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Current Opinion in Microbiology

Overloaded and stressed: whole-cell considerations for bacterial synthetic biology

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The predictability and robustness of engineered bacteria depend on the many interactions between synthetic constructs and their host cells. Expression from synthetic constructs is an unnatural load for the host that typically reduces growth, triggers stresses and leads to decrease in performance or failure of engineered cells. Work in systems and synthetic biology has now begun to address this through new tools, methods and strategies that characterise and exploit host–construct interactions in bacteria. Focusing on work in *E. coli*, we review here a selection of the recent developments in this area, highlighting the emerging issues and describing the new solutions that are now making the synthetic biology community consider the cell just as much as they consider the construct.

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Current Opinion in Microbiology 2016, **33**:123–130

This review comes from a themed issue on **Microbial systems biology**

Edited by **Ralf Takors** and **Victor de Lorenzo**

For a complete overview see the Issue and the Editorial

Available online 2nd August 2016

http://dx.doi.org/10.1016/j.mib.2016.07.009

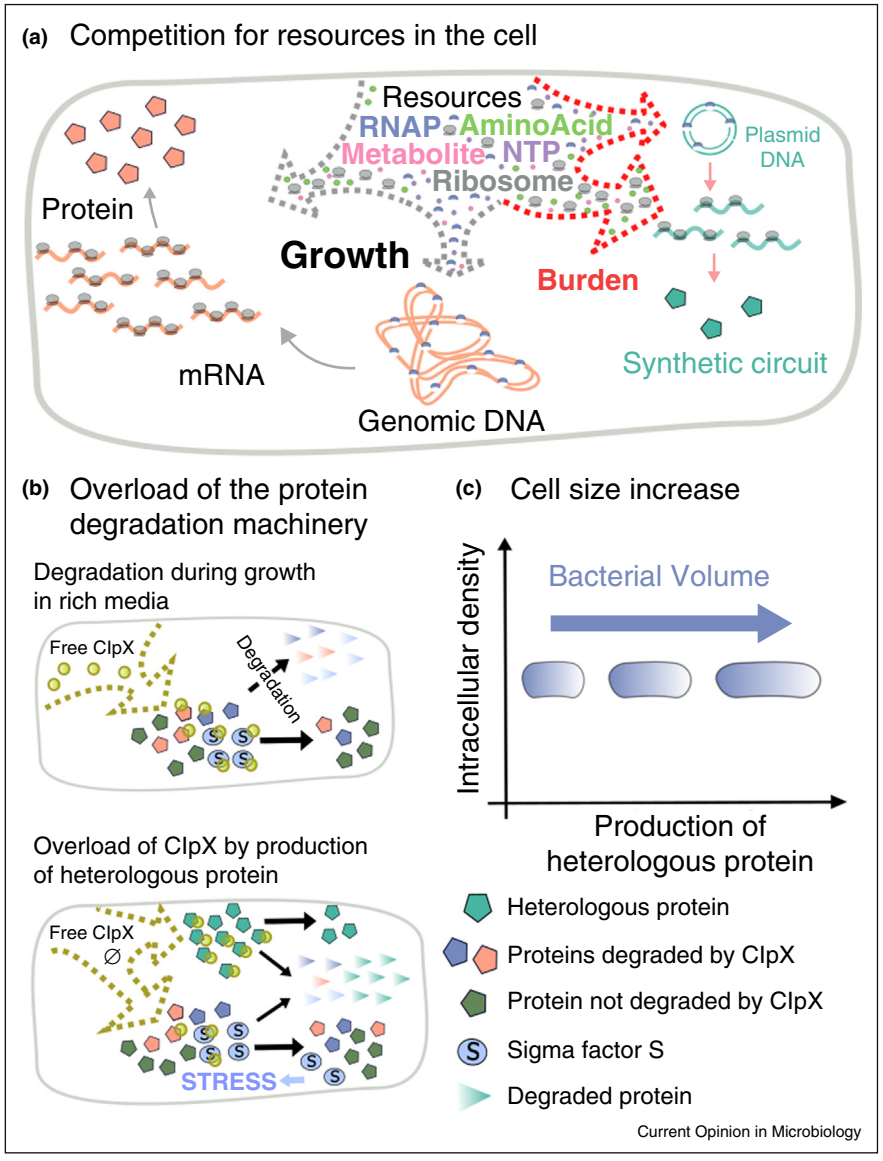
Introduction

Synthetic biology uses engineering principles to enable the rational design of genetic constructs with predictable behaviour. Many advances have been achieved towards the design of novel genetic and metabolic pathways of increasing complexity in the past decade [1,2,3••]. In recent years, research has also been devoted to design considerations where predictability is a challenge, such as situations where retroactivity, toxicity of protein products and long-term instability of DNA constructs are crucial to successful implementations [4]. While these construct-centric issues are progressively being addressed, contextual issues such as interactions between constructs and the host cell are much less understood. As a result, the behaviour of synthetic gene constructs in living cells can be hard to predict [5••] and often a large number of iterations are necessary to obtain a construct that can function adequately in the context of a specific host [6–8]. In this review we discuss the most important research advances in the characterisation of host–construct interactions in bacterial cells for synthetic biology, paying special attention to recent work that identifies insights and rules to help future design of synthetic constructs. Rather than discuss the specific interactions that various enzymes and pathways have on host cell metabolism, we focus here on interactions that are general for all synthetic constructs, such as their use of host resources for maintenance and expression.

Impact of synthetic constructs on host cell physiology

