

RESEARCH ARTICLE

Substrate-activated expression of a biosynthetic pathway in *Escherichia coli*

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Abstract

Microbes can facilitate production of valuable chemicals more sustainably than traditional chemical processes in many cases: they utilize renewable feedstocks, require less energy intensive process conditions, and perform a variety of chemical reactions using endogenous or heterologous enzymes. In response to the metabolic burden imposed by production pathways, chemical inducers are frequently used to initiate gene expression after the cells have reached sufficient density. While chemically inducible promoters are a common research tool used for pathway expression, they introduce a compound extrinsic to the process along with the associated costs. We developed an expression control system for a biosynthetic pathway for the production of D-glyceric acid that utilizes galacturonate as both the inducer and the substrate, thereby eliminating the need for an extrinsic chemical inducer.

Activation of expression in response to the feed is actuated by a galacturonate-responsive transcription factor biosensor. We constructed variants of the galacturonate biosensor with a heterologous transcription factor and cognate hybrid promoter, and selected for the best performer through fluorescence characterization. We showed that native *E. coli* regulatory systems do not interact with our biosensor and favorable biosensor response exists in the presence and absence of galacturonate consumption. We then employed the control circuit to regulate the expression of the heterologous genes of a biosynthetic pathway for the production D-glyceric acid that was previously developed in our lab. Productivity via substrate-induction with our control circuit was comparable to IPTG-controlled induction and significantly outperformed a constitutive expression control, producing $2.13 \pm 0.03 \text{ g L}^{-1}$ D-glyceric acid within 6 h of galacturonate substrate addition.

This work demonstrated feed-activated pathway expression to be an attractive control strategy for more readily scalable microbial biosynthesis.

KEYWORDS

biosensor, metabolic engineering, metabolite-responsive transcription factor, synthetic biology

1 | INTRODUCTION

Metabolic engineering of microbes introduces, alters, and leverages enzymatic reactions to produce value-added chemicals in a host cell.

A diverse array of products have been produced through microbial synthesis, including biofuels, polymer precursors, food additives, and pharmaceuticals. These processes operate at mild conditions, which are typically safer and less energy intensive compared to traditional

chemical syntheses. The potential for microbes to use renewable feedstocks for synthesis presents an additional environmental benefit.

A challenge within metabolic engineering is that overexpression of pathway enzymes imposes a metabolic burden to the host, resulting in slow growth^[1,2] and product loss.^[3] In response, common fermentation practice in research allows cells to dedicate resources to biomass accumulation before expressing pathway enzymes from chemically-inducible promoters, for example, using isopropyl β -D-1-thiogalactopyranoside (IPTG)^[4,5] or arabinose.^[6,7] Due to their simplicity and pathway-independent function, chemically inducible promoters have been widely adopted for pathway expression in recombinant microbial hosts.^[8] Despite the broad use of this approach, the practice is undesirable for industrial scale-up due to the introduction of a chemical extrinsic to the process along with the associated costs. IPTG can be the most expensive component in a fermentation by an order of magnitude.^[9] Even when less expensive alternative chemical inducers such as arabinose, lactose^[10,11] or galactose^[12] are used, the inducer could pose difficulties in separations in a bioprocess. When scaling-up bioproduction, product purification issues are best addressed early,^[13] thus the introduction of extraneous chemicals for pathway expression is in direct disagreement with bioprocess development heuristics. Industrial bioprocesses use constitutive expression of pathway enzymes to avoid the disadvantages of chemical inducers.^[14] However, constitutive expression may impose undue burden to the cells, as mentioned above. Additionally, the only tuning parameter in this scheme is pathway expression strength,^[14] thus the only way to mitigate burden is to decrease expression strength and perhaps sacrifice production. These considerations limit the real world relevance of many academically developed pathway expression strategies and hinder commercial bioprocesses from more complex expression control. As an alternative to the use of extrinsic chemical induction or constitutive expression, researchers have leveraged stationary phase promoters to express burdensome genes after substantial cell growth; however, this limits the ability of the user to specify the time of induction.^[15,16] Another approach that has been employed is to use feedback controllers to partially delay pathway expression by activating it in response to a pathway intermediate.^[17,18] This strategy requires an additional mechanism to initiate expression since intermediates must accumulate to trigger the response.

One approach to address these limitations would be to utilize components already present in the culture broth to regulate pathway expression. An example is the use of phosphate-starvation promoters, which trigger gene expression due to phosphate depletion. While used successfully in many cases,^[19] this approach places restrictions on the medium composition and can, in some cases, impact pathway productivity.^[20] In this work, we developed a feed-activated, feed-forward expression control strategy, in which the presence of the pathway substrate induces expression of heterologous pathway genes. Thus, we can achieve user-determined, delayed pathway expres-

