ACS SyntheticBiology

pubs.acs.org/synthbio Letter

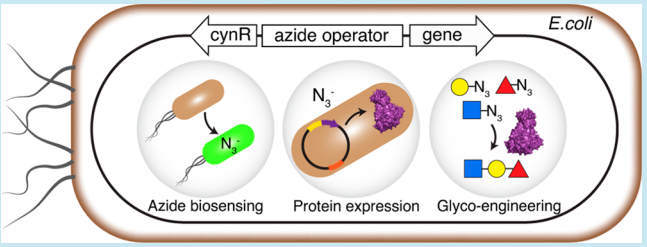
Engineered Regulon to Enable Autonomous Azide Ion Biosensing, Recombinant Protein Production, and *in Vivo* Glycoengineering

Chandra Kanth Bandi, Kyle S. Skalenko, Ayushi Agrawal, Neelan Sivaneri, Margaux Thiry, and Shishir P. S. Chundawat*

Cite This: *ACS Synth. Biol.* 2021, 10, 682–689 Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: Detection of azide-tagged biomolecules (*e.g.*, azido sugars) inside living cells using "click" chemistry has been revolutionary to the field of chemical biology. However, we currently still lack suitable synthetic biology tools to autonomously and rapidly detect azide ions. Here, we have developed an engineered synthetic promoter system called *cyn* regulon, and complementary *Escherichia coli* engineered strains, to selectively detect azide ions and autonomously induce downstream expression of reporter genes. The engineered *cyn* azide operon allowed highly tunable reporter green fluorescent protein (GFP) expression over three orders of inducer azide ion concentrations (0.01−5 mM) and rapidly induced GFP expression by over 600-fold compared to the uninduced control. Next, we showcase the superior performance of this engineered *cyn*-operon over the classical *lac*-operon for recombinant protein production. Finally, we highlight how this synthetic biology toolkit can enable glycoengineering-based applications by facilitating *in vivo* activity screening of mutant carbohydrate-active enzymes (CAZymes), called glycosynthases, using azido sugars as donor substrates.



KEYWORDS: *azide biosensor, promoter engineering, protein expression, glycoengineering, chemical biology*

Azide ion is an excellent nucleophile that readily participates in nucleophilic substitution reactions.[1] This has facilitated the discovery and synthesis of organic azides that have been ubiquitously used as building blocks in organic chemistry for over 150 years.[2] Azides have found widespread commercial applications as automobile airbag propellants, biocides, and as functional groups in fine chemicals and pharmaceutical drugs synthesis.[3,4] In particular, organic azides have been clinically approved as potent antibiotics and pharmaceutical drugs (*e.g.*, azidamfenicol, azidocillin, and zidovudine).[5−8] With the invention of biorthogonal click chemistry[9] that utilizes alkyne−azide chemistry for diverse applications in chemical biology, drug development, and bioorganic chemistry, there has been a reemergence of interest toward azides within the scientific community. Azide functionalized molecular probes are being developed as imaging reagents and biomarkers for diseased cell/tissue detection,[10] pharmaceutical drugs delivery,[11] as well as glycomics, metabolomics,[12] and chemoproteomics related applications.[13] However, *in vivo* use of azido-based drugs or molecular imaging probes necessitates monitoring organic azide stability toward degradative release of azide ions.[14] To date, spectrophotometry,[15,16] spectrometry,[17,18] fluorescence,[19−22] and redox sensing[23] have been used for *in vitro* azide ion detection. However, these methods require extensive sample derivatization and are not suited for real-time *in vivo* detection.

Glycosyl azides have also facilitated *in vivo* imaging of carbohydrates to improve our understanding of the function, localization, and metabolic role of glycans.[24] Glycosyl azides are also used as donor substrates for engineered glycosidases called glycosynthases to synthesize complex glycans.[25,26] Compared to other donor substrates (*e.g.*, glycosyl fluorides and *p*-nitrophenyl glycosides), glycosyl azides carry a small azide leaving group with strong nucleophilic character making them ideal substrates for glycoengineering applications. However, there are limited *in vivo* options available for rapid azide detection unlike other leaving groups (*e.g.*, *p*-nitrophenyl or pNP).[27] This has limited the widespread use of glycosyl azides as standard reagents in glycosciences unlike pNP-glycosides. Therefore, availability of a synthetic biology toolkit for rapid *in vivo* azide ion detection can broadly enable the fields of chemical biology and glycoengineering.

Here, we have developed a biosensing toolkit to selectively and autonomously detect azide ions inside living cells. Briefly, an *Escherichia coli* operon was engineered to generate a synthetic promoter plasmid that is selectively inducible by

