A functional perspective on phenotypic heterogeneity in microorganisms

Martin Ackermann[1,2]

Abstract | Most microbial communities consist of a genetically diverse assembly of different organisms, and the level of genetic diversity plays an important part in community properties and functions. However, biological diversity also arises at a lower level of biological organization, between genetically identical cells that reside in the same microenvironment. In this Review, I outline the molecular mechanisms responsible for phenotypic heterogeneity and discuss how phenotypic heterogeneity allows genotypes to persist in fluctuating environments. I also describe how it promotes interactions between phenotypic subpopulations in clonal groups, providing microbial groups with new functionality.

Quantitative biology
The use of mathematical tools and principles to analyse experimental data in order to test existing theories and develop new theories.

Division of labour
The division of a biological task into several different subtasks that are each executed by specialized individuals.

[1]*Institute of Biogeochemistry and Pollutant Dynamics, Swiss Federal Institute of Technology Zurich (ETH Zürich), ETH Zentrum CHN H70.3, Universitätsstrasse 16, 8092 Zürich, Switzerland.*
[2]*Department of Environmental Microbiology, Swiss Federal Institute of Aquatic Science and Technology (Eawag), Ueberlandstrasse 133, 8600 Dübendorf, Switzerland.*
e-mail: martin.ackermann@env.ethz.ch
doi:10.1038/nrmicro3491
Published online 6 July 2015

Most microorganisms do not live alone; instead, they live in groups of varying degrees of complexity: from microcolonies formed through clonal expansion of a single cell, to communities that combine organisms from all three domains of life. The properties of these groups depend on within-group diversity. This idea is familiar from complex microbial communities: one of the classic questions of microbial ecology is how the productivity of such communities[1] and their resilience to external perturbations[2] depend on genetic diversity. However, the focus of this Review is on diversity at a lower level of biological organization: phenotypic diversity that occurs independently of genetic or environmental variation and thus manifests between genetically identical individuals that live in the same microenvironment (FIG. 1). I refer to this diversity as phenotypic heterogeneity. The main message of this Review is that phenotypic heterogeneity can have important functional consequences, similar to the consequences of the biological diversity that emerges at higher levels of biological organization.

Phenotypic heterogeneity has recently developed into a major research focus in microbiology and quantitative biology. At the origin of this development were observations of cellular individuality: even when genetic and environmental differences between cells are reduced as much as possible, single cells differ from each other with respect to gene expression and other phenotypic traits[3,4]. This heterogeneity was expected on theoretical grounds[5,6] because the molecular mechanisms underlying the expression of the genotype are based on small numbers of molecules, which inevitably leads to fluctuations in the molecular composition of individual cells. Indeed, such stochastic aspects of fundamental cellular processes have now been recognized as an important factor for the generation of phenotypic heterogeneity[7–9]. In addition, several other mechanisms contribute to the observed phenotypic variation in clonal groups that reside in homogeneous environments. The molecular mechanisms that can give rise to phenotypic heterogeneity have been the subject of a number of excellent reviews (for example, REFS 7,10–12), so only the main mechanisms are summarized here, with an emphasis on the diversity of molecular mechanisms that can give rise to similar patterns of phenotypic heterogeneity.

The main focus of this Review is on the functional consequences of phenotypic heterogeneity. As I discuss below, phenotypic differences between individual cells can be beneficial for populations of microorganisms. Phenotypic heterogeneity can allow some individuals to survive sudden changes in the conditions and can thereby allow genotypes to persist in ever-changing environments; it can also lead to the division of labour between individuals and can therefore increase the rate at which populations grow and the functions that they can perform. The fundamental idea is that phenotypic heterogeneity can provide such benefits because it is a biological trait that is shaped by natural selection and can evolve. Phenotypic heterogeneity is a complex trait that does not manifest when measuring the biological properties of a single cell at one point in time; rather, it can be observed only when making comparisons between multiple individuals or when following individuals over longer periods of time. Nevertheless, phenotypic heterogeneity fulfils the criteria for evolution by natural selection because the degree of phenotypic heterogeneity in a given trait is variable and heritable

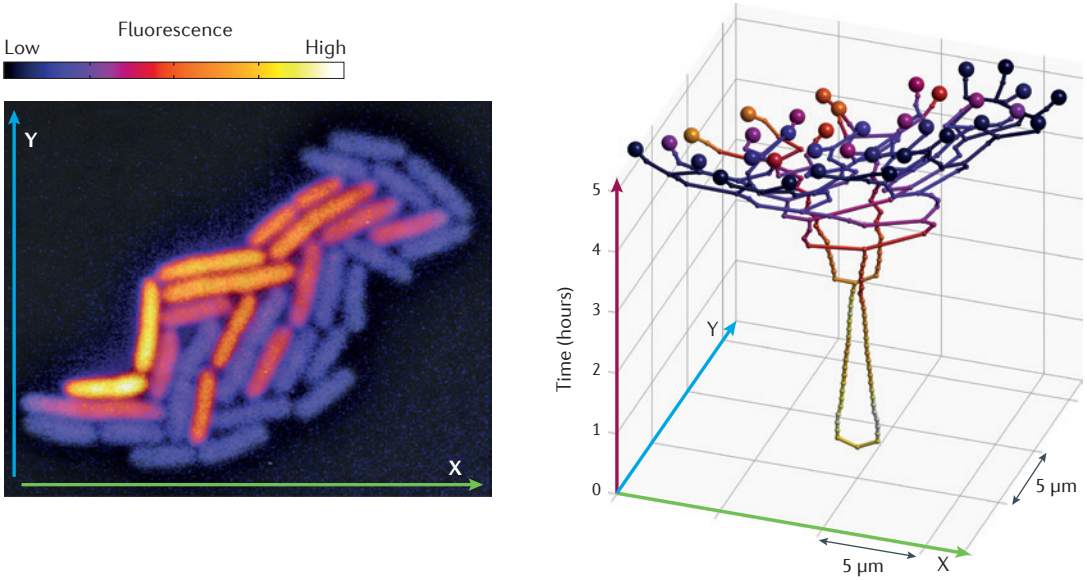


Figure 1 | **Phenotypic heterogeneity.** Time-lapse analysis with a strain of *Salmonella enterica* subsp. *enterica* serovar Typhimurium carrying a transcriptional *gfp* reporter for the promoter controlling the expression of the flagellin gene *fliC*[20]. The left panel shows a still image from a time-lapse microscopy experiment. The right panel shows a reconstruction of the lineage tree, based on the same time-lapse microscopy experiment. The root of the lineage tree is the single cell that founded the microcolony. Branching points correspond to cell division events, and the terminal branches correspond to the 35 cells that are shown in the left panel. The colour of the cells and of the branches represents the intensity of the fluorescence signal. X and Y refer to spatial dimensions, and vertical grid lines indicate 1 hour intervals. Analysis and images courtesy of S. van Vliet (Swiss Federal Institute of Technology Zurich (EHT Zurich) and Swiss Federal Institute of Aquatic Science and Technology (Eawag, Dübendorf)) and M.A..

