# SI Appendix

***Layered dynamic regulation for improving metabolic pathway productivity in Escherichia coli***

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## Supplementary Tables

Table S1. List of hybrid promoters tested in SI Appendix, Figure S1. The -35 and -10 sites are capitalized, and transcription start site denoted in red. The IpsA-binding sites are underlined.

| Promoter | Sequence |
|---|---|
| cgA | TTTACAgaattgatcgatcaagcTATTATgctagcAcgtgcaatttttaaaattaaaggcgttacccaac |
| cgAp | TTTACAgctagctcagtcctaggTATTATgctagcAaattgatcgatcaagcttaaaggcgttacccaac |
| cg44 | TTTACAgtctttattgattcagtTATTATgctagcAcgtgcaatttttaaaattaaaggcgttacccaac |
| cg44p | TTTACAgctagctcagtcctaggTATTATgctagcAtctttattgattcagtttaaaggcgttacccaac |

Table S2. List of promoters used for driving IpsA expression. All promoter sequences are derived from the Registry of Standard Biological Parts (1). The -35 and -10 sites are capitalized.

| Promoter | Sequence | Parts Registry Identifier |
|---|---|---|
| P1 | TTGACGgctagctcagtcctaggTATAGTgctagc | Bba_J23111 |
| P2 | TTTACGgctagctcagtcctaggTATAGTgctagc | Bba_J23106 |
| P3 | TTGACAgctagctcagtcctaggTATTGTgctagc | Bba_J23104 |
| P4 | TTTACGgctagctcagtcctaggTACTATgctagc | Bba_J23105 |
| P5 | TTTACGgctagctcagccctaggTATTATgctagc | Bba_J23107 |

Table S3. List of strains used in this study.

| Strain Name | Genotype | Description | Reference |
|---|---|---|---|
| | MG1655 (DE3) *ΔuxaC ΔgudD* | DE3 lysogen and *uxaC*, *gudD* knockouts | (2) |
| IB1379GA | MG1655 (DE3) *ΔendA Δzwf ΔPfkB ΔuxaC ΔgudD* | DE3 lysogen and *uxaC*, *gudD* knockouts Wildtype *pfkA* | (3) |
| L19GA | MG1655(DE3) *ΔendA Δzwf ΔpfkB ΔuxaC ΔgudD pfkA::PesaS-pfkA(LAA) HK022::104-esaRI170V 186(O)::**apFAB295-apFAB699**-esaI* | IB1379GA + *pfkA* driven by $P_{esaS}$ promoter + *esaI* driven by selected promoter and RBS sequence with predicted strength 1.7% | (4) |
| L24GA | MG1655(DE3) *ΔendA Δzwf ΔpfkB ΔuxaC ΔgudD pfkA::PesaS-pfkA(LAA) HK022::104-esaRI170V 186(O)::**apFAB296-apFAB700**-esaI* | L19GA with predicted strength 4.8% | (4) |
| L31GA | MG1655(DE3) *ΔendA Δzwf ΔpfkB ΔuxaC ΔgudD pfkA::PesaS-pfkA(LAA) HK022::104-esaRI170V 186(O)::**apFAB65-apFAB700**-esaI* | L19GA with predicted strength 1.2% | (4) |

Table S4. Glucaric acid (GA) and *myo*-inositol (MI) titer for layered regulation strains (SI Appendix, Table S3) after 72 hours fermentation in T12 with 10 g/L glucose. Values reported as described in the table legend.

| | **No IpsA** | | | **P1** | | | **P2** | | |
|---|---|---|---|---|---|---|---|---|---|
| **IB1379GA** | 0.20 ± 0.00 | 0.14 ± 0.01 | | 0.58 ± 0.01 | 0.33 ± 0.02 | | 0.65 ± 0.02 | 0.29 ± 0.01 | |
| | 0.96 ± 0.01 | 0.75 ± 0.06 | 1.71 | 2.77 ± 0.06 | 1.83 ± 0.09 | 4.60 | 3.10 ± 0.08 | 1.63 ± 0.07 | 4.73 |
| **L31GA** | 0.59 ± 0.02 | 0.19 ± 0.02 | | 0.79 ± 0.02 | 0.24 ± 0.00 | | 0.64 ± 0.03 | 0.57 ± 0.03 | |
| | 2.82 ± 0.10 | 1.07 ± 0.11 | 3.89 | 3.75 ± 0.09 | 1.35 ± 0.02 | 2.96 | 3.04 ± 0.16 | 3.19 ± 0.17 | 4.62 |
| **L19GA** | 0.43 ± 0.04 | 0.35 ± 0.03 | | 0.63 ± 0.02 | 0.19 ± 0.00 | | 1.84 ± 0.14 | 0.32 ± 0.04 | |
| | 2.03 ± 0.18 | 1.96 ± 0.17 | 3.99 | 2.99 ± 0.11 | 1.07 ± 0.02 | 4.06 | 8.75 ± 0.65 | 1.79 ± 0.20 | 10.54 |
| **L24GA** | 0.00 ± 0.00 | 0.16 ± 0.01 | | 0.45 ± 0.05 | 0.15 ± 0.01 | | 0.74 ± 0.05 | 0.20 ± 0.01 | |
| | 0.00 ± 0.00 | 0.90 ± 0.07 | 4.77 | 2.13 ± 0.23 | 0.84 ± 0.04 | 5.09 | 3.52 ± 0.22 | 1.10 ± 0.05 | 6.23 |

