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

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1   # The zero-sum game of pathway optimization: emerging

2   # paradigms for tuning gene expression

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21   Running title: Tuning expression for pathway optimization

22

23   **<u>Abstract</u>**

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.

34

Introduction

Microbial production systems display a remarkable flexibility in the diversity and

enantioselectivity of the compounds that they can generate. These compounds have

historically been natural products such as ethanol, amino acids, acetone and antibiotics.

However, with the introduction of ever more sophisticated tools, a range of natural and

unnatural products have been made in engineered hosts including compounds such as

hydroxyacids[1-3], isoprenoids[4, 5], polyketides[6, 7], and biopolymers[8, 9]. While

several of these processes have been successfully commercialized [10-12], many remain

economically infeasible and are the subject of intense optimization efforts.

In optimizing microbial pathways, the objectives are to maximize product flux,

yield and selectivity. Traditionally, this problem has been approached by an analysis of

the metabolic pathway that leads to removing branch points that lower product yield and

selectivity (gene inactivation) and increasing the flux of intermediates through the

pathway (gene overexpression). The power of such methods has improved tremendously

with the advent of computational tools such as Flux Balance Analysis (FBA) [13, 14] and

bilevel optimization [15-17] to identify flux bottlenecks, yet, they are still fundamentally

constrained by the interconnectedness and finite nature of microbial resources (Figure 1).

Gene inactivations may necessitate media supplementation, impair cellular function and

are sometimes infeasible for non-linear production pathways. Overexpression of

pathway genes, on the other hand, comes at the expense of endogenous ones due to

consumption of common precursors and titration of cellular machinery such as

polymerases and ribosomes and may lead to growth inhibition, reduced expression and

even cell death [18-20]. In certain hosts the heat shock response is stimulated by protein

58   overexpression [21, 22] further limiting the degree of overexpression possible. Moreover,

59   successfully overexpressing or knocking out genes does not guarantee improved

60   productivity. Decoupling the native regulation of flux within the pathway in these ways

61   may lead to the accumulation of intermediates that can inhibit pathway enzymes [3, 23]

62   or are bacteriostatic [1, 24, 25].   These challenges are not insurmountable, but they do

63   underscore the need for more tools in pathway optimization. This review will highlight

64   novel approaches to pathway optimization and describe emerging paradigms for flux

65   manipulation.

66

67   **<u>Downregulation of related pathways</u>**

68   Modulation of gene expression, such as downregulation of undesired branch

69   points, has been identified as a fruitful avenue for increased pathway productivity [16,

70   17]. In contrast to gene inactivation, downregulation offers the ability to redirect

71   metabolite flux into production pathways while maintaining sufficient flux for

72   endogenous processes. Moreover, in cases of drastic differences in catalytic efficiency of

73   competing enzymes, it may prove more efficient than overexpression of pathway

74   enzymes.   Downregulation may be implemented in many different ways.   One

75   promising method, amenable to implementation in a wide variety of hosts and pathways,

76   is the use of antisense RNA (asRNA) mediated inhibition of translation [26-30].

77   One such example of asRNA use in pathway optimization is found in the

78   engineering of *Clostridium acetobutylicum*. Predating the rise of petrochemical sources,

79   *C. acetobutylicum* was an industrially relevant source of solvents such as acetone and

80   butantol [33] which it naturally ferments as part of its lifecycle [31, 32]. Recent volatility

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

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.

124

125   **<u>Dynamic Expression Profiles</u>**

126   When maximizing product titers and yields for industrial scale fermentation,

127   carbon flux is shifted from the normal balance of metabolic intermediates and shunted

128   into the desired product.   This shift is frequently at odds with the goals of the cell, i.e.