Received: August 26, 2020
Published: March 22, 2021

https://doi.org/10.1021/acssynbio.0c00449
ACS Synth. Biol. 2021, 10, 682−689

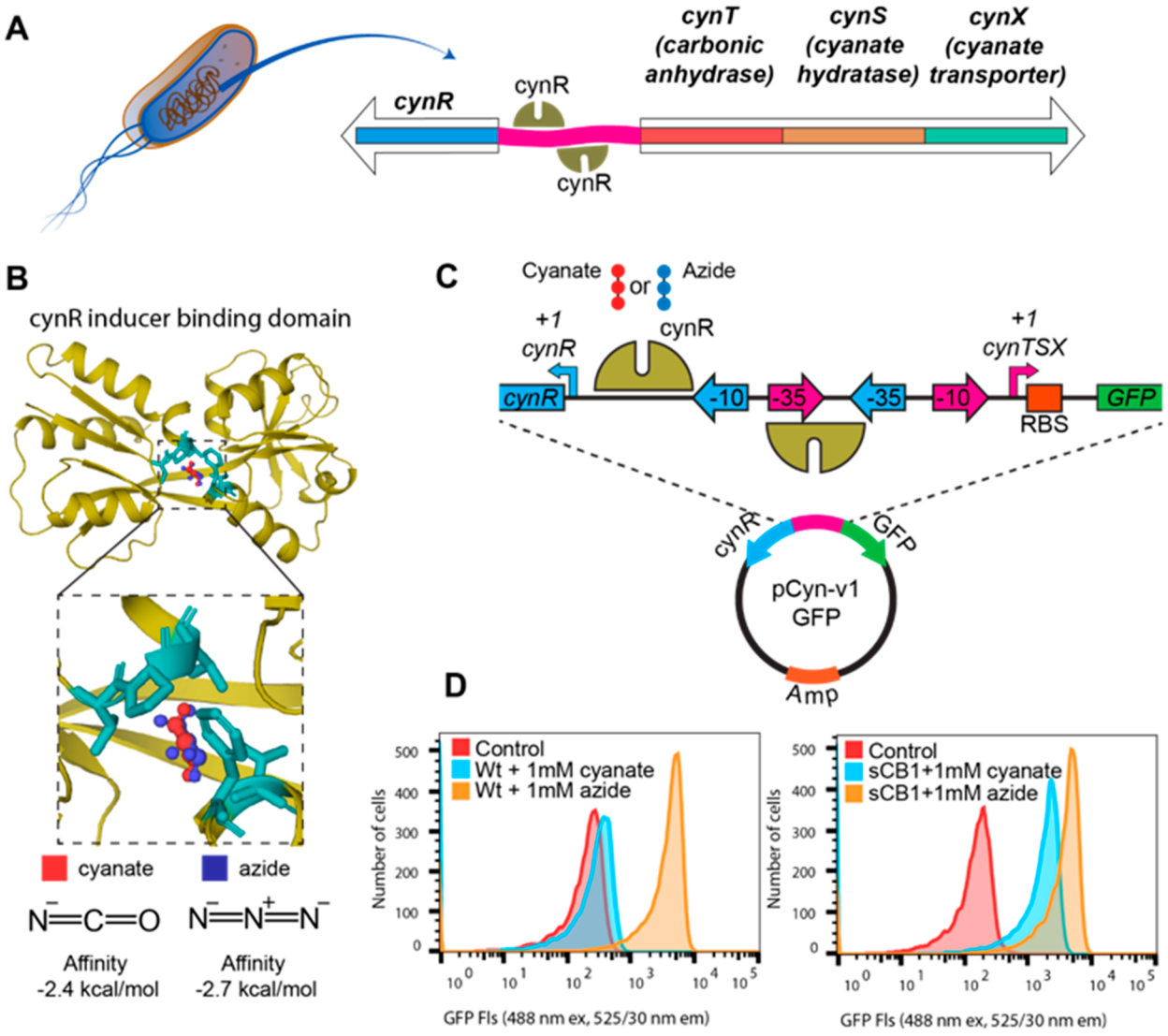


Figure 1. Promoter engineering to enable reporter *gfp* gene expression in *E. coli* using azide and cyanate as inducers. (A) Native cynTSX operon facilitates *E. coli* cell growth in a cyanate-rich environment. Cyanate molecule binds to the cynR repressor protein to regulate downstream protein expression of three essential native genes (*i.e.*, cynT, cynS, cynX). (B) Molecular docked structures of cyanate *versus* azide ions in the cynR binding pocket is shown here. Docking simulations confirmed that azide also binds tightly in the cynR binding pocket with a similar binding affinity like cyanate, a close structural homologue of azide. (C) Engineered plasmid map design of pCyn-v1-GFP containing the native cynR and *cyn* operator region cloned upstream of a GFP reporter gene. As proof of concept, the version 1 (v1) design was used first to monitor GFP expression using 1 mM azide or cyanate as inducers. (D) Flow cytometry based individual cell analysis of GFP fluorescence signal confirms cyanate *versus* azide inducible heterologous *in vivo* protein expression using both native (BW25113-wt) and engineered Δ*cynS* knockout (BW25113-sCB1) *E. coli* strains.

azide anions. The tunable expression of a model green fluorescence protein (GFP) was demonstrated using this novel azide based promoter system and compared to the standard *E. coli* lactose (*lac*) operon. Finally, the biosensing potential of this promoter system to detect azide anions, *versus* organic glycosyl azides, was showcased. In addition, a proof-of-concept glycoengineering application is showcased where we selectively monitored the *in vivo* glycosynthase enzyme activity for a mutant fucosidase that can synthesize fucosylated disaccharides using fucosyl azide as the donor sugar and pNP-xylose as the acceptor sugar.

RESULTS AND DISCUSSION

The *E. coli* genome consists of several hundred operons (~700) performing specific functions essential for bacterial metabolism and survival in harsh environments.[28] In particular, the cyanate or *cyn* operon enables *E. coli* cells to survive in cyanate-rich environments.[29] This gene had evolved in archaea and cyanobacteria for energy production and nitrogen assimilation during the early history of single-celled life in extreme marine environments.[30] The *cyn* operon, analogous to the *lac* operon, is comprised of three structural genes; *cynT*, *cynS*, and *cynX* (Figure 1A) that encode carbonic anhydrase, cyanate hydratase, and cyanate transporter proteins, respectively. These genes code for enzymes that catalyze the bicarbonate-dependent decomposition of cyanate ions into carbon dioxide and ammonia. Along with *cynTSX* genes, *cynR* repressor gene encoding cynR protein is present for regulatory purposes. The operon is under tight negative regulation of the cynR repressor protein which upon binding to the operator

