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Homogenizing bacterial cell factories: Analysis and engineering of phenotypic heterogeneity

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ABSTRACT

In natural habitats, microbes form multispecies communities that commonly face rapidly changing and highly competitive environments. Thus, phenotypic heterogeneity has evolved as an innate and important survival strategy to gain an overall fitness advantage over cohabiting competitors. However, in defined artificial environments such as monocultures in small- to large-scale bioreactors, cell-to-cell variations are presumed to cause reduced production yields as well as process instability. Hence, engineering microbial production toward phenotypic homogeneity is a highly promising approach for synthetic biology and bioprocess optimization.

In this review, we discuss recent studies that have unraveled the cell-to-cell heterogeneity observed during bacterial gene expression and metabolite production as well as the molecular mechanisms involved. In addition, current single-cell technologies are briefly reviewed with respect to their applicability in exploring cell-to-cell variations. We highlight emerging strategies and tools to reduce phenotypic heterogeneity in biotechnological expression setups. Here, strain or inducer modifications are combined with cell physiology manipulations to achieve the ultimate goal of equalizing bacterial populations. In this way, the majority of cells can be forced into high productivity, thus reducing less productive subpopulations that tend to consume valuable resources during production. Modifications in uptake systems, inducer molecules or nutrients represent valuable tools for diminishing heterogeneity.

Finally, we address the challenge of transferring homogeneously responding cells into large-scale bioprocesses. Environmental heterogeneity originating from extrinsic factors such as stirring speed and pH, oxygen, temperature or nutrient distribution can significantly influence cellular physiology. We conclude that engineering microbial populations toward phenotypic homogeneity is an increasingly important task to take biotechnological productions to the next level of control.

1. Introduction

Isogenic populations display tremendous phenotypic heterogeneity to cope with rapidly changing environments (Acar et al., 2008; Kussell and Leibler, 2005; Smits et al., 2006; Veening et al., 2008a). Hence, stochastic fluctuations (i.e., regulatory noise) in regulatory circuits have evolved to control key cellular functions such as gene expression (Eldar and Elowitz, 2010; Ozbudak et al., 2002), growth (Kiviet et al., 2014; Martins and Locke, 2015), lysogeny (Frunzke et al., 2008; Nanda et al., 2015) and sporulation (De Jong et al., 2010; Veening et al., 2009). In this way, a certain species can produce populations with multiple phenotypes with respect to metabolism, expression and growth to achieve the ultimate goal of survival in a naturally multispecies and competitive environment (Eldar and Elowitz, 2010; Lidstrom and Konopka, 2010). Vitally, population heterogeneity simply provides a basis to adaptively respond to unpredictable changes in natural habitats. This form of risk spreading, which is commonly termed bet-hedging, characterizes the phenomenon whereby diversified phenotypes bear no apparent instantaneous benefit, yet provide a significant long-term fitness advantage for the respective species during temporally

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variable conditions (Grimbergen et al., 2015; Stewart and Cookson, 2012; Veening et al., 2008a, 2008b). Moreover, differential gene expression patterns may favor a division of labor strategy to cope with complex tasks in specific environments (Healey et al., 2016; Martins and Locke, 2015; Stewart and Cookson, 2012). Furthermore, phenotypic heterogeneity, in terms of metabolically inactive subpopulations, has been shown to be beneficial in promoting survival in conditions of cellular stress. Metabolically inactive persister cells successfully enduring antibiotic treatments represent prominent examples of this persistence strategy (Allison et al., 2011; Amato and Brynildsen, 2015; Balaban et al., 2004).

In summary, phenotypic cell-to-cell variation is an innate and ultimate bacterial survival strategy in natural microbial populations to tackle suddenly changing environmental conditions in a flexible and robust fashion, thus strongly increasing their overall fitness. However, in artificial habitats such as simplified monoculture bioprocesses, interspecies competition and rapidly changing environments are minimized compared to those in natural habitats. For most artificial bioprocesses, the ultimate goal is to achieve precise control over the respective process of interest in a predictable and robust fashion. Consequently, in synthetic biology and biotechnology, homogeneous populations are favored to promote an increased degree of process stability, predictability and precise control over the balance between growth and production (Grünberger et al., 2014). As multiple phenotypes are under suspicion for lowered yields and a cause of the low robustness of bioprocesses, the aims of uncovering and diminishing phenotypic heterogeneity have gained increasing interest (Delvigne and Goffin, 2014). In the investigation of phenotypic heterogeneity in isogenic populations, different types of populations may be observed that are presumed to show a diversified impact on productivity (Huang, 2009). Most obvious phenotypic heterogeneity that is subsequently described as macro-heterogeneity depicts a multi-modal distribution behavior. Furthermore, uni-modal distributions that feature a rather broad-spread production behavior with one common maximum can be denoted as micro-heterogeneity (Fig. 1**A, left**). Thus, uni-modality does not suffice as a suited criterion to describe homogeneous productions. To distinguish between micro-heterogeneity and homogeneity and also between homo- and heterogeneity in general, heterogeneity indices based on statistical analysis are required. One such parameter that is frequently calculated for quantifying heterogeneity is the coefficient of variation (CV). For a set of inducible *E. coli* expression systems a (CV) threshold level of 25% was recently used to distinguish between phenotypic homo- and heterogeneity (Binder et al., 2016c). Similarly, Vasdekis *et al.* used a robust coefficient of variation to determine phenotypic diversity fairly neglecting outlier cells that are commonly included during single-cell analysis (Vasdekis et al., 2015). Furthermore, totally different statistic approaches such as Kolmogorov-Smirnov-based non-normality and quadratic entropy were suggested in another context to be highly appropriate for the description of phenotypic heterogeneity (Gough et al., 2014). A sharp separation of those three phenotypic states, however, might further depend on the conducted single-cell analysis approach since e.g. extra-large cells are not captured and some measurements are too insensitive to detect weak fluorescences in small bacterial cells. Moreover, it appears difficult to compare cell-to-cell variations with significantly different expression levels since expression noise is usually decreasing with elevated expression levels according to the scaling law (Baert et al., 2015).

