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The zero-sum game of pathway optimization: emerging paradigms for tuning gene expression

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Running title: Tuning expression for pathway optimization

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1
2
3 23 **Abstract**

24 With increasing price volatility and growing awareness of the lack of sustainability of
25 traditional chemical synthesis, microbial chemical production has been tapped as a
26 promising renewable alternative for the generation of diverse, stereospecific compounds.
27 Nonetheless, many attempts to generate them are not yet economically viable. Due to
28 the zero sum nature of microbial resources, traditional strategies of pathway optimization
29 are attaining minimal returns. This result is in part a consequence of the gross changes in
30 host physiology resulting from such efforts and underscores the need for more precise
31 and subtle forms of gene modulation. In this review, we describe alternative strategies
32 and emerging paradigms to address this problem and highlight potential solutions from
33 the emerging field of synthetic biology.

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35 **Introduction**

36 Microbial production systems display a remarkable flexibility in the diversity and
37 enantioselectivity of the compounds that they can generate. These compounds have
38 historically been natural products such as ethanol, amino acids, acetone and antibiotics.
39 However, with the introduction of ever more sophisticated tools, a range of natural and
40 unnatural products have been made in engineered hosts including compounds such as
41 hydroxyacids[1-3], isoprenoids[4, 5], polyketides[6, 7], and biopolymers[8, 9]. While
42 several of these processes have been successfully commercialized [10-12], many remain
43 economically infeasible and are the subject of intense optimization efforts.

44 In optimizing microbial pathways, the objectives are to maximize product flux,
45 yield and selectivity. Traditionally, this problem has been approached by an analysis of
46 the metabolic pathway that leads to removing branch points that lower product yield and
47 selectivity (gene inactivation) and increasing the flux of intermediates through the
48 pathway (gene overexpression). The power of such methods has improved tremendously
49 with the advent of computational tools such as Flux Balance Analysis (FBA) [13, 14] and
50 bilevel optimization [15-17] to identify flux bottlenecks, yet, they are still fundamentally
51 constrained by the interconnectedness and finite nature of microbial resources (Figure 1).
52 Gene inactivations may necessitate media supplementation, impair cellular function and
53 are sometimes infeasible for non-linear production pathways. Overexpression of
54 pathway genes, on the other hand, comes at the expense of endogenous ones due to
55 consumption of common precursors and titration of cellular machinery such as
56 polymerases and ribosomes and may lead to growth inhibition, reduced expression and
57 even cell death [18-20]. In certain hosts the heat shock response is stimulated by protein

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overexpression [21, 22] further limiting the degree of overexpression possible. Moreover, successfully overexpressing or knocking out genes does not guarantee improved productivity. Decoupling the native regulation of flux within the pathway in these ways may lead to the accumulation of intermediates that can inhibit pathway enzymes [3, 23] or are bacteriostatic [1, 24, 25]. These challenges are not insurmountable, but they do underscore the need for more tools in pathway optimization. This review will highlight novel approaches to pathway optimization and describe emerging paradigms for flux manipulation.

Downregulation of related pathways

Modulation of gene expression, such as downregulation of undesired branch points, has been identified as a fruitful avenue for increased pathway productivity [16, 17]. In contrast to gene inactivation, downregulation offers the ability to redirect metabolite flux into production pathways while maintaining sufficient flux for endogenous processes. Moreover, in cases of drastic differences in catalytic efficiency of competing enzymes, it may prove more efficient than overexpression of pathway enzymes. Downregulation may be implemented in many different ways. One promising method, amenable to implementation in a wide variety of hosts and pathways, is the use of antisense RNA (asRNA) mediated inhibition of translation [26-30].