https://doi.org/10.1021/acssynbio.0c00449
ACS Synth. Biol. 2021, 10, 682−689

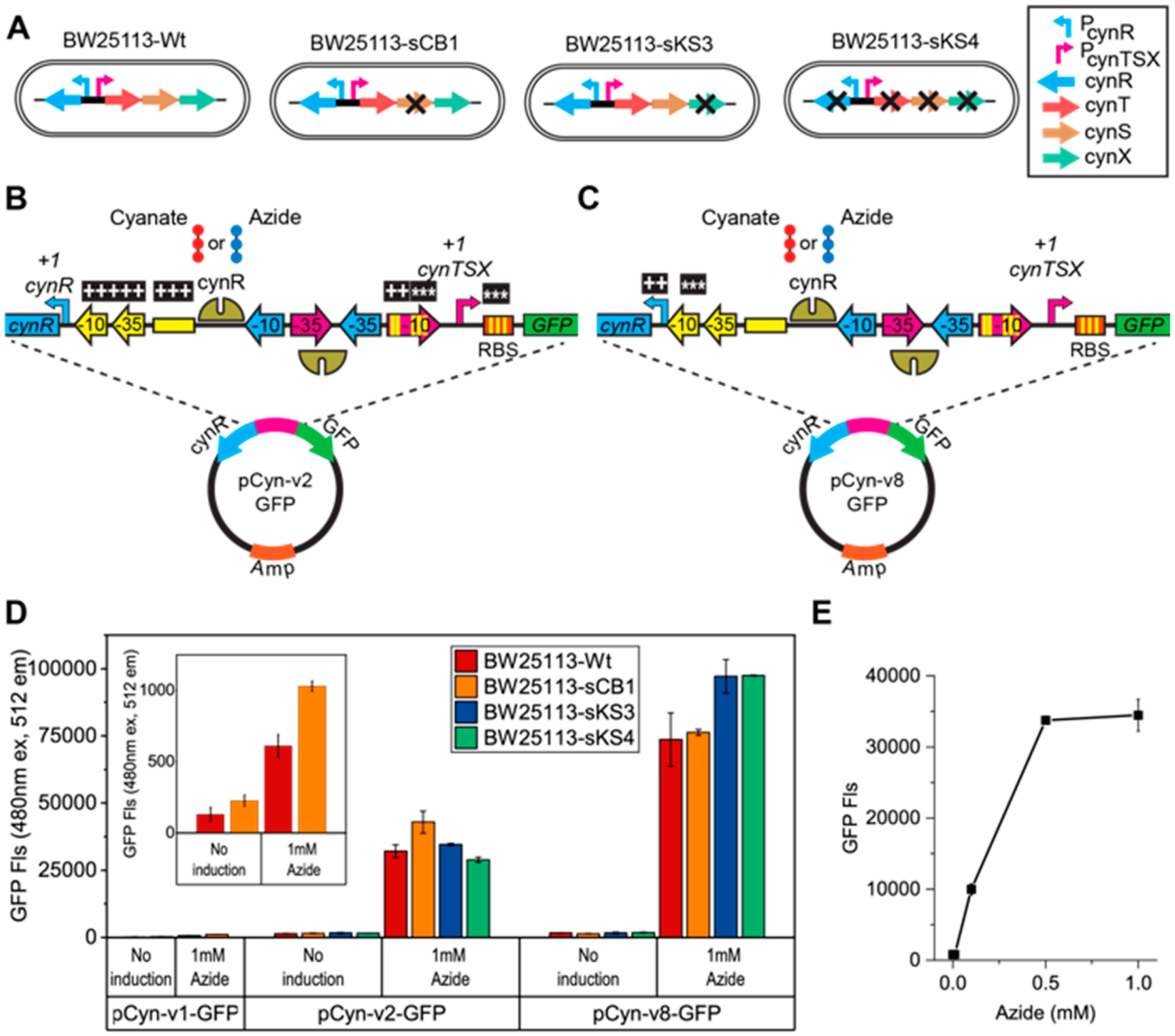


Figure 2. Strain engineering and promoter design co-optimization to increase reporter GFP protein expression upon induction by azide. (A) *E. coli* BW25113 wild type and various knockout strains are represented here in the cartoon schematics. P_{cynR} and P_{cynTSX} are promoters for cynR and cynTSX genes, respectively. Individual genes are color coded and genes knocked-out are illustrated with "X" mark. (B,C) Plasmid map design of pCyn-v2-GFP and pCyn-v8-GFP containing the engineered regulatory region between *cynR* and *gfp* genes are highlighted here. The mutational replacements in the promoter region are represented by an asterisk (*), while additions are denoted by a plus sign (+). (D) Bar graphs representing GFP fluorescence intensities from cell lysates of engineered strains containing three distinct plasmid variants (v1, v2, v8) are shown. The magnified inset shows GFP fluorescence for native promoter construct (pCyn-v1-GFP). (E) Fluorescence plot for cell lysate of BW25113-wt strain containing pCyn-v2-GFP and induced with increasing concentrations of sodium azide. Error bars indicate one standard deviation from reported mean values from three biological replicates.

region results in unfavorable DNA bending to prevent transcription of the *cynTSX* genes. The *cyn* operon is only activated when a cyanate molecule binds to the allosteric repressor protein also bound to the promoter/operator region. Exogenous cyanate can bind to the cynR inducer binding domain and cause conformational changes in the binding domain, thereby reducing the DNA bend in the operator region to facilitate transcription.[29,31,32]

Since cyanate is a linear molecular ion that is structurally homologous to azide, we hypothesized that azide could also bind to cynR and trigger the *cyn* operon. To confirm if azide can bind with similar binding affinity to cynR, Autodock vina[33] was used to perform docking simulations for both cyanate and azide in the binding pocket of cynR. The binding orientation of the docked ligands revealed a good overlap in the binding site with similarly predicted binding affinities (Figure 1B). This suggested that azide could indeed function as a gratuitous inducer for the native *cyn* operon.

Next, we subcloned the regulatory segment consisting of *cynR* gene and the *cyn* operator region into a plasmid vector with an ampicillin resistance marker and green fluorescent protein (*gfp*) as the reporter gene. The regulatory segment was placed upstream of the *gfp* reporter gene to facilitate GFP expression using either cyanate or azide ions as inducers (Figure 1C). The resultant plasmid (called pCyn-v1-GFP) was transformed into *E. coli* BW25113 wildtype (Wt) strain and induced with 1 mM of sodium cyanate or sodium azide. The supernatant of the lysed cells after induction was analyzed for fluorescence using a spectrophotometer (see Supporting Information (SI) Figure S1). In addition, the fluorescence of

https://doi.org/10.1021/acssynbio.0c00449
ACS Synth. Biol. 2021, 10, 682−689

individual intact cells was analyzed through a flow cytometer and Figure 1D presents the resultant histogram plots of the induced cells fluorescence after 19 h. Compared to the control (uninduced cells), the azide induced cell lysate had a nearly 20-fold increase in GFP fluorescence while cyanate did not result in any significant GFP expression. Specifically, there was a significant difference (two-tailed t test $P < 0.001$ for 2 biological replicates per test group) in the fluorescence observed for pCyn-v1-GFP transformed cell lysate after 19 h induction time in the presence of 1 mM sodium azide (3840 + 135; mean ± s.d.) *versus* uninduced cells (169 ± 38). The absence of GFP expression in cyanate induced cells was likely due to the breakdown of cyanate by the endogenous cyanate hydratase enzyme encoded by the native *E. coli* BW25113-Wt genome. Hence, we next tested the induction capacity of the synthetic promoter using a Δ*cynS* knockout strain (BW25113-sCB1) to clearly show GFP expression upon induction by cyanate (SI Figure S1). However, the amount of protein expressed based on total cell lysate GFP fluorescence was very low suggesting that the native *cyn* promoter strength was quite poor. This is not surprising since the native *cyn* promoter regulates associated CynTSX proteins expression required to overcome cyanate toxicity and that seldom requires high protein expression yields. The native operator region also contains suboptimal −10 sequence and Shine−Delgarno (SD) sequences (or ribosomal binding site; RBS) which could play a role in poor expression strength.

To improve the native promoter strength for enabling higher inducible protein expression levels, the native *cyn* promoter was next engineered to contain consensus −10 and SD sequences. The *cynR* gene, which is negatively regulated by *cyn* promoter, was placed under control of an independent constitutive promoter[34] (see SI Text for sequence information). Additionally to making these modifications, the promoters were separated by inserting a random DNA spacer sequence of 100 bp (pCyn-v2-GFP) and 1000 bp (pCyn-v4-GFP) to avoid any interference and steric hindrances between the two promoters (Figure 2B and SI Figure S2). A control construct that contained no spacer sequence (pCyn-v3-GFP) was also generated. The modified constructs were individually transformed into *E. coli* cells and tested for GFP expression using sodium azide as inducer. All three engineered constructs showed a greater than 50-fold increase in the fluorescence signal in the cell lysate, indicating a significant increase in GFP expression as compared to the proof-of-concept pCyn-v1-GFP version 1 promoter (Figure 2D and SI Figure S2B). Specifically, there was a significant difference (two-tailed t test $P = 0.002$ for 3 biological replicates per test group) in the fluorescence observed for pCyn-v2-GFP transformed cell lysate after 2 h induction time in the presence of 1 mM sodium azide (32 068 ± 2170; mean ± s.d.) *versus* pCyn-v1-GFP transformed cell lysate (610 ± 80). The pCyn-v4-GFP construct with longest spacer region showed around 37% increased fluorescence when compared to the no spacer pCyn-v3-GFP control. Specifically, there was a significant difference (two-tailed t test $P = 0.001$ for 3 biological replicates per test group) in the fluorescence observed for pCyn-v4-GFP transformed cell lysate after 2 h induction time in the presence of 1 mM sodium azide (65 468 ± 932; mean ± s.d.) *versus* pCyn-v3-GFP transformed cells (47 765 ± 28). However, significant leaky GFP expression was seen for the no induction controls of v4 construct (SI Figure S2C). The extra-long spacer sequences could have potentially allowed undesirable interactions in the plasmid causing a change in the DNA bending properties for CynR protein.

