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Tuning Primary Metabolism for Heterologous Pathway Productivity

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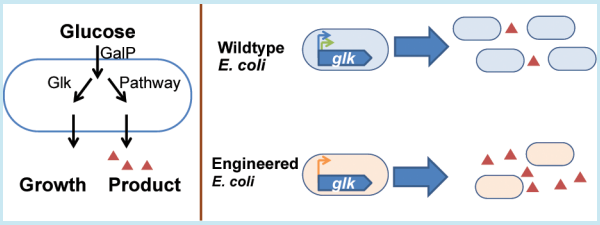
ABSTRACT: Tuning expression of competing endogenous pathways has been identified as an effective strategy in the optimization of heterologous production pathways. However, intervention at the first step of glycolysis, where no alternate routes of carbon utilization exist, remains unexplored. In this work we have engineered a viable *E. coli* host that decouples glucose transport and phosphorylation, enabling independent control of glucose flux to a heterologous pathway of interest through glucokinase (*glk*) expression. Using community sourced and curated promoters, *glk* expression was varied over a 3-fold range while maintaining cellular viability. The effects of *glk* expression on the productivity of a model glucose-consuming pathway were also studied. Through control of glycolytic flux we were able to explore a number of cellular phenotypes and vary the yield of our model pathway by up to 2-fold in a controllable manner.



KEYWORDS: *glucose utilization, primary metabolism, tuning of gene expression, flux optimization, metabolic engineering*

Metabolic engineering and microbial cell factories are powerful tools for the generation of commodity[1,2] and specialty[3−5] chemicals in a renewable manner. While new advances have diversified the product portfolio from natural products such as amino acids and acetone to unnatural products such as biopolymers[6,7] and biofuels,[8−11] there still remains the challenge of making these pathways competitive with traditional chemical synthesis. One solution to this problem is to maximize product flux, yield, and selectivity by removing competing enzymes at key pathway branchpoints and/or overexpressing pathway enzymes. However, in cases where the competing enzyme is essential or its catalytic efficiency (k_{cat}/K_m) is significantly greater than that of the production pathway, these methods may necessitate more costly medium supplementation or ultimately prove ineffective. To address these pitfalls, we examined a third strategy: tuning expression of the competing pathway.

Tuning endogenous gene expression has been previously identified as an effective strategy to increase pathway productivity[12,13] as it allows the balancing of endogenous cellular needs with that of pathway efficiency. Through dynamic and static implementations, such as codon substitution,[14,15] inducible/repressible promoters,[16,17] and antisense-mediated gene silencing,[18,19] researchers have explored this strategy with varying degrees of success. More recently, groups have begun to examine control of the nodes of central carbon metabolism.[20−23] However, there still exists a gap with regards to early intervention in central metabolism where alternate routes of carbon utilization do not exist. More importantly, the ability to introduce heterologous pathways that compete directly with central carbon metabolism at these nodes remains unexplored. We thus set out to design a system where glucose flux could be redirected from glycolysis and into a heterologous production pathway.

The first step of glucose metabolism is transport into the cell and phosphorylation for entry into glycolysis (Figure 1). In wildtype *E. coli*, this is accomplished predominantly by the phosphoenolpyruvate (PEP):carbohydrate phosphotransferase system (PTS)[24−26] where glucose is translocated across the cellular membrane and simultaneously phosphorylated to glucose-6-phosphate (G6P) with the consumption of one PEP. G6P is subsequently oxidized in glycolysis to provide ATP and other valuable metabolite precursors. This phosphorylation step, however, reduces the amount of free glucose available as a substrate for heterologous production pathways. Furthermore, the coupling of glucose transport and phosphorylation in PTS makes it unsuitable as a modulation target for the redirection of free glucose. Alternative glucose transporters, such as the low affinity galactose:H^+ symporter GalP and the ATP-dependent MglABC system, are able to internalize glucose in an unphosphorylated state,[27] while the ATP-dependent glucokinase (Glk) is able to phosphorylate glucose for glycolysis.[28] Several studies have further demonstrated that wildtype-like growth rates may be recovered in minimal medium supplemented with glucose in the absence of PTS when GalP is overexpressed.[29−31] In some strains, concomitant overexpression of Glk is required to fully restore growth.[31] In such a host, glucose transport is

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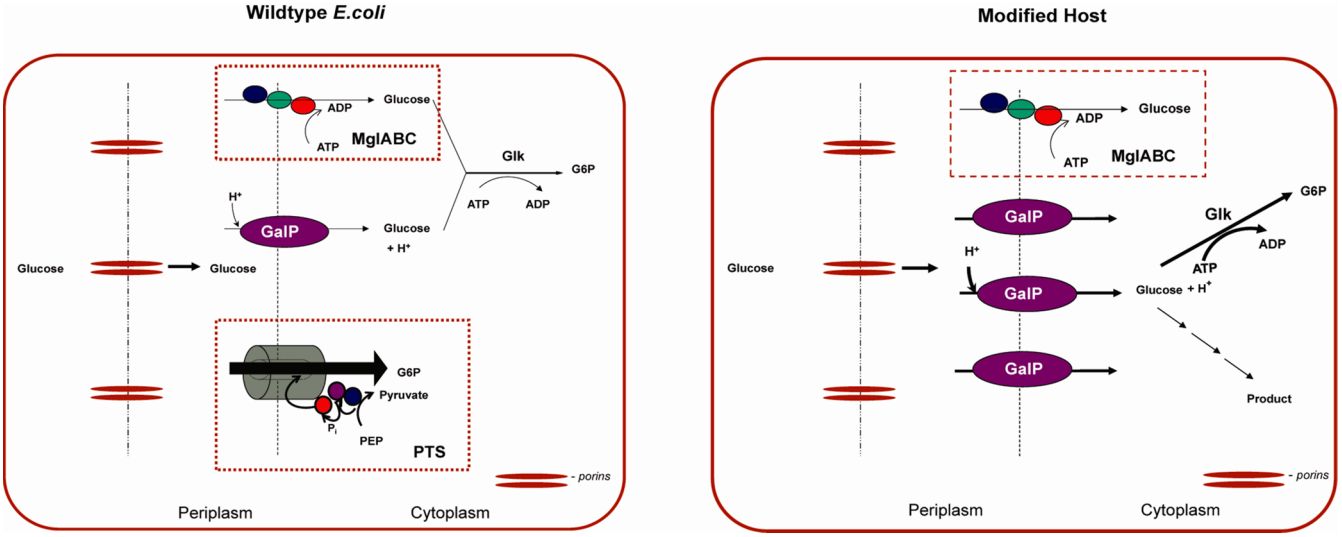

