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Metabolic variability in bioprocessing: implications of microbial phenotypic heterogeneity

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Phenotypic heterogeneity is a major issue in the context of industrial bioprocessing. Stochasticity of gene expression is usually considered to be the main source of heterogeneity among microbial population, but recent evidence demonstrates that metabolic reactions can also be subject to stochasticity without any intervention of gene expression. Although metabolic heterogeneity can be encountered in laboratory-scale cultivation devices, stochasticity at the level of metabolic reactions is perturbed directly by microenvironmental heterogeneities occurring in large-scale bioreactors. Accordingly, analytical tools are needed for the determination of metabolic variability in bioprocessing conditions and for the efficient design of metabolic engineering strategies. In this context, implementation of single cell technologies for bioprocess monitoring would benefit from knowledge acquired in more fundamental studies.

Impact of microbial phenotypic heterogeneity on microbial bioprocesses

Until now, microbial phenotypic heterogeneity has generally only been explored in basic scientific research, and few studies have taken this important phenomenon into account in the context of industrial biotechnological applications [1,2]. The production of bio-based compounds is dependent on stochastic cellular mechanisms, leading to difficulties in controlling bioprocessing. It is thus of primary importance to increase our knowledge of the mechanisms involved in phenotypic diversification. One may expect, for example, that a biopharmaceuticals manufacturer would be most interested in variability in protein synthesis. However, the true situation is more complex because other mechanisms, such as stochasticity at the level of metabolic reactions, can also be involved. We first describe the main sources of microbial phenotypic heterogeneity, with special emphasis on metabolic reactions. Then we address the actual methodologies available for the characterization of microbial phenotypic heterogeneity. Finally, technologically relevant methodologies aimed at controlling microbial phenotypic heterogeneity will be considered.

What are the sources of microbial phenotypic heterogeneity?

Although stochasticity occurs at the level of gene expression [3], stochastic effects can be observed at the level of the metabolic pathways themselves (Figure 1A and Box 1). Metabolic reactions can be directly influenced by bioprocessing conditions, and thus deeper understanding of their influence of microbial phenotypic heterogeneity would lead to technical solutions that can be implemented in the design of more robust microbial cell factories. The fact that a clonal population of microbial cells can exhibit phenotypic diversification has attracted significant attention over the past decades. Most work carried out in this area has been at a fundamental level and has shown that gene expression is subject to noise, leading to phenotypic plasticity during microbial culture [4,5]. In the field of bioprocess operations, most of this phenotypic heterogeneity is due to epigenetic mechanisms [6] or stochasticity in biochemical reactions [4], in other words non-heritable traits, whereas mutations only become an issue on timescales longer than the typical timescales of batch and fed-batch processes [5]. Noise in gene expression generally results in a Gaussian distribution of protein content across different individuals of the same population. All these sources of phenotypic heterogeneity are known to affect bioprocess performance [1,2], but the respective contributions of these different components to phenotypic diversification are not known.

However, there is an accepted picture of the importance of microbial phenotypic heterogeneity. Lack of global productivity can often be attributed to a specific subpopulation of cells exhibiting a 'non-producer' or a 'low-producer' phenotype (Figure 1B). This phenomenon has been observed in various biotechnological fields, such as the production of solvent by *Clostridia* [7], the production of

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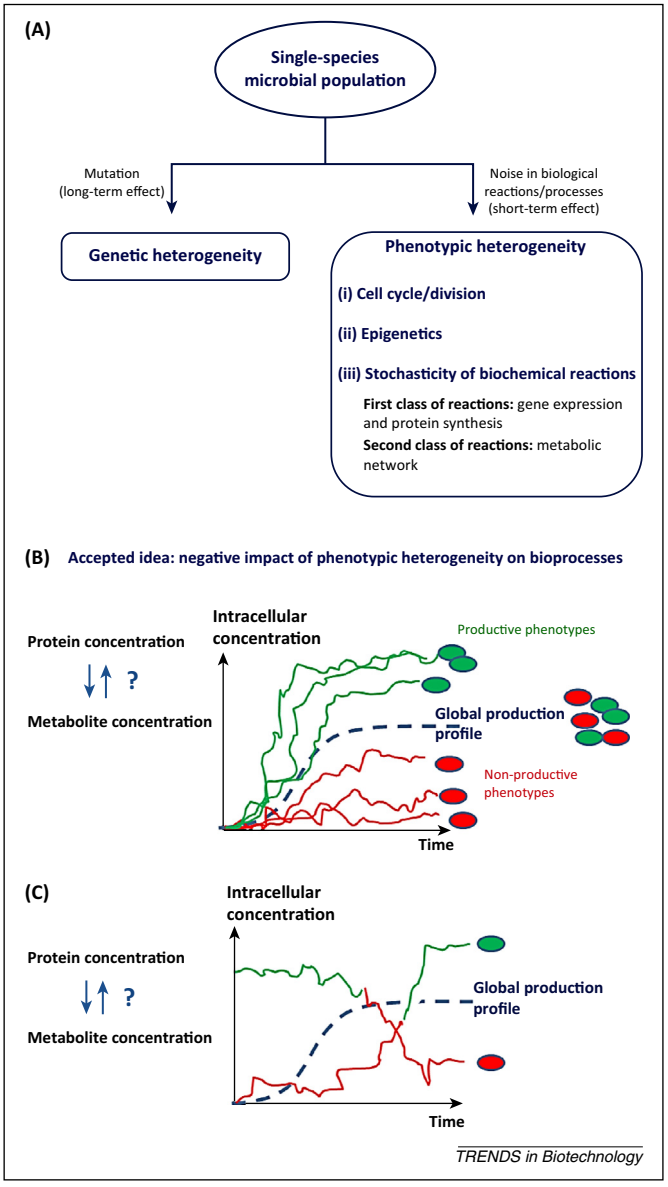