One such example of asRNA use in pathway optimization is found in the engineering of *Clostridium acetobutylicum.* Predating the rise of petrochemical sources, *C. acetobutylicum* was an industrially relevant source of solvents such as acetone and butantol [33] which it naturally ferments as part of its lifecycle [31, 32]. Recent volatility

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81 in the price of chemical feedstocks and increasing concern regarding the sustainability of
82 traditional chemical synthetic routes have led to renewed interest in the species with a
83 focus on controlling the distribution of products [27, 29, 34, 35]. The Papoutsakis group
84 used an asRNA approach to downregulate the CoA transferase which catalyzes the
85 formation of acetone (*ctfA1*) to shift these strains to a primarily alcohologenic mode of
86 production (ethanol and butanol), obtaining the highest ethanol titers reported at the time
87 in *C. acetobutylicum* [29, 34]. Similar success has been reported for the engineering of
88 glutamate synthesis from *Corynebacterium glutamicum*. *C. glutamicum* is a natural
89 overproducer of amino acids and an industrial source of several of these including
90 glutamate [36] which is produced from the transamination of α-ketoglutarate, a citric acid
91 cycle intermediate. Utilizing an asRNA approach, Kim and coworkers [28] increased the
92 cell specific productivity of glutamate by inhibiting activity of 2-oxoglutarate
93 dehydrogenase thereby allowing sufficient flux of α-ketoglutarate through the citric acid
94 cycle for energy production while diverting additional precursors to increase glutamate
95 synthesis. Finally, asRNA technology has been utilized in the synthesis of cobalamin
96 (Vitamin B_{12}) in *Bacillus megaterium* to improve titers and yields by 20% [30].
97 The use of downregulation extends beyond the realm of small molecule synthesis
98 and has similar applications in recombinant protein production where acetate has been
99 established to have an inhibitory effect on specific protein expression and bacterial
100 growth [37-40]. Controlling acetate production by inactivation of phosphotransacetylase
101 (*pta*) or acetate kinase (*ackA*) genes in *E. coli,* which shunt excess acetyl-CoA to acetate,
102 has a deleterious effect on the cellular redox state [40], carbon flux [41], and ultimately
103 growth [41]. Diverse solutions such as process-based schemes [37] and metabolic

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104 engineering of the host to shunt the excess acetyl-CoA to acetoin [39] have been
105 developed to address the issue. Nonetheless, these solutions are not scalable to all
106 methods of culture and inhibit ATP synthesis by acetate secretion. Thus, Kim and Cha
107 [42] chose an antisense based scheme to minimize detrimental physiological effects.
108 Through minor antisense inhibition of *ackA* and *pta*, Kim and Cha were able to reduce
109 acetate formation by more than 20% while simultaneously observing a 60% improvement
110 in the production of green fluorescent protein with negligible impact on cellular growth.

111 These examples of asRNA inhibition are not the only examples of pathway
112 downregulation. Alternative strategies such as those utilizing the effect of codon bias on
113 translational efficiency in *C. glutamicum* [43, 44], repressible promoters in *S. cerevisiae*
114 [45-47] and titrating inducible promoters in *E. coli* [48] have been used with great
115 success to increase product yields and/or titers. Moreover, the last decade has seen
116 intense efforts to regulate genes at the transcriptional and post translational levels
117 culminating in several novel methods such as regulated suppression of amber mutations
118 [49], inducible protein degradation [50], engineered allostery [51] and riboregulators [52,
119 53]. Despite the fact that many of these emerging technologies have yet to mature and
120 attain widespread adoption, particularly in an industrial context, the growing interest in
121 asRNA points to its relative ease of implementation. While unexplored in these studies,
122 another advantage of downregulation is the possibility of dynamic control of gene
123 expression.