Figure 1. Glycolytic uptake and utilization in wildtype *E. coli* and the modified host. In wildtype *E. coli*, glucose is primarily transported and phosphorylated through the PTS system (*ptsHIcrr*) for consumption in endogenous processes. In the modified host, constructed here, glucose transport is decoupled from phosphorylation, allowing the diversion of glucose into heterologous production pathways. MglABC, *β*-methyl galactoside transport system encoded by *mglBAC*; GalP, galctose permease encoded by *galP*; PTS, glucose- and mannose-specific components of the phosphoenolpyruvate phosphotransferase system encoded by *ptsHIcrr*; Glk, glucokinase encoded by *glk*; PEP, phosphoenolpyruvate.

decoupled from phosphorylation, allowing for intracellular redirection of glycolytic flux (Figure 1).

In this study, we hypothesized that the downregulation of glucokinase would allow for the redirection of free glucose into a competing heterologous pathway (Figure 1). Using the well characterized Anderson promoter library (http://partsregistry.org, Parts J23100–J23119), we created a family of mutants with varying expression of glucokinase to probe glucose utilization and examine the limits of cell viability. Promoter-based approaches to study phenotype and pathway productivity have been successful in the past[32] and noted to avoid issues that confound analysis such as ultrasensitivity and transcriptional heterogeneity[33] seen in some inducible systems. We then evaluated the effect of expression level on the productivity of a model pathway, the one-step oxidation of glucose to gluconic acid, a top value-added commodity chemical from biomass used in a variety of industrial processes.[34,35] In this manner, we demonstrated that control of primary metabolism over a 3-fold range is indeed possible with the potential to control heterologous pathway productivity.

RESULTS AND DISCUSSION

Generation of a Tunable Glucose Flux Platform. To enable control of intracellular glucose flux, it was necessary to generate a PTS$^-$ Glu$^+$ phenotype. We achieved this in *E. coli* DH10B by first deleting the *ptsHIcrr* operon encoding components of the glucose/mannose-specific permeases and constituents of the cascade involved in transferring a phosphoryl group from PEP to glucose.[26] As expected, the deletion resulted in a Glu$^-$ phenotype. To restore growth on glucose, *galP was* upregulated with the introduction of the strong constitutive *lacI* promoter (Part J56015 from the Registry of Standard Biological Parts, http://partsregistry.org) to replace the native *galP* promoter. Native regulation of *galP* was also removed by silent mutations of the ORF to disrupt internal GalS and GalR repressor binding sites[36,37] (see Table 4 for specific mutations). The resulting strain, KTS022, was able to recover over 60% of the parent strain's specific growth rate in glucose-supplemented minimal medium (Figure 2) and attain similar final ODs. When bearing a plasmid, a typical requirement for the expression of a heterologous pathway, specific growth rates were indistinguishable between the two strains (Figure 2).

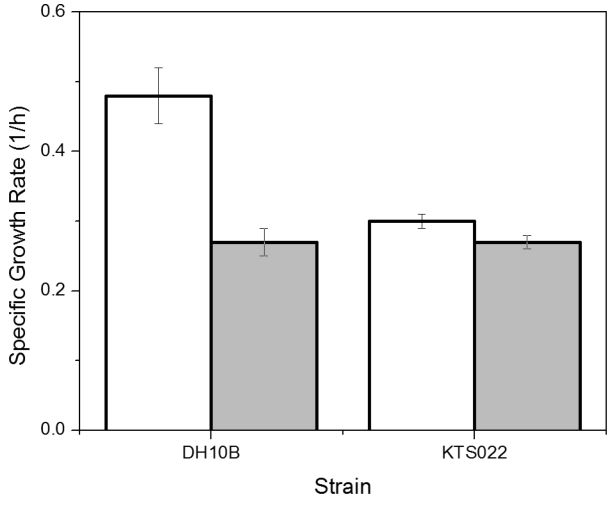

Figure 2. Growth rate recovery of Δ*ptsHIcrr galP*^{q} mutants. Specific growth rate of PTS$^-$ Glu$^+$ strain (KTS022) and its parent strain (DH10B) with and without a plasmid (pBAD30) in M9(0.4% glucose). Plasmid-bearing strains are indicated with gray bars. Graph depicts average ± standard deviation of duplicate cultures from independent experiments.

Range of *in vivo* Expression of *glk*. A family of glucokinase (*glk*) expression mutants (KTSx22) that enabled the exploration of the viable range of Glk activity was generated in the PTS$^-$ Glu$^+$ background. The native FruR regulatory binding sequence was disrupted by substitution with a sequence that had little homology to the noted consensus sequence[38] to remove endogenous regulation (Consensus sequence: RS|TGAAWC|SNTHHW → Mutated sequence: TA|GATTCA|AACGGG). The primary native promoter (P1)[39−41] was also replaced with a

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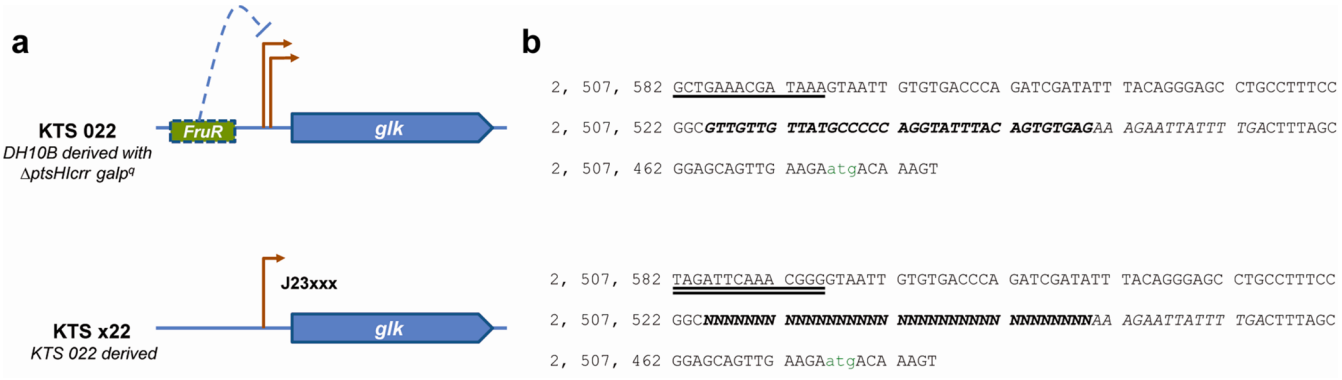