sion without the introduction of an extrinsic chemical to the process. We accomplished this by constructing a biosensor that utilizes a metabolite-responsive transcription factor (TF), for which the feed is its ligand, to control expression of one or multiple genes of interest. Biosensors employing reporters as the gene of interest are used to easily monitor the status of cells,^[21] characterize the transcriptional response to inducing conditions,^[17] or serve as a screening method in directed evolution applications.^[22] The gene(s) of interest can also encode one or multiple enzymes to couple expression, and thus enzymatic activity, to relevant cellular metabolic states.^[23] TF based biosensors specifically are widely used to sense pathway intermediates or products to trigger a cellular response.^[17,18,24] Given the wide of array of reported metabolite-responsive TFs,^[25] the relative ease of tuning transcriptional output,^[26–28] and the modularity within TFs themselves,^[29] this has the potential to be a robust strategy that can be applied to a variety of metabolites, feedstocks, pathways, and transcriptional control applications. We demonstrated our approach with a heterologous biosynthetic pathway to produce D-glyceric acid from galacturonate that was previously developed in our lab.^[30] D-Glyceric acid holds promise due to its biological activity and for its applications in surfactants and polymers.^[31] Furthermore, the pathway's galacturonate feed is the main component of pectin, a ubiquitous plant polysaccharide abundantly found in fruit and vegetable food waste streams,^[32] that could serve as a renewable feedstock for the process. Pectin makes up 24% of the 25 million metric tons of dried sugar beet pulp byproduct produced globally and 20% of the 1.2 million metric tons of dry citrus peel from Florida each year.^[33] Though these two waste streams, and other similar pectin-rich ones, are currently used as animal feed, there is potential for them to be converted into useful products. Thus, galacturonate has emerged as a desirable feedstock for microbial fermentations.^[34–36]

We began with construction and characterization of a biosensor with a galacturonate dose-dependent superfolder green fluorescent protein (sfGFP) signal output. The biosensor utilizes a galacturonate-responsive TF from *Bacillus subtilis*.^[37] We tested variants of the biosensor to select for desired response features. With our selected biosensor variant, we showed limited crosstalk with the native *E. coli* regulatory system and demonstrated robust biosensor performance in strains with varied galacturonate utilization abilities. Following a thorough analysis of the biosensor, we replaced the sfGFP reporter with genes encoding a heterologous D-glyceric acid pathway. We compared our feed-activated expression strategy to IPTG-inducible and constitutive expression circuits. Cells harboring our control circuit performed identically to those with the IPTG-inducible circuit in growth, pathway expression, and production. Furthermore, we observed that constitutive expression of the pathway resulted in poor cell growth and no production, likely due to its unmitigated metabolic burden. This work demonstrates that feed-activated pathway expression is an effective strategy for D-glyceric acid biosynthesis, and also confers benefits for scaled-up production.

2 | EXPERIMENTAL SECTION

2.1 | Strains and media

All plasmids were cloned in *E. coli* DH5 α . Biosensor variant characterization studies were performed in *E. coli* MG1655. Chromosomal deletion of *exuR* and *uxaC* from *E. coli* MG1655 (Table S1) was completed using the respective Keio strain^[38] in correspondence with the procedure described by Datsenko and Wanner.^[39] The primer pairs *dexuR*-f and *dexuR*-r; and *duxaC*-f and *duxaC*-r used for the respective knockouts were 200–500 bp away from the target gene (Table S2). D-Glyceric acid fermentations were conducted in *E. coli* MG1655(DE3) Δ garK Δ hyi Δ glxK Δ uxaC, as previously constructed.^[30] All growth and fermentation was done in Luria-Bertani (LB) broth (BD, Franklin Lakes, NJ).

2.2 | Cloning and plasmid construction

All primers, genetic parts, and plasmids used in this study are found in Tables S2–S4, respectively. Plasmids pP3XO-Ec and pP3XO-gfp, expressing the TF from promoter P3 and *sfGFP* from the proD promoter,^[40] were constructed using a modular Golden Gate strategy.^[41] pYTK095 from this strategy^[41] was used as the plasmid backbone. The *E. coli* and *B. subtilis* *exuR* genes were isolated by performing PCR with the appropriate genomic DNA as template and primers *exuR*-Ec-f and *exuR*-Ec-r; and *exuR*-Bs-f and *exuR*-Bs-r, respectively (Table S2). Downstream operators were inserted with primer pairs *exuO*-Ec-XO-f and *exuR*-Ec-XO-r; and *exuR*-Bs-XO-f and *exuR*-Bs-XO-r (Table S2) using the Q5 Site-Directed Mutagenesis Kit (NEB, Ipswich, MA).

Variants pP2XO-gfp, pP1XO-gfp, pP1OO-gfp, pP1OX-gfp, and pP1XX-gfp (Table S4) were constructed using the Q5 Site-Directed Mutagenesis Kit (NEB) and the following primers (Table S2): P2-f and P2-r; P1-f and P1-r; OO-f and OO-r; and OX-f and OX-r, respectively. The negative control containing no gene expressed from the hybrid promoter (pP1OO-empty in Table S4) was constructed using digestion with NdeI and ligation with T4 DNA Ligase; both enzymes were from NEB.