Figure 1. **(A)** Different sources of heterogeneity involved in microbial phenotypic diversification under bioprocessing conditions. **(B)** The accepted picture of the negative impact of microbial phenotypic heterogeneity on process performance. In the scheme a subpopulation with reduced protein synthesis capacity is shown that lowers the global yield of the bioprocess. **(C)** Microbial cells exhibit bistability, in other words they can switch to a producing state to a non-producing state, and vice versa [12,13].

lactobionic acid [8], and the production of recombinant proteins for biopharmaceutical applications [9,10]. Another interesting feature is the history-dependence of phenotypic heterogeneity. Several studies have shown that protein and metabolite production is dependent on the stage of the cell cycle, as observed for eukaryotes such as *Saccharomyces cerevisiae* [11] and *Pichia pastoris* [12], as well as for prokaryotic cells, for example in amino acid production by *Corynebacterium glutamicum* [13]. This phenomenon leads to microbial cell switching from a producer state to a non-producer state and vice versa (Figure 1C).

To better understand the mechanisms leading to heterogeneity in process conditions it will be necessary to integrate the phenotypic history of individual cells and also discriminate between the different sources of noise

Box 1. Does stochasticity applies for metabolic reactions?

Heterogeneities in metabolic activities are increasingly considered as a source of phenotypic heterogeneity. For example, phenotypic heterogeneity and microbial antibiotic resistance, a phenotype termed 'persistence', have been long interpreted as a consequence of stochasticity in gene expression [53]. More recently, new analyses have pointed out the role of metabolism in bacterial persistence [62]. Indeed, the entry into the persister phenotype depends mainly on the energy status of the cells, and is thus directly linked to their metabolic activity, the capacity to generate ATP, and the regeneration of cofactors (mainly shown for *Escherichia coli* and *Pseudomonas aeruginosa*). By contrast, cofactors engineering has been proposed as an efficient metabolic engineering strategy both for *E. coli* [49] and *Saccharomyces cerevisiae* [50]. One may ask if this improvement comes from a global improvement of the phenotypes or if single cell mechanisms are implicated. It is thus crucial, for a proper understanding of the impact of microbial phenotypic heterogeneity on bioprocess performances, to interpret correctly the source of stochasticity. Theoretically, two sources of noise or stochasticity in biochemical reactions can be considered:

Stochasticity in gene expression: in this case, the source of phenotypic heterogeneity is attributed solely to noise in gene expression. The term 'source' is important in this definition because gene regulation impacts upon enzymatic activities and nutrient transport inside cells, which in turn impact upon metabolic activities.

Stochasticity in metabolic reactions: generally, variability in metabolic reactions is attributed to variations in enzymes expression level, but ATP imbalance can also have a strong influence at this level. Another phenomenon that can be advanced to explain the stochasticity in biochemical reactions relates to the slow diffusion of reactants inside the cytoplasm, known as molecular crowding [63,64].

The two mechanisms of stochasticity can also occur simultaneously in microbial cells; in other words, stochasticity in gene induction has repercussions on the expression of metabolic enzymes, and this in turn induces variability at the level of metabolic reactions [21], the rates of metabolite production and consumption, as well as the rate of variation of cofactor and energy pools. It is also important to recognize that metabolic reactions can also have a significant impact on gene expression, reversing the classical flow of information [3].