Cells possess a finite pool of resources needed for biological processes such as replication, transcription, translation and enzymatic reactions. Depending on the environment and growth conditions, cells must allocate their resources to different tasks in order to maximise their fitness [9,10]. Engineered overexpression of native genes and the introduction of any heterologous DNA constructs both represent a challenge for cells that alter the fine equilibrium of their resource allocation [11,12]. This unnatural extra *load* typically leads to slower growth and lower protein yields [13], a phenomenon known as *burden* (Figure 1a). While heterologous expression can cause depletion of any of the gene expression and metabolic resources of the cell, in fast dividing organisms the use of translational and transcriptional resources (e.g. ribosomes and RNA polymerases, respectively) appears to be a major cause of burden. Scott *et al.* described the regulation of ribosome concentration as the key process that optimises the allocation of amino acids between the different functions of the cell, allowing optimal growth in different environments [14]. Gyorgy *et al.* analysed the competition for shared resources when multiple constructs are present in the cell and showed that together with ribosomes, RNA polymerases are also a limiting resource that can contribute to burden [15•]. Thus, any unnatural protein expression is an extra load that can decrease host cell fitness. One way to address this problem is through careful choice of expression determinants within a genetic construct, such as the promoter and ribosome binding site parts. These can have a significant impact on the output of the system but also on the use of

Figure 1



The impact of synthetic constructs on the host cell. **(a)** Competition for shared resources in the cell. Both the resources involved in transcription (RNAP = RNA polymerase) and in translation (ribosomes shown as grey circles) are shared between cellular processes needed for efficient cell growth and the production of proteins from synthetic constructs such as circuit-encoding plasmids. **(b)** Overload of the protein degradation machinery. The degradation machinery (yellow) degrades a wide range of proteins in the cell with different efficiencies. For example, σ^S (blue) is typically quickly degraded in rich conditions. However when a heterologous protein (light green) is produced in large amounts available proteases become limited leading to a reduction in σ^S degradation, raising its levels above the threshold to activate expression of stress-response genes [17]. **(c)** Cell size increase due to cellular overload. When a large amount of heterologous proteins are produced the size of the cell increases to keep intracellular density constant. A change in total DNA, RNA and protein per cell is also observed [18].

host resources. Consequently, designs with a lower load typically impart less burden, and often these are simply those with lower heterologous expression [16].

