BRL02. Briefly, a cassette encoding tetracycline resistance was amplified from the vector pBR322 (New England Bio Labs, Ipswich, Massachusetts) using the primers 5′-*atggcaacgaatttacgtggcgtaatggctgcactcctgactccttttga*tc atgtttgacagcttatcatcgat-3′ and 5′-*tcacccgcgctcttgcatcaactgctgggc cagcgccttcagttctggca*tccaattcttggagtggtgaatccg-3′. Primers consisted of 50 nt homology with *nanA* (italicized) and 25 nt with the tetracycline resistance gene (not italicized). The thermocycler conditions used are identical to those stated above. The tetracycline-resistance cassette was gel purified from the PCR reaction and then concentrated by ethanol precipitation. BRL01 transformed with pKD46, which harbors the lambda Red proteins necessary for recombination, were grown in Luria–Bertani (LB) media at 30 °C with shaking, 150 rpm, until an $OD_{600} = 0.1$. Expression of the lambda Red genes was then induced with 0.1% arabinose, and the induced cells continued to grow until an OD_{600} of 0.5 was reached. The cells were made electrocompetent and transformed with 100 ng DNA per 50 μL cells of the tetracycline-resistance cassette. Transformed cells were recovered in LB for 1 hour at 37 °C with shaking and then plated on LB supplemented with kanamycin (50 μg mL^{-1}) and tetracycline (10 μg mL^{-1}). Transformants were screened for *nanA* and *nanT* deletions by PCR.

Shake-flask experiments of metabolically engineered *E. coli*

F1 minimal media consisting of per liter, 6.62 g K_2HPO_4, 3.0 g KH_2PO_4, 4.0 g NH_4SO_4, 170.5 mg $MgSO_4$, and 0.5% filtered-sterilized carbon source was used for all shake-flask experiments.[25] All media was supplemented with the appropriate antibiotics unless otherwise noted: kanamycin (50 μg mL^{-1}), carbencillin (50 μg mL^{-1}) and tetracycline (10 μg mL^{-1}). All experiments were done in triplicate. Per replicate, a 10 mL starter culture was grown in LB media at 37 °C for 18 h with shaking, 250 rpm. Cells from the starter culture were harvested and diluted into 50 mL of F1 in a 250 mL Erlenmeyer flask. The inoculated cultures were grown at 30 °C with shaking, 150 rpm, and were induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when their $OD_{600} = 0.6$–0.8. The induced cultures were grown for 48 to 98 h (depending on the experiment, see below), and samples of 1 mL were taken at spaced intervals. Cells were removed from the samples by centrifugation, and the supernatants were boiled for 10 minutes. The boiled supernatants were quantified for sialic acid activity by the NanA/LDH assay (see below).

Two types of experiments were conducted: single feeding and multiple feedings. For single feeding experiments, a single initial carbon source at 0.5% was fed to the culture. Cultures were grown for 48 h post induction, and the sialic acid titers were determined. Single-feeding experiments were used to screen carbon sources or feedstocks for sialic acid production and included GlcNAc, glucose, fructose, maltose, and glycerol. Production of sialic acid was optimized for BRL02/pBRL47 cells through multiple feedings of 0.5% glucose. Doses of 0.5% glucose and ammonium hydroxide were added at 0, 12, 24, 36 and 48 h post induction, and the induced BRL02/pBRL47 cells were grown for 98 h. The ammonium hydroxide served to maintain the pH 6.9–7.1 and as a nitrogen source.

