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Controlling and exploiting cell-to-cell variation in metabolic engineering

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Individual cells within a population can display diverse phenotypes due to differences in their local environment, genetic variation, and stochastic expression of genes. Understanding this cell-to-cell variation is important for metabolic engineering applications because variability can impact production. For instance, recent studies have shown that production can be highly heterogeneous among engineered cells, and strategies that manage this diversity improve yields of biosynthetic products. These results suggest the potential of controlling variation as a novel approach towards improving performance of engineered cells. In this review, we focus on identifying the origins of cell-to-cell variation in metabolic engineering applications and discuss recent developments on strategies that can be employed to diminish, accept, or even exploit cell-to-cell variation.

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Introduction

As microscopy, flow cytometry, and other single-cell measurement technologies advance, researchers have begun to compare measurements of individual cells to bulk population averages. These studies have revealed that variability between cells can be significant, suggesting that population-level averages may obscure underlying heterogeneity [1–4]. Cell-to-cell variation can be caused by many factors including genetic differences, phenotypic heterogeneity, and differences in the local microenvironment. In natural contexts, this type of variability can play an important functional role, such as reducing burden of costly protein expression or increasing survival in changing environments [5–7]. In this review, we discuss the origins of variation relevant to metabolic engineering and highlight recent examples of strategies to control diversity that hold promise for improving production yields.

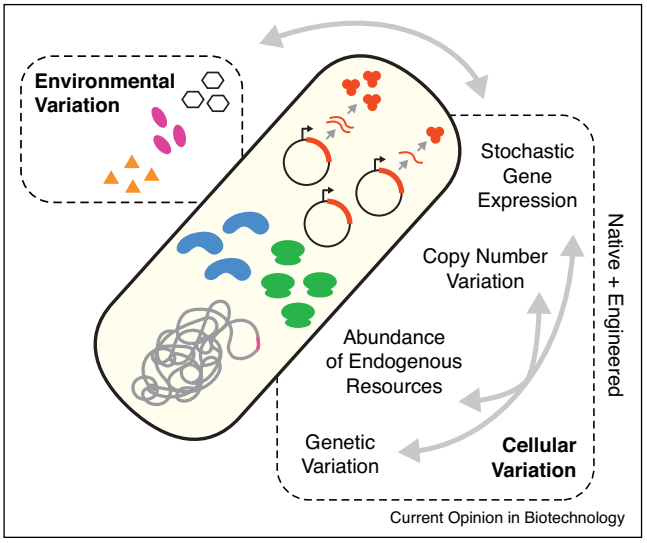
Despite the prevalence of cell-to-cell variation in nature, it is not traditionally studied in the context of metabolic engineering applications. The reasons behind this are practical, as it can be technically challenging to measure single-cell level effects, and reporters and methods for quantifying metabolically-relevant states often do not exist. However, the importance of understanding cell-to-cell differences is highlighted by recent metabolic engineering studies that have shown that cell-to-cell variation can impose a significant impact on production. For example, Xiao *et al.* demonstrated that 15% of cells in an isogenic *Escherichia coli* population of free fatty acid producers were responsible for over half of the total product [8••]. In another study, approximately a third of cells in cultures of the production host *Bacillus megaterium* were shown to persist in a low production state, regardless of culturing conditions [9]. These studies suggest that managing cell-to-cell-variation may offer a potential approach for further optimization of production pathways, which can be used in concert with traditional metabolic engineering strategies.

In this review, we discuss the origins of cell-to-cell variation in metabolic engineering and strategies to control variability. We divide the origins of variation into environmental and cellular categories, the latter of which includes variation due to native and engineered components, and the interplay between them. We then discuss strategies for controlling and exploiting variation in metabolic engineering contexts. These range from diminishing, to accepting, to actively creating variation within populations of cells. Finally, we describe technological advances that would help to facilitate quantification and the engineering of control strategies.