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125 **Dynamic Expression Profiles**

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3 126 When maximizing product titers and yields for industrial scale fermentation,
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6 127 carbon flux is shifted from the normal balance of metabolic intermediates and shunted
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8 128 into the desired product. This shift is frequently at odds with the goals of the cell, i.e.
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11 129 maintaining metabolic flux levels and maximizing biomass. Thus, genetic alterations
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13 130 that alter metabolic flux will incur a redistribution of metabolites to compensate for the
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15 131 change with some inhibition of growth. Gadkar et al. [54] studied this issue *in silico* as it
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18 132 applied to glycerol and ethanol production. In their work, they pursued a bilevel
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20 133 optimization strategy analogous to that of OptKnock [15] where product titers are
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22 134 maximized subject to growth maximization and other physical constraints to determine
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25 135 gene candidates for upregulation or deletion. However, unlike OptKnock, they also
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27 136 optimized the timing of these genetic changes. For glycerol production, simulations of a
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30 137 biphasic approach to gene expression resulted in a 30% improvement in titers over a
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32 138 static strategy. Similarly, ethanol titers were improved by 40% over a static strategy and
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34 139 90% over wildtype behavior. These cases and more were further studied by Anesiadis
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37 140 and coworkers [55] with the simulated behavior of genetic elements from synthetic
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39 141 biology, as opposed to instantaneous switching in expression, and came to a similar
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42 142 conclusion: dynamic control of gene expression may be implemented to increase pathway
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44 143 productivity.
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46 144 One of the first experimental demonstrations of this paradigm was elegantly
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49 145 performed in 2000. In trying to produce lycopene in *E. coli*, Farmer and Liao [56] sought
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51 146 to overexpress 2 key rate limiting enzymes: phosphoenolpyruvate synthase (Pps), which
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53 147 controls the pool of a glycolytic intermediate needed for lycopene biosynthesis, and
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56 148 isopentenyl diphosphate isomerase (Idi), which pulls glycolytic intermediates into the
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3 149 lycopene biosynthetic pathway. However, overexpressing them statically from a *tac*
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5 150 promoter hindered growth, yields and titers. Thus, they engineered a gene
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7 151 circuit/metabolite control system in which expression of *pps* and *idi* was directly tied to
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9 152 the availability of acetyl phosphate, a proxy for glycolytic flux and cellular health.
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11 153 Using this approach, they were able to overexpress these enzymes to higher levels than
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13 154 that seen using a static approach while maintaining cellular viability and ultimately
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15 155 improve titers by 50%, productivity three-fold and carbon yields by more than an order of
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17 156 magnitude. Similar control systems have also been developed to drive protein expression
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19 157 through the use of quorum sensing in *E. coli* [57, 58]. Such systems allow for
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21 158 coordinated delayed induction across multiple cellular populations in addition to
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23 159 transmitting the metabolic load state of the host [59] thereby mitigating potential
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25 160 challenges associated with protein overexpression. Moreover, they are modular and
26
27 161 readily amenable to integration in complex circuits [57] where Boolean logic and sensor
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29 162 functions can be implemented for tight pathway regulation in combination with other
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31 163 strategies for cumulative effects. A hypothetical example of this is presented in Figure 2
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33 164 where sensing and logic (AND) operations are used to drive expression of pathway genes
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35 165 and product only when high cell densities and carbon flux are achieved.
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39 167 **Emerging paradigms**
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41 168 With an eye towards the creation of sophisticated gene circuits and networks for
42
43 169 both pathway regulation and biosynthesis, the emerging discipline of synthetic biology
44
45 170 has established a paradigm of developing reusable modules or "parts" and "devices" to
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47 171 control gene expression [60-62]. Towards this end, libraries of sensors, control
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172 elements, promoters [63-65], and ribosome binding sites (RBS) [66] among others have
173 been developed. Many of these libraries are curated within the Registry of Standard
174 Biological Parts (http://partsregistry.org) and are freely available to the community.
175 Through these libraries of parts, network components may be individually selected, tuned
176 and regulated to achieve the necessary phenotype.