| | **P3** | | | **P4** | | | **P5** | | |
|---|---|---|---|---|---|---|---|---|---|
| **IB1379GA** | 0.53 ± 0.05 | 0.26 ± 0.02 | | 0.29 ± 0.01 | 0.29 ± 0.01 | | 0.27 ± 0.01 | 0.23 ± 0.01 | |
| | 2.51 ± 0.23 | 1.44 ± 0.11 | 3.95 | 1.40 ± 0.03 | 1.61 ± 0.03 | 3.01 | 1.28 ± 0.04 | 1.26 ± 0.03 | 2.54 |
| **L31GA** | 0.63 ± 0.03 | 0.49 ± 0.02 | | 0.19 ± 0.00 | 0.12 ± 0.01 | | 0.64 ± 0.08 | 0.20 ± 0.01 | |
| | 3.01 ± 0.15 | 2.74 ± 0.08 | 5.75 | 0.91 ± 0.02 | 0.68 ± 0.03 | 3.76 | 3.05 ± 0.36 | 1.11 ± 0.06 | 2.13 |
| **L19GA** | 1.69 ± 0.04 | 0.45 ± 0.01 | | 0.44 ± 0.03 | 0.22 ± 0.02 | | 0.17 ± 0.01 | 0.14 ± 0.00 | |
| | 8.03 ± 0.17 | 2.49 ± 0.04 | 10.52 | 2.10 ± 0.14 | 1.21 ± 0.09 | 3.30 | 0.79 ± 0.03 | 0.76 ± 0.00 | 1.55 |
| **L24GA** | 0.33 ± 0.01 | 0.18 ± 0.01 | | 0.36 ± 0.02 | 0.37 ± 0.01 | | 0.24 ± 0.03 | 0.18 ± 0.01 | |
| | 1.56 ± 0.03 | 1.02 ± 0.03 | 0.91 | 1.69 ± 0.08 | 2.07 ± 0.06 | 1.59 | 1.13 ± 0.16 | 0.99 ± 0.03 | 4.16 |

Table Legend.

| | **Promoter Driving *ipsA*** | | |
|---|---|---|---|
| **Strain Name** | GA (g/L) ± 1 S.D. | MI (g/L) ± 1 S.D. | |
| | GA (mM) ± 1 S.D. | MI (mM) ± 1 S.D. | Sum of GA+MI (mM) |

Table S5. List of primers used for plasmid construction.