(Figure 1A). Chemical engineers have integrated the fact that bioreactor scale-up induces the emergence of heterogeneous environmental conditions (e.g., in pH, nutrient, temperature, dissolved gas) which in turn induce phenotypic variability within the cultivated microbial population [1,14]. During the scaling-up procedure, mixing quality is lost to some extent, and the resulting environmental fluctuations induce specific responses at different levels of microbial physiology (transcriptional, translational, metabolic) [15,16]. In this context, classical stirred bioreactors can generate major perturbations during the scaling-up procedure, and are probably not the best choice for addressing the biological constraints [16]. Microbial cell factories exhibit reduced phenotypic heterogeneity during laboratory- and pilot-scale operations, leading to predictable and robust bioprocesses (unstable strains being discarded during the screening phase). However, variability generally occurs during large-scale operations, and it is well known that concentration gradients (e.g., spatial heterogeneities in substrate, dissolved oxygen, carbon dioxide, pH) exhibit a strong influence on cellular physiology [17]. Because microbial cells exhibit different circulation times in bioreactors, they are exposed to different concentration profiles, thereby leading to phenotypic segregation of the

microbial population. In the context of bioprocess optimization and scale-up, cellular heterogeneity must then be addressed according to the extracellular heterogeneities generated by the bioreactor [18]. However, even under well-defined environmental conditions there is evidence of physiological heterogeneity that is reflected by bimodal or multimodal distribution of relevant parameters such as growth and respiratory rates [19]. In this scenario, cells that are able to switch faster to a different phenotype could respond better to environmental changes [20].

Some researchers have proposed that the fraction of cells exhibiting phenotypic properties that differ from the average is small and not significant – except in the case where that fraction has a special influence, such as in the production of inhibitory or signal molecules [19]. Heterogeneity in glucose uptake rate in *Escherichia coli* cultivations under constant environmental conditions has also been reported [21]. If the glucose uptake rate is sufficiently high, overflow metabolism can be triggered, resulting in acetate excretion. However, acetate can also be consumed by *E. coli* if the appropriate pathways are active. By fusing fluorescent reporters to the genes coding for the glucose transporters *mglBAC* and *ptsG*, as well as to genes governing acetate assimilation and gluconeogenesis (*acs* and *pck*), it was shown that the expression of the genes encoding proteins involved in acetate assimilation, as well as those of different transporters, is significantly heterogeneous. Two phenotypic subpopulations with differential *acs* expression can also be found at glucose uptake rates close to the threshold for overflow metabolism. The coexistence of such populations can affect the performance of the bioprocess because acetate excretion and reassimilation engenders a loss of achievable energy production and reduces the efficiency of biomass and product formation. Simulations using metabolic models integrated with transcriptional regulatory data have predicted approximately the same behavior, with the existence of a distribution of growth rates in *E. coli* cultures, as well as the appearance of subpopulations exhibiting different metabolic activities: in other words, a slow-growing, acetate-secreting subpopulation; a fast-growing and CO_2-generating group of cells; and a third subpopulation of intermediate growth rate shifting from glycolysis to the Entner–Doudoroff pathway for glucose degradation to pyruvate [22].

It is important to note that very different phenotypes can be found in a clonal population of cells, and that this diversification can lead to extreme heterogeneity (Figure 2A,B) induced by major stochastic fluctuations (e.g., a bistable system governed by a genetic feedback control loop) or genetic heterogeneity (i.e., mutation [5]). Microorganisms can also display extreme heterogeneity at the level of metabolic function (Figure 2C,D). As an example, a combination of ^{13}C-based dynamic intracellular metabolic flux determinations, modeling approaches, flow cytometry, and colony plating of *S. cerevisiae* cultures revealed the coexistence of two modes of glycolysis when cells were subjected to transitions to elevated glucose levels [23]. This non-genetic metabolic variability is generally spontaneous, and up to 7% of the microbial population displayed imbalanced glycolysis upon glucose perturbation.

How to measure phenotypic heterogeneity?

Distributions of cell parameters are generally measured by optical methods such as flow cytometry or microscopy. Flow cytometry is a very promising technique because it can be coupled to the bioreactor, delivering on-line measurement of cell distributions during a bioprocess, but the technique also has some drawbacks that can be potentially avoided by complementary techniques. We now address the actual methodologies available for the characterization of microbial phenotypic heterogeneity in light of recent progress in both bioprocess engineering science and in more fundamental studies. The description of phenotypic heterogeneity in bioprocessing conditions can benefit from recent advances made at the level of miniaturized cultivation devices designed to assess microbial clone libraries in the context of systems-biology studies [24].