177 The rise of part libraries has also been accompanied by the development of
178 computer aided design (BioCAD) tools to facilitate the design of ever more complex
179 circuits [67-71]. However, they are dependent on the availability of datasheets [72] or
180 other experimental characterization to describe them which are typically context
181 dependent and not readily generalizable to all scenarios. Moreover, the current lack of
182 generic insulators for these parts results in feedback from downstream parts, or
183 retroactivity [73], which can further perturb performance from expectation. Nonetheless,
184 there has been some success with the engineering of systems from these libraries using
185 both theoretical and experimental approaches. For example, using an equilibrium
186 statistical thermodynamic model, Salis and coworkers [74] were able evaluate the effects
187 of the 5' UTR on translation culminating in the design of novel RBSs able to achieve
188 expression levels spanning 5 orders of magnitude. Their software tool, RBSCalculator
189 (https://salis.psu.edu/software/), also allows for relative expression tuning of a given
190 sequence. Empirical and combinatorial approaches to the tuning of gene expression from
191 library components have also proven successful in optimizing yields of lycopene and
192 mevalonate production pathways [63, 75]. Finally, a combination of both theoretical and
193 experimental characterization has been used to design and develop tuned systems with
194 little *post hoc* adjustment [76].

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More recently, new part classes such as engineered enzyme complexes have been developed. As discussed previously, manipulating flux gives rise to a myriad of challenges such as the physiological consequences of flux imbalance and titration of cellular machinery. These undesired effects can be attenuated with gene modulation and gene circuits with some tradeoff in selectivity due to reduced pathway intermediates. Inspired by natural solutions to minimize this tradeoff [77, 78], Deuber et al. [79] engineered an enzyme scaffold scheme to recruit multiple pathway enzymes in a single complex. By colocalizing enzymes in this way, diffusional limitations are effectively nullified and toxic metabolites can be maintained at locally high, but globally low, concentrations to maximize pathway flux with minimal disruption to endogenous processes. Furthermore, this scaffolding is scalable and generalizable to many enzymes and pathways in specified stoichiometries allowing for efficient spatial organization of pathway genes [79-81]. Using this system, mevalonate titers were improved by 70-fold when compared to scaffold free control. Moreover, with rate limiting enzymes being expressed at nominally low levels, the scaffolded constructs were able to achieve these yields without the growth inhibition seen in an unscaffolded design [79]. Alternative strategies such as direct protein fusions have also proved successful in improving pathway productivity [81].

Conclusions and Perspectives

Microbial production systems have enormous potential to synthesize many valuable chemical compounds in a sustainable manner. However, optimizing these systems for economic feasibility remains a challenge, in part, due to the zero sum nature

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3 218 of intracellular metabolites (Figure 1). Redirecting these metabolites into pathways of
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5 219 interest necessitates a loss of flux elsewhere and titration of cellular machinery away
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7 220 from endogenous processes resulting in negative physiological consequences. These
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9 221 concerns may be attenuated to some degree by microbial consortia. Such mixed
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11 222 populations of cells are able to achieve more complex tasks, are more robust to
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13 223 environmental changes and are able to be organized by function [82]. More importantly,
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15 224 this functional specialization allows for the distribution of the metabolic burden across
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17 225 populations resulting in overall healthier cultures and potentially more efficient
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19 226 pathways. This advantage is offset, however, by the recalcitrant nature of genetic
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21 227 manipulations of all but a few species and the potential for competition between
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23 228 populations making a single organism, single population solution the most tractable
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25 229 solution for the immediate future.
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27 230 Despite the limitations of finite cellular resources, the use of tools which precisely
28
29 231 modulate expression levels has led to much improvement in pathway function by
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31 232 mitigating the effects of the pathway on host physiology. Synthetic biology has further
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33 233 contributed genetic parts and tools that allow for more precise application of regulation
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35 234 through mechanisms such as basic computation [66, 83, 84], sensing[56, 85] and timed
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37 235 expression [57, 58] with demonstrated improvements in productivity. Moreover, these
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39 236 modifications may all be combined for cumulative pathway improvement (Figure 2).
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41 237 With increased understanding of the consequences of metabolic perturbations and
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43 238 evermore sophisticated regulation of expression, yields of microbial production systems
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45 239 may soon be economically competitive with traditional synthesis culminating in the
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47 240 realization of widespread microbial production.
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Figure Captions