| **Name** | **Sequence** |
|---|---|
| pHH-GFP-R | gatcttccccatcggtgatgagccagtgtgactctagta |
| cg44-RBS-GFP-F1 | tagcacgtgcaatttttaaaattaaaggcgttacccaacagaggagaaatactagatgcg |
| cg44-RBS-GFP-F2 | agtctttattgattcagttattatgctagcacgtgcaatttttaaa |
| cg44-pHH-A | gcataataactgaatcaataaagactgtaaagtcggcgatataggcgc |
| GFP-pHH-B | tactagagtcacactggctcatcaccgatggggaagatc |
| P2-IpsA-F | ctaggtatagtgctagcattcgtgcaatttttatgaggtaccgaattctcac |
| P2-pHH-R | cacgaatgctagcactatacctaggactgagctagccgtaaactcgagccaggcatc |
| P1-IpsA-F | gtcctaggtatagtgctagcattcgtgcaatttttatgaggtaccgaattctcac |
| P1-pHH-R | gctagcactatacctaggactgagctagccgtcaactcgagccaggcatc |
| P5-IpsA-F | gccctaggtattatgctagcattcgtgcaatttttatgaggtaccgaattctcac |
| P5-IpsA-F | gctagcataatacctagggctgagctagccgtaaactcgagccaggcatc |
| XhoI-P3-F | aaaaactcgagttgacagctagctcagtcctaggtattgtgctagc |
| KpnI-P3-R | aaaaaggtacctcataaaaattgcacgaatgctagcacaatacctaggac |
| XhoI-P4-F | aaaaaggtacctcataaaaattgcacgaatgctagcatagtacctaggactgag |
| KpnI-P4-R | aaaaactcgagctgatagctagctcagtcctagggattatgctagc |
| EcoRI-IpsA-F | tgcagaattcaaagaggagaaatactagatgattatgggtaggaaacaacaatacgga |
| BamHI-IpsA-R | agttggatccctagattggcgcaaccgtgg |
| NotI-pHH-F | gcggccgctactagagccaggcatcaaat |
| SacI-pHH-R | gagctctctcctctgttgggtaacgc |
| pHH-NotI-CAT-R | ctggctctagtagcggccgcttacgccccgccctg |
| pHH-SacI-CAT-F | cccaacagaggagagagctcatggagaaaaaaatcactggatatacc |
| SacI-MIOX-F | aaaaagagctcatgaaagttgatgttggtcctg |
| NotI-MIOX-R | aaaaagcggccgcttaccaggacagggtgc |
| Carb-C-RSFD | gcctcactgattaagcattggtaagaattaattcatgagcggatacat |
| Carb-N-RSFD | gacacggaaatgttgaatactcatactcttccttttttcaatattattgaagc |
| RSFD-Carb-C | atgtatccgctcatgaattaattcttaccaatgcttaatcagtgaggc |
| RSFD-Carb-N | gcttcaataatattgaaaaaggaagagtatgagtattcaacatttccgtgtc |
| cg44-flp-F | ctttacaactgaatcaataaagagtattatgctagcacgtgc |
| cg44p-RBS-GFP-F | tagcatctttattgattcagtttaaaggcgttacccaacagaggagaaatactagatgcg |
| cg44p-RBS-GFP-F2 | ctagctcagtcctaggtattatgctagcatctttattgattcagtttaaagg |
| cgAp-RBS-GFP-F | tagcaaattgatcgatcaagcttaaaggcgttacccaacagaggagaaatactagatgcg |
| cgAp-RBS-GFP-F2 | agtcctaggtattatgctagcaaattgatcgatcaagctt |

Table S6. List of plasmids used in this study.

| **Plasmid** | **Genotype** | **Reference** |
|---|---|---|
| pHHD01K | Empty vector, parent plasmid for sensors | (5) |
| pHH-cg44-GFP-P1-ipsA … pHH-cg44-GFP-P5-ipsA | pHHD01K with *gfp* expressed from hybrid promoter; *ipsA* expressed from constitutive promoters P1-P5 | this study |
| pHH-cg44-MIOX-P1-ipsA … pHH-cg44-MIOX-P5-ipsA | pHHD01K with *Miox* expressed from hybrid promoter; *ipsA* expressed from constitutive promoters P1-P5 | this study |
| pHH-cg44-GFP-Ptet-ipsA | pHHD01K with *gfp* expressed from hybrid promoter; *ipsA* expressed from aTc-inducible Ptet promoter | this study |
| pHH-cg44p-GFP-Ptet-ipsA | same as pHH-cg44-GFP-Ptet-ipsA with IpsA binding site cg0044 located downstream of the -10 region | this study |
| pHH-cgA-GFP-Ptet-ipsA | pHHD01K with *gfp* expressed from hybrid promoter variant cgA, containing one of the IpsA binding sites from *C. glutamicum* gene cg3323 located between the -35 and -10 regions; *ipsA* expressed from aTc-inducible Ptet promoter | this study |
| pHH-cgAp-GFP-Ptet-ipsA | same as pHH-cgA-GFP-Ptet-ipsA with cg3323A binding site located downstream of the -10 region | this study |
| pTrc-Ino1 | pTrc99A with *S. cerevisiae INO1* | (6) |
| pRSFDCarb-IN-Udh | pRSFDuet with carbenicillin resistance in place of kanamycin resistance, *S. cerevisiae INO1*, and *A. tumefaciens udh* | this study |
| pHH-cg44-P2-ipsA | pHH-cg44-GFP-P2-ipsA with multiple cloning site in place of *gfp* | this study |
| pHH-cg44-MIOX | pHHD01K with *Miox* expressed from hybrid promoter | this study |
| pHH-J101-MIOX-P2-ipsA | pHH-cg44-MIOX-P2-ipsA with constitutive (unregulated) promoter J23101 in place of hybrid promoter cg44 | this study |

