Engineering the robustness of industrial microbes through synthetic biology

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Microbial fermentations and bioconversions play a central role in the production of pharmaceuticals, enzymes and chemicals. To meet the demands of industrial production, it is desirable that microbes maintain a maximized carbon flux towards target metabolites regardless of fluctuations in intracellular or extracellular environments. This requires cellular systems that maintain functional stability and dynamic homeostasis in a given physiological state, or manipulate transitions between different physiological states. Stable maintenance or smooth transition can be achieved through engineering of dynamic controllability, modular and hierarchical organization, or functional redundancy, three key features of biological robustness in a cellular system. This review summarizes how synthetic biology can be used to improve the robustness of industrial microbes.

Robustness in a cellular system

Microorganisms isolated from diverse natural environments are widely used for the production of pharmaceuticals, enzymes and chemicals. To improve the production efficiency of target proteins or metabolites, these microbes often need to be engineered to meet commercial demands by modifying or introducing new cellular properties [1]. However, it is difficult to engineer some cellular properties including, for example, the stability of the desired metabolism in changing environments [2], tolerance to the toxicity of inhibitors present in complex biomass hydrolysates or of over-accumulated metabolites, and resistance to environmental stresses. These cellular properties, usually controlled by multiple genes [3–5], often dynamically interconnected and hard to improve, are principal physiological features of a complex biological system, which together are defined as biological robustness (Box 1).

Cells with biological robustness can adapt to unpredictable internal or external stresses through maintenance or transition [6,7]. This means that a cellular system can maintain functional stability and dynamic homeostasis in a given physiological state, or manipulate transitions between different physiological states, so as to adapt to the changing environment (Figure 1). Biological robustness is achieved through dynamic controllability, modular and hierarchical organization, or functional redundancy in a cellular system (Box 1). These features are similar to those in man-made complex systems, such as the automatic flight control system in a modern airplane, which has well-understood robustness control mechanisms to maintain a stable flight path (direction, altitude and velocity) regardless of perturbations in atmospheric conditions [6].

Cellular systems are much more complex than man-made complex systems. However, the basic features of cellular systems, namely topological structure, dynamic characteristics and functional complementarities, are similar to those of man-made complex systems. Although the effort towards establishing a theory of biological robustness is still in its infancy and much remains to be understood [8], some fundamental biological control elements and devices have been validated through synthetic biology [9]. Synthetic biology is defined as the application of engineering principles to biology. It disassembles, redesigns and standardizes existing biological components (parts, devices and genetic circuits) with the aim of creating novel genetic circuits, biosynthetic pathways and living system from abiotic components [10–12]. The robustness of cellular systems is a perfect field for application of synthetic biology. Recent scientific achievements have shown that the key features of biological robustness can be engineered to achieve improved productivity and yield for biotechnological manufacturing systems (Figure 1). This review shows how synthetic biology can be used to engineer the robustness of cellular systems.

Engineering the dynamic controllability of a cellular system

A cellular system is very dynamic and is modulated by complex control protocols. These natural protocols are developed during long-term evolution for the purpose of competition and survival. For example, the synthesis of most metabolites is tightly controlled to avoid excessive accumulation or metabolic imbalance. To overproduce such metabolites, tight regulation of rate-limiting enzymes has to be eliminated. This means that the existing dynamic control protocols need to be engineered. However, if heterogeneous metabolic pathways or physiological functions are to be introduced into a cellular system, the newly introduced functionality should be recognized by the existing dynamic control protocols, so that the target metabolic fluxes can be maximized regardless of environmental changes. Dynamic control of a cellular system can occur at multiple levels, including, but not limited to, single metabolic pathways,
Box 1. Cellular robustness

Robustness is one of the general design principles in all living systems and dynamically controls intracellular metabolism and physiological state in a changing environment. Cells maintain intracellular functional stability in changing environments by (i) modulating the corresponding gene expression in a stable state and (ii) transitioning between different physiological stable states (such as σ^ɛ or SOS response in E. coli and sporulation in Bacillus and Clostridium). Some features of biological robustness include dynamic control, modularity and hierarchy, and redundancy (as shown in Figure I). These features are responsible for a dynamic cellular response to a changing environment.

**Dynamic control:** the activity of rate-limiting enzymes controls the metabolic flux, sensors and transducers decide the signaling pathway under the influence of environmental stimuli, and regulatory motifs in genetic circuits control the temporal dynamics of the cellular regulatory network.