The biosensor controlled pathway and constitutive pathway plasmids (pP1OO-gli-udh and pP1XX-gli-udh in Table S4, respectively) were constructed with the PCR products of P1OO-f and P1OO-r on template pP1OO-gfp or P1XX-gfp, and gli-f and gli-r, plus udh-f and udh-r on the previously constructed pathway plasmid,^[30] using NEBuilder HiFi DNA Assembly Cloning Kit (NEB). RBS sequences for the operon (Table S3), calculated using the Operon Calculator function of the RBS Calculator,^[42] were included in the primers (Table S2). The IPTG-inducible pathway plasmid was constructed by combining the PCR product of *lacI*-Ec-f and *lacI*-Ec-r on the *E. coli* genome template and *lacO*-Ec-OO-f and *lacO*-Ec-OO-r on template pP1OO-gli-udh, using NEBuilder HiFi DNA Assembly Cloning Kit (NEB).

2.3 | Fluorescence measurements

Fluorescence characterization experiments were performed in the BioLector microbioreactor system (m2p-labs GmbH, Baesweiler, Germany). Overnight cultures were grown at 37°C with agitation at 250 rpm. One milliliter cultures were inoculated at a 1:100 dilution with the appropriate overnight culture in BioLector 48-well flower plates. Galacturonate was added at inoculation. The BioLector was set to 37°C, 1200 rpm shake speed, and 85% humidity. Continuous biomass (620 nm excitation) and GFP (488 nm excitation, 520 nm emission) measurements were taken in arbitrary BioLector units over a 24 h period.

2.4 | D-Glyceric acid fermentation

Cultures were grown in LB medium. All chemicals used for medium formulations and analytic standards were purchased from Sigma-Aldrich (St. Louis, MO). Overnight cultures were grown at 37°C with agitation at 250 rpm. Fifty milliliter fermentation cultures in 250 mL baffled flasks were inoculated from overnight cultures to an OD₆₀₀ of 0.05. The fermentation cultures were grown at 37°C with agitation at 250 rpm. 5 g L⁻¹ of D-galacturonate was added when the cultures reached an OD₆₀₀ of 0.55–0.75. For IPTG-inducible systems, 0.1 mM of IPTG was added either at inoculation or with D-galacturonate addition, approximately 3 h after inoculation. Samples were taken at regular intervals for analysis by HPLC, UV-Vis, and qRT-PCR.

2.5 | Galacturonate and D-glyceric acid quantification

Concentrations of galacturonate and D-glyceric acid in culture supernatants were determined using a 1200 Series Agilent Technologies instrument (Santa Clara, CA) with an Aminex HPX-87H Ion Exclusion Column (Bio-Rad Laboratories, Hercules, CA) and refractive index detector. The 22 min method ran an isocratic mobile phase of 5 mM sulfuric acid at 0.6 mL min⁻¹, with the column set to 65°C and the detector set to 35°C. Approximate elution times for galacturonate and D-glyceric acid were 8.6 and 11 min, respectively.

2.6 | Quantification of mRNA levels

Samples of 10⁹ cells were taken from the fermentation, assuming a conversion of OD₆₀₀ ~1 = 10⁹ cells mL⁻¹. The Illustra RNeasy Spin Kit (Cytiva, Marlborough, MA) was used for mRNA extraction. Reverse transcription was completed with the QuantiTect Reverse Transcription Kit (QIAGEN, Germantown, MD), using the kit specific protocol. Quantitative PCR reactions used Brilliant II SYBR qPCR High ROX Master Mix (Agilent Technologies, Santa Clara, CA), according to the master mix protocol, with primers for *udh* (*udh*-qRT-PCR-f and

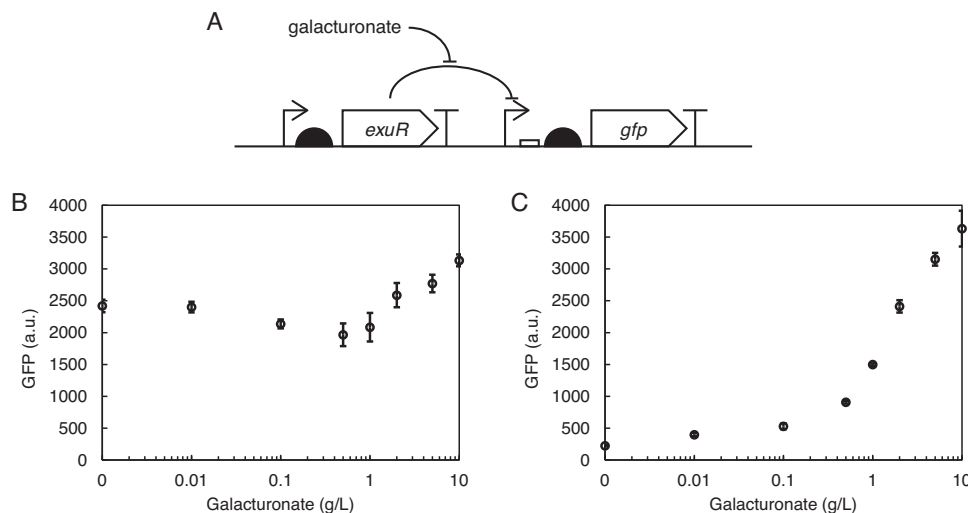


FIGURE 1 Overview of galacturonate biosensors. (A) Biosensor circuit design. A constitutively expressed *exuR* represses *gfp* expression by binding to its downstream operator in the hybrid promoter. De-repression and *gfp* expression occur in the presence of galacturonate. (B) GFP fluorescence response of the *E. coli* *exuR* biosensor to galacturonate. (C) GFP fluorescence response of the *B. subtilis* *exuR* biosensor to galacturonate. Data points are mean $\pm$ 1 SD of biological triplicates, taken 24 h after inoculation