**Supplementary Figures**

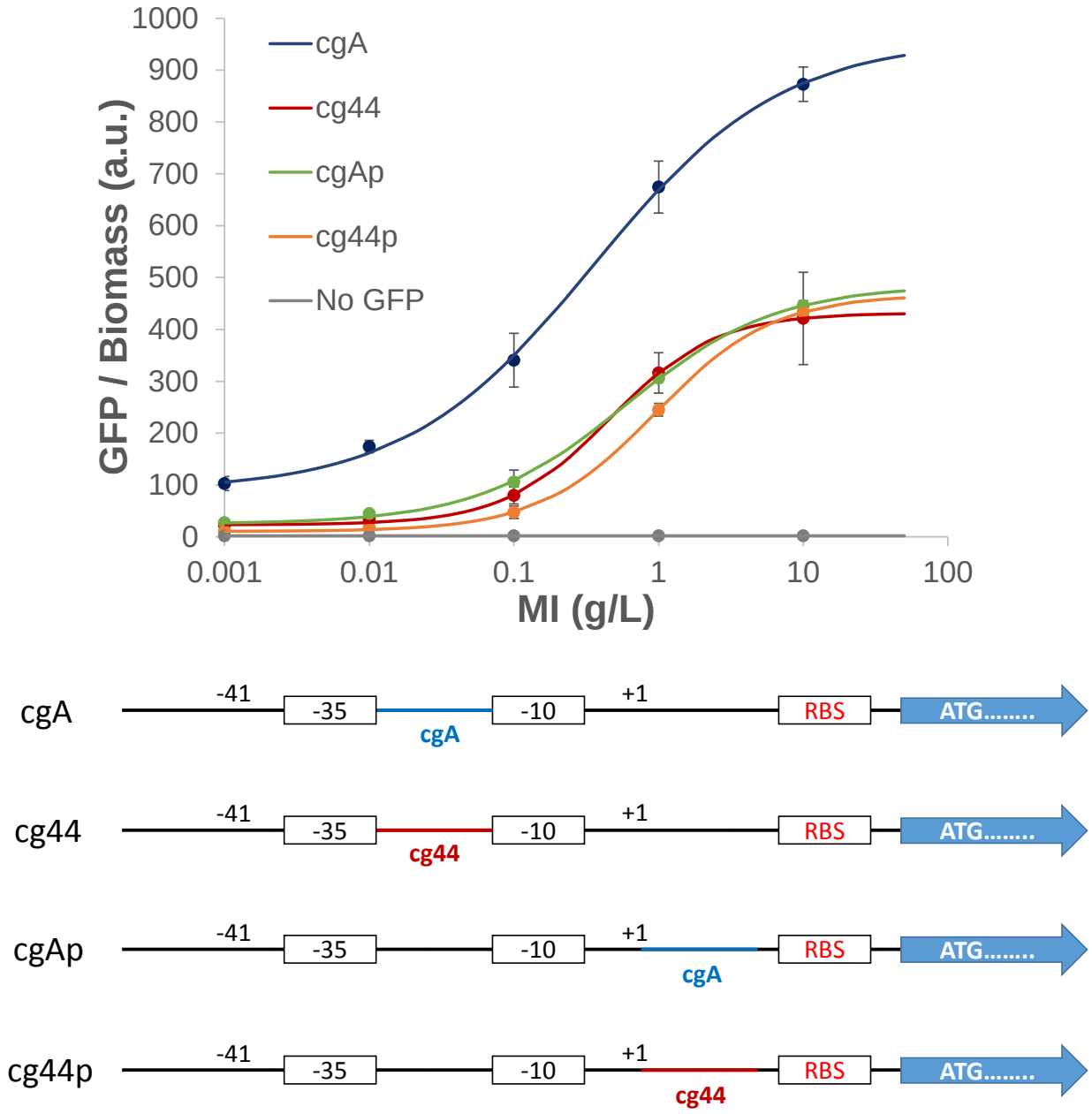


Figure S1. GFP fluorescence normalized to biomass in MG1655 (DE3) cells harboring sensor plasmids with hybrid promoter variants as a response to MI added to LB medium. Hybrid promoter designs are shown below. Sequences of the promoter variants are found in SI Appendix, Table S1. Measurements were taken 24 hours post-inoculation. "No GFP" represents the empty vector parent plasmid used to construct the sensor plasmids, without *ipsA* expressed. Individual points with error bars represent the mean ± 1 S.D. of three replicate cultures. Data points were fitted to a dose-response transfer function to yield smooth curves.

