

Host-Aware Synthetic Biology

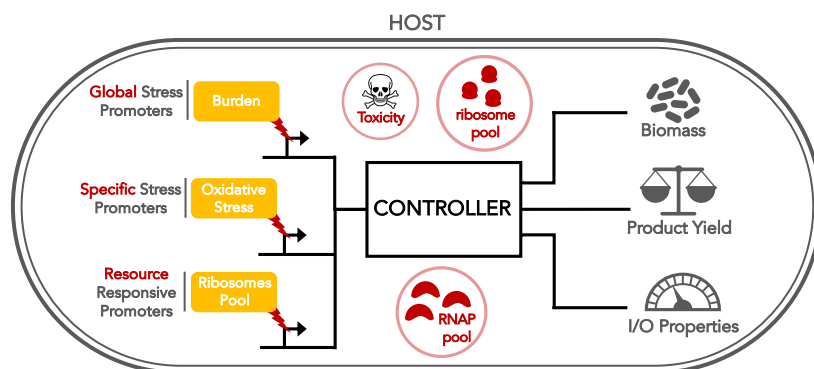
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Abstract

Unnatural gene expression imposes a load on engineered microorganisms that decreases their growth and subsequent production yields, a phenomenon known as *burden*. In the last decade, the field of synthetic biology has made progress on the development of biomolecular feedback control systems and other approaches that can improve the growth of engineered cells, as well as the genetic stability, portability and robust performance of cell-hosted synthetic constructs. In this review, we highlight recent work focused on the development of host-aware synthetic biology.



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INTRODUCTION

The fields of synthetic biology and metabolic engineering have long been confronted with a problem impairing the competitiveness and performance of their technologies: synthetic gene expression in engineered microorganisms causes native resources to be directed from the natural processes of the cell towards the synthetic constructs or pathways it hosts (**Fig.1**, [1, 2]). Because of this reallocation of cellular resources, especially limited cell machinery (e.g ribosomes, RNA polymerases), engineered cells typically grow slower than their wildtype equivalents, and this slow growth impacts performance and biosynthesis capabilities [3] (**Fig.1A**).

The fact that all natural and unnatural processes in cells are linked by common pools of resources also means that each process has the potential to affect the behaviour of all the others. More specifically, this phenomenon called *gene coupling* (**Fig.1B-C**) alters the input-output behaviour and thereby the modularity of synthetic constructs, a key factor to consider when assembling different constructs in a cell [4, 5]. Another issue arising from synthetic gene expression is that it can sometimes lead to the accumulation of toxic products [6], such as inclusion bodies that interfere with natural cellular processes and contribute to poor cell fitness (**Fig.1D**). Furthermore, cells with lower fitness due to burden often lose synthetic function due to strong positive selection for cells that acquire loss-of-function mutations, a phenomenon known as *genetic instability*.

Competition for shared cellular resources and how this leads to burden can be studied, measured and quantified [3, 7][8]. Indeed, the synthetic biology community strives to find reliable ways to build genetic constructs that minimise their impact on the cell, allowing both native and unnatural processes to work in harmony. In this review, we describe recent efforts that build technologies that reduce the impact of burden through the construction of resource-aware systems, with a particular focus on the engineering of burden-responsive biomolecular

feedback systems in *Escherichia coli*.