Figure 3. Glucokinase expression family construction. (a) Schematic of mutations introduced. (b) Sequence surrounding the 5′ UTR of the glucokinase expression family. The FruR binding site (underlined) was first replaced with a sequence of minimal homology to the natural consensus sequence (double underlined). The overlapping promoters (italicized) of *glk* were disrupted, and the primary promoter, P1 (bold), was replaced with a promoter from the Anderson library (J23xxx). The start codon of Glk is noted in lowercase green letters. Chromosomal location relative to a MG1655 chromosome is noted on the left.

Table 1. Glucokinase Expression Family Promoter Sequences[a]

Strain	Part Name	Relative Strength	Sequence
KTS022	native	N/A	GTTGTTGTTA TGCCCCCAGG TATTACAGTG TGA
KTS322	J23100	1.000	TTGACGGCTA GCTCAGTCCT AGGTACAGTG CTAGC
KTS522	J23110	0.331	TTTACGGCTA GCTCAGTCCT AGGTACAATG CTAGC
KTS722	J23115	0.152	TTTATAGCTA GCTCAGCCCT TGGTACAATG CTAGC
KTS922	J23114	0.101	TTTATGGCTA GCTCAGTCCT AGGTACAATG CTAGC
KTS622	J23117	0.064	TTGACAGCTA GCTCAGTCCT AGGGATTGTG CTAGC
KTS822	J23109	0.042	TTTACAGCTA GCTCAGTCCT AGGGACTGTG CTAGC
KTS1022	J23113	0.008	CTGATGGCTA GCTCAGTCCT AGGGATTATG CTAGC
KTS1122	J23112	0.004	CTGATAGCTA GCTCAGTCCT AGGGATTATG CTAGC

[a]Family members and their constitutive promoter part name, relative strengths, and sequences as annotated in the Registry of Standard Biological Parts, grouped and ordered by origin and relative strength. Sequence variations relative to J23100 have been highlighted in bold.

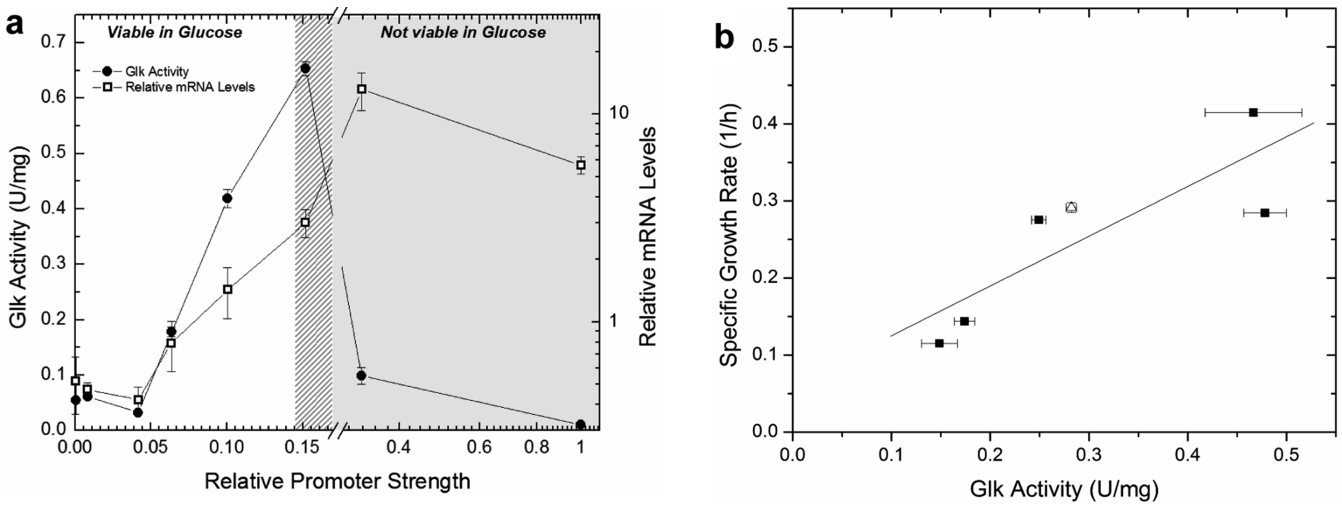

Figure 4. Expression family activity data. (a) Glucokinase activity and mRNA expression relative to the native promoter as a function of promoter activity in M9(0.4% glycerol). Region boundaries depicted are arbitrary, based on observed trends. (b) Specific growth rate as a function of Glk activity in M9(1.5% glucose) of the healthy glucose viable mutants. Wildtype activity is indicated by a triangle. Figures depict average ± standard deviation of triplicate cultures from a representative experiment.

member from the Anderson Promoter Library to tune Glk expression. These mutations (Figure 3 and Table 1) were first generated with PCR site-directed mutagenesis and cloned into pKD13 in the *Sal*I site before being introduced into the chromosome at the native locus by λ-Red mediated recombination.[42]

With the construction of the glucokinase expression family completed, the range of Glk activities that were achievable with the Anderson library variants was determined. As glycolytic flux was anticipated to be affected by glucokinase expression in a KTSx22 background, a gluconeogenic substrate was chosen for initial characterization of the library. The full dynamic range of

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Table 2. Titers of the Major Fermentation Products[a]