The limited number of enzymes available in cells can also cause crosstalk between co-existing devices inside the cells leading to unpredictable behaviours [17]. In some conditions (e.g. starvation or production of heterologous proteins) the protein degradation machinery is a limited resource and thus becomes a source of competition between proteins that need to be degraded. Cookson *et al.* showed that two apparently independent synthetic gene constructs with no interacting parts can be 'coupled' and exhibit synchronized production due to overloading of the ClpX degradation machinery causing an enzymatic 'queuing' effect [17]. The saturation of the ClpX protease

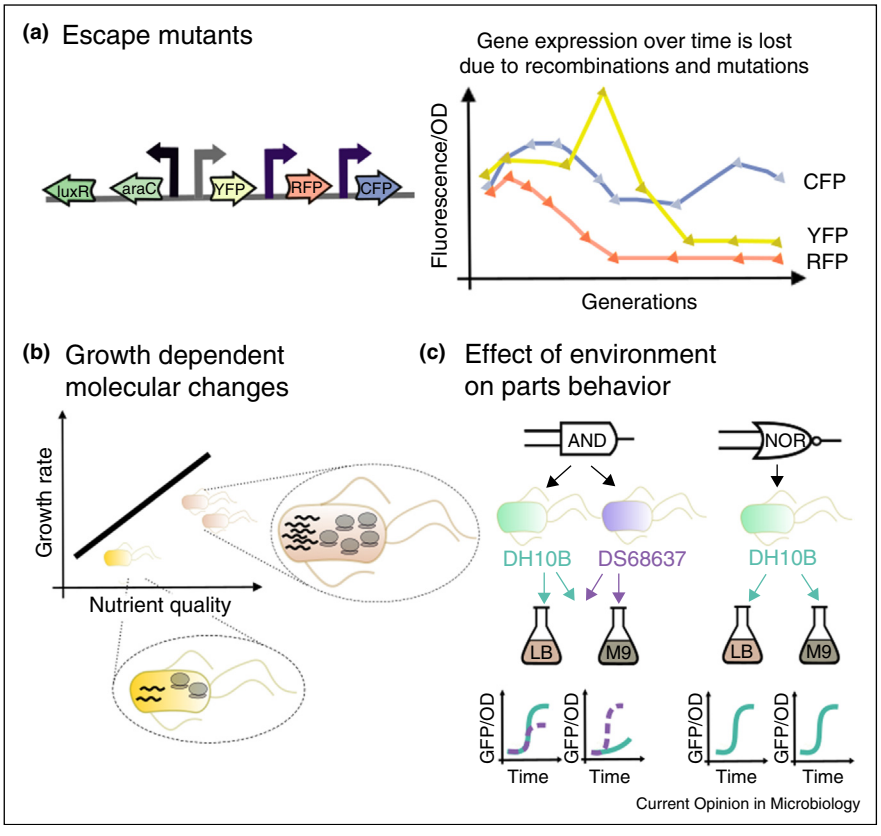
prevents turnover of highly-degraded proteins and thus modifies the relative concentration between proteins in the cell (Figure 1b). This saturation also increases the concentration of the stress sigma factor σ^S, which is usually rapidly degraded in low stress conditions. ClpX saturation increases σ^S levels and so promotes activation of the stress response (Figure 1b).

An additional parameter tightly controlled in the cell that is altered by heterologous protein production is intracellular density. A strong correlation has been observed for bacteria between cell mass and cell volume [18,19]. This implies that an increase in protein production is compensated for by an increase in cell size to keep a near constant intracellular density (Figure 1c). The increase in cell size in turns coincides with a change in the macromolecule proportions in the cell, causing a decrease in DNA and RNA concentrations [18].