— as it can be altered by genetic modifications (BOX 1) — and can influence survival and reproduction[7,13,14]. I first discuss the molecular mechanisms that underlie phenotypic heterogeneity and then focus on how phenotypic heterogeneity affects biological functions and, thus, the growth and survival of both individuals and groups of microorganisms.

Molecular causes of phenotypic heterogeneity

A number of different molecular mechanisms can give rise to phenotypic heterogeneity, and these mechanisms can lead to qualitative or quantitative phenotypic differences between individuals. In the case of qualitative differences, individual cells express two or more distinct phenotypic states; in the case of quantitative differences, individuals exhibit continuous variation in phenotypic traits.

An important question is whether phenotypic differences between cells are indeed produced independently of genetic variation and environmental signals, as implied by the common definition of phenotypic heterogeneity as variation arising between genetically identical cells in homogeneous environments. Answering this question poses two major experimental challenges. First, it is usually not possible to completely exclude a role for environmental factors because we often cannot completely control the gradients or fluctuations in environmental parameters that affect gene expression. However, there is now convincing evidence for the existence of molecular mechanisms that do not require environmental signals to produce phenotypic variation[3,15–17], rendering it plausible that phenotypic differences emerge in homogeneous environments.

Second, genetic differences can usually also not be excluded with certainty. Most experiments do not directly test whether cells that express different phenotypes are genetically identical. Some molecular mechanisms — for example, gene amplification — produce genetic variation at rates that greatly exceed the baseline mutation rate[18,19] and could explain some observations of phenotypic differences between cells. However, many cases of phenotypic differentiation arise at rates that are much higher than the rate of any known mutational mechanism, or are robust against the inactivation of known mutational mechanisms[20], indicating that many observations of phenotypic variation in populations grown from a single cell are indeed produced independently of genetic differences.

So, what mechanisms can produce phenotypic variation in the absence of genetic or environmental differences? One prominent type of mechanism is stochasticity in molecular processes (FIG. 2a). Many cellular processes are based on molecules that exist in small numbers in a cell and on processes that run at low average rates. As a consequence, the cellular state, corresponding to the molecular composition of a cell and its gene expression phenotype, varies over time and between individuals. Specific sources of this variation include stochastic gene expression[3,21] and stochastic partitioning of molecules at cell division[17]. The first direct

Gene amplification
The generation of multiple copies of a genetic region, resulting from a duplication event and subsequent homologous recombination of the duplicated region.

Stochastic gene expression
Fluctuations in the rate of mRNA and/or protein production over time. These fluctuations are a consequence of the low copy numbers of molecules in microbial cells and the burst-like nature of transcription.

Box 1 | **Genetic control over the level of phenotypic heterogeneity**

The molecular mechanisms that influence the level of phenotypic heterogeneity are directly or indirectly encoded by genes. As a consequence, one would expect the level of phenotypic heterogeneity that arises in the expression of a gene of interest to be under genetic control — that is, to be modulated by point mutations and other types of genetic modification. A number of studies have shown that this is indeed the case and have described genetic modifications that alter the level of phenotypic heterogeneity[3,4,21]. Association studies with *Saccharomyces cerevisiae* have identified loci that alter the levels of phenotypic heterogeneity in a trait of interest[89] and have shown that alleles that modulate the level of heterogeneity in particular genes or phenotypic traits segregate in natural yeast populations[90,91], potentially providing the genetic variation that is needed for an evolutionary response to selection on heterogeneity. Genome-wide studies with genetic model systems allow the levels of heterogeneity for a large number of genes to be determined and allow genes that show particularly high or low levels of heterogeneity to be identified. Studies with *S. cerevisiae*[92,93] and *Escherichia coli*[94,95] reported that genes that are essential or are evolutionarily conserved have low levels of variation[92,94,96,97], potentially as a consequence of selection against expression noise that can decrease growth rates[97]. Indeed, a number of molecular mechanisms that decrease phenotypic heterogeneity have been identified, among them negative feedback in the regulation of gene expression[98].

The second general insight from these studies is that there are consistent differences between genes belonging to different functional classes. Stress response genes and metabolic genes show higher levels of heterogeneity than genes involved in other functions in both *S. cerevisiae*[92,93] and *E. coli*[94,95]. It is possible that these types of gene are under weaker selection for lower noise levels than other types of gene, or that they are under direct selection for increased levels of noise. Alternatively, it is also possible that this pattern is a consequence of confounding variables. For example, the yeast genes that show particularly high levels of phenotypic heterogeneity also have high levels of plasticity across environmental conditions[92,99], and it is possible that phenotypic heterogeneity in these genes is a consequence of their plasticity. In *E. coli*, there is no such association[94], suggesting that selection can act on phenotypic heterogeneity and plasticity independently. Together, these genome-wide studies suggest that natural selection acts on the level of phenotypic heterogeneity and modulates the heterogeneity of individual genes differentially. Selection can act to either decrease or increase the level of phenotypic heterogeneity in genes, depending on whether the consequences of variation in these genes are beneficial to the organism.

quantification of how gene expression noise can lead to phenotypic variation between genetically identical cells came from experiments with *Escherichia coli*[3]. Two genetic elements were introduced into the chromosome of *E. coli*, one encoding a cyan fluorescent protein and the other encoding a yellow fluorescent protein; in both elements, the reporter gene was under the control of the lactose promoter. Given that both genes were controlled by the same promoter, the conventional expectation was that each cell would express identical and constant levels of the two fluorescent proteins. Instead, the authors observed marked variation between different cells, both in the absolute fluorescence levels and in the ratio between cyan and yellow fluorescence. As the observed variation is a result of fluctuations in gene expression over time and between individual cells, this work laid the foundation for the quantitative analysis of gene expression noise.

Elegant experimental work has provided direct evidence that stochastic gene expression can be a main driver of phenotypic heterogeneity. For example, genetic modifications of the bacterium *Bacillus subtilis* that resulted in larger cells, and thereby reduced fluctuations in the cellular molecular composition, decreased the level of phenotypic heterogeneity in the initiation of sporulation[16]. This outcome is in line with the assumption that phenotypic heterogeneity is caused by stochastic gene expression. Importantly, the variation in molecular composition that arises through stochastic gene expression can be modulated by gene-regulatory networks. Some networks dampen variation, whereas others — such as those containing positive feedback loops[22] — amplify variation, which can lead to the emergence of two or more phenotypic groups that differ from each other in a qualitative way (FIG. 2a).