Strain	IPTG Concn [μM]	Final OD_{600}	Gdh Activity [U/mg]	Estimated[b] Glk Activity [U/mg]	Titers			Glucose Consumed [g/L]
					Gnt [g/L]	5KG [g/L]	Acetate [g/L]	
KTS822IG	10	1.56 (0.15)	15.2 (2.3)	0.23 (0.04)	2.61 (0.09)	0.32 (0.01)	ND	6.01 (0.27)
KTS1022IG		1.34 (0.17)	10.9 (0.2)	0.27 (0.03)	2.69 (0.26)	0.28 (0.03)	ND	5.19 (0.59)
KTS622IG		1.73 (0.27)	23.5 (1.0)	0.30 (0.02)	5.00 (0.21)	0.37 (0.07)	0.79 (0.00)	10.05 (0.01)
KTS022IG[c]		1.58 (0.23)	21.2 (0.5)	0.33 (0.02)	4.88 (0.31)	0.38 (0.07)	0.41 (0.04)	10.25 (0.03)
KTS922IG		1.12 (0.06)	5.7 (0.1)	0.50 (0.03)	1.71 (0.06)	0.20 (0.02)	0.67 (0.09)	7.98 (0.01)
KTS822IG	25	0.27 (0.16)	ND	0.23 (0.04)	0.16 (0.01)	0.03 (0.02)	0.12 (0.12)	ND
KTS1022IG		0.66 (0.31)	0.8 (0.7)	0.27 (0.03)	0.21 (0.04)	0.10 (0.01)	0.17 (0.08)	1.34 (0.58)
KTS622IG		1.37 (0.04)	33.0 (1.7)	0.30 (0.02)	3.30 (0.11)	0.47 (0.12)	0.71 (0.09)	9.00 (0.09)
KTS022IG[c]		1.91 (0.36)	31.8 (3.5)	0.33 (0.02)	4.13 (0.48)	0.31 (0.02)	0.49 (0.09)	10.25 (0.89)
KTS922IG		0.98 (0.07)	22.2 (1.2)	0.50 (0.03)	1.72 (0.07)	0.21 (0.08)	0.64 (0.04)	7.51 (0.06)
KTS822IG	100	0.06 (0.02)	ND	0.23 (0.04)	ND	0.01 (0.00)	ND	ND
KTS1022IG		0.10 (0.02)	ND	0.27 (0.03)	ND	0.02 (0.01)	ND	ND
KTS622IG		1.57 (0.40)	19.2 (1.0)	0.30 (0.02)	3.04 (0.14)	0.50 (0.01)	0.01 (0.00)	6.57 (0.01)
KTS022IG[c]		1.13 (0.08)	15.2 (0.5)	0.33 (0.02)	1.30 (0.04)	0.11 (0.02)	ND	3.35 (0.01)
KTS922IG		1.09 (0.24)	27.2 (0.1)	0.50 (0.03)	2.95 (0.55)	0.22 (0.01)	0.22 (0.01)	6.28 (0.35)

[a]Final OD_{600} and titers of gluconate (Gnt), its reduced form (5-ketogluconate, 5KG), acetate, and glucose consumed at 72 h as a function of Gdh and ordered by Glk expression. Average values of at least 2 parallel cultures are reported with standard deviations given in parentheses. Flasks were grown in M9(1.5% glucose). ND = not detected or statistically insignificant (measured range encompasses zero) [b]Glk activity of the parental KTSx22IG strain at mid exponential phase in Gdh-free cells. Due to the assay chemistry, Glk activity is indistinguishable from Gdh when both are present. [c]Wildtype Glk expression.

the family was characterized using glycerol as a carbon source, minimizing concern for the impact of Glk expression on carbon metabolism and physiology. As anticipated, increasing relative promoter strength led to an increase in Glk activity (Figure 4a) over a log range. However, at high promoter strengths, the cultures grew very slowly with two family members (KTS322 and KTS522) requiring twice as much time to reach late exponential phase (OD ~1.0). Moreover, these constructs also displayed a lower than expected activity given their promoter strength. Hypothesizing a metabolic burden, the relative mRNA levels of *glk* were determined by qRT-PCR. While mRNA levels scaled well with Glk activity at low and intermediate promoter strengths, the qRT-PCR data was unable to explain the reduced activity of KTS522 (promoter strength = 0.33) suggesting an issue at the translational level. Possible explanations for this discrepancy include ribosome saturation or insufficient energy (GTP/ATP) available to meet the translational demand. A decrease in mRNA expression for KTS322 (promoter strength = 1.00) relative to KTS522 was also noted (Figure 4a), suggesting an additional transcriptional burden at extremely high expression levels.

Only relatively low expression levels of Glk are viable when the family is grown in minimal medium supplemented with glucose (Figure 4a). These mutants correspond to the weakest promoters found in the Anderson promoter library with a promoter strength of only 0.06 (KTS622) being roughly equivalent to wildtype Glk expression. Within the viable regime, Glk activity spanned a 3-fold range from 0.15 to 0.48 U/mg or roughly 0.5−2 times that of wildtype Glk activity (0.28 U/mg). This range was noticeably smaller than the corresponding span of 0.01−0.42 U/mg seen in glycerol supplemented medium. Unlike the experiments in glycerol, however, there is a direct correlation between Glk activity and specific growth rate that is approximately linear (Figure 4b), suggesting that Glk is indeed controlling glycolytic flux and thus growth. Despite the range of activities and growth rates in glucose-supplemented medium, all viable mutants attained a similar final OD_{600}. This observation is in stark contrast to previous attempts to control central carbon metabolism and/or cellular phenotype where the final OD_{600}, and not specific growth rate, is affected.[21,43] In those studies, however, metabolism was modulated at nodes where alternate, but inefficient, routes of carbon utilization exist. In such strains, it is anticipated that the biomass yield on glucose is reduced, leading to lower biomass accumulation.

KTS322 and KTS522, which were noted to be subject to high transcriptional and translational burdens, were not viable in glucose-supplemented medium. Similarly, a transitional regime was identified encompassing KTS722 (promoter strength = 0.15), which showed weak growth and was only able to attain a quarter of the maximum OD_{600} observed in the healthier, lower expressing strains (data not shown). Given the ATPase activity of Glk in the presence of glucose, we hypothesized that at these high levels of glucose phosphorylation glycolysis is saturated and the cell consumes ATP much faster than it can be regenerated aerobically. Indeed, an O_2 respiration limit of ~16 mmol/h/g dcw has been suggested,[44] potentially due to membrane space constraints in accommodating the respiratory chain.[45] Performing a stoichiometric analysis of ATP generation and consumption by central carbon metabolism, including the energetic costs in energizing the membrane, consumption of some carbon for biomass rather than energy and the dissipation of excess energy through futile cycles,[46,47] suggests that the ATPase activity of Glk at these expression levels exceeds the aerobic ATP regeneration limit of the cell and results in cell death. Factoring in the increased energy demand for DNA replication, protein synthesis and lipid biosynthesis with increasing Glk and growth rate (Figure 4b)[48] reveals that the transitional regime of cellular health approaches this energetic limit, potentially explaining the reduced biomass production.