Currently, significant advances in our understanding of microbial phenotypic heterogeneity have been achieved by using a combination of time-lapse microscopy [25] and microfluidic cultivation devices [24] that allow the history of individual cells to be tracked. However, the methods used in fundamental studies differ from those used to assess the heterogeneity of cell populations in a bioreactor. Under representative bioprocessing conditions, analyses of microbial cell population heterogeneity can be carried out on the basis of viability markers (such as the propidium iodide uptake test [26]) or on the basis of fluorescent reporter proteins (Figure 3). A great part of the experimental work on this topic has aimed at temporal profiling of the microbial response under different bioreactor configurations and operating conditions [27]. Although 'omics' techniques permit follow-up of a microbial culture carried out in bioreactor, most studies have used only a few parameters to describe the dynamics of the process (e.g., intra or extracellular metabolites, mRNA transcripts, proteins). A large amount of work has focused on characterizing the environment perceived by a microbial cell when traveling along circulation lines in a heterogeneous bioreactor [18,28]. The lifetime of a microbial cell in a large-scale bioreactor can be simulated by computational fluid dynamics (CFD) using an Euler–Lagrange referential. The Euler referential is used to simulate gradient generation (e.g., of nutrients, pH, dissolved oxygen) inside the bioreactor, whereas the Lagrange formulation accounts for the stochastic displacement of microbial cell inside these gradient fields. In this way, marked heterogeneity can be demonstrated as a result of the different paths followed by microbial cells belonging to the same population [29,30].

There is a clear need for data that combine reduced CFD models with genome-scale metabolic models, but most work has focused on systems-biology studies. Fundamental approaches focus not only on temporal but also on spatial profiling of microbial cells. One significant achievement of this research field is the high-throughput analysis of fluorescent transcriptional reporters from a clone library of a specific microorganism (Figure 3). Such studies have already been conducted on model organisms such as *E. coli* [31]. This second approach is entirely focused on cell biology, and the experimental results are often used to validate complex genome-scale models incorporating metabolic fluxes and transcriptional control of metabolic