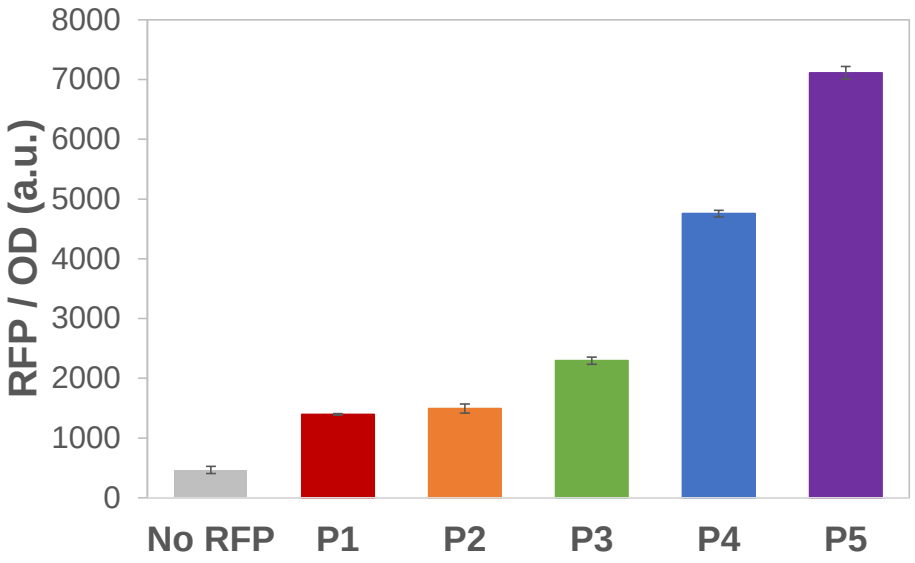


Figure S2. Relative strengths of the promoters (P1-P5) used to drive *ipsA* expression, as measured by RFP fluorescence. MG1655 (DE3) cells harboring the plasmids with promoters P1-P5 driving *rfp* were cultured in LB medium for 24 hours prior to fluorescence measurement. Error bars denote ± 1 S.D. from the mean of three replicate cultures.

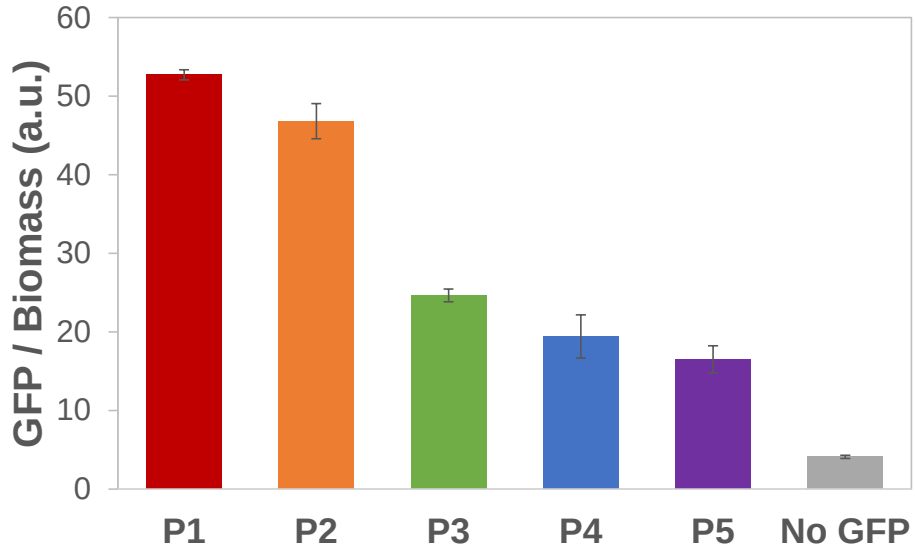


Figure S3. Leakage level (GFP fluorescence at 0 g/L MI) for each sensor variant. MG1655 (DE3) cells harboring the sensor plasmids with promoters P1-P5 driving *ipsA* were cultured in LB medium for 24 hours prior to fluorescence measurement. “No GFP” represents the empty vector parent plasmid used to construct the sensor plasmids, without *ipsA* expressed. Error bars denote ± 1 S.D. from the mean of three replicate cultures.