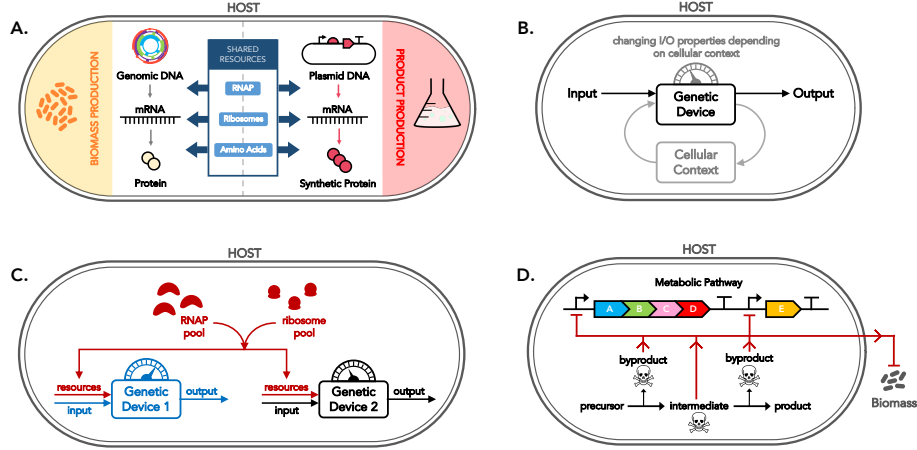


Figure 1: Current challenges leading to the development of host-aware constructs in synthetic biology. (A) Resource competition between the host cell and the synthetic constructs leads to growth rate reduction and poor system modularity, performance and robustness. (B) Resource-induced cross-talks appear due to the fact that genetic constructs not only compete for resources with the host cell but also with one another. (C) Input/output property fluctuations caused by variations of the host cellular context hamper the construction of modular constructs, i.e. constructs whose operational behaviours do not change in the presence of other constructs. (D) Toxicity arising from the expression of heterologous pathways in a host cell also leads to poor productivity and poor cell growth.

APPROACHES TO BURDEN REGULATION

A straightforward approach to reduce the burden caused by the expression of synthetic constructs is to decrease the level of expression of unnatural genes. This can be done by carefully selecting key factors influencing gene expression (e.g. promoter and ribosome binding site strengths) so as to minimise the burden and potential toxicity of synthetic constructs [3]. An important drawback of this method is that it may require several rounds of design-build-test-learn cycles and does not account for perturbations to the expression context, such as changes in the environment or phase of growth[9, 10]. A simple way to reduce burden is to integrate the constructs into the genome of the host cell, and

thus scale down their expression [11, 12]. However, this is not a viable option for applications that require high levels of gene expression. In 2010, Holtz and colleagues recommended that protein engineering, gene knockouts or simple expression level tuning systems are not effective solutions to minimise the burden of expressing complex production pathways *in vivo*, instead advocating for the development of dynamic control solutions [13, 14].

In nature, bacterial cells robustly allocate their resources to ensure survival and maximise growth. Martínez-Antonio and colleagues [15] showed that around 70% of known transcription factors in *E. coli* are autoregulated with more than two thirds of these via negative regulation. Furthermore, they found that around 40% of *E. coli* genes are negatively regulated. Negative feedback thus appears to be a key motif for robust regulation of natural gene expression in bacteria [16, 17, 18]. Negative feedback loops are widely used in engineering to attenuate the effects of unknown perturbations and design uncertainties. In synthetic biology, negative feedback biomolecular controllers of gene expression have already been widely used to alleviate burden and improve protein yields and are further discussed here [19, 20, 7, 21, 22, 23].

One of the first example of such regulatory constructs was published in 2001 when lycopene production was transcriptionally regulated through the use of a response regulator that reacts to variations in cellular energy and carbon availability by sensing intracellular glucose levels [24]. Typically, feedback regulation in systems like this are designed to respond to a specific product or metabolites [25, 26, 27, 28, 29, 7, 30]. As a result, those controllers are pathway specific and cannot be easily reused for other pathways or host cells. To build pathway-independent regulation systems, multiple studies have used quorum sensing (QS) to trigger unnatural gene expression only once a bacterial population has reached a critical cell density, thus separating a *growth phase* from a *production phase* for the engineered cells [31, 32, 33, 34]. Recently, more general approaches based on the sensing and regulation of the host cell capacity for

expression have been proposed to robustly allocate cellular resources, resulting in improved production of high value products.