Impact of host cell physiology on synthetic constructs

Just as synthetic constructs can reduce host fitness, the host cell can in turn reduce the expected performance of a construct. Fast growing bacteria are under strong selective pressure and so those transformed with synthetic plasmids are typically at a fitness disadvantage. Under this evolutionary pressure, bacteria typically escape the burden of nonessential expression by loss-of-function mutations [20•], which subsequently allow mutant cells with faster replication times to overtake the growth of the original population (Figure 2a). Increasingly, those involved in the design and modelling of synthetic constructs are paying attention to the fact that burden alters cell physiology, often leading to undesired phenotypes. In their recent work to produce stripe-forming networks in *E. coli*, Schaerli *et al.* showed that high expression levels in a subset of initial networks caused burden and yielded

Figure 2



Host cell complexity affects genetic construct behaviour. **(a)** Escape mutant profiling. The constructs developed by Sleight *et al.* [20•] are composed of the fluorescent proteins genes YFP, RFP and CFP under the control of externally-inducible promoters regulated by the *luxR* and *araC* genes and by genomic *lacI*. Expression of all three occurs when *E. coli* are grown in media supplemented with IPTG, arabinose and AHL. Tracking fluorescence expression over 200 generations of culturing allows loss-of-function mutations from different construct designs to be observed. **(b)** Growth-dependent molecular changes. Cell composition is growth-rate dependent and the amount of RNA and ribosomes per cell increases with increasing growth rate. This change results in a growth-rate dependence on gene expression [44]. **(c)** Effect of the cellular environment on part behaviour. The expression of GFP from an AND gate circuit construct shows significant differences in performance when measured in two different strains (DH10B and DS68637) and two different media (LB and M9). A NOR gate circuit construct also tested in DH10B in the two media did not show any variation [23•].

phenotypes that appeared promising, but were later determined to be failures behaving in unexpected ways due to slow growth [21].

The different conditions of typical bacterial growth also impact construct expression because the macromolecular composition of the cell, such as its transcript and protein numbers, change over media quality [19]. Klumpp *et al.* investigated this growth-rate dependence and how it affects gene expression [22]. They showed that transcription rate and ribosomal content both increase as growth rate increases, and that this also affects heterologous gene expression rates in cells (Figure 2b). Variation in the host genome also further impacts on the desired output of a system. Cardinale *et al.* investigated how host variation impacts a synthetic construct by analysing the behaviour of a simple network of three fluorescent reporters in a panel of different *E. coli* strains. In these different genetic backgrounds, often with just a single native gene removed, host metabolism and growth characteristics changed significantly leading to altered construct performance [5••]. Moser *et al.* also showed that a single construct (e.g. AND logic gate) behaved differently when transformed into alternative *E. coli* strains, and further considered the effect of different growth media on different constructs (e.g. AND and NOR gates). While the NOR gate exhibited no variation when cells were grown in rich and minimal media, the AND gate displayed no activity in minimal media but was active in rich media in strain DH10B (Figure 2c) and gave unexpected outcomes when grown at industrial scale [23•]. The unpredictable variability caused by simple changes to host or environment highlights the need for more extensive characterisation of synthetic construct performance in specific contexts.

Development of tools to measure, reduce and predict host–construct interactions

As the synthetic biology community has become more aware of the detrimental effects that synthetic constructs may have on the host cell and *vice versa*, novel tools to assess or reduce the impact of constructs on host physiology have been developed [24–26]. These now provide possible strategies to limit, 'design-around' or exploit host–construct interactions when designing new synthetic gene networks.