udh-qRT-PCR-r in Table S2) and were performed in the ABI 7300 Real Time PCR System (Applied Biosystems, Foster City, CA). The thermal cycling conditions used are as follows: 50°C for 2 min., 95°C for 10 min., and 40 cycles of 95°C for 30 s and 60°C for 1 min. The ABI software determined Ct values and the fold differences were calculated between each sample and the uninduced biosensor controlled pathway at 3 h after inoculation.

3 | RESULTS AND DISCUSSION

3.1 | Design and selection of a TF-based galacturonate biosensor

Galacturonate is a sugar acid naturally metabolized by several microorganisms. In *E. coli*, expression of galacturonate transport and catabolic genes is activated in the presence of the substrate and controlled by the transcriptional repressor ExuR.^[43,44] *Bacillus subtilis* has an ExuR that regulates its galacturonate catabolism through a repression mechanism analogous to that of *E. coli*.^[37] These naturally occurring transcription regulators provide a basis for our galacturonate-responsive biosensor. The possibility of interactions between the host *E. coli* K-12 MG1655 endogenous regulatory proteins and the engineered biosensor motivates construction of a biosensor from genetic parts taken from heterologous microbes. Thus, we constructed two versions of the galacturonate biosensor: one with the ExuR from *E. coli* and one with the *B. subtilis* equivalent. Each biosensor comprised a constitutively expressed *exuR* using promoter BBa_J23101 from the Anderson promoter library (<http://parts.igem.org/Promoters/Catalog/Anderson>) and a hybrid promoter controlling the expression of an sfGFP reporter (Figure 1A). We designed the hybrid promoter using the insulated proD promoter^[40] and the corresponding ExuR DNA

binding site (i.e., the operator), placed downstream of the transcriptional start site (Figure S1). When placed downstream of or within a promoter, the operator allows ExuR to repress or de-repress transcription in the absence or presence of galacturonate, respectively. The *E. coli* ExuR operator sequence was found in the native galacturonate regulon and confirmed through DNA footprinting;^[44] similarly, the *B. subtilis* operator was isolated experimentally.^[37]

We measured GFP fluorescence as a response to varying concentrations of galacturonate addition to characterize the *E. coli* ExuR biosensor (Figure 1B) and the *B. subtilis* biosensor (Figure 1C). Both biosensors exhibit dose-dependent responses, in which higher concentrations of galacturonate achieve more de-repression of sfGFP, thus a higher fluorescence output. It is evident from these dose-response curves that the *B. subtilis* ExuR biosensor is superior, as the uninduced and maximal outputs were 225 and 3632 a.u., compared to 2419 and 3134 a.u. for the *E. coli* ExuR biosensor. Given our concerns with orthogonality between our biosensor and the host, and superior performance of the *B. subtilis* biosensor compared to the otherwise identical *E. coli* counterpart, we made variants of the *B. subtilis* biosensor for further characterization and application.

3.2 | Characterization of *B. subtilis* ExuR biosensor variants

We constructed variants of the *B. subtilis* *exuR* biosensor to select for two performance metrics: high dynamic range and low leakiness. We defined dynamic range as the highest GFP fluorescence output divided by the uninduced output; leakiness was quantified as the uninduced GFP signal divided by the no GFP control. Our variants employed a range of constitutive promoter strengths from the Anderson promoter library (<http://parts.igem.org/Promoters/Catalog/Anderson>) to