Stochastic switching between different phenotypic states is theoretically well developed and empirically supported, but there are also other mechanisms that can give rise to phenotypic heterogeneity. A first alternative mechanism relates to periodic oscillations in cellular functions[14,23] (FIG. 2b). Some types of periodic oscillations in cellular functions are common and well studied, the primary example being the cell cycle. Another well-studied oscillation occurs in unicellular cyanobacteria, which alternate between two incompatible metabolic processes: nitrogen fixation and photosynthesis.

A second alternative mechanism relates to cellular age. Two cells emerging from division often differ from each other in a systematic way; that is, cell division is asymmetrical[24] (FIG. 2c). One important case of asymmetrical cell division occurs in microorganisms that are rod shaped or in those that divide through budding. In such cases, the two cells emerging from division differ in age as defined by the time of cell pole or cell wall synthesis, resulting in a distribution of cell ages within a clonal population. Importantly, ageing effects that are based on asymmetrical cell division arise in exponentially growing populations, in contrast to the effects of 'chronological ageing', which concern cellular changes in non-replicating cells during extended periods of starvation[25]. If the phenotypic traits expressed by individuals depend on cellular age, this leads to phenotypic heterogeneity. Such age dependence has been demonstrated for a number of cellular traits in budding yeast (*Saccharomyces cerevisiae*)[26,27] as well as in bacterial systems. For example, in the alphaproteobacterium *Methylobacterium extorquens*, both cell size and the timing of cell division depend on cellular age[28]. In *Mycobacterium tuberculosis*, cellular age has been found to influence survival following exposure to antibiotics, although the direction of this effect is inconsistent between studies[29]. These observations raise the question of whether age-dependent mechanisms are common molecular causes of phenotypic heterogeneity.

A third alternative mechanism giving rise to phenotypic heterogeneity involves cell–cell interactions mediated by diffusible molecules or through physical contact between individual cells[30,31] (FIG. 2d). The currently dominant view on phenotypic heterogeneity is that each cell makes its own gene expression 'decisions'. However, it is likely that the cellular decisions of an individual are often influenced by the phenotypes of other cells in the population. This is because the expression of many genes is modulated by signals from the environment, including signals from neighbouring cells. Although quorum sensing is the most commonly studied form of intercellular

Periodic oscillations
Changes in phenotypic traits that occur at regular time intervals.

Cellular age
The age of a cell as measured by the time since synthesis of particular subcellular structures. During cell division, these structures can be asymmetrically segregated into the daughter cells, resulting in two cells of different cellular ages.

Quorum sensing
The regulation of gene expression in response to changes in bacterial population density. Quorum sensing is mediated by intercellular chemical signalling.