129   maintaining metabolic flux levels and maximizing biomass.   Thus, genetic alterations

130   that alter metabolic flux will incur a redistribution of metabolites to compensate for the

131   change with some inhibition of growth. Gadkar et al. [54] studied this issue *in silico* as it

132   applied to glycerol and ethanol production. In their work, they pursued a bilevel

133   optimization strategy analogous to that of OptKnock [15] where product titers are

134   maximized subject to growth maximization and other physical constraints to determine

135   gene candidates for upregulation or deletion. However, unlike OptKnock, they also

136   optimized the timing of these genetic changes. For glycerol production, simulations of a

137   biphasic approach to gene expression resulted in a 30% improvement in titers over a

138   static strategy. Similarly, ethanol titers were improved by 40% over a static strategy and

139   90% over wildtype behavior. These cases and more were further studied by Anesiadis

140   and coworkers [55] with the simulated behavior of genetic elements from synthetic

141   biology, as opposed to instantaneous switching in expression, and came to a similar

142   conclusion: dynamic control of gene expression may be implemented to increase pathway

143   productivity.

144   One of the first experimental demonstrations of this paradigm was elegantly

145   performed in 2000. In trying to produce lycopene in *E. coli*, Farmer and Liao [56] sought

146   to overexpress 2 key rate limiting enzymes: phosphoenolpyruvate synthase (Pps), which

147   controls the pool of a glycolytic intermediate needed for lycopene biosynthesis, and

148   isopentenyl diphosphate isomerase (Idi), which pulls glycolytic intermediates into the

149   lycopene biosynthetic pathway. However, overexpressing them statically from a *tac*

150   promoter hindered growth, yields and titers. Thus, they engineered a gene

151   circuit/metabolite control system in which expression of *pps* and *idi* was directly tied to

152   the availability of acetyl phosphate, a proxy for glycolytic flux and cellular health.

153   Using this approach, they were able to overexpress these enzymes to higher levels than

154   that seen using a static approach while maintaining cellular viability and ultimately

155   improve titers by 50%, productivity three-fold and carbon yields by more than an order of

156   magnitude. Similar control systems have also been developed to drive protein expression

157   through the use of quorum sensing in *E. coli* [57, 58]. Such systems allow for

158   coordinated delayed induction across multiple cellular populations in addition to

159   transmitting the metabolic load state of the host [59] thereby mitigating potential

160   challenges associated with protein overexpression. Moreover, they are modular and

161   readily amenable to integration in complex circuits [57] where Boolean logic and sensor

162   functions can be implemented for tight pathway regulation in combination with other

163   strategies for cumulative effects. A hypothetical example of this is presented in Figure 2

164   where sensing and logic (AND) operations are used to drive expression of pathway genes

165   and product only when high cell densities and carbon flux are achieved.

166

167   **Emerging paradigms**

168   With an eye towards the creation of sophisticated gene circuits and networks for

169   both pathway regulation and biosynthesis, the emerging discipline of synthetic biology

170   has established a paradigm of developing reusable modules or "parts" and "devices" to

171   control gene expression [60-62].   Towards this end, libraries of sensors, control

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].

195   More recently, new part classes such as engineered enzyme complexes have been

196   developed. As discussed previously, manipulating flux gives rise to a myriad of

197   challenges such as the physiological consequences of flux imbalance and titration of

198   cellular machinery. These undesired effects can be attenuated with gene modulation and

199   gene circuits with some tradeoff in selectivity due to reduced pathway intermediates.

200   Inspired by natural solutions to minimize this tradeoff [77, 78], Deuber et al. [79]

201   engineered an enzyme scaffold scheme to recruit multiple pathway enzymes in a single

202   complex. By colocalizing enzymes in this way, diffusional limitations are effectively

203   nullified and toxic metabolites can be maintained at locally high, but globally low,

204   concentrations to maximize pathway flux with minimal disruption to endogenous

205   processes. Furthermore, this scaffolding is scalable and generalizable to many enzymes

206   and pathways in specified stoichiometries allowing for efficient spatial organization of

207   pathway genes [79-81]. Using this system, mevalonate titers were improved by 70-fold

208   when compared to scaffold free control. Moreover, with rate limiting enzymes being

209   expressed at nominally low levels, the scaffolded constructs were able to achieve these

210   yields without the growth inhibition seen in an unscaffolded design [79]. Alternative

211   strategies such as direct protein fusions have also proved successful in improving

212   pathway productivity [81].