75 BURDEN-REGULATED CONSTRUCTS

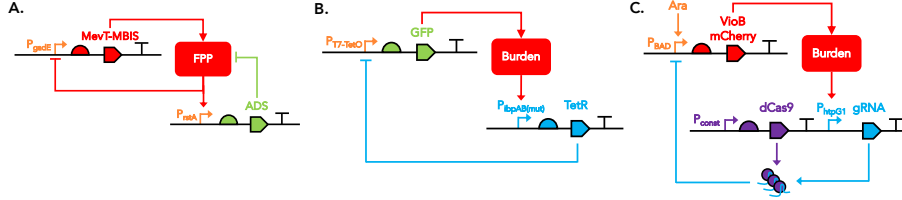


Figure 2: Constructs using negative feedback regulation to improve cell fitness. (A) A stress-specific feedback control system to improve cell growth [7]. The construct regulates farnesyl pyrophosphate (FPP) production in the isoprenoid biosynthetic pathway in *E. coli* to improve the yield of the final product: amorpha-4,11-diene. Without amorpha-4,11-diene synthase (ADS) expression, cells accumulate the toxic FPP intermediate, while in the presence of ADS, FPP is converted into nontoxic amorpha-4,11-diene. FPP is sensed by promoters identified via transcriptome analysis. In the presence of FPP, the FPP-repressed *gadE* promoter downregulates expression of the *MevT-MBIS* operon that produces FPP, while the FPP-activated *rstA* promoter is used to upregulate ADS expression. The two regulatory loops improve amorpha-4,11-diene yields by more than 2-fold. (B) A transcription factor-based feedback system for optimising toxic overexpression of protein, that utilises a stress-responsive promoter (*ibpAB*)[19]. Burdensome GFP expression is negatively regulated by the TetR repressor binding to its promoter (the hybrid T7-TetO promoter). TetR is expressed from the *ibpAB* promoter, which is known to activate in response to inclusion body formation. A degradation tag is fused to TetR to improve feedback loop dynamics. (C) A CRISPRi-based burden-driven feedback system for gene expression, utilising a general burden-induced promoter (*htpG1*) identified by RNA-seq [35]. Burdensome expression of a fusion protein (VioB-mCherry) can be regulated by a dCas9-based negative feedback controller. Burden induces the *htpG1* promoter which triggers expression of a small guide RNA (gRNA) designed to target constitutively expressed dCas9 to whichever promoter is suspected of producing the burden. Here the gRNA:dCas9 complex represses transcription from the *araBAD* promoter used to express VioB-mCherry, thereby closing the burden-based feedback loop.

Burden can be used to dynamically control expression of synthetic constructs. Ideally the controller should down-regulate unnatural expression when

burden impairs the cell giving a desired balance between unnatural gene expression and host cell growth [25, 26, 27, 1]. Most approaches used to build
80 host-aware synthetic constructs aim to keep burden within acceptable bounds and/or redistribute shared cellular resources to improve growth, productivity and construct modularity [19, 5, 35, 36, 37].

Two types of burden-regulated constructs have been demonstrated. The first
85 type focuses on sensing specific types of stresses and improving host tolerance to these. Using this approach, Dahl and colleagues (**Fig.2A**) built a construct that regulates the level of farnesyl pyrophosphate (FPP) and HMG-CoA, which are both toxic intermediates of the isoprenoid biosynthetic pathway [7]. Promoters downregulated by FPP were used to repress the expression of the isoprenoid
90 pathway. Conversely, promoters upregulated in toxic conditions were used to promote the expression of an enzyme (amorphadiene synthase, ADS) that consumes the toxic FPP intermediate, improving cell fitness. Siu and colleagues, followed a similar approach to improve *E. coli* tolerance to pinene [22]. They identified that expression of efflux pumps can help improve host tolerance to
95 pinene. However, significant efflux pump expression is toxic for the host, reducing cell growth. As a solution, expression of efflux pumps was directed by an engineered pinene-responding promoter. As a result, the stress of efflux pump expression was reduced and cells grew better in fluctuating concentrations of pinene.