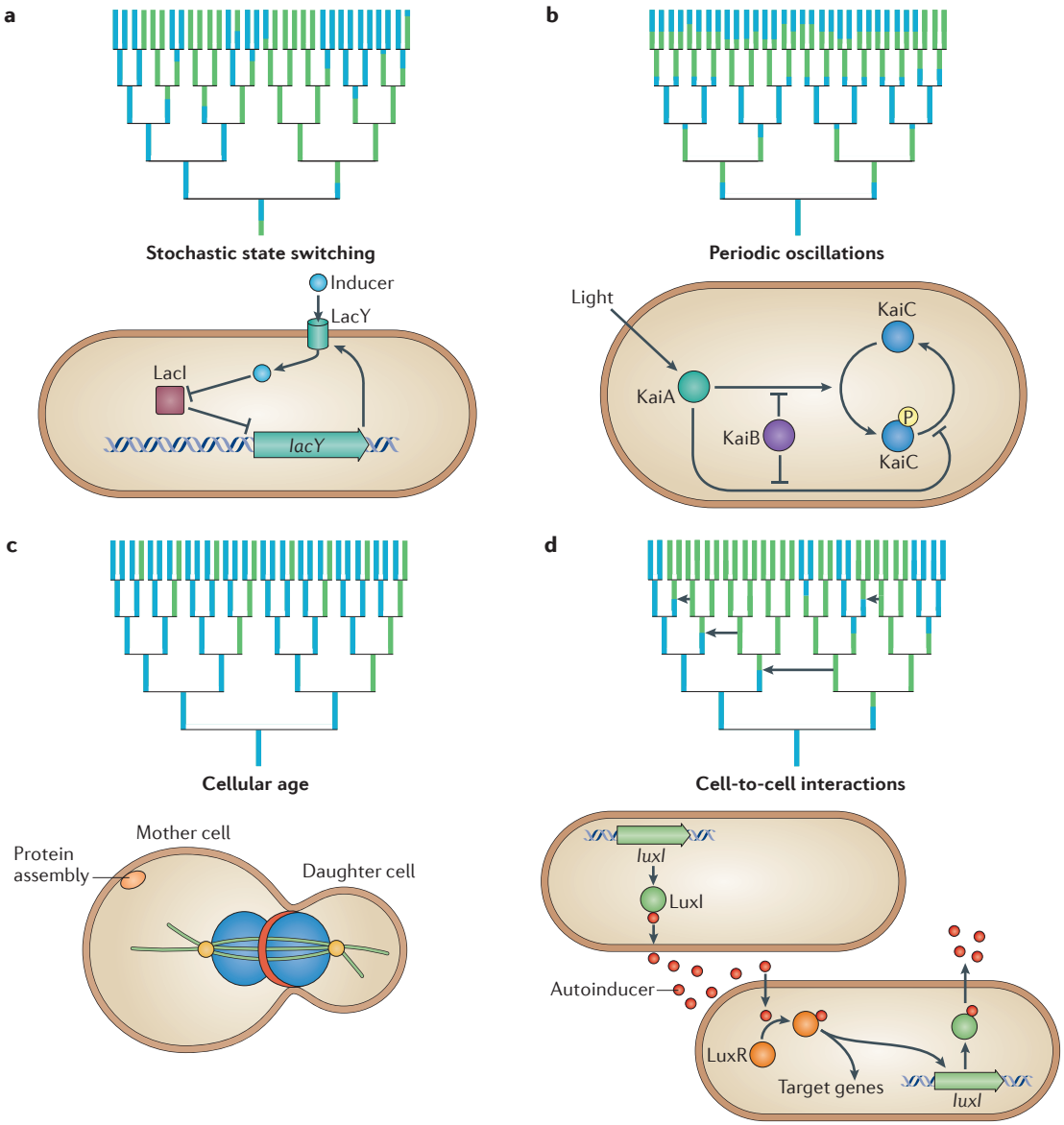


Figure 2 | **Molecular mechanisms of phenotypic heterogeneity.** Simplified schematic representations of phenotypic patterns on lineage trees (not based on experimental data) and schematic diagrams of key molecular components are shown for different mechanisms of phenotypic heterogeneity. The lineage trees map phenotypes and division events in a clonal population with individual cells that, for simplicity, are depicted as being in one of two distinct phenotypic states (blue and green), as opposed to the continuous variation in phenotypic traits that these mechanisms can also give rise to. Each population is derived from a single founder cell, which is at the bottom of each tree. **a** | Stochastic state switching of the lactose utilization system, which switches between ON and OFF as a result of stochastic events at the level of the promoter. State switching is based on a positive feedback loop. Expression of the transporter lactose permease (LacY) increases the intracellular concentration of the inducer; this leads to increased expression of LacY via inhibition of lactose operon repressor (LacI). **b** | Periodic oscillations in the cyanobacterium *Synechococcus elongatus*, involving a non-transcriptional oscillator that forms the basis of the circadian clock in this organism. The circadian variation in light input into this circuit results in periodic oscillation in phenotypic outputs. The oscillator is based on phosphorylation of the protein KaiC, which is regulated by KaiA (in response to light) and KaiB. Depending on the light input, KaiA will either enhance or inhibit KaiC phosphorylation, whereas KaiB blocks both of these effects of KaiA on KaiC. **c** | Cellular ageing in microorganisms that are rod-shaped or divide by budding. In such organisms, the age of cell poles and cell walls can differ between the two cells emerging from division, which can lead to phenotypic heterogeneity[27]. Here, asymmetrical segregation of protein assemblies in *Saccharomyces cerevisiae* is shown. Asymmetry in the segregation of such assemblies can drive phenotypic differences between cells of different ages[27]. **d** | Phenotype switching in response to cues received from other cells. The canonical example is quorum sensing, shown here for *Vibrio fischeri*. *N*-acyl homoserine lactone synthetase (LuxI) produces a diffusible molecule known as an autoinducer. The autoinducer is taken up by other cells, in which it modulates gene expression via binding to LuxR. This modulation includes the induction of LuxI expression and, therefore, the production of more autoinducer molecules. The black arrows in the lineage tree indicate moments when the phenotype expressed by one cell influences gene expression in another cell.

Box 2 | **Experimental approaches to study phenotypic heterogeneity**

Investigating phenotypic heterogeneity requires one to measure properties of individual cells. Some of the seminal discoveries of heterogeneity were based on measuring traits that are accessible by direct microscopic inspection, such as motility, growth and survival[39,100]. Most of the recent studies have used fluorescent proteins as gene expression reporters, in combination with microscopy[101] or flow cytometry[21]. To analyse how the expression of different genes co-varies at the level of single cells, and thereby to gain insights into heterogeneity in complex phenotypes involving many genes, one needs to follow the expression of different genes at the same time, in the same cell. Combinations of fluorescent proteins allow such experiments for a small number of genes[102]. Measuring the transcriptome[103] and proteome[92] of single cells extends such analyses to the genome-wide scale, although this approach is limited in bacterial systems by the small number of mRNA molecules and proteins per cell[104].

New approaches enable researchers to go beyond genetic model systems and laboratory environments, and thereby to investigate the relevance of phenotypic heterogeneity in natural situations. A first step is to analyse phenotypic heterogeneity with natural isolates in the laboratory. Phenotypic heterogeneity can be analysed in natural isolates when working on phenotypic traits that can be measured without using fluorescent proteins as reporters. Motility, growth and survival can be directly measured, and recent technological developments also allow quantifications of mRNA levels[105], protein levels[106] and the assimilation of isotope-labelled nutrients[107,108]. These approaches can be used to analyse microorganisms in more natural environments: for example, by sampling microbial aggregates from natural sources and analysing metabolic activities at the single-cell level in the laboratory[109], or by incubating samples from natural communities *in situ* with isotope-labelled nutrients and analysing isotope content at the single-cell level[110,111]. In such studies, the degree of genetic variation in the cell population that is being analysed is usually unknown, and it is thus not clear whether the variation in phenotype within this population is a manifestation of phenotypic heterogeneity (as defined in this Review) or of genetic variation. However, new experimental approaches can resolve this complication. One such approach is to encapsulate single cells in gel microdroplets so that these cells can grow in clonal microcolonies while their chemical coupling to their microenvironment is preserved[112]. Another approach can be applied for studies on host interactions: experimental host systems allow the study of microorganisms in their natural environment — the host — under well-controlled laboratory conditions, and such experiments have shed light on the role of phenotypic heterogeneity in these host–microorganism interactions[41,77,113].

modulation of gene expression[32], a large number of other mechanisms can also couple cellular decisions to the phenotype of neighbouring cells, such as contact-dependent signalling based on signalling molecules that remain anchored to the bacterial cell envelope[33].

Another mechanism that can produce phenotypic heterogeneity is the epigenetic modification of chromatin through changes in DNA methylation and/or histone composition. Epigenetic modifications are an important source of phenotypic heterogeneity in some eukaryotic microorganisms[11,34], but are not discussed here.

The diversity of molecular mechanisms that can give rise to phenotypic heterogeneity has an important consequence: detailed experimentation is often required to determine which molecular mechanisms are responsible for a given example of phenotypic heterogeneity[35] (BOX 2). Furthermore, although all of these different molecular mechanisms can lead to phenotypic variation, they are not equivalent in all respects. For example, the histories of single cells — the phenotypic pattern expressed along a cellular lineage — differ depending on the particular molecular mechanism responsible for phenotypic heterogeneity (FIG. 2). Theoretical[36] and experimental[37] studies indicate that these differences can be relevant; for example, the formation of multicellular chains in *B. subtilis* depends on cells switching between two phenotypic states simultaneously: that is, on the timing of their cell histories[37].

Consequences of phenotypic heterogeneity

One of the most important questions relating to phenotypic heterogeneity is whether it is beneficial for organisms: that is, whether it provides individuals or groups with advantageous properties that would not occur in a phenotypically homogeneous clonal population.

The identification of adaptive benefits is challenging in general[38], including in the case of phenotypic heterogeneity. Specifically, it is difficult to identify the likely selective forces that favour the evolution of gene-regulatory networks that produce heterogeneity. However, one useful approach is the analysis of the functional consequences of a given case of phenotypic heterogeneity. First, one needs to establish that the observed phenotypic differences between individuals are functionally relevant — that phenotypic heterogeneity measured by a phenotypic proxy does indeed lead to functional differences between cells[13]. This functional relevance has been established for some specific traits in a number of organisms through experiments that have analysed the survival, growth and behaviour of cells that express different phenotypes; such experiments have shown that genetically identical cells can differ in their tolerance to stress[26] and to antibiotics[29,39,40] and in the expression of virulence traits that determine behaviour inside a host[41]. When functional relevance is established, experimentation and mathematical modelling can further probe the consequences of phenotypic heterogeneity on the evolutionary success of a genotype in an environment of interest. This combination has led to the identification of several potentially beneficial consequences of phenotypic heterogeneity.