213

214   **Conclusions and Perspectives**

215   Microbial production systems have enormous potential to synthesize many

216   valuable chemical compounds in a sustainable manner. However, optimizing these

217   systems for economic feasibility remains a challenge, in part, due to the zero sum nature

218   of intracellular metabolites (Figure 1).   Redirecting these metabolites into pathways of

219   interest necessitates a loss of flux elsewhere and titration of cellular machinery away

220   from endogenous processes resulting in negative physiological consequences. These

221   concerns may be attenuated to some degree by microbial consortia. Such mixed

222   populations of cells are able to achieve more complex tasks, are more robust to

223   environmental changes and are able to be organized by function [82]. More importantly,

224   this functional specialization allows for the distribution of the metabolic burden across

225   populations resulting in overall healthier cultures and potentially more efficient

226   pathways. This advantage is offset, however, by the recalcitrant nature of genetic

227   manipulations of all but a few species and the potential for competition between

228   populations making a single organism, single population solution the most tractable

229   solution for the immediate future.

230   Despite the limitations of finite cellular resources, the use of tools which precisely

231   modulate expression levels has led to much improvement in pathway function by

232   mitigating the effects of the pathway on host physiology. Synthetic biology has further

233   contributed genetic parts and tools that allow for more precise application of regulation

234   through mechanisms such as basic computation [66, 83, 84], sensing[56, 85] and timed

235   expression [57, 58] with demonstrated improvements in productivity. Moreover, these

236   modifications may all be combined for cumulative pathway improvement (Figure 2).

237   With increased understanding of the consequences of metabolic perturbations and

238   evermore sophisticated regulation of expression, yields of microbial production systems

239   may soon be economically competitive with traditional synthesis culminating in the

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.