**Modularity and hierarchy:** function-related enzymes are spatially colocalized by noncovalent interactions or by membrane anchoring in a cell, and their expression is generally coregulated by transcription factors. In the whole cellular network, modules can be divided into submodules and global regulators regulate local regulators, which forms a hierarchical organization structure. The hierarchy of a cellular system makes thousands of components in a living cell behave as a whole and controls a tremendously dynamic response within a large space of possible phenotypes.

**Redundancy:** cells usually have alternative functional enzymes, pathways or even catalytic domains as backups that have the advantage of aiding in competition and survival in changing environments.

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crosstalk between intracellular pathways, and extracellular signal communication, which can be engineered (Figure 2).

Assembling a robust biosynthesis pathway

Native pathways are usually controlled by driving forces including the thermodynamic characteristics of biochemical reactions (e.g. in the form of release of gaseous molecules, polymerization of monomers or hydrolysis of pyrophosphate) and the characteristics of the enzymes catalyzing individual biochemical reactions (amount or specific activity) [13]. The dynamic controllability of a biosynthesis pathway can be engineered by modifying the characteristics of the native enzymes [14] or by replacing these with enzymes with distinct characteristics (Figure 2a) [15]. A recent example is the construction of an efficient n-butanol biosynthesis pathway in Escherichia coli [15]. When crotonyl-CoA reductase from Treponema denticola (Ter), which irreversibly converts crotonyl-CoA to butyryl-CoA, was used to replace the crotonyl-CoA reductase from Streptomyces coelicolor (Ccr), the final butanol titer reached 4.65 g/l, a 1500-fold increase. The forward reaction rate catalyzed by Ter is significantly higher than the reverse reaction rate, so the driving force for butanol biosynthesis was greatly enhanced. Dellomonaco et al. recently assembled another robust butanol biosynthesis pathway using a different control protocol (Figure 2a) [16]. They utilized the highly effective enzymatic reactions in the β-oxidation cycle and achieved redox balance for butanol synthesis under microaerobic conditions. The efficiency of this synthetic pathway surpassed that of its native producer Clostridium acetobutylicum or engineered E. coli with a clostridial butanol production pathway [17]. In the native producer C. acetobutylicum, the reducing power generated by conversion of pyruvate to acetyl-CoA (catalyzed by pyruvate-ferredoxin oxidoreductase) is used to produce hydrogen by hydrogenase. In E. coli, one molecule of glucose can be oxidized to two molecules of acetyl-CoA with four molecules of NADH generated under aerobic conditions, which is theoretically sufficient to drive the reaction from acetyl-CoA to butanol. The novel butanol biosynthesis pathway led to a significant redirection of carbon flow. As a consequence, an excitingly high titer of butanol (14.5 g/l, 33% yield) was produced during microaerobic growth in minimal medium. Such a robust butanol biosynthesis pathway was assembled with reverse engineering of a fatty acid degradation pathway, and suggests that the dynamic control protocols of a metabolic pathway in a natural system can be exploited for assembly of robust biosynthesis pathways.

Controlling target metabolic flux by sensing intracellular metabolites

When a synthetic metabolic pathway is introduced into a cellular system, instability is often observed if its regulation is independent of the native regulatory system. The commonly used fine-tuning approaches, which target promoters...
or ribosome-binding sites, can help to achieve balanced expression of enzymes to maximize metabolic flux under static conditions [18]. However, maximal metabolic flux may not be maintained when the environmental conditions change. For example, simply introduction of the lycopene biosynthetic pathway into *E. coli* resulted in production of only a minor amount of lycopene. Overexpression of the two rate-limiting enzymes (phosphoenolpyruvate synthase, Pps, and isopentenyl diphosphate isomerase, Idi) did not improve lycopene production because cell growth was inhibited [19]. In addition, accumulation of acetic acid has long been recognized as a limiting factor for high-density cell cultures of *E. coli*. Frimer *et al.* developed an elegant ‘one stone, two birds’ approach for increasing lycopene productivity (Figure 2b) [19]. They introduced a synthetic device consisting of an engineered Ntr regulator with only NRI...
activity, whereas the NRII kinase was inactivated. NRI and NRII kinase comprise a two-component system, encoded by \textit{glnG} and \textit{glnL}, respectively. The synthetic device sensed changes in the intracellular concentration of acetyl phosphate (ACP, the precursor of acetate) and consequently controlled the biosynthesis of lycopene. When the ACP concentration increased, NRI was phosphorylated by ACP, which in turn activated transcription of \textit{pps} and \textit{idi} from the \textit{glnAp2} promoter. This led to redirection of the carbon flux from acetate formation to lycopene biosynthesis. The engineered strain produces 18-fold higher lycopene, indicating the importance of considering the dynamic interactions between different intracellular metabolic pathways. Therefore, a dynamic control strategy that regulates the expression of pathway enzymes by sensing the changing intracellular environment would greatly improve the productivity for target metabolites.