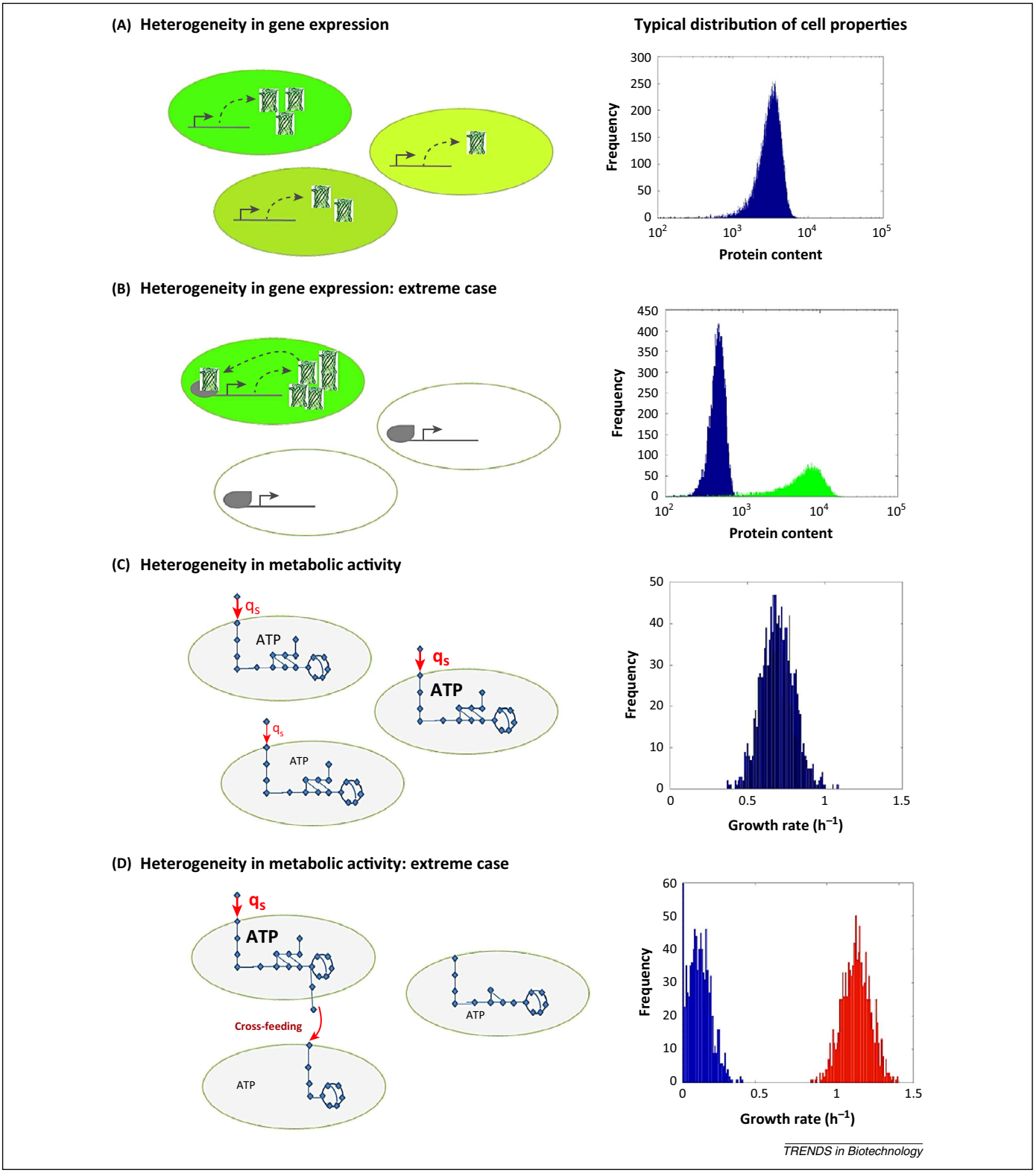

Figure 2. Heterogeneity in gene expression and metabolic activities. **(A)** Microbial population displays phenotypic heterogeneity as a result of stochasticity in gene expression **(B)** An extreme case of phenotypic heterogeneity where a subpopulation exhibiting a very high level of expression of a given gene, and another exhibiting a very low expression level (or no expression) of the same gene, depicted as a bistable state. **(C)** Intracellular metabolite content (cofactors, energy, metabolite pool) are unevenly distributed among a clonal population, leading to heterogeneity at the level of metabolic function. **(D)** An extreme case of metabolic variability can be observed in a natural environment where microbial cells display metabolic specialization in a division-of-labor perspective [54].

enzymes. Some researchers are considering the possibility of combining the two approaches, such as the omic characterization of specific subpopulations [32] following flow cytometry and cell sorting. Single cell omics for prokaryotic systems in bioreactors is generally limited to this special configuration and has been restricted to proteome analysis. Indeed, the half-life of mRNA transcripts is short in comparison to the time required for sample preparation (1–2 h when considering a classical cell sorting apparatus). For the bioprocessing development, single cell omics tools with