Decreasing the length between the promoters largely reduced the leaky expression under no induction while maintaining the largely improved GFP yield upon azide induction (Figure S2). For all subsequent work, pCyn-v2-GFP design was chosen to keep the promoters at optimal distance from each other. The nearly 626-fold increase in GFP fluorescence observed was now promising to be able to utilize the pCyn-v2-GFP engineered design for autonomously sensing azide ions. Specifically, there was a significant difference (two-tailed t test $P < 0.001$ for 3 biological replicates per test group) in the fluorescence observed for pCyn-v2-GFP transformed cell lysate after 4 h induction time in the presence of 1 mM sodium azide (105 179 ± 3802; mean ± s.d.) *versus* uninduced cells (168 ± 17). To determine the minimal azide amount required to induce GFP protein expression, we induced cells transformed with the pCyn-v2-GFP plasmid with varying concentrations of azide ions. The maximum amount of azide used for induction was limited to 5 mM since higher amounts had a negative influence on bacterial cell growth (Figure S3). GFP fluorescence of cell lysate showed a strong correlation in protein expression as a function of inducer dosage. GFP expression increased until 1 mM azide concentration induction after which there was reduction in the GFP signal for 5 mM inducer concentration likely due to cellular toxicity of azide (Figure 2E). The lowest tested azide concentration (10 μM) showed marginal GFP expression indicating that the detection limit for this synthetic promoter would have to be greater than 10 μM. The bacterial growth phase during the time of induction also played an important role and we observed that induction at early exponential phase yielded higher expressed protein amounts (Figure S4).

CynR protein acts as a repressor for the promoter by causing a bend at the −35 site hindering the binding of RNA polymerase. The cynR protein consists of two domains: an inducer binding domain and a DNA binding domain. The inducer binding domain binds to the inducer (cyanate or azide) which decreases the bend at the promoter site to facilitate transcription. For regulating protein expression levels, along with the inducer amounts the basal level of cynR protein present is also critical. We therefore further engineered the cynR constitutive promoter to adjust the background level of repressor protein expressed. Four different constructs (v5, v6, v7, and v8) were created by introducing mutations at the promoter −10 site, regions between −10 and −35 site, and between RBS and translation start site (Figure 2C and SI Figure S5A). The expression strength of these modified promoters was tested in the wild type (BW2113) and several additional knockout strains (sCB1, sKS3, sKS4) by monitoring GFP fluorescence in cell lysates. The knockout strains sKS3 and sKS4 were generated to remove the native *cynX* gene and *cyn* operon (SI Table S1), respectively, to minimize the export of azide ions using native transporter proteins and reduce interference from the endogenous *cyn* operon. The pCyn-v8-GFP construct, which had reduced efficiency at −10 site but optimal length between RBS and translation start site, gave about 120−160 fold higher reporter GFP fluorescence as compared to the native promoter, with the maximum fold increase observed in Δ*cynX* (sKS3), Δ*cynR*, and Δ*cynTSX* (sKS4) knockout strains (Figure 2D). Specifically, there was a significant difference (two-tailed t test $P = 0.006$ for 3 biological replicates per test group) in the fluorescence