Production of sialic acid was confirmed by proton NMR in D_2O. Sialic acid was purified from a cultural broth by removing the cells *via* centrifugation and then isolating the sialic acid in the supernatant by ion-exchange chromatography as described.[35] Sialic acid: ^{1}H NMR (300 MHz, D_2O) $\delta = 3.98$–3.91 (m, 2H, H6 and H4), 3.82, (dd, $J = 10$, 10 Hz, 1H, H5), 3.74 (dd, $J = 11.5$, 1.5 Hz, 1H, H9), 3.68–3.62 (ddd, $J = 9.0$, 6.0, 2.5 Hz, 1H, H8), 3.52 (dd, $J = 11.5$, 6.5 Hz, 1H, H9′), 3.45 ($J = 9.0$, 1.0 Hz, 1H, H7), 1.77 (dd, $J = 13.0$, 4.5 Hz, 1H, H3β), 1.95 (s, 3H, $COCH_3$), 1.77 (dd, $J = 13.0$, 11.5 Hz, 1H, H3α); MS (ESI) calc'd for $C_{11}H_{20}NO_9$ (M + H$^+$) 310.1, observed 310.0, calc'd for $C_{11}H_{19}NO_9Na$ (M + Na$^+$) 332.1, observed 332.0. Purity was calculated to be >90% based on ^{1}H NMR. Product characterization agreed with literature characterization.[36]

NanA/LDH assay for measuring sialic acid concentration

Sialic acid concentrations were determined using the sialic acid aldolase-lactate dehydrogenase (NanA/LDH) assay as described.[37] LDH was purchased from Roche (Penzberg, Germany), and NanA was isolated from recombinant *E. coli*. Absorbances were measured in a GENESYS 20 spectrophotometer (Thermo Electron Corporation, Madison, Wisconsin). The dynamic range of the assay was found to be 0.035 to 0.002 mg mL^{-1} (0.11 to 0.006 mmol L^{-1}) sialic acid. Reaction conditions consisted of 100 mM Tris buffer (pH 8.0), 0.15 mM NADH, 2.5 μg mL^{-1} LDH, 5 μg mL^{-1} NanA, and 0.010–0.030 mg mL^{-1} of sialic acid. Reactions were carried out with all the above components except NanA at 37 °C for 1 h. Initial absorbances at 340 nm were then recorded. NanA was then added to the reactions, and the reactions were incubated at 37 °C for 3 h. The final absorbances were measured, and the differences between initial and final absorbance values were fitted against a standard curve to quantify the sialic acid concentration in the samples.

Synthesis of *N*-butanoylglucosamine

Glucosamine hydrochloride (5.0 g, 23 mmol, 1.0 equiv.) and triethylamine (16 mL, 115 mmol, 4.8 equiv.) were suspended in DMF (48 mL) at room temperature. The reaction mixture was stirred for 30 minutes at which butyric anhydride (4.5 mL, 27 mmoL, 1.2 equiv.) was added dropwise. The reaction was stirred for 18 h at room temperature. Ethyl acetate and hexane (800 mL, 1 : 3 v : v) was added to precipitate the product. The mixture was stirred for 30 minutes, filtered and the solid was repeatedly washed with hexane. The solid was dissolved in

methanol (200 mL) filtered and dried under vacuum to afford 5.1 g (20 mmol, 87% yield) of a 1 : 1 mixture of the α : β anomers of *N*-butanoyl glucosamine. ^{1}H NMR (300 MHz, D_2O) $\delta = 5.09$ (d, $J = 3.5$ Hz, 0.5H, α-H1), 4.60 (d, $J = 8.0$ Hz, 0.5H, β-H1), 3.84–3.57 (m, 4H, H3, H4, H5), 3.40–3.32 (m, 1H, H2), 2.18 (t, $J = 7.0$ Hz, 1H, $COCH_2CH_2CH_3$), 2.17 (t, $J = 7.0$ Hz, 1H, $COCH_2CH_2CH_3$), 1.52 (sextet, $J = 7.0$ Hz, 2H, $COCH_2CH_2CH_3$), 0.82 (t, $J = 7.5$ Hz, 1.5H, $COCH_2CH_2CH_3$), 0.81 (t, $J = 7.5$ Hz, 1.5H, $COCH_2CH_2CH_3$); MS (ESI) calc'd for $C_{10}H_{20}NO_6$ ($M + H^+$) 250.1, observed 250.0. Product characterization agreed with literature characterization.[38]