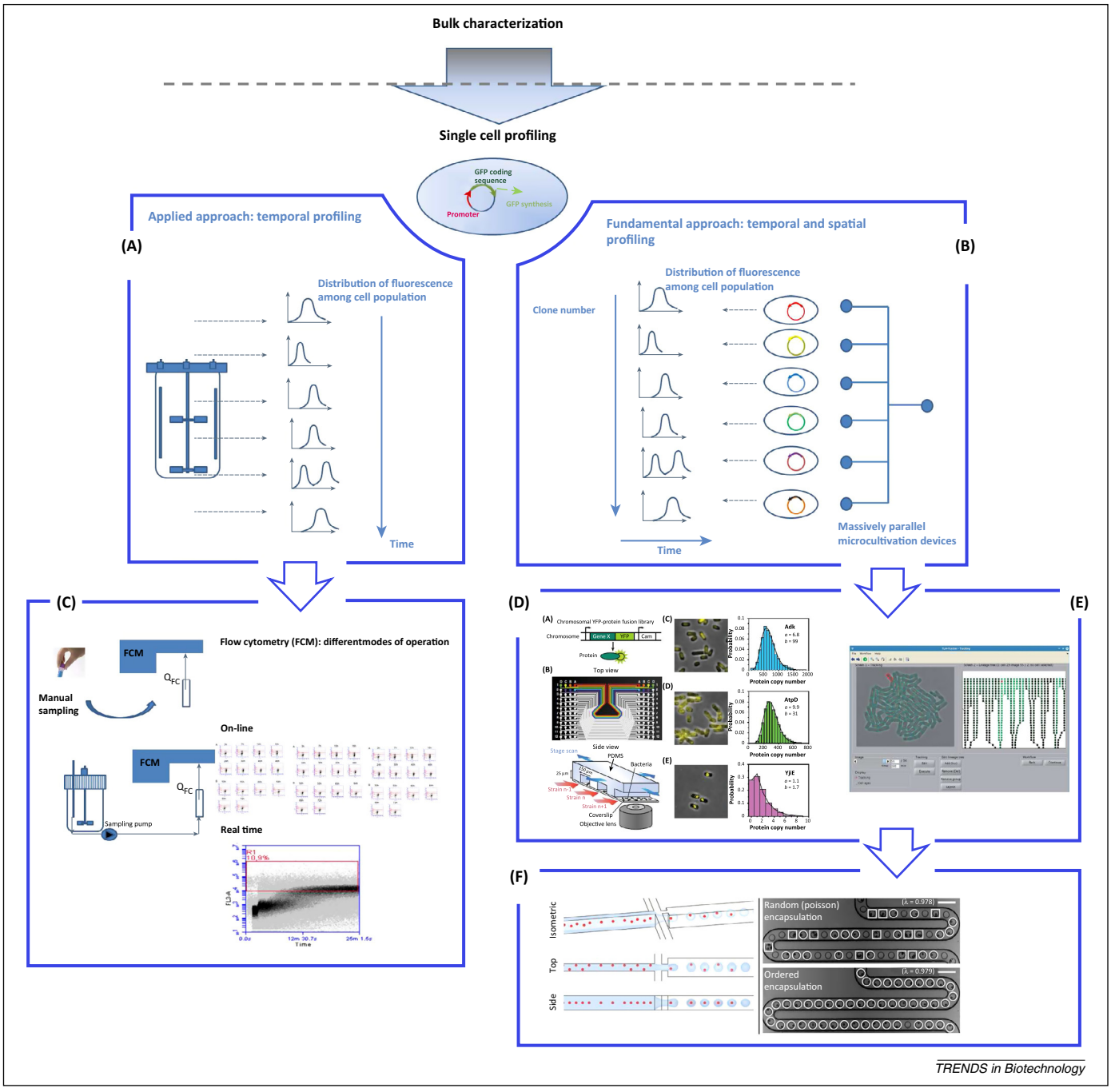

Figure 3. Applied versus fundamental approaches for the characterization of microbial physiology at the single cell level using a fluorescent reporter system. **(A)** Temporal profiling. **(B)** Temporal and spatial profiling. **(C)** Single cell analytics in bioprocessing measured by flow cytometry off-line [65], on-line [34], or in real time [33]. **(D)** Highly parallel microcultivation devices that allow the imaging of single cells over time. Reproduced with permission from the American Association for the Advancement of Science [31]. **(E)** Specific software (e.g., TLM-tracker [66]) designed for processing single cell imaging data to determine cell lineages and address extrinsic sources of noise. Reproduced, with permission, from Oxford University Press. **(F)** Microfluidic devices that allow the encapsulation of single microbial cells in picoliter droplet of cultivation medium; these can be connected to cell sorter, automatic lysis system, and single cell omics devices (lab-on-a-chip approach [67]). Reproduced, with permission, from the Royal Society of Chemistry [68].

temporal and spatial resolution are thus needed. To date, the most promising single cell technology that can be used for bioprocess monitoring and control is flow cytometry [33]. This technique can be coupled directly to a bioreactor for on-line monitoring with single cell resolution. Most studies addressing the heterogeneity of gene expression involve the use of fluorescent reporters. Over recent years these reporters have been refined to highlight the metabolic potential of microbial cell factories (Box 2). From a process control perspective, data analysis must be addressed more specifically because it is difficult to quantify the degree of segregation of a microbial population on the basis of on-line parameters [34,35].

Following a single cell in the lab and in an industrial bioreactor: not the same job

Because the cultivation devices used for fundamental single cell studies must be compatible with microscopy imaging, such devices are miniaturized to allow parallel culture of thousands of microbial cells [36]. In addition,

Box 2. Evolution of experimental techniques for investigating microbial phenotypic heterogeneity

Phenotypic heterogeneity in the field of bioprocess engineering is often analyzed by using flow cytometry. The main drawback of this technique is that the history of specific microbial cells cannot be followed. In addition, flow cytometry requires specific fluorescent labels that are not always representative of the metabolic potential of the cells. By contrast, fundamental studies involving microfluidics can give access to cellular history and offer the possibility to perform 'omic' analyses in the case of droplet-based systems. In time, several variations of these approaches have appeared and are described below.

Promoter-based fluorescent reporters: the first application of fluorescent reporters exploited the induction of stress-related promoters [69] (e.g., the induction of the general stress response regulon by using a *prpoS::gfp* sensor [70]). In view of the importance of real metabolic activity at the single cell level, further studies have focused on the use of promoters closer to those involved in metabolism; for example, those involved in glucose transport system and cellular metabolism [21].

Fluorescent biosensors for intracellular metabolites: new, promoter-independent, fluorescent biosensors are available for the detection of intracellular cofactors (ATP, NADH, . . .) [2,71] or the presence of key metabolites [13,72]. These systems are valuable for the determination of the metabolic potential of microbial cell factories, and have notably been exploited for high-throughput selection of a high amino acid producer for *Corynebacterium glutamicum* [73]. These biosensors have subsequently been used for the design of new metabolic engineering strategies in the same context [51].

Subpopulation 'omics' techniques: the limitation regarding cellular content can be partially overcome by cell sorting and omic analysis of the sorted subpopulation (Figure I, top). However, this approach is dependent on the use of fluorescent probes for phenotypic discrimination. Promising results have been obtained by performing subpopulation proteomic profiling [32].

Single cell 'omics' techniques: it is now possible to consider applying transcriptomics, metabolomics, and proteomics at the single cell level. These analytical technologies are generally applied to cells extracted from microfluidic devices, such as encapsulated cells in picoliter droplets (Figure I, bottom). The advantage of this approach is that it does not require a fluorescent biomarker. However, the applicability of such techniques is limited in the case of microbial cells because of the small amount of intracellular molecules in these biological systems; further developments are therefore needed before single cell omics techniques can be more widely applied in the field of microbial bioprocesses [74], and the convergence between omics biology and single cell biology is currently limited to eukaryotic cells in specific microfluidic devices [75].