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The examples above illustrate the use of stress-specific biosensors tailored to specific biosynthesis scenarios. Such sensors are ideal for the host-aware production of specific products of interest but not reusable for other applications. If the end goal instead is to minimise general burden irrespective of its root cause
105 then global burden biosensors need to be used. Regulation constructs that use global burden sensing are not optimised to respond to specific stresses but their strength comes from their portability across different genetic contexts. As they are sensitive to universal effects of burden, they can reduce the effects of burden

when expressing a large variety of genetic constructs. An early burden-driven
110 feedback controller was built in 2012, when Dragosits and colleagues (**Fig.2B**)
reasoned that the stress-related *ibpAB* promoter could be used to regulate stress-
ful expression of proteins. The promoter was chosen as a sensor based on its
known upregulation when heterologous protein over-expression causes inclusion
bodies to form within *E. coli* [19]. To link sensing to regulation they used the
115 TetR protein, with this used to repress a specific T7 promoter variant that drives
the stressful expression. Increased TetR expression in response to burden de-
creased T7-driven expression and led to growth rate recovery and improvement
in protein fraction solubility.

120 Recent work that utilizes CRISPR-based regulation has significantly im-
proved on this approach by enabling general targeting of any promoter driving
stressful expression [35]. Ceroni and colleagues developed a feedback system
that uses an RNA-mediated actuator to be able to quickly respond to a stress
without each time needing to translate a regulator protein (**Fig.2C**). To chose a
125 promoter to act as the burden sensor in their system, they performed transcrip-
tome analysis by RNA-seq to identify native promoters upregulated when cells
are burdened from the induction of various different synthetic constructs. The
native burden-responsive promoters that were identified by this work, could be
repurposed as a set of biosensors that detect and react to burden. One of these,
130 the *htpG1* promoter, was used in a feedback system to express synthetic guide
RNA (gRNA), which directs the deactivated Cas9 protein (dCas9) to bind to se-
quences in target promoters and sterically-repress expression from these. This
system automatically regulates expression from a chosen targeted promoter,
maintaining burden within reasonable bounds. They demonstrated that their
135 system quickly acted to reduce the production rate of the burdensome protein
when stress was detected, and in doing so freed up resources to provide better
host cell growth.

Feedback systems like these that are driven by the sensing of global burden

140 offer various advantages: they are robust to fluctuations in growth conditions
and perturbations to expression context, they can be applied to systems express-
ing different heterologous proteins, and they can even be ported to different host
strains. These systems also enable a way to optimally express different heterol-
ogous proteins without having to first go through many rounds of optimisation
145 to find the ideal promoter and RBS combinations.

REDUCING HOST-CONSTRUCT INTERACTIONS

The design of modular constructs that retain their input/output properties
in vivo remains a difficult problem due to the complex nature of the interac-
tions between the construct and the host, but also between different synthetic
150 constructs, for example through pools of shared cellular resources. Shopera and
colleagues (**Fig.3A**) hypothesised that negative feedback systems could be used
to reduce the coupling between different genetic constructs [5]. They observed
linear as well as nonlinear correlations depending on construct regulatory ar-
chitectures, revealing that resource-associated couplings arise from construct
155 topology. To investigate how to best construct a negative feedback loop to min-
imise gene coupling due to resource sharing, they built a system composed of two
independent constructs exhibiting a linear coupling correlation that minimally
impacted growth. While both constructs are inducible, the second is also reg-
ulated by a protein-based sequestration system to maintain its protein output
160 constant independently of the induction level of the first construct. Through
careful fine-tuning of the feedback regulator, coupling effects could be reduced.
However when the protein regulator was expressed in high amounts to increase
negative feedback gain, Shopera and colleagues observed an increase in resource
coupling as its expression also uses cellular resources. This highlights the impor-
165 tance of carefully tuning of the feedback loop to ascertain its advantage over an
open-loop configuration. In more recent work, Huang and colleagues (**Fig.3B**)
used a sRNA-based controller to build a similar system capable of reducing gene
coupling effects [38]. They demonstrated how quasi-integral negative feedback

can reject fluctuations in free ribosome availability by treating these fluctuations
170 as a disturbance that impacts the performance of synthetic constructs.