Origins of cell-to-cell variation in metabolic engineering

Cell-to-cell variation in metabolic engineering applications can be divided into two categories. First, environmental variation, which is due to the impact of gradients in local conditions, such as nutrient availability or extracellular product levels. Second, cellular variation, which is due to properties internal to the cell, such as

Figure 1



Origins of cell-to-cell variation in metabolic engineering. Sources of variation can be divided into environmental and cellular variation. Environmental variation originates from heterogeneity in the local environment, such as due to poor-mixing in a large-scale bioreactor. Cellular variation can result from both native and engineered pathways due to genetic diversity or phenotypic heterogeneity. Significant interplay exists between environmental and cellular variation, and between native and engineered pathways.

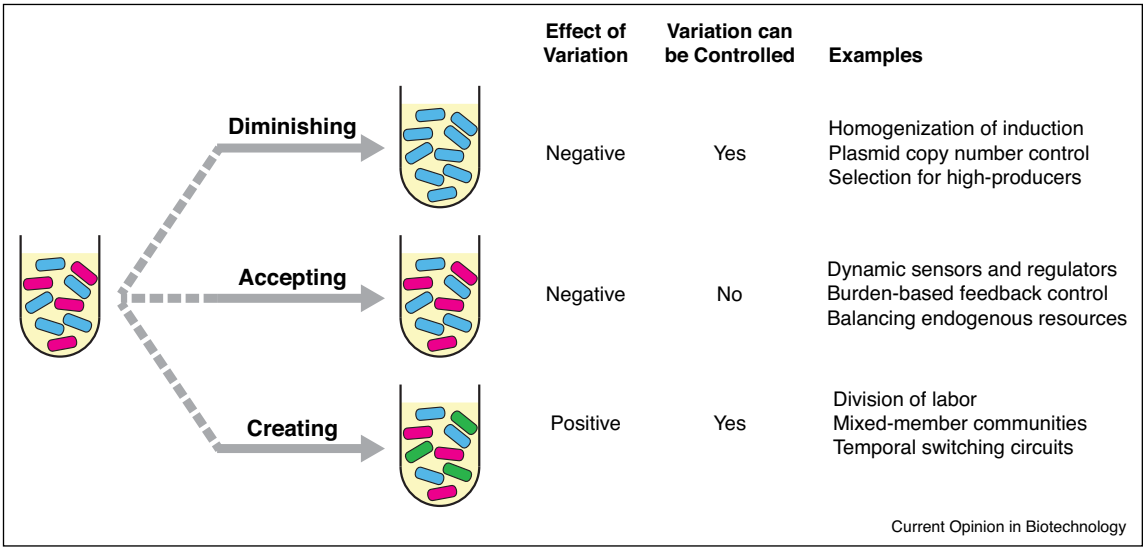
heterogeneity in cellular resources or intracellular product levels (Figure 1).

Although we generally assume that production environments are homogeneous, several studies have demonstrated that this assumption is not entirely valid as volume scales are increased. Within industrial scale bioreactors, mixing becomes challenging due to high viscosities and large volumes [10]. Consequently, production variation can occur even within isogenic cell populations due to the fact that cells are exposed to different local conditions within the same bioreactor. Most approaches for increasing homogeneity within scaled-up bioreactors are mechanical and aim to ensure even mixing [11–13], however it is also possible to design genetic circuits that work to mitigate the effects of this nonuniformity.

Cellular variability is impacted by both native properties, such as ribosome and ATP levels, and heterologous factors, such as expression of a burdensome, non-native enzyme. In addition, diversity can arise due to the interplay between these native and engineered components. For instance, some cells may have a higher capacity for expression of a synthetic circuit than others due to single-cell level differences in transcription or translation machinery.