Enzymatic synthesis of *N*-butanoyl sialic acid

Enzymatic synthesis of *N*-butanoyl sialic acid was carried out in 60 mM Tris, pH 8.0, 20 mM ATP, 20 mM $MgCl_2$, 15 mM *N*-butanyolglucosamine, 750 mM pyruvate, 0.2 mg mL^{-1} NanA and 0.2 mg mL^{-1} NanE (*N*-acetylmannosamine-6-phosphate epimerase). The NanA and NanE proteins were prepared as described.[39] The enzymatic reaction was incubated at 37 °C. After 3 h a 20 μL sample was removed and quenched with 200 μL of methanol. Catalytic amounts of Amberlyst 15 (5 mg) were added and the mixture was shaken for 18 h at room temperature. Mixtures were centrifuged at 16000 × *g* for 10 min. The supernatant was removed and dried under vacuum. The sample was suspended in 50 μL water and analyzed by LC-MS (Analysis performed in duplicate. Stationary phase, Alltima C_{18} LC-MS, 3 u, 150 mm × 2.1 mm; Mobile phase A, 5.0% acetonitrile, 0.05% formic acid in H_2O; Mobile phase B, 5.0% H_2O 0.05% formic acid in acetonitrile: Flow rate, 0.100 mL min^{-1}; gradient, 10 min at 0% B then a linear gradient from 0% B to 95% B over 10 min). MS (ESI) calc'd for $C_{14}H_{26}NO_9$ ($M + H^+$) 352.2, observed 352.1; calc'd for $C_{14}H_{25}NO_9Na$ ($M + Na^+$) 374.1, observed 374.0.

N-Butanyol sialic acid production from BRL02/pBRL47 *E. coli*

Analog production experiments were done as described (see "Shake-flask experiments of metabolically engineered *E. coli*"). *N*-Butanoyl sialic acid production was optimized from BRL02/pBRL47 cells by multiple-feedings of 0.3% glycerol and either 0.3% *N*-butanyolglucosamine or *N*-acetylglucosamine (negative control). Doses of feedstock and ammonium hydroxide were added at 0, 12, 24, 36 and 48 h post induction, and the induced BRL02/pBRL47 cells were grown for 98 h. The ammonium hydroxide served to maintain the pH 6.9–7.1 and as a nitrogen source. Combined sialic acid and *N*-butanoyl sialic acid titers were determined by the NanA/LDH assay (see above). Average combined titer from *N*-butanoylglucosamine feedings was 67 ± 26 mg L^{-1}. Average combined titer from *N*-acetylglucosamine feedings was 822 ± 23 mg L^{-1}. *N*-Butanyol sialic acid product was verified by LC-MS (in duplicate) using the following protocol. From 50 mL of cell-free broth, a 2 mL aliquot was dried under vacuum and suspended in 100 μL H_2O. Methanol (3 mL) and Amberlyst 15 (50 mg) were added and the reaction stirred for 12 h. A 200 μL aliquot was dried under vacuum, resuspended in 50 μL water and analyzed by LC-MS (Stationary phase, Alltima C_{18} LC-MS, 3 u, 150 mm × 2.1 mm; Mobile phase A, 5.0% acetonitrile, 0.05% formic acid in H_2O; Mobile phase B, 5.0% H_2O 0.05% formic acid in acetonitrile: Flow rate, 0.100 mL min^{-1}; gradient, 10 min at 0% B then a linear gradient from 0% B to 95% B over 10 min). A 2 : 1 ratio of sialic acid to *N*-butanoyl sialic acid (from the *N*-butanoylglucosamine feeding experiment) was approximated from the LC-MS analysis. MS (ESI) calc'd for $C_{14}H_{26}NO_9$ ($M + H^+$) 352.2, observed 352.0; calc'd for $C_{14}H_{25}NO_9Na$ ($M + Na^+$) 374.1, observed 374.0.

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