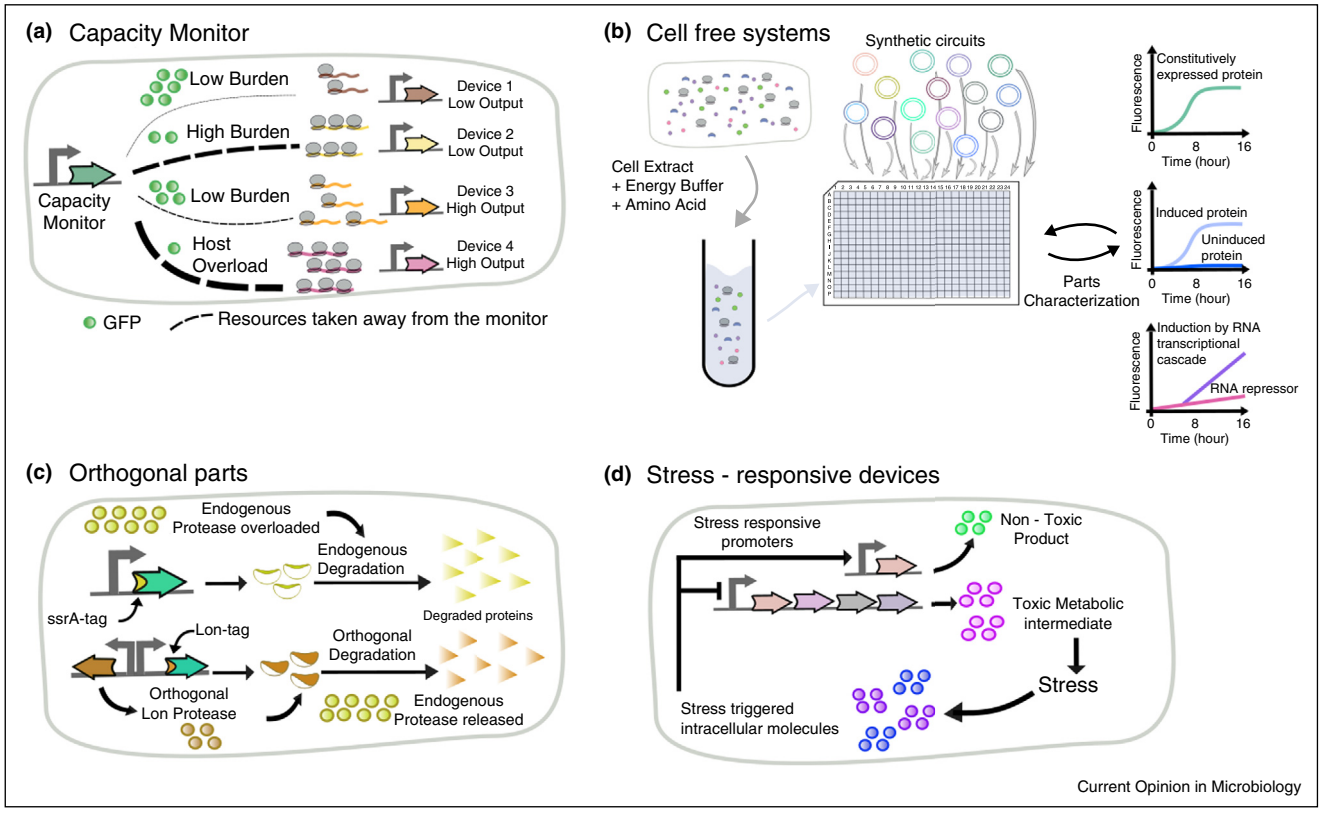
Firstly, to measure the burden caused by different genetic parts when added to *E. coli*, a method was recently described where cells are equipped with a 'capacity monitor' that reports how much gene expression capacity is taken-up by a construct [27••]. The monitor is a synthetic cassette placed in the bacterial chromosome that constitutively expresses a green fluorescent protein (GFP). When constructs are added to the cell, their burden can be calculated from subsequent changes in green fluorescence. By having a way to measure burden from different constructs, efficient designs that consume fewer resources can then be chosen (Figure 3a).