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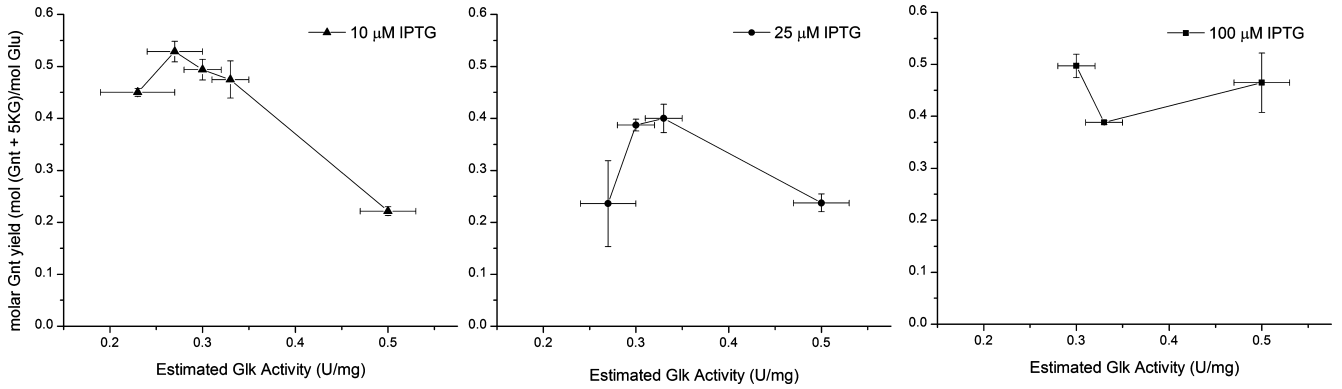

Figure 5. Gluconate molar yield on glucose as a function of Glk activity and IPTG induction at 72 h. Gluconate molar yield represents the yield of the major gluconate derived species (gluconate (Gnt) and 5-ketogluconate (5KG)). Plots represent averages ± standard deviations of at least 2 parallel cultures in a representative experiment. Due to the similar assay chemistries, Glk activity cannot be measured in the presence of Gdh. Estimated Glk activities presented here represent those of the host strain in the absence of any plasmid.

Glk Expression Controls Gluconate Productivity. Having established the dynamic range of Glk activities possible, we then probed the efficiency with which the glucose-viable strains could synthesize a small molecule product that competes with Glk for glucose as a substrate. The single-step oxidation of glucose to gluconic acid (gluconate), catalyzed by glucose dehydrogenase (Gdh) with the consumption of one $NAD(P)^+$, was selected. Gdh was expressed in a gluconate catabolism negative background (*idnK gntK*, named KTSx22IG)[49,50] to facilitate accumulation of product. In this background, the accumulation of gluconic acid is thermodynamically favored;[49] however, small amounts may be reversibly reduced to 5-keto-gluconic acid.

Gdh was expressed at one of three induction levels from a high copy, IPTG-inducible pTrc99-derived vector. To minimize issues with synchronization due to the strain family's wide range of growth rates, these vectors were induced upon inoculation. Preliminary studies showed no differences in the results obtained in this manner as compared to more traditional protocols where cultures are induced prior to the onset of stationary phase. After 72 h, the cultures were assayed for Gdh activity, growth, and titers of several major fermentation products (Table 2). Induction level appeared to have a strong effect on cellular growth, expression, and titers. We observed an inverse correlation between final OD and induction for mutants expressing Glk significantly below wildtype levels. This effect may be attributed to the metabolic burden of expression and reduced carbon flux through Glk for endogenous activities. The burden of induction also manifested in the ability of the mutants to express Gdh, with most strains exhibiting maximum Gdh expression at lower induction levels. Counterintuitively, although not unexpectedly,[51] the highest Gdh activity observed did not correspond with the highest product titers. These titers, ∼5 g/L, were obtained at intermediate Gdh expression levels at the lowest induction tested (10 μM). At this induction level, all strains tested produced significant amounts of gluconate (∼1.7−5 g/L).

Examining titers more closely at specific induction levels, trends are more difficult to discern as gluconate production is a nonlinear function of cellular phenotype, namely, Gdh and Glk levels as well as intracellular glucose, $NAD(P)^+$, and ATP pools. The high variability in cellular phenotype across strains and induction levels is echoed in the acetate levels (Table 2), typically indicative of excess carbon flux and/or cellular stress. However, if we evaluate the conversion of glucose to gluconate and 5-ketogluconate as a function of Glk activity (Figure 5), trends begin to emerge despite variations in these other parameters. At the lowest induction level tested, which generated the highest product titers and gluconate yields, there is a clear inverse correlation between gluconate yield and Glk activity with gluconate yields doubling to more than 0.5 mol of gluconate per mol glucose while Glk activity is decreased ∼50% going from KTS922IG to KTS1022IG (0.50−0.27 U/mg). However, as we decreased Glk activity further (i.e., KTS822IG, 0.23 U/mg), there appeared to be a slight decrease in yield, likely due to the limited availability of intracellular resources and compromises in cellular health. At the next induction level tested, 25 μM, a similar pattern emerges where there is a 50% increase in yield with only 40% decrease in Glk activity (0.5−0.3 U/mg). Further suppression of Glk compromises production in KTS1022IG (0.27 U/mg) and impairs production and growth of KTS822IG (0.23 U/mg) altogether, presumably due to the increased metabolic burden of Gdh expression with reduced carbon flux. At the highest induction level tested, the trends disappear altogether and both KTS822IG and KTS1022IG show negligible growth. We hypothesize that at this high level of induction the metabolic burden begins to impinge on the health of all the strains tested resulting in poor growth and/or pathway limitations other than glucose availability.