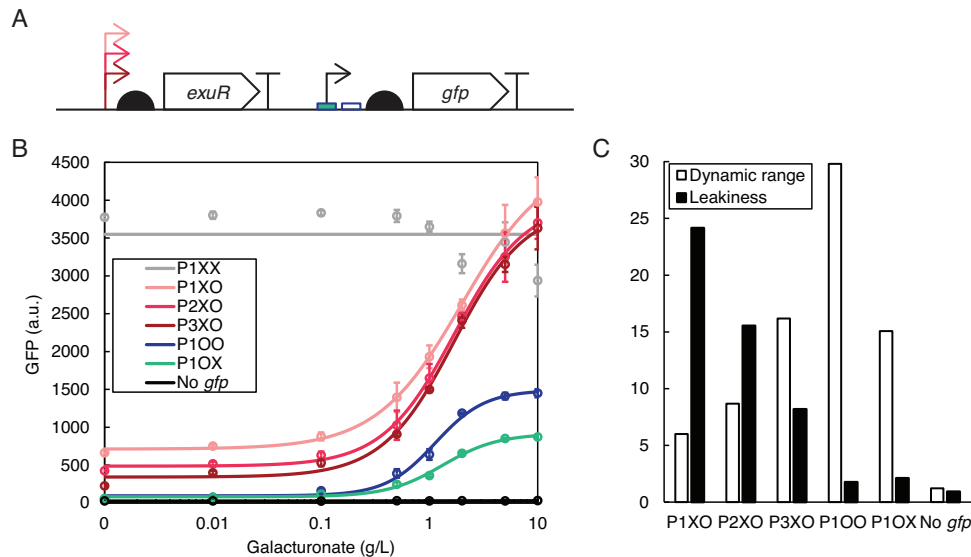


FIGURE 2 Comparison of *B. subtilis* *exuR* biosensor variants. (A) Overview of biosensor variant circuit. Variants expressed a constitutive *exuR* from a weak (P1/light pink), medium (P2/pink), or strong (P3/red) promoter. The presence or absence of an operator in the core or downstream of the -35 and -10 RNAP binding sites is denoted with an “O” or “X,” respectively. Variants of hybrid promoters contained an operator downstream of the -35 and -10 RNAP binding sites (XO/open rectangle), in the core position between the RNAP binding sites (OX/filled rectangle), or two operators placed in both positions (OO/blue rectangles). The hybrid promoter variant with no operators (XX) serves as a constitutive expression positive control. See Figure S1 for a more detailed schematic of the hybrid promoters used. (B) GFP fluorescence response of the biosensor variants to galacturonate. Data points are mean $\pm$ 1 SD of biological triplicates, taken 24 h after inoculation. (C) The average dynamic range and leakiness of each variant from its dose response curve

express *exuR* and altered the number and placement of operator sites in the hybrid promoter (Figure 2A). The promoters have reported relative strengths of 0.06, 0.36, and 0.7 and are referred to as P1, P2, and P3, respectively (Table S3). The placement of an operator is denoted with an O in our naming convention and a place without an operator is denoted with an X. The hybrid promoters had operators downstream of the promoter (XO), in the core position between the -35 and -10 σ^{70} RNA polymerase binding sites (OX), or in both positions (OO) (Figure S1). We did not test upstream placement of the operator as this position yields the least effective repression.^[45] The absence of an operator (XX) should leave nowhere for the ExuR to bind and regulate transcription, and acts as our constitutive, positive control (Figure S1). Figure 2B shows the galacturonate dose response and Hill fit curves of the biosensor variants. Decreasing the *exuR* promoter strength results in weaker repression and higher leakiness, as expected, since there are fewer repressor molecules in the cell (Figure 2C). The downstream operator variants have similar maximal GFP outputs, which was expected given that the hybrid promoter is identical among them. The placement of a core operator decreases the leakiness and results in stronger overall repression of the hybrid promoter, even with weak *exuR* expression. When there is an operator in both positions, the strong repression and low leakiness matches that of the core operator variant, but the maximum fluorescence is higher. Thus, the P1OO variant is our best performer, with the highest dynamic range and lowest leakiness (Figure 2C). These results are in agreement with previously characterized hybrid and natural promoter systems.^[45,46]

3.3 | Effect of endogenous regulation and catabolism on biosensor response

Though we identified robust biosensor variants with desirable performance in the wild type (WT) *E. coli* MG1655 host, we sought to characterize the effects of endogenous regulation and catabolism of the substrate on biosensor response. The homology of the *B. subtilis* putative galacturonate regulon to that of *E. coli* aided in the elucidation of its ExuR regulatory functions.^[37] Thus, it is plausible that the synthetic biosensor could have crosstalk with the *E. coli* host and its endogenous galacturonate-response ExuR. To examine the effect of the endogenous *E. coli* ExuR on our P1OO biosensor, we compared fluorescence characterization in *E. coli* MG1655 Δ *exuR* to the response in WT (Figure 3A). Using a Student's *t*-test with $\alpha = 0.01$ and a Bonferroni correction for each of the eight concentrations tested, we determined that the response in these two strains is not significantly different. These data indicate that the endogenous ExuR has no appreciable effect on the biosensor. The dynamic range and leakiness in the WT and Δ *exuR* strains are also highly similar: 29.8 and 28.9 -fold average dynamic range and leakiness values of 1.8 and 1.6, respectively (Figure 3B). We conclude that endogenous transcriptional regulation does not directly affect biosensor performance and the biosensor acts orthogonally to its host.

In addition to the endogenous *exuR*, the galacturonate catabolic genes remain intact in the WT host. Though the enzymes encoded by these genes do not directly interact with our biosensor, native catabolism of the substrate reduces the availability of the inducer to