Examples of endogenous sources of cell-to-cell variation are genetic diversity and phenotypic heterogeneity in expression of native pathways. Genetic diversity can arise from mutations accumulated during the production process, which can lead to production differences between cells [14••]. Alternatively, genetic differences may be specifically engineered, such as in applications that employ different stains or species in co-cultures for biosynthesis [15••]. In contrast to variation due to genetic changes, phenotypic heterogeneity, which is commonly

Figure 2



Strategies for coping with cell-to-cell variation in metabolic engineering. Depending on the circumstances, the optimal engineering strategy may be to diminish variation, accept variation but mitigate its negative impact, or create and exploit cell-to-cell variation.

referred to as 'noise,' exists even in isogenic cells due to stochasticity in how genes are expressed [16]. Phenotypic heterogeneity can have a major impact on the physiological state of the cell, and has been shown to affect growth rate, ATP levels, overall protein abundance, and metabolism in single cells [17–21]. Endogenous variation ultimately impacts biosynthesis, leading to differences in production between cells. The addition of heterologous elements can further exacerbate cell-to-cell variation, as these components can impose burden, introduce toxicity, and redirect metabolic flux, thereby affecting native pathways [22,23•,24,25].

Strategies for coping with cell-to-cell variation in metabolic engineering

Depending on the circumstances, it may be advantageous to reduce variability, to design strategies to accommodate it, or even to increase and exploit variability (Figure 2).

Diminishing variation

Some variation in engineered constructs is a by-product of noisy regulatory elements. For example, the arabinose inducible P_{BAD} promoter is commonly used to control gene expression. In the arabinose regulatory network, unequal expression of the transporters AraE and AraFGH can create heterogeneous expression from the P_{BAD} promoter [26]. Cells with more transporters take up more arabinose and further induce transporter expression, forming a positive feedback loop which creates a bimodal distribution of cells with the P_{BAD} promoter ON and OFF. Overexpressing AraE produces more uniform P_{BAD} promoter expression, effectively homogenizing the response [27,28]. Similar response heterogeneity effects and homogenizing strategies have also been described for lactose/IPTG [29,30] and aTc [31,32] inducible promoters. These examples demonstrate how heterogeneity can be removed when native noisy regulatory elements are repurposed.

Expression heterogeneity caused by differences in plasmid copy number is another source of variation relevant for metabolic engineering. Plasmid numbers can vary widely, even in clonal populations, due to stochastic fluctuations in partitioning and replication [33] and environmental perturbations [34–36]. For example, a recent study on the production host *B. megaterium* found that asymmetric plasmid distribution was responsible for approximately 30% of the engineered cells existing in a low-production state [9,37]. The uneven distribution of plasmids can cause detrimental effects to both low and high copy number cells, where cells with fewer plasmids have decreased productivity, while those with more plasmids become overloaded, consequently ceasing growth and stopping production. Integrating pathways into the chromosome does not fully resolve the problem due to variation in single-cell replication states in fast dividing populations, which can result in genes located closer to the origin of replication having higher effective copy numbers [38]. A recent study developed a novel method for maintaining stable expression even in the face of varying copy number [39••]. Segall-Shapiro *et al.* engineered incoherent feedforward loops in *E. coli* promoters using transcription-activator-like effectors (TALEs) to detect changes in plasmid copy number and tune promoter activity accordingly. Promoters equipped with the genetic circuit were able to maintain constant expression despite widely varying plasmid copy numbers and environmental perturbations.

More general methods that do not aim at any specific source of variation have also been developed to homogenize populations by isolating high-performing cells. Xiao *et al.* developed a population quality control circuit in *E. coli* for free fatty acid and tyrosine production that allows for continuous selection of high-producing variants [8••]. The genetic circuit links end-product synthesis with expression of the tetracycline resistance gene using a synthetic promoter controlled by a biosensor. The production reaction is conducted under conditions with tetracycline, thus low-production cells, which also exhibit low antibiotic resistance, are eliminated, leaving only high-production cells. Using this approach, the study achieved a four-fold increase in free fatty acid production and two-fold increase in tyrosine production over conditions without the selection. In a second example, Rugbjerg *et al.* built a genetic circuit in *E. coli* to link production of mevalonic acid with expression of the essential genes, *glmM* and *folP*, limiting growth of low-production cells [14••]. These strategies serve to homogenize the population and eliminate underperforming cells.