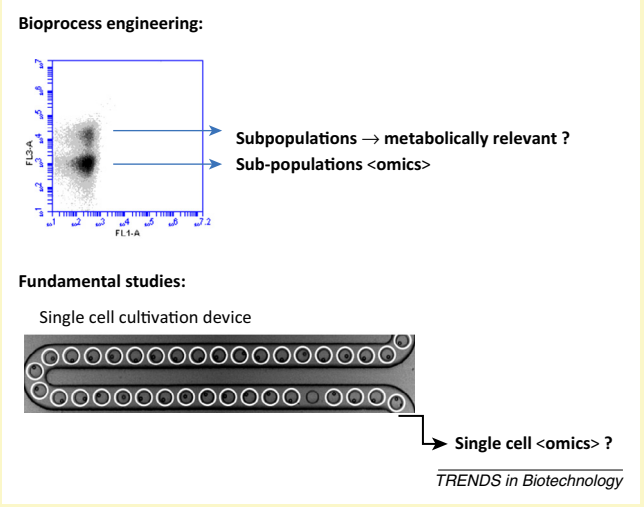

Figure I. Methods for examining single cells. Reproduced, with permission, from the Royal Society of Chemistry [68].

because the experiments are generally carried out in microfluidic flow-cells, the entire population can be followed over time, giving access to the history of each cell. Important segregation phenomena highlighted in this way include stochastic switching between phenotypes [12]. This phenomenon is also important in bioprocesses because recombinant systems can exhibit transient expression of the target protein followed by a period with no production [37]. It would be tempting to translate the results gained by using single cell microfluidic cultivation devices to bioreactors, but several studies have reported that cell density has a significant effect on population heterogeneity. Indeed, it has been shown that a single cell cultivated in an environment without any competition displays increased growth rate [38]. Another study revealed that more competitive phenotypes, in other words those showing better substrate utilization versus cell growth, can be selected for by using individually encapsulated single cells [39]. This again demonstrates that metabolic behavior is distributed across a population of microbial cells. By contrast, individual cells cultivated in picoliter microfluidic chambers exhibit an enhanced quorum-sensing response owing to the accumulation of signaling molecules which would be more dilute in large-scale cultivation systems [40].

How to control microbial phenotypic heterogeneity?

Technologically relevant methodologies aimed at controlling microbial phenotypic heterogeneity will now be considered. Metabolic engineering can be designed to reduce phenotypic plasticity. To control metabolic variability, cofactor engineering, in particular, must be considered. In addition, synthetic biology may provide access to specific DNA components and devices aimed at controlling noise in gene expression [41,42]. Bioreactor design can also be modified to exploit phenotypic heterogeneity. For example, single-species biofilm reactors are now being used for biotechnological processes, and these show extended robustness and productivity.

Suppression of phenotypic heterogeneity: the use of genetically encoded biosensors and metabolic engineering strategies

The modification of cellular capabilities through rational manipulation of genetic information has allowed the overproduction of a variety of commercially important molecules, ranging from small (amino acids, aromatic compounds, organic acids) to large molecules (plasmid DNA, recombinant proteins, biopolymers). Commonly applied genetic strategies include gene deletion and overexpression, the introduction of new or increased enzymatic activities by the expression of heterologous genes, and the expression of genes for modified proteins with higher activity or reduced sensitivity to product inhibition [43]. In some cases, an entire pathway for the synthesis of a product not naturally produced by the cell factory has been introduced by synthetic biology [44].

However, clones selected as the 'best producers' in batch cultivations are not necessarily good producers in the fed-batch schemes typical of industrial applications [45]. Such observations emphasize that the vast majority

of the published work on metabolic engineering aims to develop cell factories that overproduce a given molecule or have improved product yields. By contrast, cellular modification to cope with large-scale production environments is generally not considered. This is, however, a very relevant factor because environmental heterogeneities owing to ineffective mixing in large-scale bioreactors can lead to heterogeneous cell populations, which in turn lower productivity [2]. Recent work has pointed out that most of the heterogeneity can arise from the heterogeneous expression of a relatively small group of key genes [22]. Such genes constitute potential metabolic engineering targets for the control of microbial phenotypic heterogeneity. From a scale-up perspective, it can be assumed that cells with reduced metabolic flexibility to environmental fluctuations would be more robust for biotechnological applications. For instance, the successive deletion of genes involved in glucose transport of *E. coli* resulted in a variety of growth and acetate production rates [46]. Strains with reduced acetate production rates were also able to produce higher amounts of an experimental plasmid DNA vaccine [47]. It is therefore possible that careful selection of the glucose transporter may reduce population heterogeneity and improve the production of the desired molecule. Despite this, the application of metabolic engineering strategies to microbial phenotypic heterogeneity has been largely overlooked, carving out space for an entirely new field of research.