A strategy to cope with dynamic environments. The best-known adaptive benefit of phenotypic heterogeneity is that it can allow genotypes to persist in the face of fluctuating environments[42–47] (FIG. 3a,b). The conventional perspective on microbial adaptation to fluctuating environments is that individuals sense environmental cues and respond to them through signal transduction and the regulation of gene expression, thereby attaining a phenotype that performs well in the current environment. However, it is likely that this strategy — sensing and responding — is not always possible, such as when the number of possible environmental conditions that an organism encounters is so large that it is not possible to evolve a signal transduction pathway for every relevant condition[48], or when environmental impacts arise so rapidly that organisms cannot express phenotypic features that would accommodate the impact fast enough.

One solution to this scenario is that organisms can evolve to express these protective features probabilistically and with a low probability, independently of environmental cues[42–45]. In this case, at any point in time, the majority of the population will not express the features; these individuals will grow well in the absence of environmental impacts but poorly during impacts. A

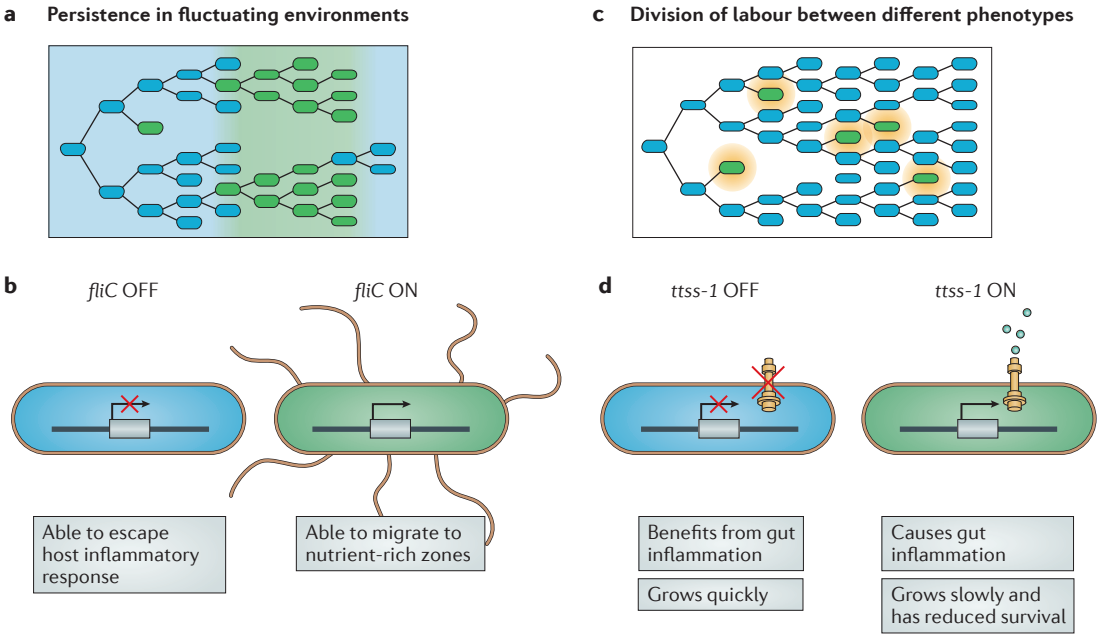


Figure 3 | **Different functions of phenotypic heterogeneity.** Phenotypic heterogeneity can promote persistence in fluctuating environments and lead to the division of labour in clonal groups. These two functions of phenotypic heterogeneity manifest in infections with *Salmonella enterica* subsp. *enterica* serovar Typhimurium, used as an example here. **a** | A genotype that expresses two different phenotypes (blue and green cells) can persist in an environment that fluctuates between two states (light blue and light green) in which only individuals that express the matching phenotype can survive. **b** | S. Typhimurium exhibits phenotypic heterogeneity in the expression of the flagellin gene *fliC*, and this allows this organism to persist in an environment that fluctuates between favouring flagellation (*fliC* ON) and selecting against it (*fliC* OFF)[54]. **c** | Phenotypic heterogeneity can lead to interactions and the division of labour within clonal populations. For a genotype that expresses two different phenotypes (blue and green cells), individuals that express the green phenotype do not continue to grow but do produce a resource (orange) that promotes the growth of the blue phenotype. **d** | S. Typhimurium exhibits phenotypic heterogeneity in the expression of the virulence locus type three secretion system 1 (*ttss-1*), which encodes a multiprotein secretion apparatus. This leads to a division of labour between *ttss-1* OFF and *ttss-1* ON subpopulations. The *ttss-1* ON subpopulation invades host tissue and causes inflammation but suffers a reduction in growth and survival. The *ttss-1* OFF subpopulation benefits from the inflammation and proliferates. Interestingly, phenotypic heterogeneity in flagellin genes and in *ttss-1* expression is correlated at the level of single cells[127].

minority of the individuals in a clonal population will express these features, and they will potentially grow more slowly in the absence of environmental impacts if these features are costly to express but will survive environmental impacts. Thus, this type of phenotypic heterogeneity, in which phenotypic variants are produced independently of environmental signals, allows organisms to persist in fluctuating environments under conditions in which 'sensing and responding' is not possible. This evolutionary strategy is known as bet hedging[49] (FIG. 3a). The notion that phenotypic heterogeneity can allow organisms to persist in fluctuating environments through such a bet-hedging mechanism is supported by a number of empirical studies. A first line of experimental support comes from experiments that analysed the role of phenotypic heterogeneity in response to adverse conditions. For example, growth rate heterogeneity in populations of *E. coli* contributes to survival on exposure to antibiotics[39]. A small subpopulation of cells (known as persisters) in clonal populations grows very slowly or not at all, and these cells have a higher chance of surviving sudden exposure to antibiotics. The slow growth that confers tolerance to antibiotics can be caused by a range of different exterior or interior factors, including nutrient shifts[50] or expression of virulence genes[40]. The source of the phenotypic heterogeneity that determines survival when exposed to antibiotics has in some cases been traced to changes in the expression of single specific genes. For example, in *E. coli*, the formation of persisters has been linked to fluctuations in the expression of an intracellular toxin called HipA; cells in which the level of HipA exceeds a threshold become dormant and tolerant to antibiotics[51].

A role for phenotypic heterogeneity has also been demonstrated when cells are challenged with other types of environmental insults. The stochastic switching to competence or sporulation in *B. subtilis*[52,53] suggests that this organism uses heterogeneity as a biological strategy to survive stressful conditions. Switching to competence in *B. subtilis* is mediated by phosphorylation of the master regulator stage 0 sporulation protein A (Spo0A). Intercellular variation in phosphate flux leads to differences in the levels of phosphorylated Spo0A, and these levels determine whether a cell will become competent.

Persisters
Cells that are phenotypically tolerant to antibiotics without being genetically resistant.