Accepting variation and mitigating its detrimental impact

Due to the complex nature of cell-to-cell variation, it may not always be practical to homogenize the population. Instead, methods that accept the variation and mitigate its negative impact are often a strategic choice. Dynamic control strategies that combine gene circuits with biosensors can allow cells to respond to variation that arises from either engineered or native pathways, and fine-tune heterologous (and other) pathways to achieve individualized optimal production levels. For recent reviews on dynamic control, see [40–42].

Intermediate and enzyme levels vary from cell to cell, thus an ideal production process will allow each individual cell to turn on production as substrates accumulate in order to achieve maximal efficiency. As an example, Zhang *et al.* developed a dynamic sensor-regulator system to allow engineered *E. coli* to sense the existence of acyl-CoA and then turn on a production pathway to convert this intermediate to fatty acid ethyl ester [24]. This method allowed cells to produce three-fold higher titers over those without the control system.

Differences in heterologous enzymes, pathway intermediates, and end-product concentrations can also lead to cell-to-cell differences in the burden and toxicity imposed by the pathway. One way to mitigate this is to tune down expression when toxic compounds accumulate in each individual cell using dynamic control [22,24,25]. In addition to controlling production, it is also possible to turn on detoxifying mechanisms to cope with product accumulation. As an example, Siu *et al.* developed a dynamic control strategy to counteract biofuel toxicity. When biofuel accumulates, the host cell turns on expression of an efflux pump to export biofuel, thereby increasing tolerance and growth [23•]. A recent review covered engineering strategies for tackling burden and toxicity of heterologous pathways [43].

Most dynamic control circuits are designed to cope with one specific type of burden due to the specificity of their biosensors. An alternative, more general approach is to sense and regulate the overall cellular burden rather than a particular product. Using RNA-seq to identify major transcriptional changes that occur during burdensome foreign protein expression, Ceroni *et al.* found that native promoters related to heat-shock response can actively respond to a wide range of heterologous pathway burdens, including expression of an inducible reporter, a large heterologous protein, and a metabolic pathway [44•]. The researchers used a dCas9-based feedback-regulation system to downregulate pathway expression in response to burden sensed by the heat shock promoters. Therefore host cells equipped with these controllers tune production based on an individual cell's burden, instead of in response to a specific compound.

In addition to dynamic control strategies that target variation that arises from the addition of engineered pathways, an alternative approach is to individualize control in cells based on their endogenous physiological states, such as based on the availability of ribosome [45,46] and RNA polymerase [47] resources. For example, Darlington *et al.* recently developed a ribosome allocator in *E. coli* to enable dynamic partitioning of the limited ribosome pool between native and heterologous pathways [48•]. The system was able to tune expression of an orthogonal ribosome-specific 16S rRNA gene to steer ribosome resources between native and engineered pathways depending on the demand. A similar strategy has also been used to build a RNA polymerase allocator to direct transcriptional resources using orthogonal sigma factors [49]. In addition, Venturelli *et al.* developed a global cellular resource allocator by controlling the overall decay rate of host mRNA to reduce competition between endogenous and heterologous pathways [50•].

Creating, engineering, and exploiting variation

Under certain conditions, cell-to-cell variation can be desirable and may be a feature that can be exploited in engineering applications. Indeed, native pathways take advantage of diversification, suggesting the potential untapped benefits of this line of research. Examples where there are fitness advantages to heterogeneous populations include diversification to counter uncertain environments and division of labor among cells [6].

Microbes can use cell-to-cell variation to increase their fitness by hedging against environmental uncertainty [51]. For example, during the glucose-cellobiose diauxic shift, *Lactococcus lactis* populations diversify to have cells that can metabolize cellobiose (Cel^+) and cells that cannot (Cel^-). Although Cel^- cells are not able to grow in the cellobiose environment, when introduced into a new environment with galactose they are able to divide much faster than Cel^+ cells. Thus, the non-growing population (Cel^-) plays a bet hedging role to jump-start growth on a potential future carbon source [52]. When there is uncertainty about the future, population diversity can serve as a potential mechanism for insuring against environmental perturbations.