Figure 1

The zero sum challenges with traditional pathway optimization strategies. In the original pathway (A), only one media supplement (yellow circle) is needed to generate product (blue circle) and essential metabolites (aqua circle). However, gene inactivation (B) necessitates additional supplementation to generate the essential metabolite while overexpression (C) increases the pool of desired intermediate at the expense of expression and flux through the other enzymatic steps potentially limiting growth. The sum of these effects on the host's health controls the degree of success on overall pathway production. Metabolite flux is proportional to the line thickness while metabolite pool size is represented by the circle area. Dashed lines indicate an absence of metabolite flux/pools when compared to wildtype.

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Figure 2

A hypothetical example of a complex regulatory circuit utilizing multiple modules or parts. The LuxR/LuxI quorum sensing system (*luxI* not shown), mediated by N-acyl homoserine lactone (AHL), is used to drive the expression of *glnAp2*, an acetyl phosphate (ACP) sensor [86]. Sufficient carbon flux through central metabolism will lead to accumulation of ACP. The presence of GlnAp2 and ACP serve as inputs to an AND gate (binding of ACP to GlnAp2) whose output is expression of pathway genes and, ultimately, synthesis of product (triangles). Product is only produced when biomass *and* carbon flux is high, i.e. from a healthy culture.

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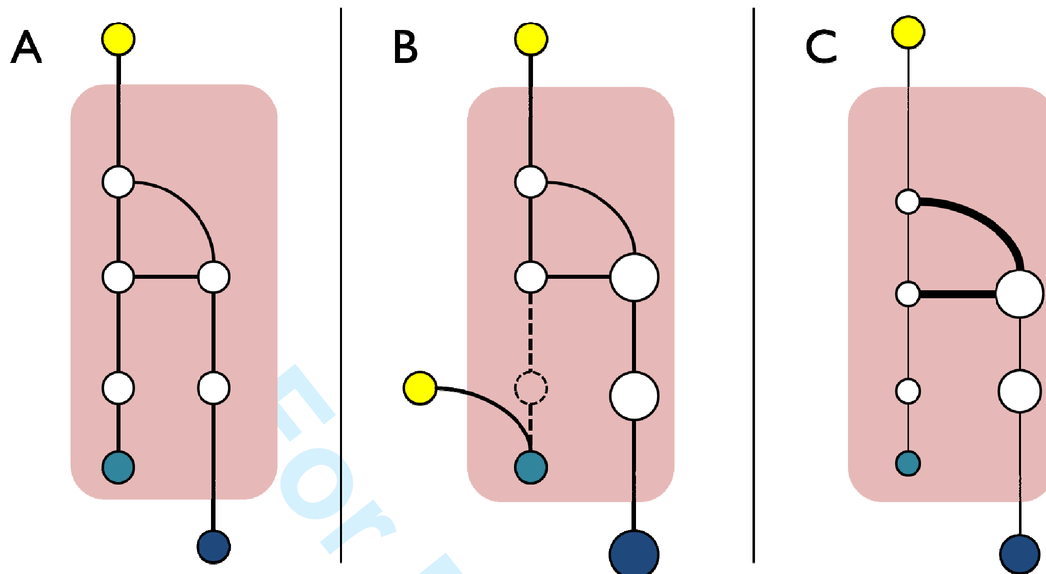
Figure 1