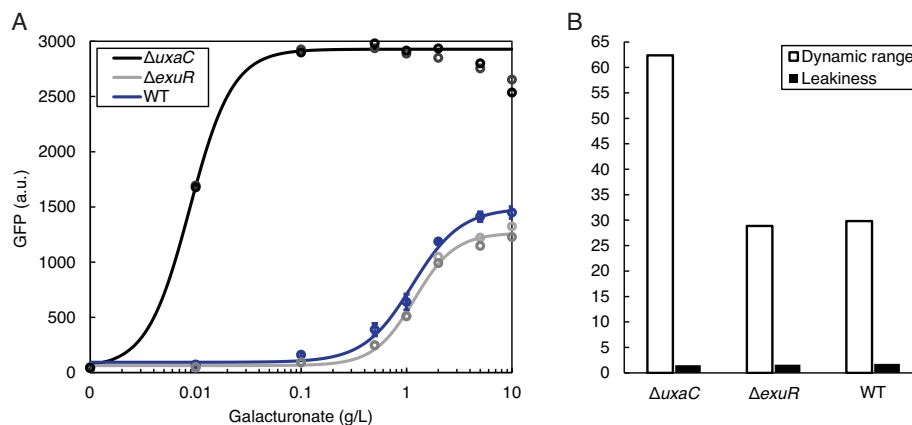


FIGURE 3 Effect of endogenous *exuR* and *uxaC* on biosensor response. (A) GFP fluorescence response of the P100 biosensor to galacturonate, in *E. coli* MG1655 $\Delta uxaC$ (black) and MG1655 $\Delta exuR$ (grey), compared to *E. coli* MG1655 WT (blue). Data points from the knockout strains are of individual biological duplicates, taken 24 h after inoculation. (B) The average dynamic range and leakiness of P100 in each strain from the corresponding dose response curve

de-repress *sfGFP* expression. Galacturonate was fully consumed by 24 h, regardless of initial concentration added, in all characterization experiments performed in the WT strain (data not shown). We expect galacturonate consumption in the end application of the biosensor, since it was constructed to control expression of a galacturonate-utilizing pathway. However, knocking out the endogenous *uxaC*, the galacturonate isomerase that implements the first step of catabolism, allowed us to compare P100 biosensor induction in the presence and absence of galacturonate catabolism. The latter represents a performance ceiling for the biosensor where galacturonate can achieve its full de-repression potential. From the dose response curves of the biosensor in *E. coli* MG1655 and *E. coli* MG1655 $\Delta uxaC$ (Figure 3A), we see that more initial galacturonate is required to achieve higher GFP fluorescence in the WT strain containing *uxaC*. This result is expected since catabolism of galacturonate lowers the effective concentration within the cell. Though there is a stark difference between the response curves of the two strains, both have favorable performance metrics,

as defined above: 29.8 and 62.4-fold average dynamic range and leakiness values of 1.8 and 1.5, for *E. coli* MG1655 and *E. coli* MG1655 $\Delta uxaC$, respectively (Figure 3B). The favorable performance of the biosensor in both the WT and $\Delta uxaC$ strains demonstrates that the choice of strain is flexible, regardless of whether *uxaC* is necessary for the desired application. Additionally, the biosensor could serve as a chemical induction system in which galacturonate induces expression, similarly to IPTG.

3.4 | Feed activated expression of a D-glyceric acid production pathway

The galacturonate biosensor can be used to control the expression of a galacturonate-utilizing pathway in a feed responsive manner, as demonstrated by the high dynamic range of *sfGFP* reporter output; the low leakiness prevents unwanted expression of pathway enzymes before galacturonate addition. We demonstrated this application with

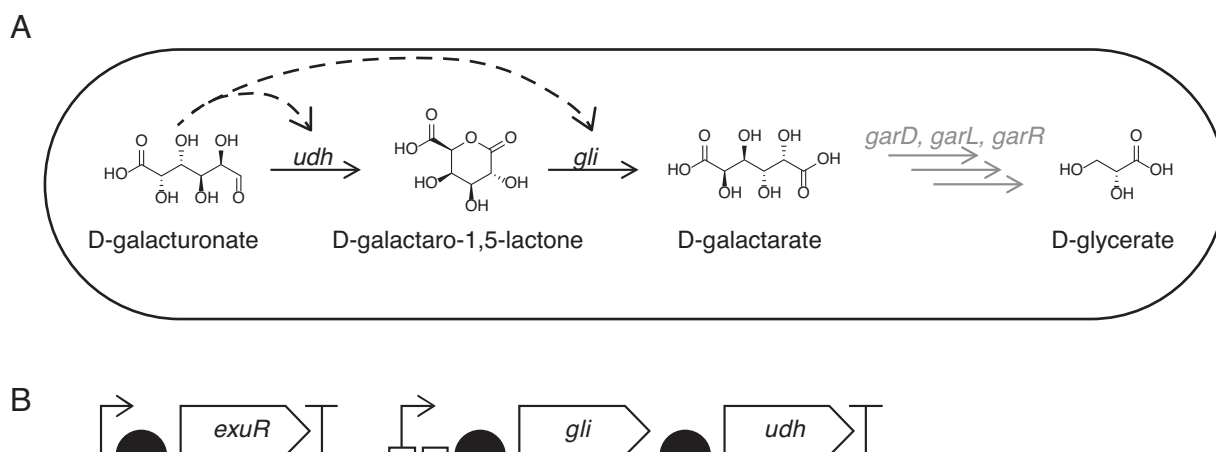


FIGURE 4 Overview of galacturonate activated expression of D-glyceric acid biosynthetic pathway. (A) Schematic of feed activated pathway expression. Galacturonate induces the expression of the heterologous pathway genes, *udh* and *gli*; endogenous enzymes complete the pathway to produce D-glyceric acid. (B) Genetic circuit of P100 controlling the D-glyceric acid pathway. The heterologous pathway genes form an operon