Recent work has also shown that Cell-Free Systems (CFS) can be useful as rapid, simplified prototyping environments that mirror *in vivo* conditions. These are often referred to as transcription-translation systems ('TX-TL') and typically consist of bacterial cell extracts or purified gene expression machinery, mixed with buffer/energy mix optimised for gene expression. Synthetic construct DNA is added to this *in vitro* and performance data for construct expression can be quickly measured. Due to their simplicity and versatility, CFS are starting to be used to test and characterise out-of-context behaviours of synthetic systems to quickly identify unfavourable designs not suited for *in vivo* work [28,29]. CFS not only allow in depth investigation of condition-dependent behaviours by directly varying the concentrations of DNA, amino acids, ATP or sugars, but also give data not confounded by bacteria adapting to the added construct, for example, by changing their growth rate, size or by adapting via stress responses. As an example, Siegal-Gaskins *et al.* used CFS to analyse resource competition of synthetic constructs and were able to delineate the relative contribution of transcription and translation to the load of synthetic gene expression [30•] (Figure 3b).

Together with strategies to measure burden, tools have also been developed to take into account and reduce host–construct interactions. Using orthogonal protein degradation machinery, for example, provides a solution to the aforementioned coupling problem [31••]. By expressing in *E. coli* an orthogonal Lon protease taken from a different bacterium that recognises an alternative peptide tag, Cameron *et al.* demonstrated that they could specifically degrade only proteins from their synthetic constructs and thus avoid overloading the host protein degradation machinery (Figure 3c). A further alternative is to exploit regulatory system that the cell would normally use to adjust to burden. Bacteria have native mechanisms to adapt to new stresses and to combat the insertion of unwanted DNA (e.g. from phages). When a synthetic construct imposes burden, these mechanisms can trigger intracellular stress responses via activation or repression of genes [32]. The molecules and regulators involved are effectively intracellular sentinels of burden and are useful indicators of the status of the cell. Promoters that themselves respond to burden can be used to trigger repression of expression from synthetic constructs, thus creating a negative feedback loop to automatically keep burden within tolerable limits [33]. This approach can also be used to control the level of a toxic intermediate in a metabolic pathway, and so can help optimise biosynthesis of the final product [34••] (Figure 3d).

Finally, to overcome the burden imposed by plasmid-based devices, techniques can be used to integrate DNA