https://doi.org/10.1021/acssynbio.0c00449
ACS Synth. Biol. 2021, 10, 682−689

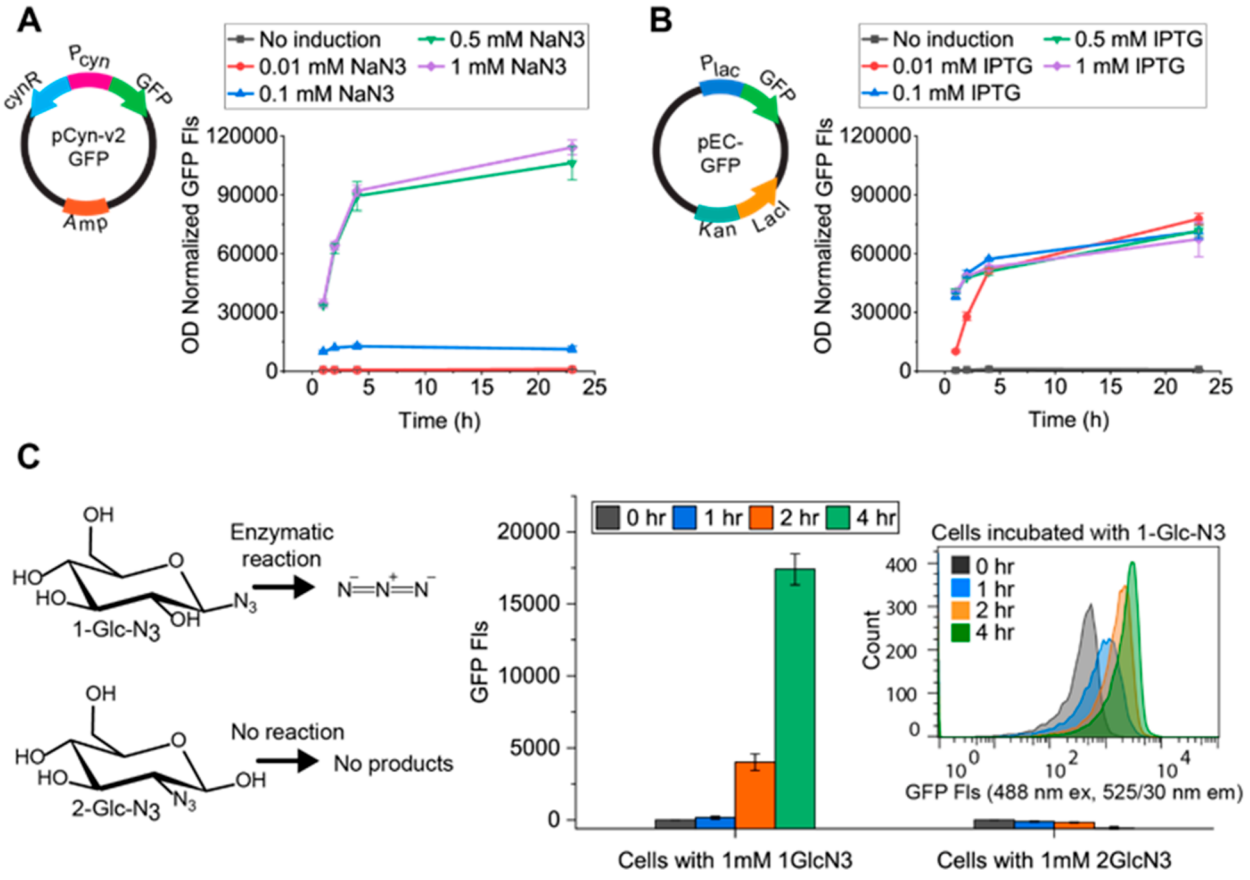


Figure 3. Utility of novel synthetic azide promoter system toward heterologous protein expression and biorthogonal chemical biology applications. (A) *E. coli* BW25113-Wt containing pCyn-v2-GFP plasmid was induced with varying azide concentrations for 24 h and GFP fluorescence of cell lysate was measured at various time points. (B) *E. coli* BL21 containing pEC-GFP plasmid was induced with different IPTG concentrations for 24 h and GFP fluorescence of the cell lysate was measured at different time points. (C) *E. coli* BW25113-Wt cells with pCyn-v2-GFP plasmid was incubated with 1-azido-β-D-glucopyranosyl azide (1-Glc-N_3) and 2-deoxy-2-azido-β-D-glucopyranosyl azide (2-Glc-N_3) and GFP fluorescence of respective cell lysates are shown as bar graphs. The inset depicts the flow cytometry analysis data for cells incubated with 1-Glc-N_3 for 4 h. Error bar indicates one standard deviation from reported mean values from three biological replicates.

observed for pCyn-v8-GFP transformed cell lysate after 2 h induction time in the presence of 1 mM sodium azide (73 686 ± 9930; mean ± s.d.) *versus* pCyn-v1-GFP transformed cell lysate (610 ± 80). On the other hand, while other designs (v5, v6, v7) showed comparable fluorescence with respect to the v2 design, there was undesirable leaky background GFP expression seen even without inducer addition (SI Figure S5). The optimized plasmid designs (pCyn-v2-GFP and pCyn-v8-GFP) could now function as highly tunable synthetic biosensors for rapid *in vivo* detection of azide ions. The amount of GFP expressed within 2 h of 1 mM azide induction in BW25113-Wt strain was estimated to be 0.4 mg and 0.9 mg from 1 mL culture for pCyn-v2-GFP and pCyn-v8-GFP, respectively, based on the protein concentration and GFP fluorescence calibration curve (SI Figure S6).

Next, the heterologous protein expression efficiency of the engineered promoter (pCyn-v2-GFP) was compared against the *E. coli lac* operon system.[35,36] The synthetic *cyn* promoter (P_{cyn}) in BW25113-Wt (Figure 3A) and *lac* promoter (P_{lac}) in BL21 cells (Figure 3B) were induced using sodium azide and isopropyl-β-D-1-thiogalactopyranoside (or IPTG), respectively, at various concentrations ranging between 0.01 mM to 1 mM. The amount of protein expressed in the P_{lac} system was higher during the early time points after induction and at the lowest inducer concentrations. Even at lower IPTG concentrations (0.01 mM and 0.1 mM), GFP expression increased with induction time and reached a maximum after 24 h of induction, whereas we see a relatively lower GFP expression at similar low azide concentrations (Figure 3A and 3B). However, we see a highly tunable reporter gene expression during azide induction as the amount of GFP expressed was closely proportional to inducer concentration, unlike IPTG. Also, the maximum GFP expression achieved after 24 h was at least 2-fold higher for the *cyn* promoter than *lac* promoter at inducer concentrations higher than 0.5 mM (Figure 3A and 3B). Specifically, there was a significant difference (two-tailed *t* test $P = 0.005$ for 3 biological replicates per test group) in the fluorescence observed for pCyn-v2-GFP transformed cell lysate in the presence of 1 mM sodium azide (114 238 ± 3721; mean ± s.d.) *versus* pEC-GFP transformed cell lysate in the presence of 1 mM IPTG (67 336 ± 8892) after 23 h induction time. These results showcase the utility of the novel engineered plasmid harboring the P_{cyn} promoter as an alternative system for heterologous protein expression and other biotechnology applications.

In addition to application as a heterologous protein production system, the developed azide promoter can be useful for chemical biologists especially in glycobiology and glycosynthase engineering related applications. The proposed system is a powerful synthetic biology toolkit that will allow

https://doi.org/10.1021/acssynbio.0c00449
ACS Synth. Biol. 2021, 10, 682−689

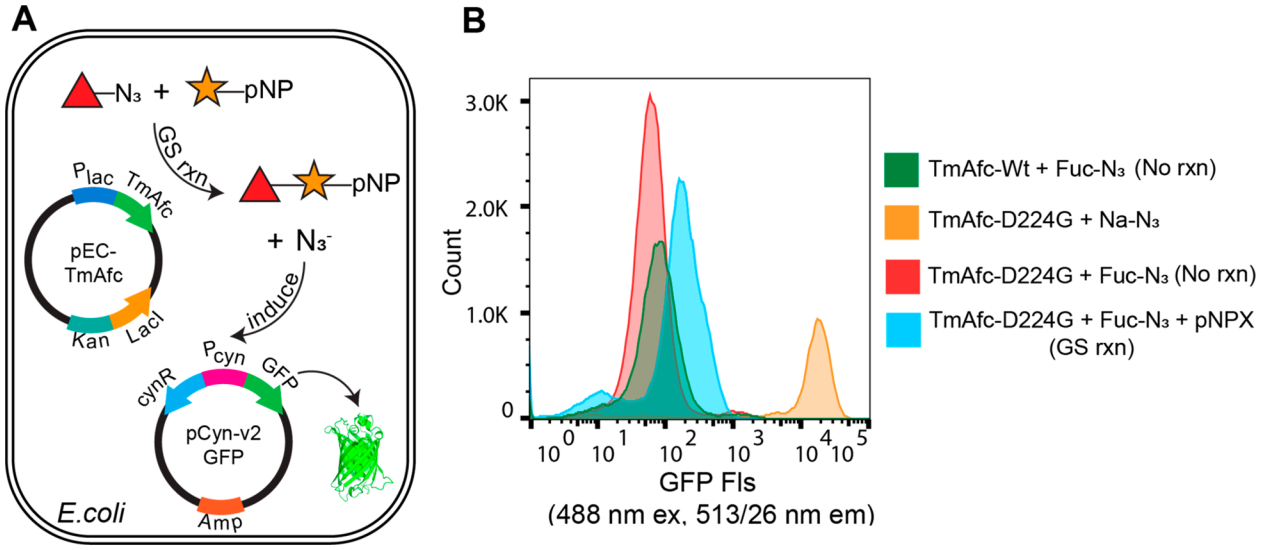


Figure 4. Glycosynthase enzyme engineering and screening using flow cytometry is enabled using azide promoter system. (A) *E. coli* containing two plasmids (pEC-TmAfc and pCyn-v2-GFP) was incubated with glycosynthase reaction substrates; fucosyl-azide (red triangle) and pNP-xylose (orange star). TmAfc-D224G mutant alone catalyzes the glycosynthase (GS) reaction releasing azide ion as byproduct, which then induces the azide promoter on the secondary plasmid (pCyn-v2-GFP) resulting in GFP expression. (B) Flow cytometry analysis of *E. coli* cells containing dual plasmids were incubated with glycosynthase substrates and various controls to demonstrate the utility of the azide promoter system. Only cells expressing the TmAfc-D224G mutant and in the presence of both GS reaction donor/acceptor sugar substrates gave a clear shift in cell fluorescence (blue) due to the release of azide ion during the GS reaction.