In contrast to macro- and micro-heterogeneous populations, a homogeneous population exhibits a single bell-shaped distribution of cells with equal production levels and a narrow spread, thus exhibiting minor deviations from the population average (Fig. 1**A, right**). Although all three types of single-cell distribution profiles may lead to exactly the same production average within a population, single-cell productivities and their impact on overall productivity can be highly dissimilar (Fig. 1**B**).

Whereas both macro- and micro-heterogeneous distribution profiles consist of low- and high-producing cells, the production behavior in homogeneous populations is uniform, with significantly smaller deviations (Fig. 1**B, right**). Besides homogeneous populations that solely consist of high-producing cells, it may be further crucial to create intermediate or rather well-adjustable homogeneous expression responses if toxic or difficult-to-express proteins come into play (Medema et al., 2011; Rosano and Ceccarelli, 2014; Saïda et al., 2006). Here, a precise and homogeneous adjustment is supposed to be key for toxic gene products or complex metabolic pathways; and “cheater cells”, whose production is too low, too high or simply not in-time, would significantly hamper overall productivity. Furthermore, production heterogeneity is a significant impediment for bioprocesses, whereby a tight interconnection between growth and production exists and rapidly dividing non-producers tend to overgrow slower-growing producer cells. Currently, it is hypothesized that the overall productivity of bioprocesses can be optimized if cells are forced into a homogeneous production state with high or rather optimally adjusted productivity, in contrast to populations consisting of cells in producing and non-producing states.

Thus, unraveling and diminishing phenotypic heterogeneity have emerged as key aspects in bioprocess optimization and are of the utmost relevance for critical and complex productions, wherein fine adjustments of gene expression levels in time and magnitude are essential.

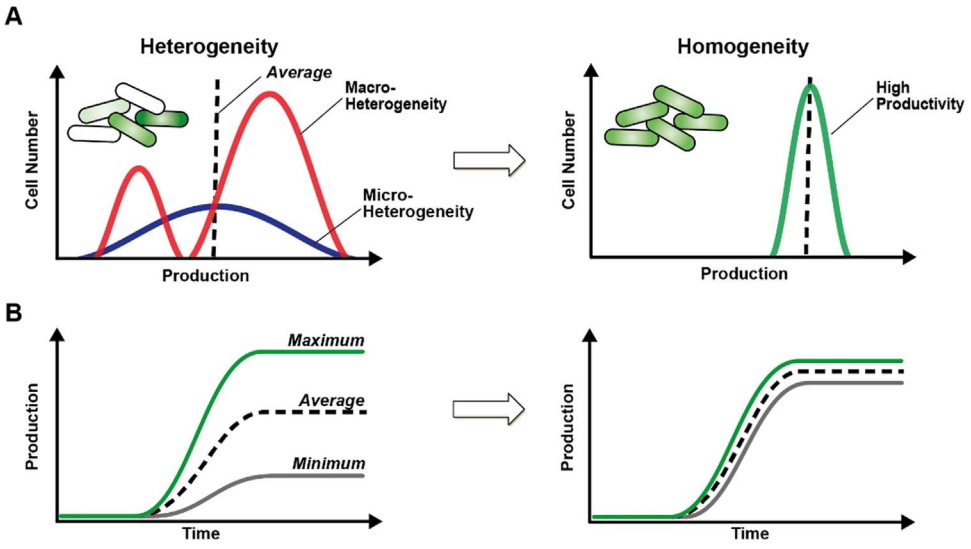

Fig. 1. Types of phenotypic heterogeneity and their impact on productivity. **A**) Obvious and multi-modal macro-heterogeneity manifests in clearly different phenotypes. Less obvious micro-heterogeneity exhibits no clear phenotypes and is characterized by large deviations from the mean value. Homogeneity, by contrast, features a unimodal response with significantly lower deviations from the mean value. **B**) In heterogeneous populations, diverse cellular production levels are obtained, whereby average values might not reflect single-cell productivities, especially in the case of macro-heterogeneity. In homogeneous populations, single-cell productivities show only small deviations from the average. Here, a higher level of control over single-cell productivity is assumed, which might be essential for toxic gene products or complex metabolic pathways in particular.