Figure 3



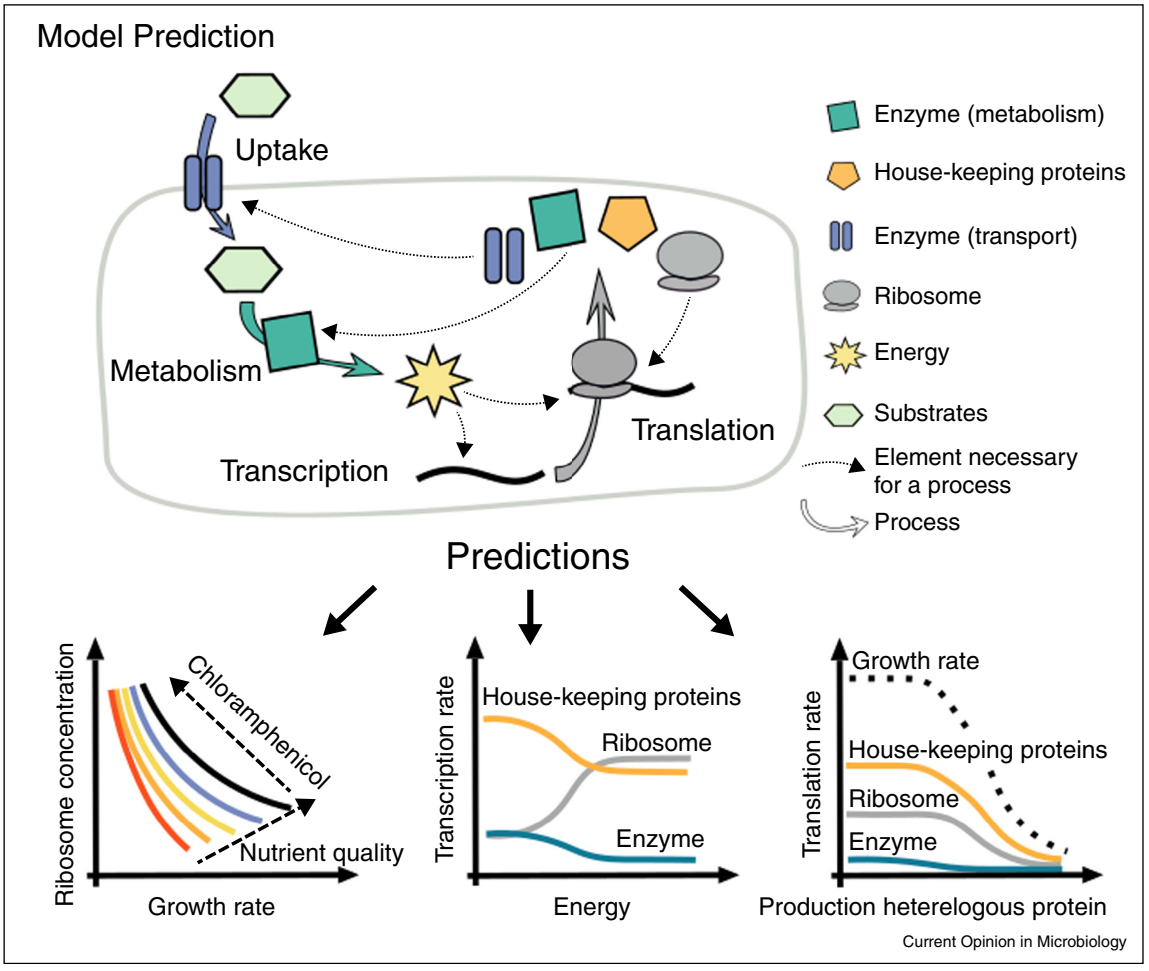
Synthetic biology tools for the characterisation of host–construct interactions *in vivo*. **(a)** Experimental results in Ceroni *et al.* [27••] showed that GFP production from a chromosomically-encoded capacity monitor (green circle) acts as a proxy measure for resource availability in bacteria. Different designs for synthetic devices have different resource consumption and the load of each construct can be measured using the capacity monitor. **(b)** Cell-free system (CFS) is a simplified platform for protein expression using a mix of *E. coli* extracts, amino acids and an energy source solution. It is used for rapid construct characterisation and can also be used to determine resource use [31••]. **(c)** Orthogonal constructs and parts. An orthogonal Lon protease (brown circles) has been used as an efficient tool to control the concentration of proteins expressed from synthetic constructs in *E. coli* without overloading the host degradation machinery (yellow circle) [31••]. **(d)** Stress-responsive devices. Heterologous metabolic pathway expression can lead to the production of toxic metabolites (pink circles). Stress-responsive promoters have been used as toxic metabolite detectors that automatically optimise the isoprenoid biosynthetic pathway to not overstress the host cell [34••].

constructs into the host chromosome. Most established methods exploit recombination for the integration of DNA fragments [35], such as the RAGE (recombinase-assisted genome engineering) method which can be used for the integration of whole metabolic pathways into the *E. coli* chromosome [36]. Recently, CRISPR/Cas9 technology has also proven to be a useful tool for DNA integration in bacteria and offers both flexibility and a rapid protocol [37].

With rapid characterisation methods and ever-increasing options for using different DNA parts in constructs, the final challenge for synthetic biology is to develop new computational tools that capture the essential dynamics necessary to predict host–construct interactions and enable them to be considered in designs. An elegant example of this was recently shown by Nielsen *et al.* who developed a software package called *Cello* that automates the design of Boolean logic networks in cells. One of the features of *Cello* is that it analyses the design space for a desired network and automatically identifies (and rejects) unfavourable designs more likely to cause burden when implemented in *E. coli*. By incorporating considerations of burden and other likely causes of network failure, *Cello* was able to design networks that behaved as desired 90% of the time when implemented *in vivo* [3].