selective identification of inorganic azides among other chemically linked organic azides. Enzymatic reactions which result in the release of azide ion (as leaving group) can be potentially identified using the proposed *cyn* promoter system. Carbohydrate-Active enZymes (CAZymes) such as glycosyl hydrolases and transglycosidases have been shown to release azide from azido-based sugars during glycosidic bond hydrolysis[37] and synthesis,[26,37−40] respectively. Multiple glycosyl azides such as galactosyl-, glucosyl-, and mannosyl-azides have been reported to be hydrolyzed by galactosidases, glucosidases, and mannosidases, respectively.[37] Similarly, engineered glycosidases (*i.e.*, transglycosidases and glycosynthases) have been shown to use azido-hexoses and other N-acetyl derivatives as donor sugars for oligosaccharides synthesis.[38,40] β-glucosidases from *Aspergillus* sp. and *Agrobacterium* sp. belonging to glycosyl hydrolase (GH) families GH3 and GH1 were reported to actively release azide ion from glucosyl azides.[39] Similarly, homologous β-glucosidases belonging to the GH1 and GH3 families present in native *E. coli* can potentially also show similar substrate specificity to release azide ions when incubated with glucosyl azides.

Here, we incubated *E. coli* BW25113-Wt cells containing the engineered pCyn-v2-GFP plasmid with 1-azido-β-D-glucopyranosyl azide (1-Glc-N_3) for 4 h. As illustrated in Figure 3C, a small change in GFP fluorescence was seen in the first hour, after which a rapid increase in fluorescence was observed within 1−4 h owing to the release of azide when using 1-Glc-N_3. Specifically, there was a significant difference (two-tailed *t* test $P = 0.035$ for 3 biological replicates per test group) in the fluorescence observed for pCyn-v2-GFP transformed cell lysate in the presence of 1 mM 1-Glc-N_3 after 1 h induction time (170 ± 93; mean ± s.d.) *versus* 0-h induction time (0 ± 15). To verify if the detected GFP fluorescence is due to release of azide ions and not due to presence of azido-glucose, we also incubated cells with 2-deoxy-2-azido-β-D-glucopyranosyl azide (2-Glc-N_3) analogous to the 2-deoxy-2-fluoro glycoside based β-glucosidase inhibitors.[41] No change in fluorescence was detected in the control samples since 2-Glc-N_3 is not hydrolyzed by β-glucosidases and hence cannot release free azide ions for induction of the engineered promoter. The preferential sensitivity of the designed promoter toward azide ions can therefore be used for *in vivo* glycoengineering to identify efficient CAZymes such as glycosyl hydrolases (GH) for glycosidic bonds synthesis and/or hydrolysis.[27]

Glycosynthases (GS) are engineered/mutant glycosyl hydrolases or glycosidases that can synthesize oligosaccharides by forming glycosidic bonds between suitable donor sugars and acceptor glycone or aglycone groups. The donor sugars are often activated sugars containing fluoride or azide moieties as leaving groups on the anomeric carbon that can be readily released in the enzyme active site in the presence of incoming acceptor substrates to form a glycosidic bond. The fluoride or azide ions are hence released as byproducts of the glycosynthase reaction. Higher concentrations of byproduct release are expected to be strongly correlated with higher glycosynthase activity. Our engineered azide specific promoter system can therefore be used for screening enzyme activity and identifying improved GS enzyme mutants. As proof of concept, a previously engineered fucosynthase mutant (TmAfc-D224G), isolated from GH29 family fucosidase enzyme (TmAfc-Wt),[38] was selected to determine the applicability of the azide biosensor for glycosynthase enzyme engineering and screening activity in live cells using flow cytometry. *E. coli* cells containing two plasmids (*i.e.*, pEC-TmAfc and pCyn-v2-GFP) were induced for TmAfc enzyme expression and incubated with fucosyl-azide (donor) and pNP-xylose (acceptor) for conducting the fucosynthase reaction along with several controls. The resultant *E. coli* cells were analyzed for GFP expression using flow cytometry (Figure 4). The cells containing wild-type enzyme (TmAfc-Wt) incubated with donor sugar alone was used as a baseline control. This baseline control represents the response expected for inactive or

https://doi.org/10.1021/acssynbio.0c00449
ACS Synth. Biol. 2021, 10, 682−689

nonglycosynthase based samples, whereas the glycosynthase active variant (TmAfc-D224G) was also incubated with sodium azide alone as another control to depict the theoretical maximum glycosynthase activity that would be possible if 100% of the added fucosyl azide was converted by the enzymes into products. TmAfc-D224G showed very minor hydrolysis when cells expressing this D224G enzyme mutant were incubated with only donor sugars (red). However, the active GS mutant (TmAfc-D224G) showed a significant increase in GFP fluorescence when both the donor and acceptor sugars were added to the cell milieu (blue). Specifically, there was a significant difference (two-tailed t test $P < 0.001$) in the fluorescence observed for transformed cells incubated with donor + acceptor substrates (230 ± 148; mean ± s.d.; n = 71191; raw data shown in blue histogram in Figure 4) *versus* cells incubated with only donor substrate (68 ± 37; n = 84 611; raw data shown in red histogram in Figure 4). Our recent work has shown that although the D224G single-point mutation results in an active fucosynthase unlike the wild-type enzyme, the total reaction yield is still well under 10% based on the donor sugar as limiting reagent.[27] This explains why TmAfc-D224G expressing cells gave an increase in GFP fluorescence that did not reach the maximum expected theoretical concentration (based on observed GFP fluorescence shown in orange histogram in Figure 4), if 100% of the fucosyl azide was consumed in the GS reaction. Regardless, these proof-of-concept results clearly show that dual plasmids carrying *E. coli* cells (*i.e.*, pEC-TmAfc and pCyn-v2-GFP) can be theoretically used for screening and isolating active glycosynthase mutants that release azide ions as the GS reaction byproduct.

In summary, we have demonstrated the design and engineering of a novel azide (and cyanate) inducible promoter system for *E. coli*. This azide promoter system allows tunable expression by varying the inducer (*i.e.*, azide ion) concentrations that also outperformed the conventional lactose/IPTG based system for heterologous reporter GFP expression. Additionally, the developed toolkit functions as a biosensor for detecting the presence of azide ions using living cells. This allowed us to employ this toolkit for engineering CAZymes such as glycosyl hydrolases (and/or glycosynthases) which use azido-sugars as substrates and autonomously monitor released azide ions upon substrate hydrolysis or during transglycosylation reactions. Furthermore, this biosensing platform can be evolved in the future for use with other prokaryotic or eukaryotic cells. Adaptation of an azide specific promoter system for diverse cell types would be also beneficial for various chemical biology based research applications like azido-based drug development and molecular probes for real-time multicolor fluorescence imaging.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.0c00449.