For Peer Review

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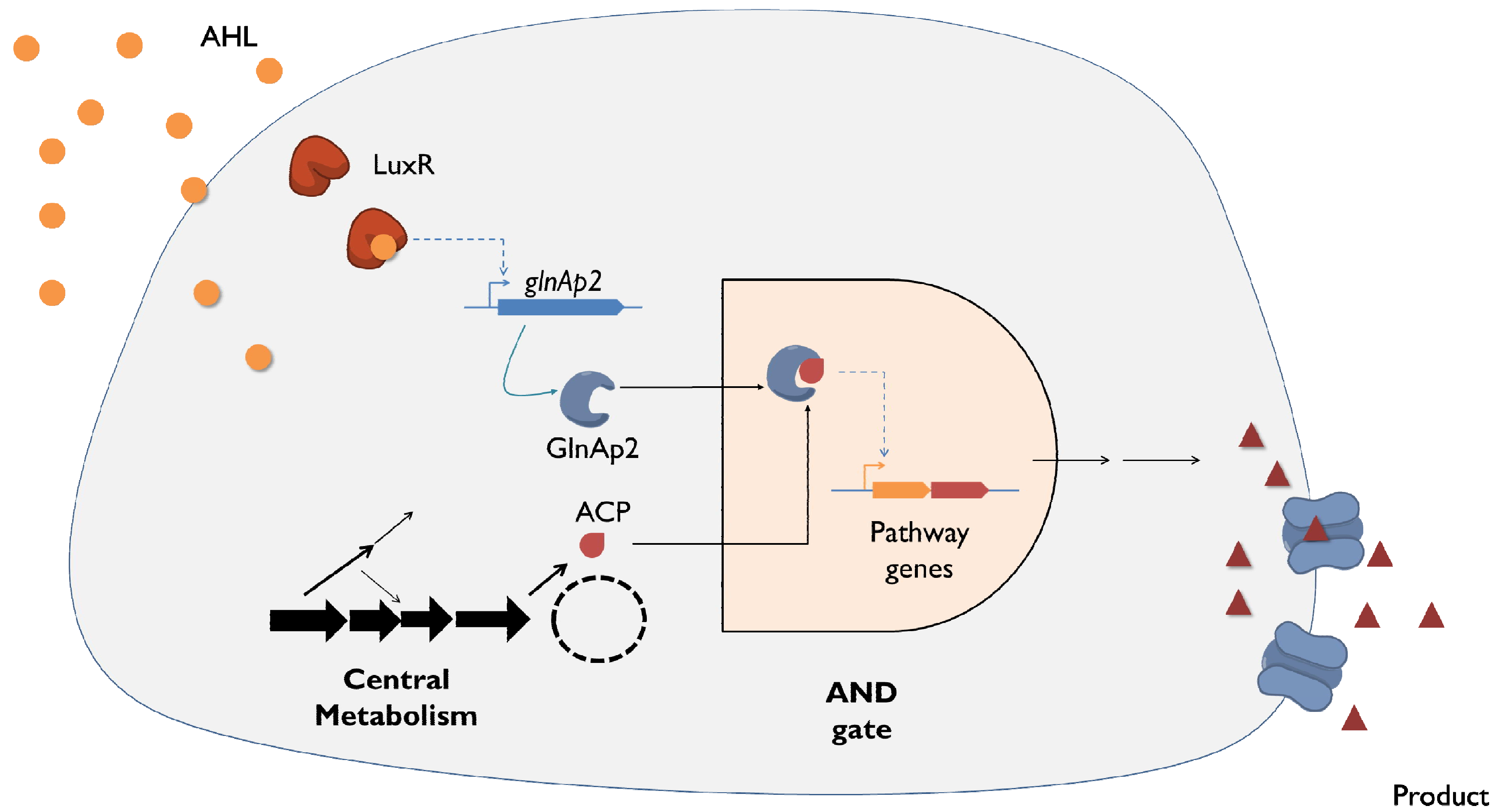