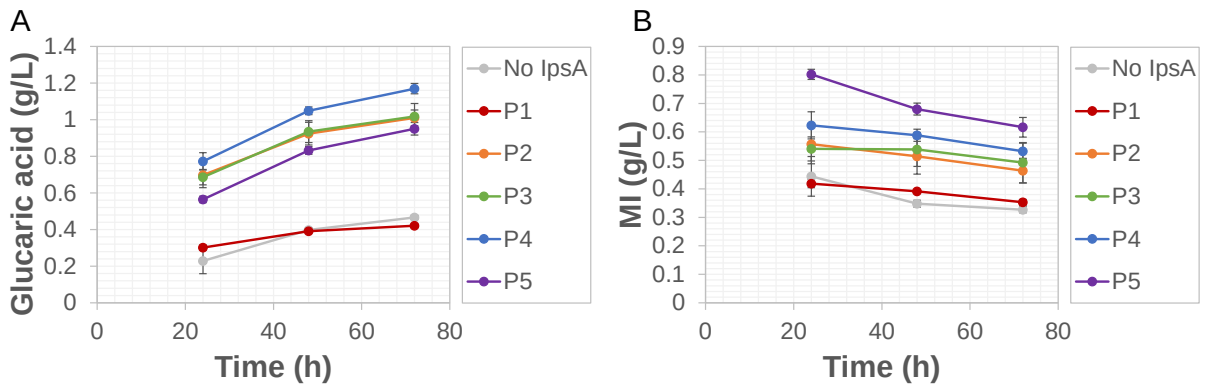


Figure S4. Production of (A) glucaric acid and (B) MI over time from MI-controlled expression of *Miox* in MG1655 (DE3) Δ*gudD* Δ*uxaC*. IpsA is expressed from promoters P1-P5. No IpsA: constitutive (unregulated) *Miox* expression. Error bars represent ± 1 S.D. from the mean of three replicate flasks.

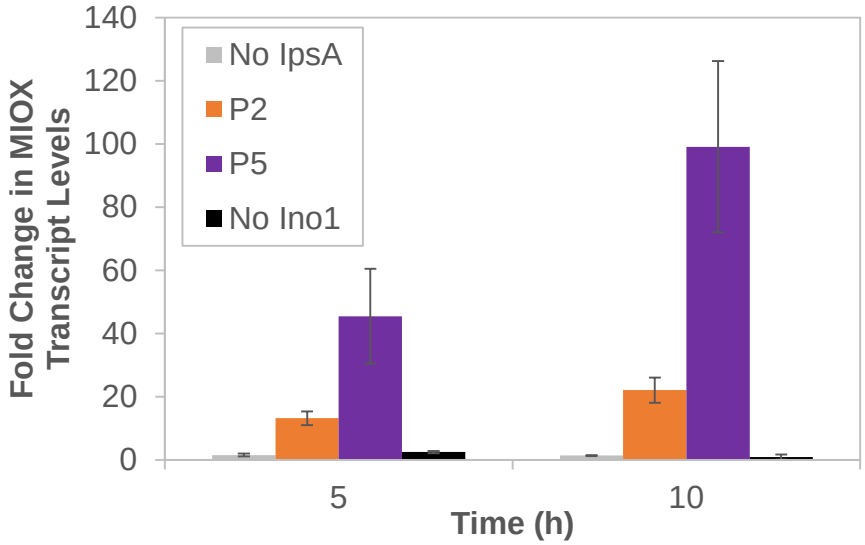


Figure S5. Fold change of *Miox* transcript levels at 5 and 10 hours post-inoculation for MG1655 (DE3) *ΔuxaC ΔgudD* as measured by qRT-PCR. Values reported as the fold change over the initial time point for each strain. IpsA is expressed from promoters P2 and P5. No IpsA: constitutive *Miox* control. No Ino1: no MI production control, which ensures continuous repression of *Miox*. Error bars represent ± 1 S.D. from three replicate flasks.