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

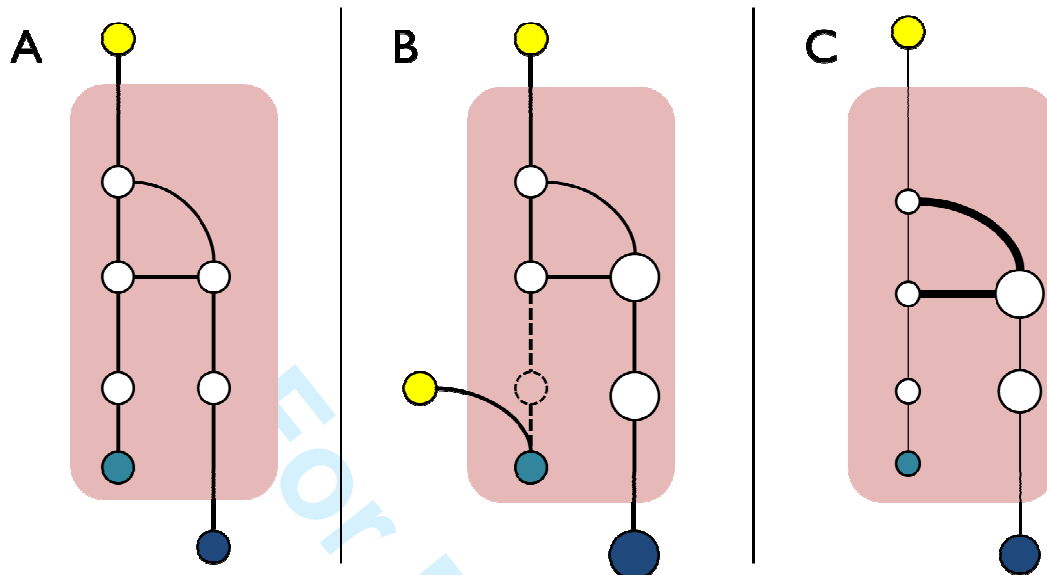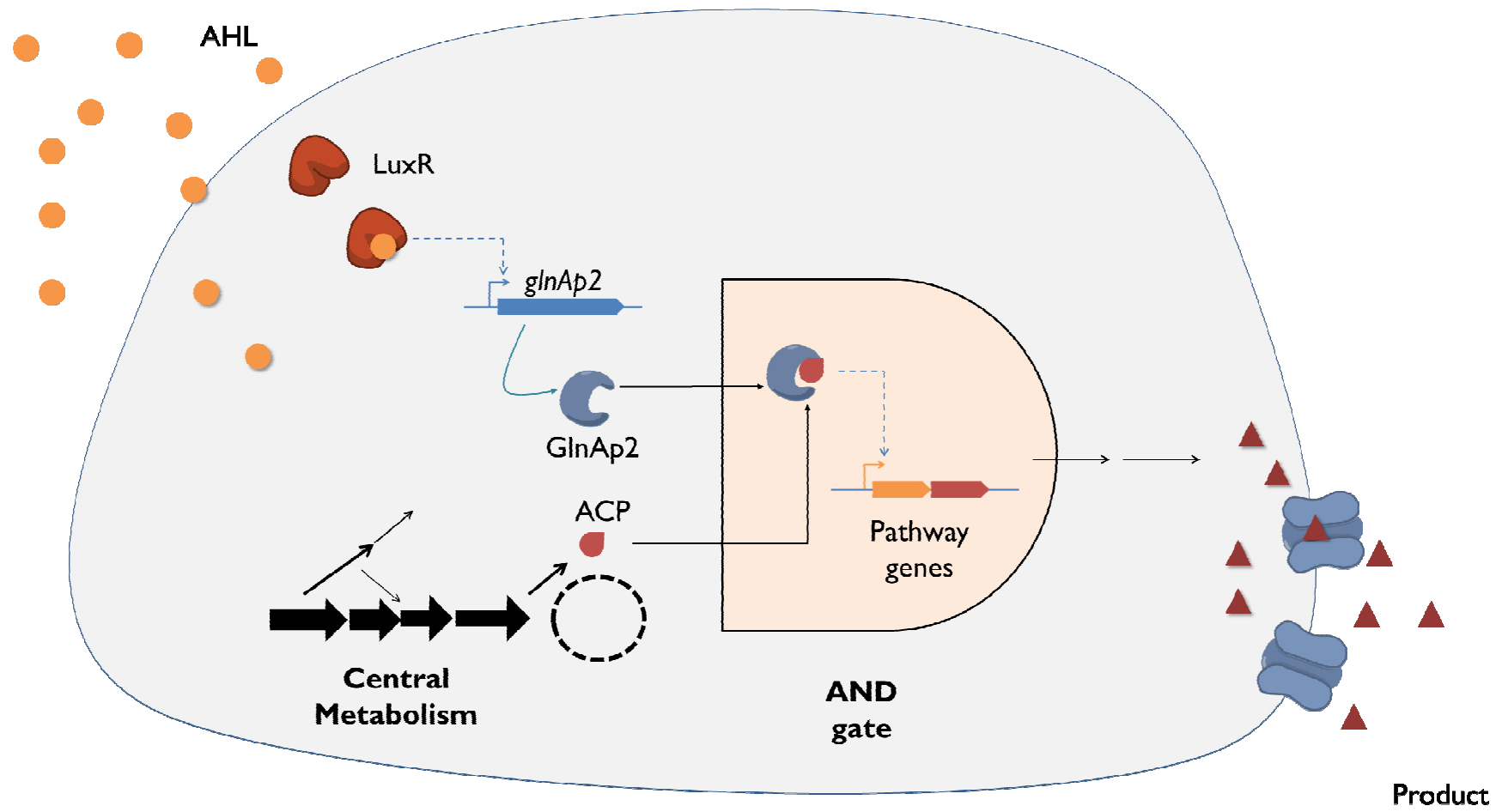*and* carbon flux is high, i.e. from a healthy culture.