Experimental Methods, DNA Sequences, Figures S1−S6, and Tables S1−S2 (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Shishir P. S. Chundawat − *Department of Chemical & Biochemical Engineering, Rutgers, The State University of New Jersey, Piscataway, New Jersey 08854, United States;* orcid.org/0000-0003-3677-6735; Email: shishir.chundawat@rutgers.edu

Authors

Chandra Kanth Bandi − *Department of Chemical & Biochemical Engineering, Rutgers, The State University of New Jersey, Piscataway, New Jersey 08854, United States;* orcid.org/0000-0001-6273-8068

Kyle S. Skalenko − *Department of Genetics and Waksman Institute, Rutgers, The State University of New Jersey, Piscataway, New Jersey 08854, United States*

Ayushi Agrawal − *Department of Chemical & Biochemical Engineering, Rutgers, The State University of New Jersey, Piscataway, New Jersey 08854, United States*

Neelan Sivaneri − *Department of Chemical & Biochemical Engineering, Rutgers, The State University of New Jersey, Piscataway, New Jersey 08854, United States*

Margaux Thiry − *Department of Chemical & Biochemical Engineering, Rutgers, The State University of New Jersey, Piscataway, New Jersey 08854, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acssynbio.0c00449

Author Contributions

CKB and SPSC conceived the project. CKB, KSS, AA, NS, MT contributed toward experimental work. CKB and SPSC performed data analysis and contributed toward manuscript writing. CKB generated all figures and TOC graphics using Adobe Illustrator. All authors have given approval to the final version of the manuscript.

Notes

The authors declare the following competing financial interest(s): SPSC and CKB have filed a US Provisional Patent Application filed June 30, 2020 titled 'Biosensors for Selectively Identifying Azide Ions' (Rutgers Provisional Application No. RU 2019-159).

■ ACKNOWLEDGMENTS

SPSC acknowledges support from the US National Science Foundation (CBET Award No. 1704679 and Chemistry Award No. 1904890) and Rutgers School of Engineering.

■ ABBREVIATIONS

CAZymes, carbohydrate-active enzymes; GFP, green fluorescent protein; GH, glycosyl hydrolase; IPTG, isopropyl β-D-1-thiogalactopyranoside; pNP, *para*-nitrophenyl.

■ REFERENCES

(1) Soli, E. D., Manoso, A. S., Patterson, M. C., DeShong, P., Favor, D. A., Hirschmann, R., and Smith, A. B. (1999) Azide and Cyanide Displacements via Hypervalent Silicate Intermediates. *J. Org. Chem.* *64* (9), 3171−3177.

(2) Brse, S., and Banert, K. (2009) *Organic Azides: Syntheses and Applications* (Brse, S., and Banert, K., Eds.) John Wiley & Sons, Ltd., Chichester, U.K. DOI: 10.1002/9780470682517.

(3) Madlung, A. (1996) The Chemistry behind the Air Bag: High Tech in First-Year Chemistry. *J. Chem. Educ.* *73* (4), 347.

(4) Dyukarev, E. A., and Knyazeva, A. G. (1999) Model of Detonation of Lead Azide (Pb(N3)2) with Regard to Fracture. *Int. J. Fract.* *100* (2), 197−205.

(5) Wereide, K. (1975) Sensitivity to Azidamphenicol. *Contact Dermatitis* *1* (4), 271−272.