**Rewiring signaling pathways by sensing extracellular stress**

Cellular physiological properties are often affected by changes in the environment, such as changes in nutrients, products and physical stresses. Desirable industrial microbes are expected to work efficiently as cell factories, regardless of environmental changes. Thus, cellular systems should sense environmental changes and adjust themselves to maintain desired metabolic functions. The sensor and interconnected proteins dictate which signals are transmitted. Therefore, sensors and sensor–transducer interactions need to be engineered when reconstructing signaling pathways (Figure 2c).

Several synthetic signaling pathways have been developed to program cells in response to changing environments [10,20]. Novel signal molecule specificity for sensor proteins that respond to new signal molecules can be obtained by mutation and screening or by rational design [21]. Looger et al. created a novel sensor (a periplasmic sugar-binding protein of \textit{E. coli}) through computer-aided rational design [22]. In addition to engineering of sensors, the sensor–transducer interactions could also be manipulated to modify the dynamic response of cells to the extracellular environment. For example, the synthetic sensor Cph8 made non-photo-synthetic \textit{E. coli} respond to the intensity of light. The light signal was transmitted through a synthetic osmotic shock regulation system (the \textit{EnvZ}–\textit{OmpR} two-component system) that controls the production of an indicatory protein [23]. Other environmentally responsive synthetic signaling pathways were created in response to cell density, DNA-damaging stimuli and temperature changes [10,24]. In addition, synthetic RNA devices can be engineered to control cellular information processing in response to the extracellular and intracellular environment [25,26]. Based on an understanding of the input–output relationship and the construction of synthetic signaling pathways, the development of robust industrial microbes capable of dynamic adaptation to environmental changes and stable maintenance of target metabolic function might be expected in the near future.

![Figure 2. Engineering of the dynamic controllability of cellular systems. To maximize the target metabolic flux in engineered cells regardless of changing environments, the existing dynamic control protocols in the natural strain can be engineered via synthetic biology from a single pathway to the whole system. (a) Engineering of the dynamic control of a single pathway by generating a strong driving force, such as replacement of a reversible reaction by an irreversible reaction, or assembly of a non-natural synthetic pathway with balanced cofactors. (b) Engineering of the dynamic crosstalk between metabolic pathways to make a heterogeneous pathway modulate itself by responding to changes in the intracellular environment. The enzymes in the target metabolic pathway are regulated by a synthetic sensor, which can sense changes in the concentration of a specific intracellular signal molecule. The intracellular signal molecule is denoted by a red solid circle. (c) Rational design of a synthetic signal transduction pathway responding to changes in the extracellular environment. Synthetic sensors can be engineered to recognize a new extracellular signal molecule, and then activate specific transducers or transcription factors, which in turn regulate the expression of target genes. (d) Optimization of a genome-scale regulatory network to modulate different dynamic temporal responses to cope with perturbations.](https://trends-in-microbiology.com/trends-microbiology-2012/vol-20-is-2)
Engineering large-scale cellular regulatory network

The temporal dynamics of gene expression in cellular systems respond to different perturbations across a broad range of timescales and behave in different patterns, such as single pulse patterns in response to transient stimuli, sustained state-transition patterns in response to constant stimuli, and oscillating patterns in response to cell division and periodic stimuli [27]. These temporal responses can be applied in engineering of cellular robustness. For example, the temporal response characteristics of a feed-forward loop controller are expected to achieve a higher butanol yield than those of three other controllers in response to butanol toxicity [28]. Industrial strains are expected to cope with different perturbations. Therefore, different temporal dynamic responses need to be modulated on the genome scale to cope with perturbations (Figure 2d).