Summary and Concluding Remarks. In this study, we generated a family of *glk* expression mutants through the use of promoters found in the Anderson promoter library. Only the weakest promoters in this collection, with relative strengths ranging from 0.008 to 0.101 were found to be viable in minimal medium supplemented with glucose. Within this range, however, growth rate was controlled from 38% to 141% of wildtype with a corresponding 3-fold change in Glk activity (0.15−0.48 U/mg). Despite the diversity of specific growth rates possible, we were able to achieve comparable levels of biomass accumulation. Moreover, in this library, we were able to identify an upper bound to glucose utilization through Glk of at least twice that of wildtype levels. As we moved beyond this limit, cells failed to grow and/or attain maximal cell density, which we hypothesize is due to the unregulated ATPase activity of Glk. Similarly, we suspect that there is a floor to Glk activity based on the observed correlation of growth rate and Glk activity.

Within this Glk family, we were then able to examine the ability of the cell to redirect glucose to a heterologous pathway. Over a subset of Glk activities, there is a clear inverse correlation

between endogenous glucose utilization and the efficiency of a model pathway, suggesting a successful redirection of carbon flux. However, reducing endogenous metabolism also had the deleterious effect of reducing metabolic flexibility, the ability of the cell to support exogenous loads. At high induction levels, impaired cells failed to grow or express recombinant protein. Moreover, while product molar yield increased with decreasing Glk, these increases were not necessarily reflected in improved titers, suggesting that the metabolic burden imposed on these metabolically inflexible cells had an uncharacterized impact on other intracellular parameters such as glucose uptake rate and cofactor pools. The existence of these inferred effects implies that there exists a more complex relationship than that between carbon flux redirection and biomass production (specific growth rate). This issue, however, may potentially be addressed by a more dynamic "valve" or "switch",[20−22,43,52,53] such as regulated promoters, which allows cells to accumulate the necessary endogenous components to support production before flux redirection is implemented.

Despite these challenges and limitations, we were able to create a microbial host that allows for the incorporation of unphosphorylated glucose directly into heterologous production pathways enabling the creation of unnatural pathways. The existence of community sourced and, more importantly, curated tools, in this case the Anderson Promoter Library, proved to be invaluable in allowing us to probe the glucose utilization landscape and identify general viability regimes. Despite its 2-log range, however, the low number of weak promoters available limited our ability to precisely identify and define phenotypic boundaries. Nonetheless, with this library we were able not only to demonstrate the ability to redirect carbon and control product conversion yields but also to examine the flexibility of cells to tolerate perturbations in central carbon metabolism without alternate routes of carbon flux.

METHODS

Strains and Plasmids. *E. coli* strains and plasmids used in this study are listed in Table 3. All molecular biology manipulations were carried out according to standard practices.[54] Chromosomal manipulations were achieved through λ-Red mediated recombination[42,55] using pKD46[42] or a derivative containing *recA*. Sequence specific mutations (e.g., promoter replacements, binding site disruption) were introduced either directly through the primers used for PCR generation of the recombination cassette (Table 4) or first cloned into the suicide vector pKD13[56] (CGSC, New Haven, CT) in the *Sal*I site using standard techniques before PCR amplification. In all cases, the *kan* selection cassette was cured from the final mutants with FLP recombinase expressed from pCP20 (CGSC).[56,57] The plasmid pTrc99ACm-gdh, containing genes for the expression of glucose dehydrogenase (*gdh*, EC 1.1.1.47) from *B. subtilis*,[58] was used to enable gluconic acid production. The vector pTrc99ACm was generated by first PCR amplifying *cat* (Cm^R) from pMMB206[59] (ATCC, Manassas, VA), then blunt ligating the fragment with pTrc99A[60] linearized with *Sca*I, disrupting *bla* (Amp^R). *gdh* was then amplified from *B. subtilis* genomic DNA with the addition of appropriate restriction sites and inserted between the *Xba*I and *Hin*dIII sites of pTrc99ACm. All mutants were identified and isolated by colony PCR with appropriate primers; knock-ins and other sequence specific mutations were further verified with sequencing and restriction digests of colony PCR products. PCR amplifications were performed with Phusion High-Fidelity DNA Polymerase (NEB, Ipswich, MA) and oligonucleotides from Sigma-Genosys (St. Louis, MO; Table 4). All plasmids were cloned and propagated in *E. coli* DH10B with the exception of pKD13-derived plasmids which were propagated in EC100D pir-116 (Epicenter Biotechnologies, Madison, WI).

Table 3. Main Strains and Plasmids Used[a]

Name	Relevant Genotype	Source
	E. coli Strains	
Electromax DH10B	*F⁻ mcrA* Δ*(mrr-hsdRMS-mcrBC) φ80lacZ*Δ*M15* Δ*lacX74 recA1 endA1 araD139* Δ*(ara, leu)7697 galU galK λ⁻ rpsL nupG*	Invitrogen
Transformax EC100D pir-116	as above, *pir-116(DHFR)*	Epicenter Biotechnologies
KTS002	DH10B Δ*ptsHIcrr*	this study
KTSx22 family	as above, P_{glk}::P_{con*} *galP*^{q}	this study
KTSx22IG family	as above, Δ*gntK* Δ*idnK*	this study
	Plasmids	
pBAD30	*p15A, bla* (Amp^R)	ref 61
pCP20	*λ* p_r Rep^{ts}, *bla*(Amp^R), *cat*(Cm^R), FLP expressed by *λ* p_r under control of *λ* cI857 (ts)	CGSC #7629
pTrc99ACm-gdh	ColE1(pBR322) *ori, cat* (Cm^R), *lacI, gdh* from *B. subtilis* under the control of P_{trc}	this study
pKD13	*oriRγ, bla*(Amp^R), *kan*	CGSC #7633
pKD46	*oriR101, repA101*^{ts}, *bla* (Amp^R), *araC, araBp-λ*$_\gamma$*-λ*$_\beta$*-λexo*	CGSC #7739
pKD46RecA	as above, *recA*	This study

[a]con* = constitutive promoter from the Anderson promoter library.