HipA
An intracellular bacterial toxin that is part of the toxin–antitoxin module *hipBA*.

Other examples of phenotypic heterogeneity mediating the response to environmental stimuli include resistance to heat killing in *S. cerevisiae*[26] and evasion of the host immune response by *Salmonella* spp., a strategy that is based on the formation of a subpopulation of bacteria that do not express flagella and thereby evade eukaryotic defence pathways[54] (FIG. 3b). Finally, a recent study provided evidence that phenotypic heterogeneity could also have an important role in coping with fluctuating conditions in natural environments. Yeast strains isolated from environments with high levels of lead pollution have higher levels of cell-to-cell variation in lead tolerance than strains from unpolluted environments[55].

A second line of experimental support for phenotypic heterogeneity as a strategy in fluctuating environments comes from the analysis of metabolic functions. In a natural environment, organisms need to frequently switch to new sources of ephemeral nutrients. These transitions might be difficult if individuals lack the energy or other cellular resources to express new metabolic pathways. In such cases, phenotypic heterogeneity in metabolic functions can help clonal populations to continue to grow after nutrient shifts, as some of the individuals express alternative metabolic pathways that allow them to consume the newly available nutrients[44]. Recent studies with *E. coli*[50], *Lactococcus lactis*[56] and *S. cerevisiae*[57] showed that phenotypic heterogeneity plays an important part in metabolic transitions from one carbon source to another: only a minority of individuals in clonal populations enact the metabolic transition required for growth on the second substrate. The majority of the clonal population does not grow on the second carbon source, possibly because the energy state of these cells is too low for the cells to express the second catabolic pathway[56]. The study with *E. coli*, which focused on metabolic transitions from glucose to gluconeogenic substrates, showed that the molecular decision of growing versus not growing on the second, gluconeogenic substrate depended on metabolic flux: cells grew if, immediately after the switch, the metabolic flux attributable to utilization of the gluconeogenic substrate exceeded a threshold level[50].

Two experimental-evolution studies provide complementary and more direct support for the idea that increased levels of phenotypic heterogeneity can evolve in response to fluctuating selection. A recent study in *S. cerevisiae*[58] showed that regimens that alternate between two carbon sources lead to the evolution of genotypes in which a larger proportion of the individuals resume growth after the nutrient switch than in the founder genotype. An earlier study observed the rapid evolution of increased levels of phenotypic heterogeneity in a non-metabolic trait: experimental populations of *Pseudomonas fluorescens* rapidly evolved the capability to switch between two states that differed in colony morphology, driven by fluctuating selection of this trait[59]. These two experiments directly show that increased levels of heterogeneity in a single trait can evolve in response to natural selection.

These results suggest that phenotypic heterogeneity is an important way for microorganisms to cope with environmental fluctuations. In these examples, signal transduction and the regulation of gene expression might still be important for consolidation of the phenotypic state of those individuals that survived an environmental impact because they were in the subpopulation that, by chance, expressed the right features; however, the initial expression of the phenotypic features that proved decisive for survival is mediated by phenotypic heterogeneity[60]. Importantly, phenotypic heterogeneity in the form of continuous variation in phenotypic traits (as opposed to the examples of binary variation discussed above, in which each cell expresses one of two discrete phenotypic states) can also promote growth and survival in environments that vary across different time and length scales[47].

The defining features of these bet-hedging strategies are that their benefit arises in fluctuating environments and that the benefit does not require any interaction between individuals. This is in contrast to other adaptive benefits of heterogeneity (see below).

Differentiation and the division of labour. Although bet hedging is the most intensively studied benefit of phenotypic heterogeneity, there are other benefits that are fundamentally different. One important adaptive benefit is based on interactions and the division of labour between phenotypically different individuals in a clonal group[30,61] (FIG. 3c,d). In the case of phenotypic heterogeneity that promotes interactions and the division of labour between individual cells, the characteristic feature is that the benefits for a given cell (in terms of resources or other factors that promote growth and survival) depend on the phenotypes of the other cells in the same microenvironment. I first discuss two examples of the division of labour that are asymmetrical, in the sense that one cell type expresses a behaviour that is beneficial for a second cell type, without the first cell type receiving a direct benefit in return. A first example is the production and secretion of metabolites or other cellular products that are accessible for the whole clonal group. Under nutrient-limiting conditions, *B. subtilis* produces the secreted protease subtilisin E, which degrades proteins outside of the cell. As subtilisin E and its products are freely diffusible, all cells in the microenvironment are expected to have access to the benefits of subtilisin E, irrespective of whether they contribute to its secretion[62]. Single-cell measurements of gene expression revealed that only a minority of cells in *B. subtilis* populations express the gene that encodes subtilisin E, *aprE*[62]. This suggests that only a minority of individuals in a clonal population engage in the secretion of a compound that is potentially costly to produce but provides benefits to all individuals in the population.

A second example has been shown in the bacterial pathogen *Salmonella enterica* subsp. *enterica* serovar Typhimurium. Clonal populations of *S.* Typhimurium in the gut differentiate into two subpopulations, one of which expresses a set of virulence genes known as type three secretion system 1 (*ttss-1*)[63] (FIG. 3d). The two subpopulations (*ttss-1* ON and *ttss-1* OFF) have different and complementary roles in the infection process[41];

Metabolic flux
The rate at which molecules flow through a metabolic pathway.

Type three secretion system 1
(*ttss-1*). The genetic locus encoding a multiprotein secretion apparatus that some Gram-negative bacteria use to translocate effector proteins into host cells.

the *ttss-1* ON subpopulation invades the gut epithelium and elicits inflammation of the gut[64], whereas the *ttss-1* OFF subpopulation does not invade the epithelium and remains in the gut lumen. This *ttss-1* OFF subpopulation benefits from the inflammation elicited by the *ttss-1* ON subpopulation, allowing the *ttss-1* OFF cells to outcompete the native gut microbiota, thus promoting their proliferation and potential transmission to new hosts[41].

A third example of division of labour is the phenotypic heterogeneity observed in metabolic functions in filamentous (and thus multicellular) cyanobacteria, in which nitrogen fixation and photosynthesis are segregated into different cells (see below)[65]. Recent single-cell measurements of metabolic activities indicate that this differentiation might also occur in colonies of unicellular cyanobacteria[66].