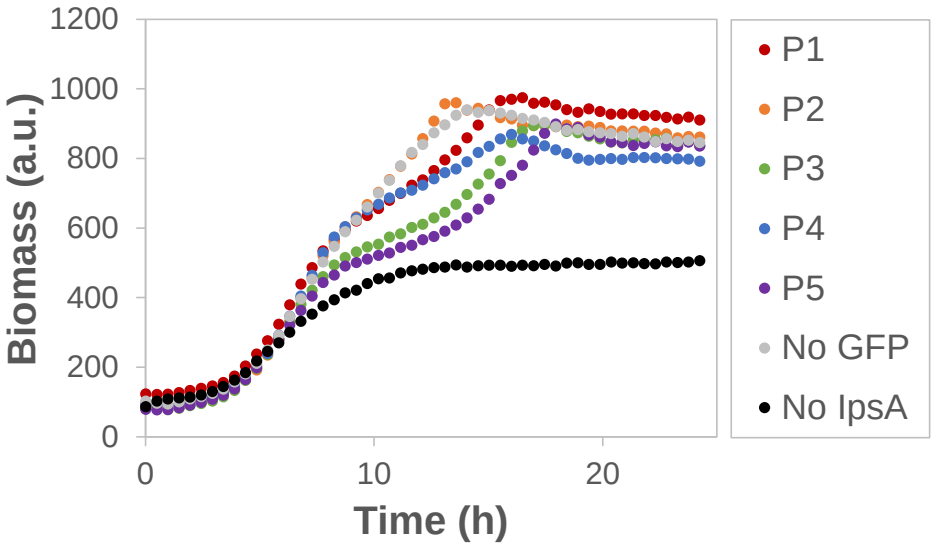


Figure S6. Growth curves for MG1655 (DE3) cells expressing the sensor circuit and *INO1*, measured in BioLector biomass units (a.u.). The GFP negative control using the parent plasmid for the sensor (denoted "No IpsA") demonstrated poor growth compared to strains with promoters P1-P5 driving *ipsA*. A GFP negative control with *ipsA* expressed from P2 (denoted "No GFP") demonstrated comparable growth curves to strains with promoters P1-P5, suggesting that expression of *ipsA* leads to a boost in growth when *INO1* is simultaneously expressed from a separate plasmid. The boost in growth was not observed in strains expressing all three heterologous glucaric acid pathway enzymes (SI Appendix, Fig. S6). Values represent the mean of three replicate cultures.

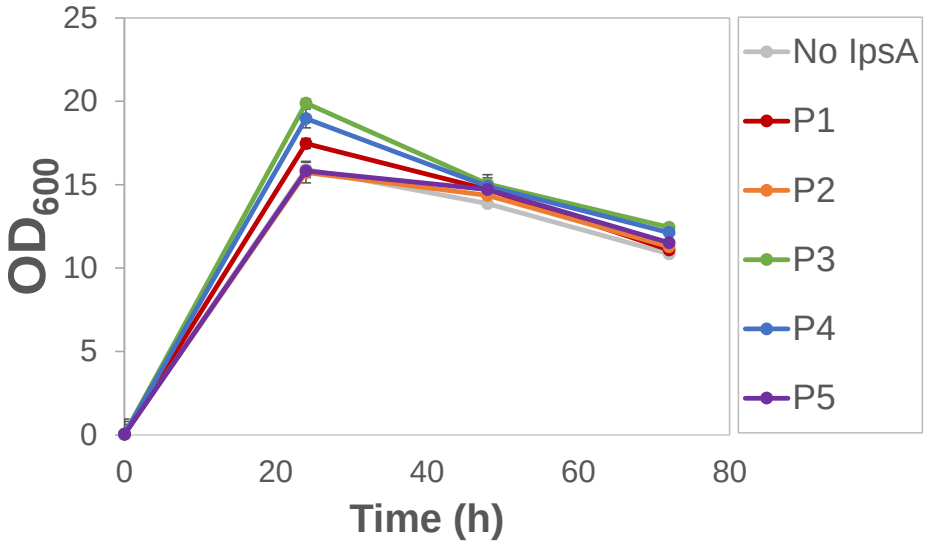


Figure S7. Optical density measurements at 24, 48, and 72 hours post inoculation for MG1655 (DE3) Δ*uxaC* Δ*gudD* cells harboring plasmids encoding the glucaric acid pathway genes with *Miox* regulated by IpsA. Cells were cultured in T12 medium with 10 g/L glucose. Corresponding MI and glucaric acid titers are shown in Fig. 3. Error bars denote ± 1 S.D. from the mean of three replicate cultures.