(6) Bengtsson, E., Strandell, T., Svanbom, M., and Tunevall, G. (1972) Azidocillin Treatment of Enterococcal Septicemia. *Scand. J. Infect. Dis. 4* (2), 143−148.
(7) Blum, M. R., Liao, S. H., Good, S. S., and de Miranda, P. (1988) Pharmacokinetics and Bioavailability of Zidovudine in Humans. *Am. J. Med. 85* (2A), 189−194.
(8) Glassford, I., Teijaro, C. N., Daher, S. S., Weil, A., Small, M. C., Redhu, S. K., Colussi, D. J., Jacobson, M. A., Childers, W. E., Buttaro, B., Nicholson, A. W., MacKerell, A. D., Cooperman, B. S., and Andrade, R. B. (2016) Ribosome-Templated Azide-Alkyne Cycloadditions: Synthesis of Potent Macrolide Antibiotics by in Situ Click Chemistry. *J. Am. Chem. Soc. 138* (9), 3136−3144.
(9) Devaraj, N. K. (2018) The Future of Bioorthogonal Chemistry. *ACS Cent. Sci. 4* (8), 952−959.
(10) Devaraj, N. K., Thurber, G. M., Keliher, E. J., Marinelli, B., and Weissleder, R. (2012) Reactive Polymer Enables Efficient in Vivo Bioorthogonal Chemistry. *Proc. Natl. Acad. Sci. U. S. A. 109* (13), 4762−4767.
(11) Au, K. M., Wang, A. Z., and Park, S. I. (2020) Pretargeted Delivery of PI3K/MTOR Small-Molecule Inhibitor−Loaded Nanoparticles for Treatment of Non-Hodgkin's Lymphoma. *Sci. Adv. 6* (14), eaaz9798.
(12) Laughlin, S. T., and Bertozzi, C. R. (2009) Imaging the Glycome. *Proc. Natl. Acad. Sci. U. S. A. 106* (1), 12−17.
(13) Medina-Cleghorn, D., and Nomura, D. K. (2014) Exploring Metabolic Pathways and Regulation through Functional Chemoproteomic and Metabolomic Platforms. *Chem. Biol. 21* (9), 1171−1184.
(14) Dunge, A., Chakraborti, A. K., and Singh, S. (2004) Mechanistic Explanation to the Variable Degradation Behaviour of Stavudine and Zidovudine under Hydrolytic, Oxidative and Photolytic Conditions. *J. Pharm. Biomed. Anal. 35* (4), 965−970.
(15) Adhikari, S., Guria, S., Ghosh, A., Pal, A., and Das, D. (2017) A Curcumin Derived Probe for Colorimetric Detection of Azide Ions in Water. *New J. Chem. 41* (24), 15368−15372.
(16) Tsuge, K., Kataoka, M., and Seto, Y. (2001) Rapid Determination of Cyanide and Azide in Beverages by Microdiffusion Spectrophotometric Method. *J. Anal. Toxicol. 25* (4), 228.
(17) Wang, L., Dai, C., Chen, W., Liu Wang, S., and Wang, B. (2011) Facile Derivatization of Azide Ions Using Click Chemistry for Their Sensitive Detection with LC-MSwz. *Chem. Commun. 47* (47), 10377−10379.
(18) Dillen, L., Sips, L., De Vries, R., Borgmans, C., Vliegen, M., Leclercq, L., and Cuyckens, F. (2013) Quantitative LC-MS/MS Analysis of Azide and Azidoalanine in in Vitro Samples Following Derivatisation with Dansyl Chloride. *Anal. Methods 5* (12), 3136−3141.
(19) Sahana, A., Banerjee, A., Guha, S., Lohar, S., Chattopadhyay, A., Mukhopadhyay, S. K., and Das, D. (2012) Highly Selective Organic Fluorescent Probe for Azide Ion: Formation of a "Molecular Ring. *Analyst 137* (7), 1544.
(20) Dhara, K., Saha, U. C., Dan, A., Sarkar, S., Manassero, M., and Chattopadhyay, P. (2010) A New Water-Soluble Copper(II) Complex as a Selective Fluorescent Sensor for Azide Ion. *Chem. Commun. (Cambridge, U. K.) 46* (10), 1754−1756.
(21) Wang, K., Friscourt, F., Dai, C., Wang, L., Zheng, Y., Boons, G.-J., Wang, S., and Wang, B. (2016) A Metal-Free Turn-on Fluorescent Probe for the Fast and Sensitive Detection of Inorganic Azides. *Bioorg. Med. Chem. Lett. 26* (7), 1651−1654.
(22) Puthiyedath, T., and Bahulayan, D. (2017) A Click-Generated Triazole Tethered Oxazolone-Pyrimidinone Dyad: A Highly Selective Colorimetric and Ratiometric FRET Based Fluorescent Probe for Sensing Azide Ions. *Sens. Actuators, B 239*, 1076−1086.
(23) Lim, J. Y. C., and Beer, P. D. (2017) A Halogen Bonding 1,3-Disubstituted Ferrocene Receptor for Recognition and Redox Sensing of Azide. *Eur. J. Inorg. Chem. 2017* (2), 220−224.
(24) Baskin, J. M., Prescher, J. A., Laughlin, S. T., Agard, N. J., Chang, P. V., Miller, I. A., Lo, A., Codelli, J. A., and Bertozzi, C. R. (2007) Copper-Free Click Chemistry for Dynamic in Vivo Imaging. *Proc. Natl. Acad. Sci. U. S. A. 104* (43), 16793−16797.
(25) Bojarová, P., and Kren, V. (2010) Azido Leaving Group in Enzymatic Synthesis-Small and Efficient. *Carbohydr. Chem.*, 168−175.
(26) Fialová, P., Carmona, A. T., Robina, I., Ettrich, R., Sedmera, P., Přikrylová, V., Petrásková-Hušáková, L., and Křen, V. (2005) Glycosyl Azide—a Novel Substrate for Enzymatic Transglycosylations. *Tetrahedron Lett. 46* (50), 8715−8718.
(27) Agrawal, A., Bandi, C. K., Burgin, T., Woo, Y., Mayes, H. B., and Chundawat, S. P. S. (2020) Click-Chemistry Enabled Directed Evolution of Glycosynthases for Bespoke Glycans Synthesis. *bioRxiv*, Mar 25, 2020, 2020.03.23.001982. .
(28) Salgado, H., Moreno-Hagelsieb, G., Smith, T. F., and Collado-Vides, J. (2000) Operons in Escherichia Coli: Genomic Analyses and Predictions. *Proc. Natl. Acad. Sci. U. S. A. 97* (12), 6652−6657.
(29) Sung, Y.-C., and Fuchs, J. A. (1988) Characterization of the Cyn Operon in Escherichia Coli K12* Young-Chul Sung And. *J. Biol. Chem. 263* (29), 14769−14775.
(30) Palatinszky, M., Herbold, C., Jehmlich, N., Pogoda, M., Han, P., von Bergen, M., Lagkouvardos, I., Karst, S. M., Galushko, A., Koch, H., Berry, D., Daims, H., and Wagner, M. (2015) Cyanate as an Energy Source for Nitrifiers. *Nature 524* (7563), 105−108.
(31) Sung, Y.-C., and Fuchs, J. A. (1992) The Escherichia Coli K-12 Cyn Operon Is Positively Regulated by a Member of the LysR Family. *J. Bacteriol. 174* (11), 3645.
(32) Lamblin, A.-F. J., and Fuchs, J. A. (1994) Functional Analysis of the Escherichia Coli K-12 Cyn Operon Transcriptional Regulation. *J. Bacteriol. 176* (21), 6613−6622.
(33) Trott, O., and Olson, A. J. (2019) Autodock Vina: Improving the Speed and Accuracy of Docking. *J. Comput. Chem. 31* (2), 455−461.
(34) Stringer, A. M., Singh, N., Yermakova, A., Petrone, B. L., Amarasinghe, J. J., Reyes-Diaz, L., Mantis, N. J., and Wade, J. T. (2012) FRUIT, a Scar-Free System for Targeted Chromosomal Mutagenesis, Epitope Tagging, and Promoter Replacement in Escherichia Coli and Salmonella Enterica. *PLoS One 7*, e44841.
(35) Studier, F. W., and Moffatt, B. A. (1986) Use of Bacteriophage T7 RNA Polymerase to Direct Selective High-Level Expression of Cloned Genes. *J. Mol. Biol. 189* (1), 113−130.
(36) Eames, M., and Kortemme, T. (2012) Cost-Benefit Tradeoffs in Engineered Lac Operons. *Science (Washington, DC, U. S.) 336* (6083), 911−915.
(37) Bojarová, P., Petrásková, L., Ferrandi, E. E., Monti, D., Pelantová, H., Kuzma, M., Simerská, P., and Křen, V. (2007) Glycosyl Azides - An Alternative Way to Disaccharides. *Adv. Synth. Catal. 349* (8−9), 1514−1520.
(38) Cobucci-Ponzano, B., Conte, F., Bedini, E., Corsaro, M. M., Parrilli, M., Sulzenbacher, G., Lipski, A., Dal Piaz, F., Lepore, L., Rossi, M., and Moracci, M. (2009) Beta-Glycosyl Azides as Substrates for Alpha-Glycosynthases: Preparation of Efficient Alpha-L-Fucosynthases. *Chem. Biol. 16* (10), 1097−1108.
(39) Müllegger, J., Jahn, M., Chen, H.-M., Warren, R. A. J., and Withers, S. G. (2005) Engineering of a Thioglycoligase: Randomized Mutagenesis of the Acid-Base Residue Leads to the Identification of Improved Catalysts. *Protein Eng., Des. Sel. 18* (1), 33−40.
(40) Cobucci-Ponzano, B., Zorzetti, C., Strazzulli, A., Carillo, S., Bedini, E., Corsaro, M. M., Comfort, D. A., Kelly, R. M., Rossi, M., and Moracci, M. (2011) A Novel -d-Galactosynthase from Thermotoga Maritima Converts -d-Galactopyranosyl Azide to -Galacto-Oligosaccharides. *Glycobiology 21* (4), 448−456.
(41) Rempel, B. P., and Withers, S. G. (2008) Covalent Inhibitors of Glycosidases and Their Applications in Biochemistry and Biology. *Glycobiology 18* (8), 570−586.

https://doi.org/10.1021/acssynbio.0c00449
ACS Synth. Biol. 2021, 10, 682−689