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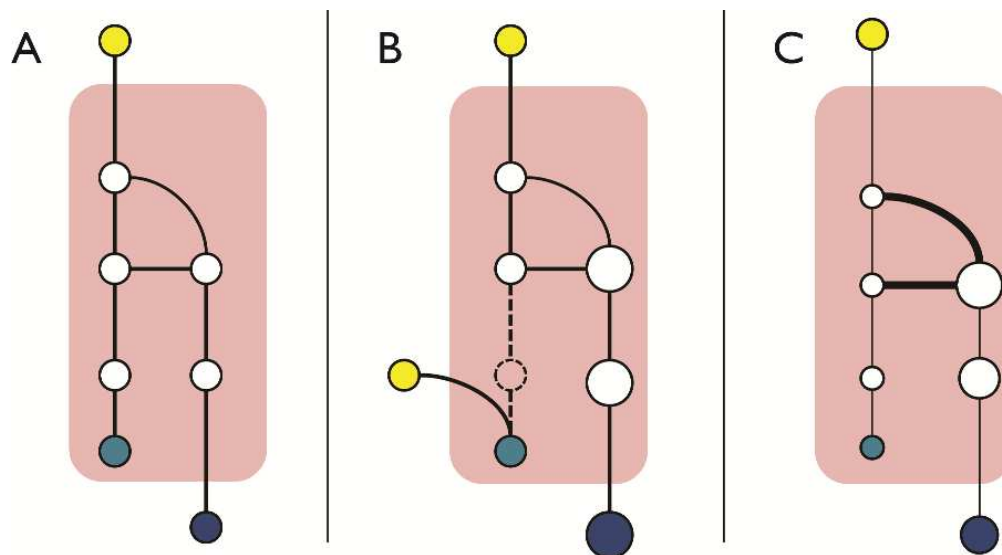59
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Figure 1

Figure 2

Fig 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.
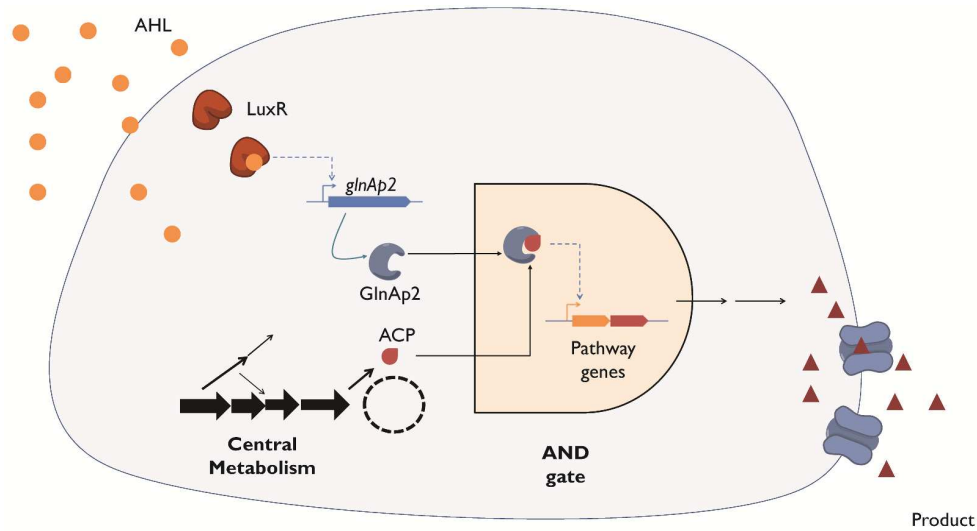76x42mm (300 x 300 DPI)

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)