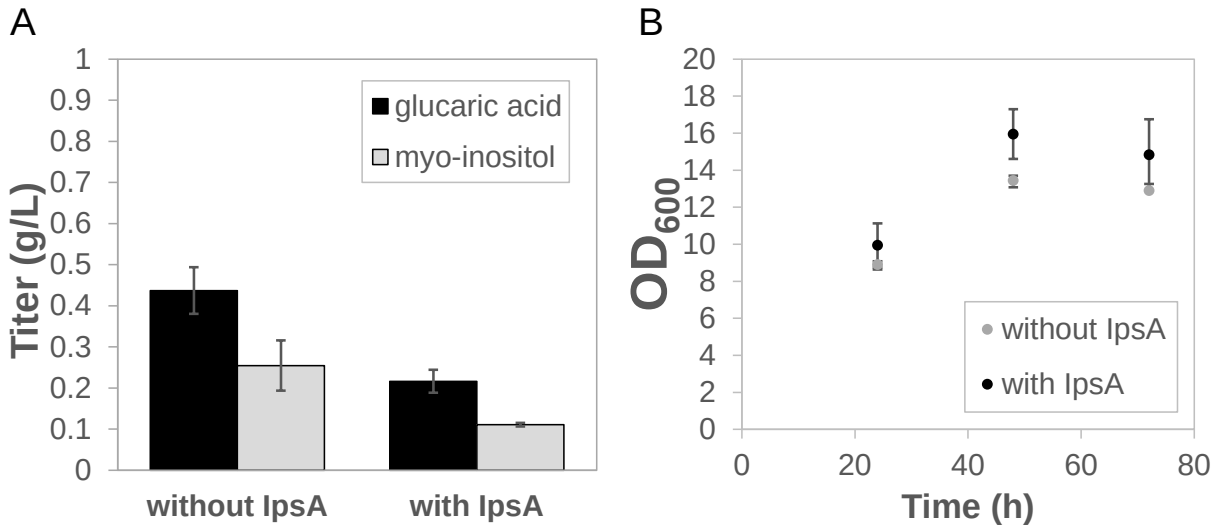


Figure S8. Glucaric acid production without MIOX control in the presence and absence of IpsA. MG1655 (DE3) Δ*uxaC* Δ*gudD* harboring plasmids encoding the glucaric acid pathway genes with and without IpsA were cultured in T12 medium with 10 g/L glucose. To remove IpsA-mediated regulation of *Miox*, the parent promoter without the IpsA binding site was used to drive *Miox* expression, and *ipsA* was expressed from promoter P2. (A) Glucaric acid and MI titer (g/L) and (B) optical density ($OD_{600}$) in the presence and absence of IpsA. No significant increase in titer or growth was observed from solely expressing *ipsA*. Error bars denote ± 1 S.D. from the mean of three replicate cultures.

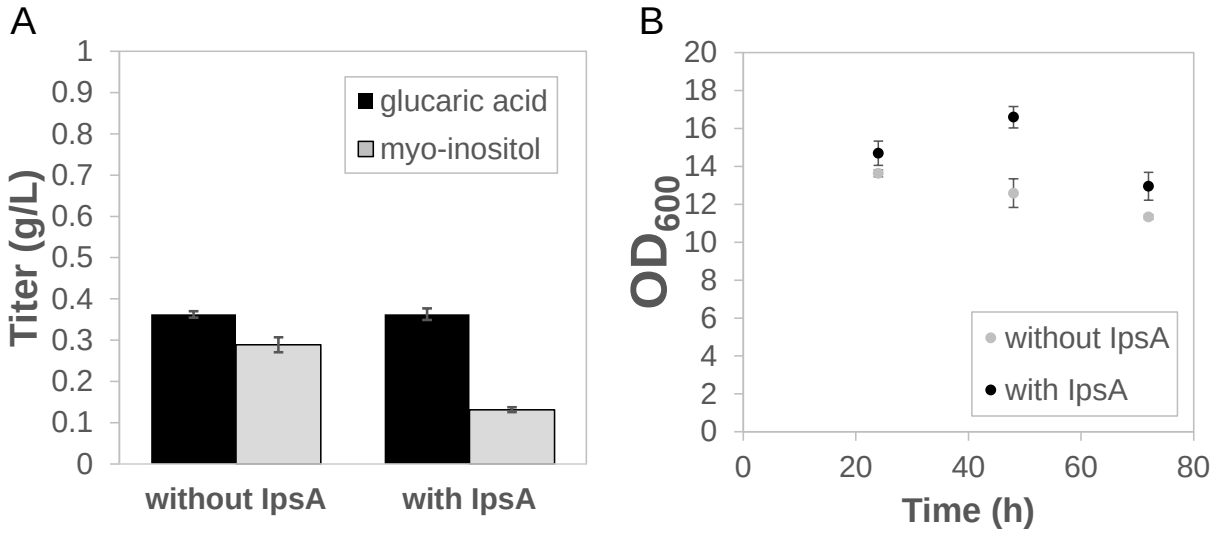


Figure S9. Glucaric acid production without MIOX control in the presence and absence of IpsA. Strain L19GA harboring plasmids encoding the glucaric acid pathway genes with and without IpsA were cultured in T12 medium with 10 g/L glucose. For the strain without IpsA, IpsA-mediated regulation of *Miox* was removed as in SI Appendix, Fig. S7. (A) Glucaric acid and MI titer (g/L) and (B) optical density ($OD_{600}$) in the presence and absence of IpsA. No significant increase in titer or growth was observed from solely expressing *ipsA*. Error bars denote ± 1 S.D. from the mean of three replicate cultures.

**References**


1. Registry of Standard Biological Parts Available at: http://www.partsregistry.org/ [Accessed September 21, 2015].
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3. Brockman IM, Prather KLJ (2015) Dynamic knockdown of E. coli central metabolism for redirecting fluxes of primary metabolites. *Metab Eng* 28:104–13.
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6. Moon TS, Yoon S-H, Lanza AM, Roy-Mayhew JD, Prather KLJ (2009) Production of glucaric acid from a synthetic pathway in recombinant Escherichia coli. *Appl Environ Microbiol* 75(3):589–95.