The cellular regulatory network is remarkably flexible and contains some regulatory motifs, and constantly reconfigures itself by manipulating motifs in response to perturbations [27]. To engineer the cellular regulatory network, regulatory motifs should be resolved and standardized with a specific function assigned. The first synthetic regulatory motif was a toggle switch, composed of two repressible promoters and corresponding repressors arranged in a mutually inhibitory network. This type of artificial regulatory motif can form a typical sustained state-transition temporal pattern to regulate gene expression [10,29]. A new synthetic genetic timer can produce various tunable sustained state-transition dynamic patterns based on computer-aided design [30]. Several other synthetic regulatory motifs were created to achieve quantified diversiform oscillating temporal patterns for the control of gene expression, including a tunable synthetic gene oscillator [31], a genetic clock [32] and a genetic counter [33]. More synthetic regulatory motifs can be found in other reviews [9,24]. These examples provide methods to standardize regulatory motifs and rationally synthesize temporal dynamic patterns for the control of large-scale dynamic responses when programming cells. Recently, Clancy et al. proposed the use of computer-aided design to deal with the complexity of cellular systems and developed an automated ‘genetic compiler’ to program large-scale regulatory networks in a manner similar to the building of electronic circuits using Boolean algebra [34]. Thus, synthetic biology provides tools and methods for optimizing intracellular information transmission in response to environmental perturbations to achieve robust functions.

Figure 3. Engineering of the modularity and hierarchy of cellular systems. (a) Construction of protein-based or RNA-based scaffolds to assemble spatial metabolic modules (E1-E4). (b) Optimization of the hierarchy of the cellular network by introducing an exogenous global regulator (blue).

Engineering the modularity and hierarchy of the cellular network

Modular and hierarchical organization is one of the key features of biological robustness [6]. The creation of new functional modules with special hierarchical control can be used to engineer the robustness of microbial cells.

Spatial modular control of metabolic pathway enzymes

In the native cytoplasm, enzymes in a metabolic pathway are often colocalized together by noncovalent interactions or by membrane anchoring to form a metabolic channel. Such a channel can limit the diffusion of intermediates into the surrounding milieu, facilitate fast turnover of labile or toxic intermediates, and prevent undesired crosstalk between different metabolic pathways [35]. For example, the αβ2 complex in tryptophan synthase forms a channel for the intermediate metabolite indole [35]. Without spatial limitation, indole produced by engineered E. coli severely inhibited the biosynthesis of a taxol precursor [36]. The assembly and disassembly of enzyme complexes in a plant biosynthesis pathway can control the metabolic direction and produce different major metabolites in cells [37]. Therefore, colocalization of pathway enzymes and the synthesis of metabolite channels are important to obtain robust metabolic function in engineered cells. Several strategies have been applied to spatially control metabolic pathways, including the fusion of multifunctional proteins and the assembly of protein scaffolds [35,38].

Two typical methods for spatial modularization of metabolic pathways are protein-based and RNA-based scaffold assembly (Figure 3a). Based on assembly of the natural complexes of cellulosomes for synergistic degradation of cellulose, synthetic minicellulosomes can be constructed by assembling a few different cellulases that coordinately act on sequential degradation of cellulose in noncellulolytic E. coli [39]. Another synthetic protein scaffold was used to spatially recruit enzymes in a metabolite biosynthetic pathway of interest to maximize pathway flux [40]. Three key enzymes in the mevalonate biosynthesis pathway were appended with different ligands that incorporated them into the corresponding sites in an artificial protein scaffold in E. coli. Assembly of the three colocalized enzymes was optimized in terms of the amount of individual enzyme and domain orientation in scaffold architecture. The optimal synthetic complex resulted in a 77-fold improvement in product titer at low enzyme expression levels, which reduced the physiological burden on host cells. RNA-based
scaffold assembly was also developed to constrain metabolic flux in vivo [41]. Synthetic RNA was designed and assembled into functional discrete and one- and two-dimensional scaffolds. The engineered RNA scaffolds contain RNA aptamer binding domains (such as PP7 and MS2 aptamer domains), which can spatially colocalize PP7 and MS2 fusion proteins to form a multidimensional protein complex in cells. Two proteins ([FeFe]-hydrogenase and ferredoxin) involved in biological hydrogen production were captured by these synthetic RNA scaffolds to form a hydrogen-produced spatial module in cells. The module architecture was controlled with nanometer precision in a discrete and one- and two-dimensional manner, which resulted in 4-, 11- and 48-fold increases, respectively, in hydrogen production. The construction of synthetic scaffolds could therefore provide a simple platform for spatial modular control over the target pathway flux.

**Engineering hierarchy by introducing artificial regulators**

To date, rational rewiring of a synthetic regulator with many target genes and construction of a special hierarchical topological structure for desired output are still in their infancy. However, engineering of global regulators through mutation and screening provides a simple approach for optimizing the hierarchy of a cellular network to obtain a robust phenotype (Figure 3b).