Culture Conditions. For construction, strains were propagated in Luria−Bertani (LB) medium at 37 °C (30 °C for intermediate recombination steps). Temperature-sensitive plasmids were cured at 42 °C. All experimental cultures were grown at 37 °C in a M9 minimal medium supplemented with 0.8 mM L-leucine and either glucose or glycerol as indicated. For all experiments, starter cultures were grown to an OD measured at a wavelength of 600 nm (OD_{600}) of at least 0.2 before being transferred to a 50 mL flask of the same media. For experiments involving Gdh, IPTG was added as indicated to both the starter culture and 50 mL flasks at inoculation. In enzyme activity and qPCR studies, 50 mL shake flasks were inoculated to a final OD_{600} of 0.001, and 5 mL samples were taken at OD_{600} of ∼0.5 for flasks supplemented with glucose or OD_{600} of ∼1.0 for flasks supplemented with glycerol. These samples were then pelleted and stored at −20 °C until assaying. For gluconate experiments, shake flasks were inoculated to a final OD_{600} of 0.005 with M9 medium supplemented with glucose and monitored for 72 h before samples were taken and clarified by centrifugation. The supernatant was then stored at 4 °C until HPLC analysis. As appropriate, the antibiotics ampicillin, kanamycin, and chloramphenicol were used at concentrations of 100, 25, and 50 μg/mL, respectively.

Quantification of mRNA Levels. Total RNA from culture samples grown in M9 medium supplemented with glycerol were extracted using the illustra RNAspin Mini Isolation Kit (GE Healthcare Bio-Sciences, Piscataway, NJ) with an on-column DNaseI treatment following the kit protocol. Using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA) according to manufacturer's instructions, 0.5 μg of total RNA was treated to remove trace DNA contamination before cDNA was synthesized with random primers. The synthesized cDNA was then amplified with primers *glk_qPCR_for* (5′-TTGCGGGCGGTATCGT-3′) and *glk_qPCR_rev* (5′-

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Table 4. Oligonucleotides Used

Name	Sequence 5′→3′[a]
Glk Mutant Construction and Integration	
glk1FruR	CTATGTCGAC**TAGATTCAAACGGGG**TAATTGTGTGACCCAGA
glk2_J23100	CTATGTCGAC**GCTAGCACTGTACCTAGGACTGAGCTAGCCGTCAA**GCCGGAAAGGCA
glk2_J23110	CTATGTCGAC**GCTAGCATTGTACCTAGGACTGAGCTAGCCGTAAA**GCCGGAAAGGCA
glk2_J23117	CTATGTCGAC**GCTAGCACAATCCCTAGGACTGAGCTAGCTGTCAA**GCCGGAAAGGCA
glk2_J23115	CTATGTCGAC**GCTAGCATTGTACCAAGGGCTGAGCTAGCTATAAA**GCCGGAAAGGCA
glk2_J23109	CTATGTCGAC**GCTAGCACAGTCCCTAGGACTGAGCTAGCTGTAAA**GCCGGAAAGGCA
glk2_J23114	CTATGTCGAC**GCTAGCATTGTACCTAGGACTGAGCTAGCCATAAA**GCCGGAAAGGCA
glk2_J23113	CTATGTCGAC**GCTAGCATAATCCCTAGGACTGAGCTAGCCATCAG**GCCGGAAAGGCA
glk2_J23112	CTATGTCGAC**GCTAGCATAATCCCTAGGACTGAGCTAGCTATCAG**GCCGGAAAGGCA
glk_kan	*CTATTCCTTATGCGGGGTCAGATACTTAGTTTGCCCAGCTTGCAAAAAGGCATCGCTGCAATTGGT*GTGTAGGCTGGAGCTGCTTC
J23100_insert	*CACATCACCGACTAATGCATACTTTGTCATTCTTCAACTGCTCCGCTAAAGTCAAAATAATTCTTT*GCTAGCACTGTACCTAGGACTGAGCTA
J23110_insert	*CACATCACCGACTAATGCATACTTTGTCATTCTTCAACTGCTCCGCTAAAGTCAAAATAATTCTTT*GCTAGCATTGTACCTAGGACTGAGCTA
J23117_insert	*CACATCACCGACTAATGCATACTTTGTCATTCTTCAACTGCTCCGCTAAAGTCAAAATAATTCTTT*GCTAGCACAATCCCTAGGACTGA
J2311S_insert	*CACATCACCGACTAATGCATACTTTGTCATTCTTCAACTGCTCCGCTAAAGTCAAAATAATTCTTT*GCTAGCATTGTACCAAGGGCT
J23109_inset	*CACATCACCGACTAATGCATACTTTGTCATTCTTCAACTGCTCCGCTAAAGTCAAAATAATTCTTT*GCTAGCACAGTCCCTAGGACTGA
J23114_insert	*CACATCACCGACTAATGCATACTTTGTCATTCTTCAACTGCTCCGCTAAAGTCAAAATAATTCTTT*GCTAGCATTGTACCTAGGACTGAGCTA
J23113_insert	*CACATCACCGACTAATGCATACTTTGTCATTCTTCAACTGCTCCGCTAAAGTCAAAATAATTCTTT*GCTAGCATAATCCCTAGGACTGAGC
J23112_insert	*CACATCACCGACTAATGCATACTTTGTCATTCTTCAACTGCTCCGCTAAAGTCAAAATAATTCTTT*GCTAGCATAATCCCTAGGACTGAGC
ptsHIcrr Deletion[b]	
pts-For-1st	*TTAGTTCCACAACACTAAACCTATAAGTTGGGGAAATACA*GTGTAGGCTGGAGCTGCTTC
pts-Rev-1st	*TGATGCGGATAACCGGGGTTTCACCCACGGTTACGCTACC*TCCGTCGACCTGCAGTTCG
pts-For-2nd	*GCTAACAATACAGGCTAAAGTCGAACCGCCAGGCTAGACTTTAGTTCCACAACACTAAACCTATAAGTTG*
pts-Rev-2nd	*CCGATGGGCGCCATTTTTCACTGCGGCAAGAATTACTTCTTGATGCGGATAACCGGGGT*
galP Mutation[c]	
galP-For-1st	**TGGTGCAAAACCTTTCGCGGTATGGCATGATAGCGCC**CACAATAAAAAATAACCATATTGGAGGGCATC
galP-Rev-1st	**A**CCTGCGAT**AACG**CCGATATCCAGGCCAAAGAGTAATCCCGCCAGAG
galP-For-2nd	GTCGACTGGTGCAAAACCTTTCGCGG
galP-Rev-2nd	GTCGACACCTGCGATAACGCCGATAT
galP-For-Rep	*AATTATTTTCATGCACTTAAATCATAACTAAGATAAATGTGTGTAGGCTGGAGCTGCTTC*
galP-Rev-Rep	*AGTAATCTGGAATTCATCTGCAATAAACGGCAGTGCACCTGCGATAACGCCGATAT*
Gluconate Catabolism Deletion	
didnK_F	*AAATTATTATGCCGCCAGGCGTAGTATCGCAGCAGGTAAGATGATTCAGGAGATTTTAAA*GTGTAGGCTGGAGCTGCTTC
didnK_R	*CAGCATGTGCGCGACGGTAAGGCGCGTTACCGCGTGGTGTTGAAAGCCGATTTTTGAAAA*TCCGTCGACCTGCAGTT
gntKF2	*CTGATATTGTCCGGCTGGACAATGTTACCGATAACAGTTACCCGTAACATTTTTAATTCTTGTATTGTGGGGGCACCACT*GTGTAGGCTGGAGCTGCTTC
gntKR2	*ATACGCGCCTTCATGACTAAAAACAGCAGCAGTAAAACAGACCCTACTGCTGTTAAAACAAGCGTTAATGTAGTCACTAC*TCCGTCGACCTGCAGTT
Gdh Cloning	
For_Cm_pMMB206	TGGTGTCCCTGTTGATACC
Rev_Cm_pMMB206	CGCTTATGGCAGAGCAG
For_gdh_subtilis	TACATATAAGTCTAGATAACAAATGGAGGAGGATG
Rev_gdh_subtilis	CAAGTAACTAAAGCTTTCATGTCTGGGTCGCT