Another solution to the problem of competition for shared cellular resources consists in creating separate, host-specific and construct-specific pools of resources. This idea gained popularity in the last decade with work focusing
175 on the engineering of orthogonal pools of resources for transcription (e.g. T7 polymerase [40]) and degradation (e.g. proteases [41]). Ribosomal capacity, represented by the amount of free ribosomes in a cell, has recently been shown to represent a key limiting factor for gene expression in fast dividing organisms [4, 42, 3, 43]. Darlington and colleagues (**Fig.3C**) created a feedback controller
180 to dynamically regulate the number of orthogonal ribosomes dedicated to the translation of native processes or to the translation of synthetic constructs [36]. Synthetic ribosomes, or o-ribosomes, carry a synthetic 16S rRNA sequence that competes with the native 16S rRNA to form o-ribosomes instead of native ribosomes. If the demand for o-ribosomes by synthetic mRNAs is low, high amounts
185 of LacI regulator mRNA are produced, leading to a decrease in the production of synthetic 16S rRNA and thus of o-ribosomes. On the contrary, if demand for o-ribosomes is high, 16S rRNA production will no longer be downregulated. They found that their controller could reduce gene coupling by 50%. They recently expanded the current implementation of their controller to design a
190 quasi-integral controller for dynamic allocation of translational resources [44].

Finally, Venturelli and colleagues (**Fig.3D**) developed a more global approach to the problem of resource reallocation. They have made use of the native MazF ribonuclease to cleave unprotected mRNA and by doing so reallocate
195 all the cellular resources that translate mRNAs [39]. They found that recoding synthetic constructs to lack MazF recognition sequences greatly improved the translation rate of these protected constructs when MazF was expressed, and this could be used to dramatically increase synthetic expression of enzymes that drive gluconate production. This is because once MazF is expressed, host native

200 mRNAs are degraded *en masse* and so the synthetic RNA becomes saturated
with the native ribosomes, thus reallocating these ribosomes from host growth
functions to synthetic construct over-expression. Synthetic gene expression was
further enhanced by protecting key host factors for translation and RNA de-
cay, and by devising a negative autoregulatory feedback loop on MazF to more
205 precisely titrate MazF levels in the system. Compared to CRISPR, MazF can
easily regulate the expression of numerous genes, offering an exciting tool to
build global resource reallocation systems.

PREDICTING HOST-CONSTRUCT INTERACTIONS

Computational models describing host-construct interactions are a valuable
210 tool for the design and optimisation of host-aware synthetic constructs, and
especially dynamic controllers that robustly balance the allocation of cellular
resources between unnatural expression and biomass [42, 45]. In work described
in 2015, Weisse and colleagues built a coarse-grained whole-cell model in an
attempt to capture the impact of synthetic constructs on cell growth [46]. This
215 simple model considers finite pools of shared cellular resources (e.g. energy,
building blocks, and transcriptional and translational resources) that are used
for synthetic construct expression and host growth processes. This theoretical
framework has been expanded recently to provide more detailed host-construct
interactions and now includes other shared pools of resources. For example,
220 Darlington and colleagues have expanded the Weisse model to incorporate ri-
bosome synthesis and two quasi-orthogonal pools of ribosomes [36]. Hopefully,
in the future, genetic designers will make full use of these whole-cell models to
produce educated designs of robustly optimal host-aware constructs.

CONCLUSIONS

225 Synthetic biology has now reached a point where it is no longer possible to
ignore host-construct interactions and designing host-aware systems should now
be a priority. The goal is to have engineered systems that automatically adapt

their level of host resource consumption and toxicity in order to robustly perform their function while maintaining cell growth rates within tight bounds. As
230 the complexity of synthetic constructs increases, new challenges also arise. In the field of metabolic engineering, the core objective is often to increase production yields at all costs. Logic circuit constructs on the other hand require high accuracy and precision when it comes to signal transduction. Hence, synthetic modules should ideally behave in a predictable manner taking into account tox-
235 icity and the availability of shared cellular resources.