Division of labour can resolve incompatibilities. One scenario in which phenotypic heterogeneity may confer a benefit through division of labour is a situation in which two (or more) incompatible cellular processes are required to achieve a biological function[14,67,68]. Simultaneous activity of these processes in single cells is either not possible or inefficient, but this can be resolved by segregating the processes into different cells. The classic example of incompatible cellular processes is nitrogen fixation and photosynthesis in cyanobacteria, in which the oxygen released by photosynthesis damages the enzyme required for nitrogen fixation. As discussed above, these two processes are segregated into different cells in cyanobacteria, which allows multicellular filaments to perform both processes at the same time[65]. A special case of incompatibility occurs when a cellular process precludes the cell from future division or survival (FIG. 3c,d). One example is the production of proteins or other cellular compounds that act extracellularly but can be released only if the cell producing the compound lyses. Release through lysis has been reported for a number of toxins in bacterial pathogens[69,70], as well as for bacteriocins[71], and it can be mediated by phages[69] or several other cellular processes. For example, a recent study reported a new principle of the release of compounds from bacterial cells (including the toxin pneumolysin, which is produced by the pathogen *Streptococcus pneumoniae*): many bacterial strains contain a gene encoding the enzyme PezT, which inhibits cell wall synthesis. PezT is usually inactivated by the antitoxin PezA, a protein that cells express at the same time as PezT. External stresses such as starvation lead to the degradation of PezA, which activates PezT and consequently blocks the formation of the cell wall, leading to lysis during growth and division and the release of toxins from the cell[72]. The lytic effect of PezT depends on the cellular growth rate[72], which might restrict lysis to only a minority of cells in clonal populations. Another example of a cellular process that impairs growth and survival is the expression of *ttss-1* in *Salmonella* spp. during infection of a host, which leads to growth retardation[73] and killing of some of these bacteria on invasion of the host tissue[41].

It might not be immediately obvious how cellular processes that preclude future growth and survival can be sustainably carried out by microorganisms, as cells that engage in these processes leave no copies of their genomes to future generations. However, phenotypic heterogeneity offers a solution[41]: if these processes are active in only a minority of cells within a clonal population, but all cells derive a benefit, then the clonal population as a whole can grow. The cells that carry out these costly processes (and therefore do not survive or grow) can continually be regenerated from the surviving cells through phenotypic heterogeneity.

The importance of spatially structured environments. Benefits of phenotypic heterogeneity that arise through the division of labour can emerge only if individuals remain spatially close to each other, for two reasons. First, proximity is often a prerequisite for cell-to-cell interactions because it is necessary for the exchange of molecules through diffusion[74]. Second, spatial proximity can play an important part in protecting clonal groups from mutants that benefit from the group division of labour without contributing to these interactions[75]. Such mutants are often referred to as cheaters[76]. Many of the interactions mediated by phenotypic heterogeneity that are discussed above are based on the formation of a valuable resource, such as nutrients released by exoenzymes[62], or on a modification of the host environment that is conducive for bacterial growth during infection[41,77]. Importantly, these resources are typically accessible to cells irrespective of whether these cells contribute to the resources themselves. This leads to the 'public goods dilemma' that has been characterized in other examples of microbial interactions[78–80]: genotypes that do not contribute to the production of the shared resource — or, in the case of phenotypic heterogeneity, those that never express the cooperative phenotype that produces the resource — have a net growth advantage and increase in number. Spatial structure can prevent this from happening, as it restricts the movement of individuals and the diffusion of compounds. Such structure confines the genotypes that never express the cooperative phenotype to a local microenvironment that is poor in the shared resource and can therefore prevent these genotypes from spreading and taking over microbial populations. This stabilization of the production of a shared resource is also known as kin selection[81].

These considerations are in line with the observation that examples of phenotypic heterogeneity promoting interactions between individuals are often associated with spatially structured habitats. For example, microorganisms in biofilms often show phenotypic heterogeneity in the production of secreted, and thus potentially shared, compounds[82,83]. Associations with hosts also provide spatial structure to microbial groups through physical barriers to the movement of microorganisms and to the diffusion of chemicals secreted by these microorganisms. As explained above, a role for phenotypic heterogeneity in host modifications has been demonstrated for *S.* Typhimurium[41,64] and is likely to occur in other pathogens, as the above examples on the

Cheaters
Individuals that contribute less than others to a collectively produced public good but still benefit from that good.

Biofilms
Microbial communities that are attached to surfaces. Biofilms provide spatial structure by limiting the movement of microorganisms and the diffusion of molecules.

release of toxins through bacterial lysis[69,70,72] indicate. In these examples, groups of microorganisms show aspects of multicellularity: namely, phenotypic differentiation and specialization, and division of labour between cells that express different phenotypes. The spatial cohesion of these host-associated populations is mediated not necessarily by direct physical attachment between cells but rather by the environment that keeps cells together as groups.

Division of labour leads to collective functionality. The main idea that emerges from the discussion above is that interactions and division of labour between genetically identical microorganisms can provide clonal groups with new functionality and promote collective behaviour. This idea is an extension of the 'social' perspective that has recently attracted a lot of attention in microbiology[76,78–80]. Social interactions are defined as behaviours that have consequences (in terms of affecting growth or survival) for both the individual that shows the behaviour and other individuals in the same environment. Many studies on social interactions, with the notable exception of the work on biofilms[82], have so far implicitly assumed that all individuals in a clonal population would express the same phenotype (such as the production of exoenzymes, toxins or metal chelators) and that all individuals therefore pay the same metabolic cost. However, as mentioned above, there are situations in which only a minority of individuals in a clonal population express a social trait, but other individuals benefit without contributing. In these cases, the properties of microbial groups are shaped by interactions between individuals that differ in phenotype, and these interactions lead to collective functionality at the level of the group.

Importantly, the different adaptive benefits of phenotypic heterogeneity do not exclude each other (FIG. 3). For example, specialization in different metabolic pathways in a clonal population can be advantageous in a static environment through the reduction of competition or the promotion of metabolic interactions between individuals (BOX 3), but the same specialization can also help persistence of the genotype in the face of environmental fluctuations[44,45]. A recent experimental study addressed this point directly and found that phenotypic heterogeneity in one single trait can have different functional consequences. In this example, phenotypic heterogeneity in virulence gene expression in *S.* Typhimurium leads to the division of labour during infection and also promotes persistence during exposure to antibiotics[40]. The functional consequence of phenotypic heterogeneity is thus potentially more complex than is often assumed.

Outlook

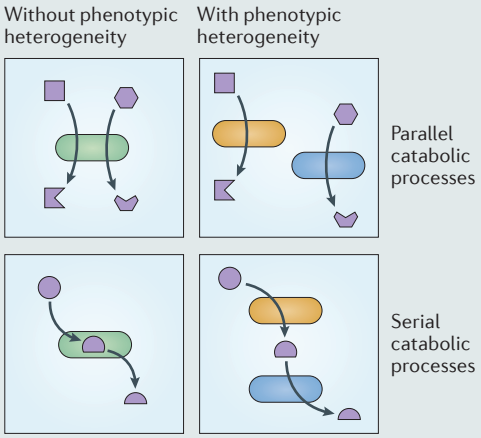
The notion of phenotypic heterogeneity has changed how we look at microbial populations. Microbial cells are individuals that differ from each other in terms of their behaviour and their properties, and this individuality is based on a number of molecular mechanisms that generate phenotypic differences between cells even in the absence of genetic and environmental variation. Phenotypic heterogeneity can have important functional consequences and can provide individuals and groups with new functionality.