Another potential benefit of creating variation is through division of labor [53–55]. Division of labor allows separation of pathways either spatially or temporally, exploiting advantages of different species and reducing complexity and burden imposed on one cell type [15••].

Distributing production pathways within a synthetic consortium can allow for division of labor in the spatial regime. This approach has been employed for the biosynthesis of various compounds such as oxygenated taxanes, ferruginol, isobutanol, benzylisoquinoline alkaloids, and flavonoids [56–61]. For example, Zhou *et al.* engineered an inter-species microbial consortium of *E. coli* and *Saccharomyces cerevisiae* to exploit the advantages of each microbe. In this system, *E. coli* is used for rapid production of intermediates based on its fast growth rate, while *S. cerevisiae* catalyzes oxygenation reactions due to its complete protein expression system. This approach yielded 33 mg/L of oxygenated taxanes and also resulted in the highest titer (18 mg/L) of ferruginol that had been reported in the literature [56].

Production pathways can also be divided temporally. For example, Xu *et al.* developed a method allowing cells to separate different pathways in the temporal regime [22]. To do this, they developed a genetic circuit in *E. coli* that responds to accumulation of the intermediate malonyl-CoA. After malonyl-CoA has accumulated, the upstream pathway that produces it is shut down, and the downstream pathway is turned on to convert malonyl-CoA to fatty acids. Once malonyl-CoA is depleted, the upstream pathway is turned on again. This genetic circuit implements sequential switching between upstream and downstream production pathways, therefore using a temporal division of

labor, which resulted in a more than two-fold improvement in fatty acid titer.

Conclusions

Cell-to-cell variation in metabolic engineering applications can have distinct and even opposing impacts. Depending on the circumstances, the optimal engineering approach may be to diminish, accept, or create cell-to-cell variation. Strategies that diminish variation should be employed when diversity has a negative impact on production and in circumstances where it is straightforward to design noise reduction circuits. However, in many cases, the most practical choice may be to accept that variation exists and to design gene circuits that mitigate its detrimental impact. This can be achieved by employing dynamic control strategies to fine-tune heterologous (or other) pathways corresponding to the variation. Finally, creating and exploiting variation can be beneficial for robust growth and improved production and can be achieved by dividing pathways spatially or temporally in order to exploit advantages of different genotypes or to reduce complexity and burden in one cell. Ultimately, it may be possible to combine subsets of these strategies for distinct parts of the metabolic engineering process, for example, exploiting temporal variation to avoid overloading cells while diminishing the impact of negative variation in well-characterized genetic control elements.

Advances in this area will benefit from new technologies for single-cell level quantification of variation in metabolic pathways. For instance, there are tools to quantify various intracellular compounds at the single-cell level, such as riboswitches or RNA that enable sensing of thiamine 5'-pyrophosphate [62], 5-diphosphate and *S*-adenosylmethionine [63]; expanding this toolkit will make quantification of variability more straightforward. These tools can join biosensors coupled with expression of reporters to read out single-cell levels of engineered products, for instance L-methionine and branched-chain amino acids [64,65]. A potential downside of these methods is that they rely on indirect measurements or require identification of specific biosensors. An alternative is to use chemical imaging methods to more directly quantify pathway intermediates and end-products [66], and this represents an important future area for technological development. In addition, microarray and microfluidic methods also show promise for quantifying variation in a high throughput manner [67,68].

Researchers are just beginning to appreciate and quantify cell-to-cell variation and to develop strategies for managing variation in metabolic engineering applications. We anticipate that the continued development of technologies that enable single-cell level understanding will provide insight and new avenues for engineering cells for improved production.

Conflict of interest statement

Nothing declared.

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