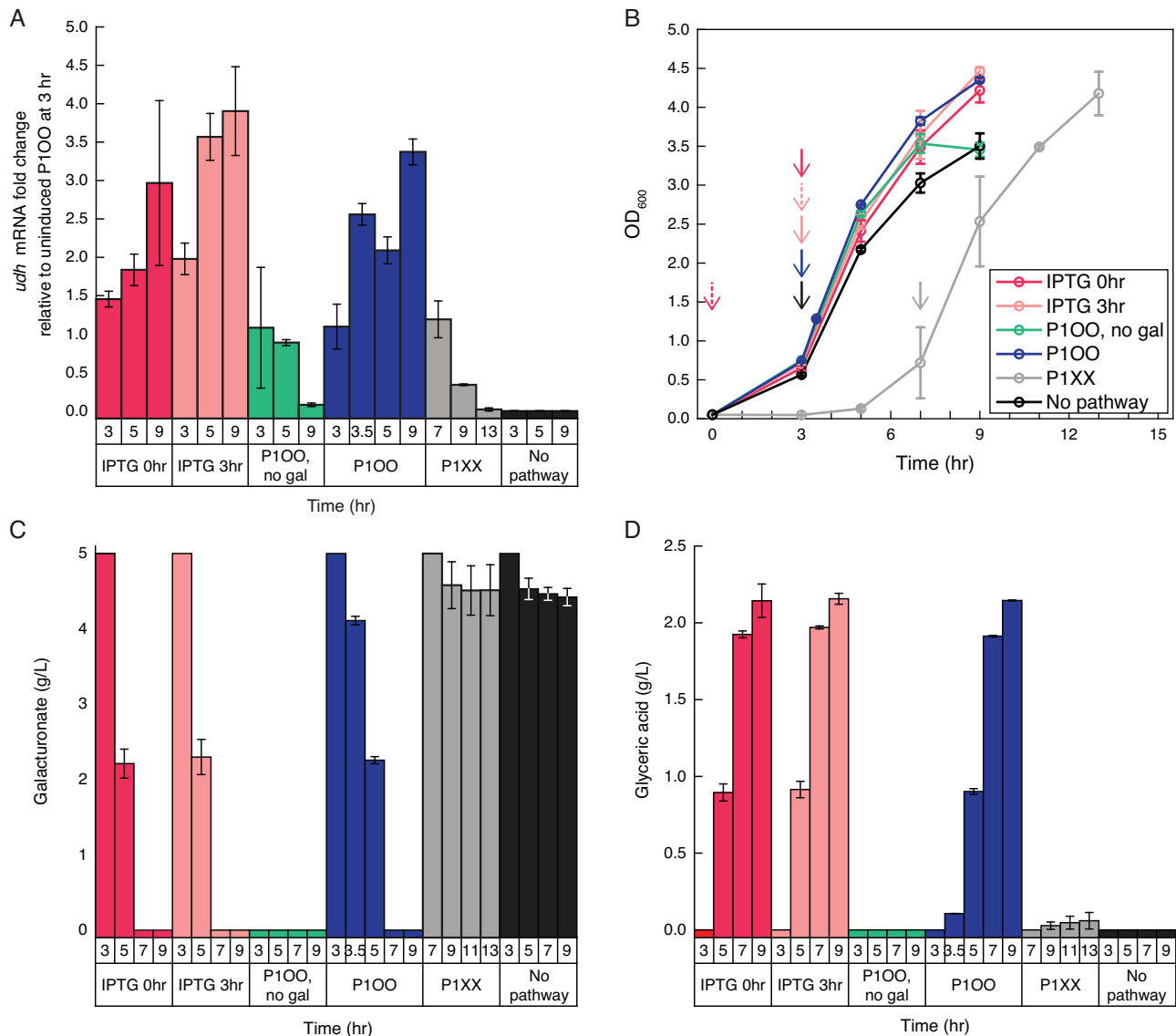


FIGURE 5 Fermentation time series data of mRNA fold change, biomass, galacturonate, and D-glyceric acid. (A) *udh* mRNA fold change relative to the uninduced P1OO levels 3 h after inoculation, over time. (B) OD₆₀₀ over time. Dotted arrows indicate IPTG addition; solid arrows indicate galacturonate addition. (C) Galacturonate concentration over time. (D) D-Glyceric acid concentration over time. Data are mean $\pm$ 1 SD of biological duplicates

the D-glyceric acid production pathway previously developed in our group.^[30] The pathway utilizes enzymes encoded by two heterologous genes, uronate dehydrogenase (*udh*) and galactarolactone isomerase (*gli*), to generate the intermediate D-galactarate. D-Galactarate is converted to D-glyceric acid by enzymes encoded by the endogenous genes galactarate dehydratase (*garD*), 5-keto-4-deoxy-D-glucarate aldolase (*garL*), and 2-hydroxy-3-oxopropionate reductase (*garR*) (Figure 4A, Table S1). The *udh* and *gli* genes were expressed from the P1OO biosensor circuit as an operon (Figure 4B). Consequently, the presence of the galacturonate feed de-represses ExuR from the hybrid promoter and induces transcription of the heterologous pathway genes (Figure 4A). To characterize this system, we conducted fermentations with the MG1655(DE3) Δ gark Δ hyi Δ glxK Δ uxaC strain used previously.^[30]