Global transcription machinery engineering (gTME) is an example of the engineering of cellular hierarchy in this sense. A mutated hub transcription factor (SPT15) resulted in a change in the expression of hundreds of genes in *Saccharomyces cerevisiae* [42], which led to increased ethanol tolerance and improved ethanol yield and productivity. Engineering of the sigma factor in other bacteria (such as σ^50 in *E. coli* and *Lactobacillus plantarum*) also resulted in robust phenotypes [43,44], suggesting that this is an effective approach with broad application. IrrE from an extremely radiation-resistant bacterium, *Deinococcus radiodurans*, was also recently engineered to improve resistance against multiple stresses in *E. coli*. Constitutive expression of the *irrE* gene in *E. coli* promotes DNA repair and protects the host against oxidative, osmotic and thermal damage. Further analysis showed that introduction of this exogenous hub regulator regulated the expression of more than 120 genes [45]. The wild-type IrrE protein cannot confer higher ethanol or butanol tolerance to *E. coli*. However, through mutation and screening, a laboratory-evolved IrrE mutant was selected and conferred enhanced host tolerance to multiple chemicals, including ethanol, butanol, isobutanol, pentanol, isopentanol and acetate [46]. The mutation and screening of mutant IrrE can be considered an optimization of the hierarchical regulatory network. Rational optimization of hierarchical structures in regulatory networks using synthetic methods requires detailed knowledge about the hierarchical mechanism in the network, which should be addressed in future work.

**Engineering functional redundancy of cellular physiology**

Redundancy helps biological systems to adapt to environments or perturbations via functional compensation by duplicate genes, alternative metabolic pathways and even similar domains [47]. Different functional redundancy shapes special cellular physiology in different ecological niches [48]. For example, yeast cells possess a redundant set of membrane hexose transporters with varying affinities and different transport efficiencies for glucose that allows them to adapt to a wide range of glucose concentrations. For industrial strains, redundant functions that can increase stress resistance and metabolic capability need be strengthened, whereas redundant functions that decrease the genetic stability of heterogeneous genes need to be removed. For example, overexpression of L-1,2-propenedioli oxidoreductase (FucO), which represents an alternative to aldehyde/alcohol dehydrogenase activity, strengthened the reduction of butyryl-CoA to butanol [16]. In addition, overexpression of a putative acyltransferase (YqeF) supported conversion of acyl-CoA to acetocetyl-CoA. Simultaneous overexpression of YqeF and FucO significantly increased the butanol titer. This suggests that metabolic capabilities can be strengthened by introducing enzymes with overlapping functions in metabolic pathways, so-called engineering of metabolic redundancy.

**Strengthening functional redundancy by introducing synthetic pathways**

Many environmental stresses may affect multiple physiological functions. For example, butanol interferes with proteins involved in nutrient transport, ATP synthesis and respiratory functions [4]. Strengthening of the functional redundancy of damage protection can improve cellular tolerance. Glutathione (GSH) is a thiol compound that plays an important role in oxidative stress resistance. Recent studies using the Gram-positive bacterium *Lactococcus lactis* as a model system showed that GSH may protect key enzymes in glycolysis, conferring cellular resistance to multiple environmental stresses [49]. When a synthetic pathway for GSH biosynthesis was introduced into the solvent-producing *C. acetobutylicum*, increased oxidative resistance and solvent tolerance were observed, leading to an increased solvent titer [50]. Furthermore, introduction of a synthetic pathway for polyhydroxyalkanoate (PHA) biosynthesis into non-PHA-producing microbes can confer improved survival ability on host cells under adverse conditions such as starvation, desiccation, UV radiation, high osmotic pressure and the presence of organic solvents [51]. The introduction of new biosynthetic pathways capable of producing stress-related molecules may confer redundancy for damage protection on host cells, thus increasing their stress resistance. However, the constitutive expression of enzymes involved in synthetic pathways could impose a physiological burden on the host, so an increase in the dynamic controllability of the synthetic pathway introduced, using the strategies illustrated above, seems necessary.

**Reducing negative functional redundancy by reducing genome size**

Redundant genetic elements including mobile DNA elements, such as transposons, prophage genes, virulence genes and repeat sequences, can be used for mutation...
and evolution. These redundant genetic elements often affect genetic stability, which is not desirable for an industrial strain, and thus need to be removed by genome reduction.