Box 3 | **Phenotypic heterogeneity in metabolic functions**

The metabolic activities of a cell are a type of cellular function for which phenotypic heterogeneity could be of particular importance. Metabolic genes often have higher levels of phenotypic variation than other groups of genes[92,94,95], and there is evidence that this can provide adaptive benefits to an organism. Phenotypic heterogeneity can promote persistence in environments in which the availability of nutrients fluctuates over time or can promote metabolic specialization and interactions in clonal groups. Here, I focus on the second scenario and summarize the main ideas and the experimental and theoretical evidence to support these theories.

The basic idea of metabolic specialization in clonal groups is that individual cells specialize phenotypically in a subset of metabolic processes, rather than performing all metabolic processes that are observed at the level of the clonal population. Phenotypic specialization in a smaller set of metabolic processes can potentially lead to the resolution of incompatibilities and biochemical conflicts and thereby increase the rate of metabolic processes in the cell[67,68]. Microorganisms can specialize in different anabolic processes (which synthesize building blocks) and can exchange building blocks with complementary specialists, or they can specialize in different catabolic processes (which break down nutrients to yield energy and biomass building blocks). Catabolic specialization can occur either in parallel pathways — for example, when individuals specialize in different nutrients that are broken down in separate sets of enzymatic processes (see the figure) — or in serial pathways, such that individuals specialize in different consecutive steps in the breakdown of a nutrient (see the figure). Empirical and theoretical evidence suggests that specialization in metabolic functions is beneficial under some conditions because specialization in a smaller set of processes can increase the rate of these processes[67] and reduce competition between individuals in clonal groups. Support for this idea comes from a number of studies with *Escherichia coli*[114–117]; these studies analysed the growth characteristics of pairs of two different genotypes that specialize in different sets of metabolic processes. Although these experiments did not directly investigate phenotypic specialization in metabolism, they established that some pairs grew faster or reached a higher biomass yield than the wild type. Theoretical studies have made specific predictions about which combinations of metabolic processes need to be separated in different cell types in order to increase growth[118–121].

Together, these studies provide a general insight: in some cases, segregating metabolic processes into separate cells, rather than running them in the same cell, can increase growth rates or yield. In these cases, one would expect that genotypes that segregate these processes into different cell types by means of phenotypic heterogeneity should outgrow genotypes that result in each cell performing the whole set of these processes[118,121]. Recent investigations have tested this scenario experimentally. These investigations have reported that, under some conditions, there is evidence for phenotypic heterogeneity in metabolism in clonal populations of *E. coli*[122], *Pseudomonas putida*[123] and *Saccharomyces cerevisiae*[124]: gene expression reporter systems using fluorescent reporters revealed conditions under which only a subpopulation of cells expresses genes involved in specific parts of serial or parallel metabolic pathways, suggesting the formation of two phenotypic subpopulations that each specialize in different steps in metabolic pathways and that complement each other[88]. New methods to quantify the assimilation of isotope-labelled compounds in single cells[107,125,126] now allow the direct testing of predictions about phenotypic heterogeneity in metabolism as an evolutionary strategy that increases growth rates or yield.



Almost all of our current knowledge about phenotypic heterogeneity is based on experiments performed in the laboratory and on mathematical modelling. One key question in the field is whether phenotypic heterogeneity is likely to be important in populations and communities of microorganisms that reside in natural environments. How much of the phenotypic variation expressed by bacteria in natural environments is produced independently of genetic and environmental differences (BOX 2)? And does this variation have biological consequences by, for example, promoting persistence in the face of environmental fluctuations[55] or promoting cell-to-cell interactions that are beneficial for the clonal group?

One might think that phenotypic heterogeneity does not have an important role in natural environments. Microbial communities usually have high levels of genetic diversity on all phylogenetic levels[84], and it therefore seems conceivable that the contribution of phenotypic heterogeneity within each species to the overall diversity within a community is not substantial. Furthermore, natural environments are often characterized by microscale chemical and physical gradients, both temporal and spatial[85], and microorganisms continuously adjust their phenotypes in response to these gradients. One might expect that little additional phenotypic variation is produced independently of these gradients.

However, there are arguments for why phenotypic heterogeneity could be important for microbial physiology irrespective of the high levels of genetic and environmental variation that characterizes many natural environments. The main argument is that phenotypic heterogeneity can have unique beneficial consequences. One benefit pertains to the link between diversity and stability in microbial communities. As discussed above, phenotypic heterogeneity can promote the persistence of a genotype in the face of environmental impacts. This is superficially analogous to the relationship between genetic diversity and the stability of microbial communities, a topic that has received a lot of attention in microbial ecology[2]. However, and importantly, the genetic diversity of a community can be depleted through repeated environmental impacts[86]. By contrast, variation that is expressed by a single genotype will always be replenished as long as the genotype persists, and it can thus promote continuing diversification in dynamic environments[45].

Another possible benefit of phenotypic heterogeneity in nature is that it can promote interactions that would not be stable between genetically distinct partners. As discussed above, phenotypic heterogeneity sometimes leads to a division of labour such that one of the two subpopulations invests without receiving a return, reducing the growth and reproduction of those cells[41,87,88]. Such interactions are not stable between cells with different genotypes, as the genotype that does not receive a return could not persist. Here, phenotypic heterogeneity offers a solution: the phenotype that does not receive a return is continuously replenished from the other phenotypic subpopulation (which is of the same genotype), leading to maintenance of the phenotypic variation and the division of labour that it promotes.

These considerations suggest that phenotypic heterogeneity is an important aspect of the biology of microorganisms in their natural environment and that it contributes functionally relevant biological diversity to microbial populations. Probing the importance of phenotypic heterogeneity in nature remains a challenge for the field, but experiments with genetic model systems in the laboratory, combined with mathematical modelling, are instrumental for developing hypotheses that then can be tested in natural settings. Such an integrated research effort has the potential to change our perspective on a fundamental issue in microbiology: the importance of the properties and behaviours of individual cells.

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Acknowledgements
The author gratefully acknowledges fruitful collaborations and important discussions with current and former members of the research group, and with a number of colleagues (in particular W.-D. Hardt and M. Doebeli). The author also thanks the referees and S. van Vliet for helpful comments on the manuscript. This work was supported by the Swiss National Science Foundation, the Swiss Federal Institute of Technology Zurich (ETH Zürich), the Swiss Federal Institute of Aquatic Science and Technology (Eawag) and the Marie Curie Program of the European Commission.

Competing interests statement
The author declares no competing interests.