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Table 4. continued

Name	Sequence 5′→3′[a]
Colony PCR and Sequencing	
002check_F (pts)	GCTAACAATACAGGCTAAAGTCGAAC
002check_R (pts)	CCGATGGGCGCC
022check_F (galP)	AATTATTTTCATGCACTTAAATC
022check_R (galP)	GTAATCTGGAATTCATCTGC
glkchkF	AAAGGCATCGCTGCAAT
glkasfor500	CTATGAATTCCTATTCGGCGCAAAATCAAC
glkasfor100	CTATGAATTCAATAGGTCTTAGCCTGCGAG
gntchkF	AAGTAGCTCACACTTATACACTTAAG
gntchkR	TGACTAAAAACAGCAGCAG
idnchkF	GTGAAATTATTATGCCGC
idnchkR	TGACTAAAAACAGCAGCAG

[a]Introduced mutations (promoters or other) are in **boldface** type; homologous sequences for recombination are *underlined and italicized*; restriction sites used for cloning are underlined. All oligonucleotides were purchased from Sigma-Genosys, St. Louis, MO. [b]PCR for the *ptsHIcrr* deletion cassette performed in two stages where a 2nd round of PCR was used to increase the degree of homology to facilitate recombination. [c]galP mutations were performed in three parts: mutations were introduced in the first round before a *SalI* site was added for cloning into pKD13. The final primer set (galP-xxx-Rep) was then used to generate the linear DNA cassette for recombination.

GGAAACCGGAGGCTTTGAAG-3′) in a qPCR reaction with Brilliant II Sybr Green High ROX QPCR Mix (Agilent Technologies, Santa Clara, CA) on an ABI 7300 Real Time PCR System instrument (Applied Biosystems, Beverly, MA). Transcript levels were quantified in duplicate with appropriate no-template and no-RT controls and are relative to that of native *glk* expression levels (KTS022) as determined from a standard curve. Reported levels are the averages of triplicate flasks, each measured in duplicate.

Enzyme Activity Assays. All enzyme activity assays were performed on crude lysates generated by resuspending frozen cell pellets in 500 μL of 10 mM Tris-HCl (pH = 8.0) and sonicating at ~8 W in 5 × 5 s pulses on ice. The resulting lysates were clarified by centrifugation before being used as follows. Glucokinase (Glk) activity was measured in a coupled enzymatic assay as first described by DiPietro and Wein-house.[62] Glk phosphorylates glucose in the presence of ATP to glucose-6-phosphate. Glucose-6-phosphate is in turn oxidized to 6-phospho-D-gluconate by glucose-6-phosphate dehydrogenase (G6PDH) with the generation of a spectrophotometric NADPH signal at 340 nm. One unit of Glk activity will phosphorylate 1.0 μmol/min of D-glucose at pH 7.5 and room temperature in the presence of 3.33 U/mL G6PDH, 60 mM Tris-HCl, 20 mM magnesium chloride, 8.0 mM ATP, 12.0 mM glucose, and 0.9 mM $NADP^+$. Glucose dehydrogenase (Gdh) activity was measured directly from the oxidation of glucose to gluconolactone with the production of a NADH signal at 340 nm.[63] One unit of Gdh activity oxidizes 1.0 μmol/min of D-glucose at pH 7.6 at room temperature in 60 mM potassium phosphate buffer and 0.67 mM NAD^+. As Gdh is able to accept NAD^+ and $NADP^+$ as cofactors[64] with the generation of a common spectrophotometric signal at 340 nm, Glk activity cannot be measured independently in the presence of Gdh. All enzyme activities were normalized by total protein levels as measured in a modified Bradford assay described by Zor and Selinger.[65]

Metabolite Analysis. Culture supernatant was analyzed on an Agilent 1100 series HPLC instrument with separation on an Aminex HPX-87 H anion exchange column (Bio-Rad Laboratories, Hercules, CA) with 5 mM H_2SO_4 as the mobile phase at 55 °C and a constant flow rate of 0.6 mL/min. A refractive index detector (RID) set to 55 °C was used to monitor glucose levels while a diode array detector (DAD) was used to measure acetate and 5-ketogluconate (a gluconic acid derived species) at 210 nm, and gluconic acid at 230 nm. As gluconic acid and glucose coelute (~ 9 min) and are both detected by the RID, the DAD signal at 230 nm was used to resolve the two species. Concentrations were determined from standard curves of each analyte prepared from commercial standards.

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Notes

The authors declare no competing financial interest.

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