We verified that the control circuit modulates transcription in response to galacturonate by quantifying the relative mRNA levels of *udh*, the second gene in the operon, over the course of a fermentation using qRT-PCR (Figure 5A). For all samples, the fold change of mRNA was analyzed relative to P1OO levels before galacturonate addition. We constructed an IPTG-inducible variant of the P1OO control circuit that employs LacI in place of ExuR and two *lac* operators^[47] to benchmark the galacturonate-inducible circuit. IPTG addition was tested in two conditions: at inoculation and 3 h after inoculation. Both cases show an increase in *udh* expression over time. The 3 h IPTG addition resulted in higher overall *udh* levels (3.9 ± 0.6 fold change compared to 3 ± 1 for 0 h addition, at 9 h post inoculation), likely due the growth advantage of delayed pathway induction, discussed further below. Galacturonate added at 3 h post inoculation to the P1OO controlled pathway resulted

in a 3.4 ± 0.2 *udh* fold change at 9 h post inoculation. The sample taken just after induction with galacturonate shows a spike in *udh* expression that falls slightly as cell growth slows. The 5 h *udh* level matches those of the IPTG induced cells. We can conclude that the changes in pathway mRNA expression result from the transcription factor and hybrid promoter interactions in response to galacturonate. Thus, the galacturonate controlled circuit is a direct and effective replacement for the IPTG inducible expression system.

As expected, the uninduced P1OO-controlled pathway maintains consistently low levels of *udh* transcript, which drops over the course of the fermentation as is common with constitutive type expression.^[48] This behavior is also seen in the constitutive P1XX case. While the drop in transcription was expected, the low values of mRNA present in the P1XX containing cells surprised us. We expected high relative *udh* levels based on the sfGFP fluorescence characterization. The cause of this became clear as we looked at the biomass, and galacturonate and D-glyceric acid concentrations profiles taken throughout the fermentations (Figure 5B-D).

The growth profiles of all the strains are similar (Figure 5B), with the exception of the constitutive pathway harboring strain. The final OD is lower for the strain with no pathway and the strain that was not fed galacturonate. This can be explained by the pyruvate byproduct produced by the D-glyceric acid pathway, which gives cells a growth advantage. We see a substantial lag in the constitutive expression strain, suggesting the pathway introduces significant burden to cell growth. The slow growth and low mRNA presence in the constitutive pathway strain indicate that the perpetual presence and generation of the heterologous enzymes is detrimental to cell health. This is further supported by observed inability of the strain to consume galacturonate and produce D-glyceric acid (Figure 5C,D). Thus, cell health and D-glyceric acid production require an inducible production pathway, as demonstrated by the IPTG-inducible and P1OO controlled cases. The galacturonate utilization and D-glyceric acid production profiles are almost identical when the pathway is induced (Figure 5C,D). These strains consumed all 5 g L^{-1} of galacturonate within 4 h of addition. IPTG induction at inoculation, 3 h after inoculation, and galacturonate induction 3 h after inoculation resulted in titers of 2.1 ± 0.1 , 2.16 ± 0.04 , and $2.146 \pm 0.003 \text{ g L}^{-1}$ D-glyceric acid and molar yields of 78%, 79%, and 78%, respectively. From these results, we see that we can effectively replace the IPTG-inducible system controlling the D-glyceric acid pathway with one that only requires the pathway feed, galacturonate. This was achieved without compromising pathway performance, as observed by feed utilization and product formation.

4 | CONCLUSION

Metabolite-responsive regulatory systems can be leveraged to build substrate-inducible, feed-forward expression control strategies. We constructed a galacturonate biosensor with a heterologous metabolite-responsive TF from *B. subtilis* and its cognate DNA binding sites, and used fluorescence characterization to select a top per-

forming variant. The biosensor has minimal crosstalk with the native *E. coli* galacturonate-responsive regulator and exhibits favorable output despite full consumption of the galacturonate feed. Thus, the biosensor is an effective induction system that removes extrinsic chemical inducers for applications in which galacturonate is the feed substrate, but it could also be used generally as a chemical induction system. To demonstrate the former case, we used the biosensor to activate expression of a D-glyceric acid production pathway in the presence of its galacturonate feed. We determined that pathway induction is necessary, as constitutive expression resulted in poor cell growth and no product formation. Strains harboring the D-glyceric acid pathway expressed from our galacturonate-induced system performed identically to an IPTG-induced system in growth, feed utilization, and production. This work demonstrates that feed-activated pathway expression enables product biosynthesis while removing extrinsic chemical inducers from a microbial production system.

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CONFLICT OF INTEREST

The authors declare no commercial or financial conflict of interest.

AUTHOR CONTRIBUTIONS

Cynthia Ni: conceptualization; data curation; formal analysis; investigation; methodology; visualization; writing-original draft; writing-review & editing. Kevin J. Fox: conceptualization; data curation; investigation; methodology; writing-original draft. Kristala L. J. Prather: conceptualization; data curation; formal analysis; funding acquisition; methodology; project administration; resources; supervision; visualization; writing-review & editing.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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