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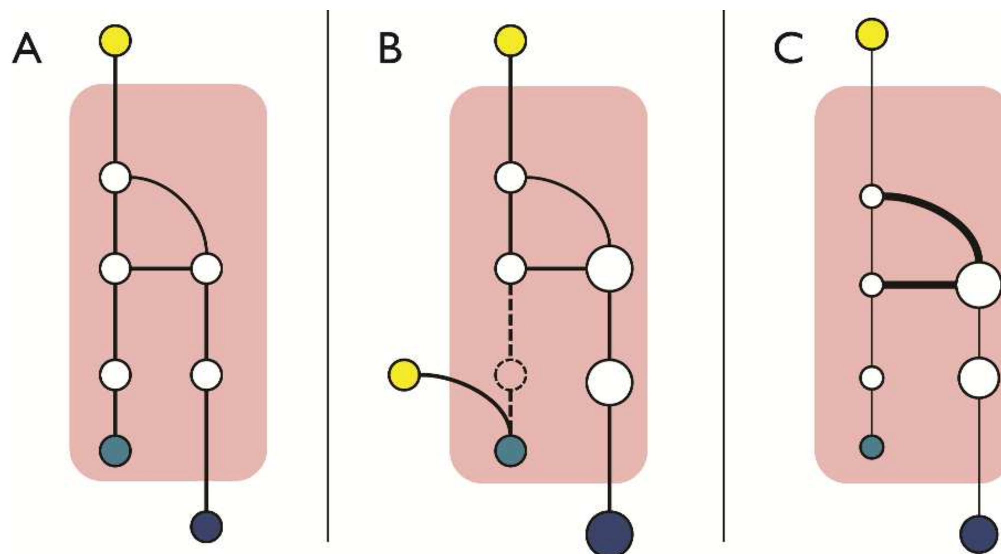

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view

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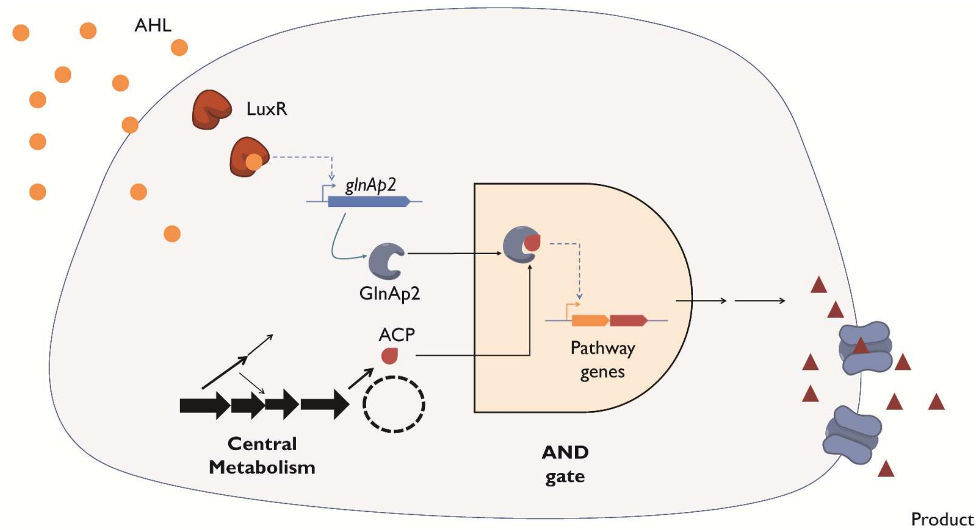

Figure 2. A hypothetical example of a complex regulatory circuit utilizing multiple modules or parts. The LuxR/LuxI quorum sensing system (luxI not shown), mediated by N-acyl homoserine lactone (AHL), is used to drive the expression of glnAp2, an acetyl phosphate (ACP) sensor [86]. Sufficient carbon flux through central metabolism will lead to accumulation of ACP. The presence of GlnAp2 and ACP serve as inputs to an AND gate (binding of ACP to GlnAp2) whose output is expression of pathway genes and, ultimately, synthesis of product (triangles). Product is only produced when biomass and carbon flux is high, i.e. from a healthy culture.
138x73mm (300 x 300 DPI)

Review