Predictability of construct behaviour *in vivo* is a hot topic, with a particular focus on the development of whole-cell models that predict host-construct and construct-construct interactions. Further development of such frameworks
240 are necessary for the design and understanding of resource-aware synthetic biology. Recent work has demonstrated that burden-driven negative feedback can improve robustness to genetic and environmental perturbations and increase genetic stability of synthetic constructs. Such biomolecular feedbacks are also useful to reduce gene coupling between different genetic constructs hosted by
245 the same cell and to reduce construct-host competition for shared cellular resources. The main advantage of these feedback systems is their dynamic nature, which is still to be fully demonstrated experimentally. There is also a need for studying the effect of different pools of shared resources beyond ribosomes such as orthogonal RNA polymerase, tRNA or proteases pools.

250 Increased use of transcriptome, proteome and metabolome analysis tools will further improve our understanding of engineered cells and their contextual effects, and will no doubt increase the predictive capabilities of resource-based models. It may not be possible to ever eliminate burden when programming
255 cells to do extra tasks, however engineering efforts such as those described here can at least mitigate its impact and allow the development of synthetic biology systems that work in better harmony with their host.

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DECLARATION OF INTERESTS

Declarations of interest: none.

Mini Summaries for the References

265 Papers of particular interest, published within the period of review, have been highlighted as:

(●) of special interest (●●) of outstanding interest

- 270 1. (●) **Cell-free prediction of protein expression costs for growing cells [8]**

The authors demonstrated how cell lysate-based assays can be used to quantify the burden of genetic constructs and how, coupled with a mathematical model, this can be used to predict the burden imposed by the same constructs in *E. coli*. Through this approach, they could predict
275 host behaviour in response to the expression of different designs of the beta-carotene pathway, especially when cellular burden arises from competition of shared cellular resources (expression burden).

- 280 2. (●) **Design and selection of a synthetic feedback loop for optimising biofuel tolerance [22]**

To improve cell tolerance to pinene, the authors dynamically regulated the production of efflux pumps known to impose a large burden on engineered cells. Pump production was triggered in the presence of pinene

285 stress. Promoter engineering was performed to reduce leakiness, so that,
in the absence of pinene, the growth rate would not be impacted by the
production of the efflux pumps.

290 3. (●) **Layered dynamic regulation for improving metabolic pathway
productivity in *E. coli* [34]**

By building two layers of regulation, the authors improved the production
of D-glucaric acid in *E. coli*. A first layer uses quorum sensing to switch
between “growth” and “production” modes when the density of cells is suf-
ficiently high. The second layer triggers the production of a downstream
295 enzyme (Miox) only when the concentration of an intermediate product
(myo-inositol) is sufficiently high.

4. (●●) **Burden-driven feedback control of gene expression [35]**

300 The authors demonstrated how the htpG1 promoter, identified via RNA-
seq, can be used as a burden biosensor when a range of synthetic con-
structs are expressed in *E. coli*. They then used the htpG1 promoter to
drive a dCas9-based negative feedback loop to keep burden within reason-
able bounds and improve synthetic production yields.

305 5. (●●) **Dynamic allocation of orthogonal ribosomes facilitates un-
coupling of co-expressed genes [36]**

The authors expanded Weisse’s model [46] to predict if assigning different
ribosome pools to native or synthetic processes can reduce translational
resources couplings between multiple synthetic constructs. They exper-
310 imentally validated that their dynamic allocation system of orthogonal
ribosomes reduces gene coupling in *E. coli*.

6. (●) **Synthetic control systems for high performance gene expres-
sion in mammalian cells [37]**

315 In mammalian cells, the authors found through the implementation of

incoherent feedforward, negative feedback and hybrid incoherent feedforward and negative feedback motifs that both variations in plasmid take-up and transactivator concentration lead to different cell-to-cell behaviours in transient mammalian transfection experiments. They demonstrated that robustness, yield, and burden impact could be improved by these motifs.

7. (••) **A quasi-integral controller for adaptation of genetic modules to variable ribosome demand [38]**

Using an sRNA-based controller, the authors showed that resource coupling between synthetic constructs can be mitigated. Their solution relies on the implementation of a quasi-integral feedback loop that treats fluctuations in the number of free ribosomes in the cell as a perturbation.