Mathematical modelling has so far been used to capture variability caused by intracellular composition [38] and to understand how bacterial gene expression, resources and growth are all linked together [39•]. This work is now leading to the development of new models able to more comprehensively capture global physiological changes and parameters [22], different metabolic needs [40] and the impact of foreign DNA constructs on the gene expression machinery [41]. For example,

Figure 4



In silico predictions of host–construct interactions. Mathematical modelling can be used to simulate cellular processes and how they interact with different constructs. Weiße *et al.* [42] developed a simplified whole-cell model which described a cell producing just four type of proteins: metabolic enzymes (green), housekeeping proteins (orange), transporters (blue) and ribosomes (grey) and performing only four processes: substrate uptake, metabolism, transcription and translation. This model could predict the production of the four protein types in different growth conditions and successfully predicts the known burden of several different synthetic constructs.

by considering that cells must balance a finite pool of proteins, ribosomes and energy to undergo different parallel tasks, Weiße *et al.* developed a mathematical model that predicts how the host is impacted by different synthetic constructs [42] (Figure 4). This model is effectively a coarse-grained whole-cell model, considering just the fundamental processes of the cell when a construct is present. In principle, more advanced and detailed whole cell models encapsulating and simulating all the processes within a cell would offer *in silico* prototyping for the analysis and predictive design of synthetic constructs in specific hosts. The first bacterial whole cell model working at this scale has recently been described for *Mycoplasma genitalium* and takes into account all gene functions known for this bacteria and is supported by experimental data [43]. Such highly-detailed models are desirable but are very complex and remain difficult to implement, use or modify.

Conclusions and future directions

The work described here shows that there has been encouraging progress on many fronts in the past five years in terms of considering how synthetic constructs interact with their host cells and vice versa. The combination of new design strategies, models, software and measurement methods, along with new orthogonal and burden-responsive genetic parts, means that a researcher building a new construct for a desired task can now use a plethora of new tools to improve the predictability and health of the final living synthetic system. This is especially important for bacterial synthetic biology, where many downstream

applications require engineered cells to grow robustly in large volumes and in changing environments without failing due to stresses and subsequent mutations.

As we move forward, our ability to predict and exploit the many interactions between host cells and synthetic construct will improve further as our systems-level understanding of bacterial cells improves. Next-generation whole-cell models with sufficient detail to capture global effects like resource use, as well as specific issues like protein function, will undoubtedly improve predictability, engineering efficiency and complexity. In parallel, more extensive characterisation of different constructs in different cells and varied conditions should give a deeper understanding of the host–construct interactions and the limits of our current abilities to predict performance. Synthetic biology has so far had an over-reliance on using GFP and multiwell plates to grow and measure everything, so new characterisation approaches and more application-relevant growth conditions are a priority for the future. And while some of the greatest achievements in synthetic biology in recent years have been in producing the many new orthogonal parts that now allow more complex constructs, perhaps the greatest achievements of the next decade will come from engineering host cells to provide a set of orthogonal resources for their synthetic constructs, so that the host and construct can be made to benefit one another.

Acknowledgements

This work was supported by awards EP/J021849/1, EP/M002306/1 and EP/M002187/1 from the UK Engineering and Physical Research Council (EPSRC).

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A coarsed-grain mathematical model was developed to capture the fundamental processes of an *E. coli* cell (transport, translation, transcription, metabolism) and the proteins involved in these (ribosomes, transporters, metabolic enzymes, among others). The model is able to recover Monod's law, the growth laws described by Scott *et al.* [36] and also predicts host–construct interactions.

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