The first reduced E. coli genome was developed by deletion of 12 K-islands (E. coli K-12 strain-specific regions) and the genome size was reduced by 8.1% (376 kbp deleted) [52]. By further deletion of mobile DNA elements and cryptic virulence genes, a series of genome-reduced strains was constructed in which the genome size was reduced by up to ~15%. These strains were designated multiple-deletion series (MDS) strains [53]. These insertion sequence-free MDS strains retained their original growth performance, but exhibited stronger protein production capability, higher electroproporation efficiency and improved maintenance of toxic exogenous plasmids. Because the mobile DNA elements were deleted, the genetic stability improved and the evolvability was reduced. These MDS strains are therefore an appropriate chassis for the construction of robust engineered cells [54]. Bacillus subtilis is an important microorganism with a superior ability to secrete various industrial enzymes. By deleting an 874-kbp genomic sequence including prophage and prophage-like sequences, 20% of the genome was reduced to create the B. subtilis MGB874 mutant [55]. This strain retained good growth performance and showed improved capability for the production of industrial proteins. The activities of the recombinant thermostable alkaline cellulase and alkaline protease in MGB874 cell cultures were approximately 1.7- and 2.5-fold higher, respectively, than those in wild-type cells.

Reducing redundant metabolic options can contribute to improving the yield of target metabolites. Streptomyces is an important microorganism capable of producing numerous secondary metabolites. Genes encoding multiple secondary metabolic pathways are often found on the linear chromosomes. Streptomyces avermitilis produces the anthelmintic agent avermectin and has been systematically engineered by deletion of nonessential genes [56]. A segment of more than 1.4 Mb located on the left subtelomeric region of the S. avermitilis genome was deleted; this segment contains genes encoding major endogenous secondary metabolite pathways and most transposons, as well as insertion sequences. In addition, genes encoding the secondary metabolite oligomycin and terpene biosynthesis pathways were deleted. The genome-reduced strains were designated SUKA strains and exhibited enhanced intrinsic genetic stability and significantly improved production of multiple exogenous secondary metabolites such as streptomycin, cephamycin C and plant terpenoid compounds. Genome-reduced strains are less able to mutate and evolve and contain fewer metabolic options, which means that they can easily maintain the stability of engineered functions in changing environments. Such a property is desirable for industrial applications; therefore, it is expected that genome-reduced strains will be widely used for further engineering.

Concluding remarks
The development of a bio-based economy calls for more pharmaceuticals, chemicals, materials and fuels to be produced from renewable resources. The extreme diversity of microbial metabolism provides a basis for the development of artificial synthetic pathways. To fully utilize these resources, the principles and rationales for assembling a robust biosynthesis pathway need to be better understood. Metabolite-responsive biosensors and artificial signal transduction pathways are promising for the dynamic control of metabolic pathways by monitoring changes in the environment [26]. Future work will focus on the screening and engineering of natural sensors and the design of artificial sensors. The robustness of complex systems is related to redundant design, plug-and-play modules and hierarchical organization. Engineering of the robustness of cellular systems requires an in-depth understanding of the relationship between cellular organizational structure and phenotype, especially the temporal dynamics of gene regulation in response to stimuli and related molecular mechanisms [27]. Such an understanding is currently lacking. These are the main challenges for the engineering of biological robustness, and many questions remain to be addressed (Box 2).

To obtain ideal industrial microbes [1,57], the robustness of natural microbes needs to be engineered so that bioprocesses can efficiently compete with existing chemical processes. Synthetic biology offers scientists tremendous opportunities to generate engineered microbial strains with desired properties using engineering principles to deal with biological complexity. Engineering of the robustness of industrial strains via synthetic biology could reshape the whole-cell response to stresses and optimize dynamic control of a target metabolic flux regardless of changes in the environment. In this regard, engineering principles for the creation of robust controls, as in the automatic flight control system in modern airplanes, may help microbiologists and biotechnologists to create man-made complex microbial systems with defined control protocols, a rational hierarchical structure and suitable redundant functions.

Box 2. Outstanding questions

- How can we understand biological robustness in a simple mathematical way so that we can predict the impact of genetic modification on robustness?
- For native pathways with low yield or a weak driving force, what principles determine which genes need to be replaced and which genes can be used for replacement? Could a standardized library of well-characterized parts, devices and genetic circuits help in understanding this?
- What dynamic control protocols can be used to improve the robustness of a given metabolic pathway?
- How can a specialized hierarchical organization of a cellular network be designed to achieve a desired phenotype?
- How can we determine which redundancy is beneficial in improving industrial properties?
- What is the minimum size that a genome can be reduced to without affecting its industrial properties?
- How can a hub regulator be designed to modify regulatory networks on a large scale and achieve a predictable output?

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