8. (••) **Programming mRNA decay to modulate synthetic circuit resource allocation [39]**

Using the MazF sequence-dependent endonuclease, the authors demonstrated that protecting the mRNA of synthetic constructs against MazF degradation leads to improved productivity and titer of gluconate due to translational resource reallocation.

9. (•) **On the design of integral translational resource allocation controllers for synthetic cellular circuitry [44]**

The authors studied the robustness of different controllers for resource allocation. They used a geometric formulation of the μ analysis problem, proposing an approach broadly applicable to study the robustness of synthetic constructs.

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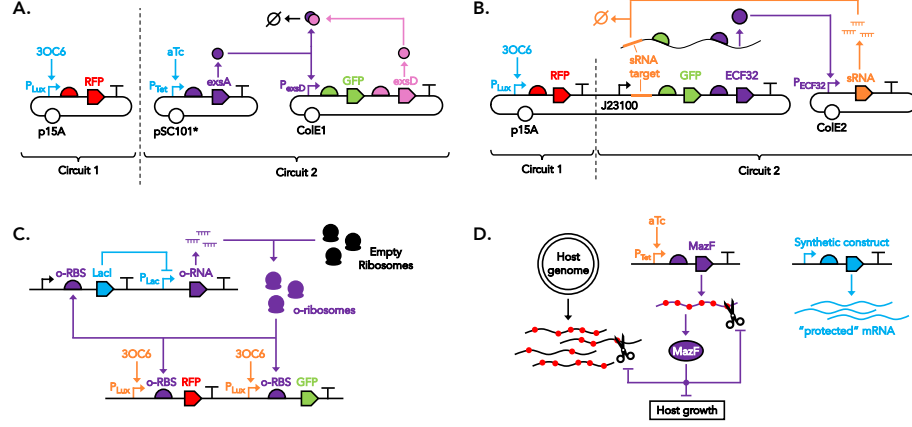


Figure 3: Engineered systems designed to improve construct modularity and host fitness by reallocation of shared resources. (A) Decreased gene coupling via a protein-based feedback system [5]. Two independent constructs are co-expressed in *E. coli*. RFP expression is induced by the AHL molecule 3OC6, which activates the Lux promoter, and aTc binds the ExsA activator to induce the exsD promoter to produce GFP. The anti-activator protein ExsD is also expressed from the exsD promoter, and acts as a proxy for the abundance of GFP and thus of host expression resources. The exsA-exsD control system reduces GFP expression when its resource consumption impacts RFP expression, thereby improving modularity (i.e. operational independence). (B) Decreased gene coupling via a feedback controller that treats variation in free ribosomes as disturbances of resource competition [38]. RFP is regulated by AHL (3OC6), while GFP expression is affected by the availability of free ribosomes. A sigma factor (ECF32) co-expressed with GFP binds to its cognate promoter to produce a small RNA that binds and activates degradation of GFP mRNA, hence downregulating GFP expression. (C) Dynamic control of the size of the natural and orthogonal ribosome pools depending on ribosome demand [36]. Orthogonal ribosomes (o-ribosomes) are produced by expressing a synthetic 16S rRNA (o-RNA) that binds ribosomes lacking 16S rRNA. As demand for translational resources from two independent constructs (RFP and GFP) increases, o-RNA production increases providing more o-ribosomes. To regulate o-ribosome levels, the LacI, RFP and GFP constructs compete for o-ribosomes, such that, when demand for o-ribosomes is high, less LacI repressor is expressed, and hence more o-RNA are produced. (D) General reallocation of cellular resources by MazF ribonuclease cleavage [39]. The aTc-induced MazF endoribonuclease recognises the RNA sequence 'ACA' shown here as red dots. If synthetic constructs are codon-optimised to remove all such recognition sites, then MazF only targets native mRNAs. Upon aTc induction, MazF degrades native transcripts and host translational resources are reallocated almost exclusively to the expression of the synthetic constructs.