Harnessing phenotypic heterogeneity: use of genetically encoded biosensors

In some cases heterogeneity could be beneficial for a microbial population cultivated in large-scale bioreactor because it can confer robustness to extracellular perturbations [48]. Harnessing phenotypic heterogeneity in the context of industrial bioprocesses requires the use of specific sensors and actuators for effective feedback regulation of the mechanisms involved in phenotypic plasticity. At this time, effective genetically encoded biosensors are available for the detection of intracellular metabolite concentrations or cofactor levels [13,42]. Because the content of cofactor has been recognized as a major source of heterogeneity, the actuator can be internal, for example using cofactor engineering [49] (such as by increasing the availability of NADH [50]) to reduce metabolic variability among the population, or external, for example by implementing detection by on-line flow cytometry (Figure 3) and external cofactor substrate feeding as an actuator. It should be noted that a feedback regulation loop based on detection by on-line flow cytometry is not presently available; this would provide an interesting direction for future innovations. The same principle can be used for the control of intracellular metabolite concentrations at the single cell level because synthetic biology can be used to fine-tune a metabolic pathway on the basis of the biosensor response [51], and on-line flow cytometry may also be adapted for this type of measurement.

Harnessing microbial phenotypic heterogeneity: spatial control of phenotypic heterogeneity through the design of a single-species biofilm reactor

Another strategy for the control of microbial phenotypic heterogeneity in bioprocesses can be derived from microbial ecology studies. Indeed, in the environment, diversification of fitness strategies through phenotypic heterogeneity is a common strategy used by microbial populations to thrive even in adverse conditions. In this way, diversity of fitness strategies allows a heterogeneous population to have a higher probability of survival in the face of environmental fluctuations [52,53]. Bacterial populations in the environment are far more efficient when they operate according to the division of labor strategy [54,55]. In their natural environment, microorganisms exploit genetic and phenotypic heterogeneity to survive. This picture is very different from the bioreactor where all the microorganisms are expected to behave in a similar way to achieve the production of the targeted compound. Given the different mechanisms involved, single cell studies in the field of microbial ecology have therefore not been applied directly to bioreactor optimization. Notably, genetic heterogeneity would be expected to play a greater role in microbial ecology where the timescales are large in comparison to those of typical bioprocessing operations.

Nevertheless, significant work has been carried out in the design of single-species biofilms that can be used for biocatalysis or for the production of secondary metabolites [56]. Indeed, microbial cells cultivated in biofilm reactors exhibit improved robustness and productivity. These improvements can be attributed to the fact that a 3D biofilm structure creates gradients of temperature and pH, as well as of minerals and nutrients, oxygen, metabolites, and quorum-sensing signals. This creates specific conditions in every point of space, to which a bacterial cell reacts by changing its physiological state [57]. As the bacterial cells adapt to growth in these hydrated surface-associated communities, they express phenotypic traits that are often distinct from those expressed in the planktonic state. Owing to the spatial structure of biofilms, both the nutrients that bacteria consume and the metabolic waste products they release are only transported slowly by diffusion, creating a range of microenvironments within the same biofilm. Therefore, bacteria residing in different regions of the same biofilm structure can experience distinct extracellular redox conditions, leading to functional stratification, as has been reported for mono-species biofilms.

A biofilm can thus be considered as a structuring environment in which phenotypic and metabolic heterogeneity can be stratified. The robustness of such biofilms has been proposed for the design of biofilm reactors for the synthesis of secondary metabolites [58–60]. However, studies on microbial phenotypic and metabolic heterogeneity in a biofilm are limited to microscale investigations, involving fluorescent reporter systems and microfluidic devices (Figure 3), in view of the technical challenges of studying biofilms at the single cell level under real process conditions (meso- and macroscale) [56,61]. However, understanding biofilm formation and the stratification that can be obtained with such devices will be invaluable in the design of efficient biofilm reactors on the basis of the spatial control of metabolic heterogeneity.

Concluding remarks and future perspectives

Microbial phenotypic heterogeneity clearly plays an important role in bioprocess robustness, but additional

data will be necessary to address its implications more fully. Indeed, recent results have revealed stochasticity at the level of metabolic reactions without any intervention of gene expression mechanisms, multiplying the possible sources of phenotypic heterogeneity. Dedicated single cell analytical tools are thus needed for this purpose, and solutions are already available for this purpose (i.e., on-line flow cytometry with robust data treatment) or are under development (microfluidic cultivation tools). The most promising methods for improving the control of microbial phenotypic heterogeneity include metabolic engineering, and cofactor engineering in particular, because cofactor imbalance is known to be involved in metabolic variability from one cell to another. The combination of single cell cultivation devices with appropriate metabolic engineering strategies may lead to more robust microbial cell factories with reduced phenotypic heterogeneity.

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