Review


# Phenotypic Heterogeneity in Bacterial Quorum Sensing Systems

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## Abstract

Quorum sensing is usually thought of as a collective behavior in which all members of a population partake. However, over the last decade, several reports of phenotypic heterogeneity in quorum sensing-related gene expression have been put forward, thus challenging this view. In the respective systems, cells of isogenic populations did not contribute equally to autoinducer production or target gene activation, and in some cases, the fraction of contributing cells was modulated by environmental factors. Here, we look into potential origins of these incidences and into how initial cell-to-cell variations might be amplified to establish distinct phenotypic heterogeneity. We furthermore discuss potential functions heterogeneity in bacterial quorum sensing systems could serve: as a preparation for environmental fluctuations (bet hedging), as a more cost-effective way of producing public goods (division of labor), as a loophole for genotypic cooperators when faced with non-contributing mutants (cheat protection), or simply as a means to fine-tune the output of the population as a whole (output modulation). We illustrate certain aspects of these recent developments with the model organisms *Sinorhizobium meliloti*, *Sinorhizobium fredii* and *Bacillus subtilis*, which possess quorum sensing systems of different complexity, but all show phenotypic heterogeneity therein.


## Introduction

For orchestrating broader changes in their lifestyle, bacteria often make use of small diffusible molecules called autoinducers. These autoinducers accumulate in the environment while the producing cells grow, and once a certain threshold is reached, they trigger processes like biofilm formation, production of exofactors or virulence. Many of these processes are not only far-reaching and costly; they are also supposed to be effective only when cooperatively performed by a larger number of cells. As autoinduction is commonly seen as the means to determine when this sufficient population size—the quorum—is reached, and to then induce collective target gene expression in the group, the process has been termed quorum sensing [1].

This original and still prevalent image of autoinduction implies two major concepts: a role of autoinducer concentrations solely as a proxy for cell density; and homogeneity of both autoinducer production and target gene activation. However, already in 2002, Redfield [2] had argued for a more direct benefit of autoinducer molecules for the producing cells, namely to sense diffusion rates in the environment and subsequently regulate the production of secreted proteases, siderophores, antibiotics or other exofactors, whose synthesis would be worthwhile only if they remained in somewhat close proximity to their producers. This view spurred a discussion on the function of autoinduction

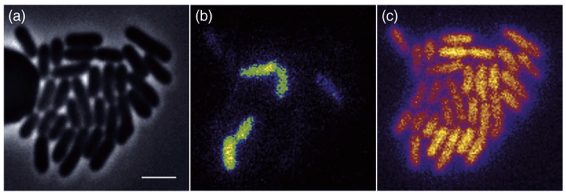


**Fig. 1.** Phenotypic heterogeneity in quorum sensing-related gene expression. (a) Phase contrast microscopy image of an *S. meliloti* microcolony displaying (b) heterogeneous activation of the promoter of the autoinducer synhase gene *sinI*, controlling expression of the fluorescent reporter gene *mVenus*, and (c) homogeneous expression of the fluorescent reporter gene *mCherry* from the constitutive T5 promoter. Both promoter–reporter gene fusions are located on the same plasmid. The scale bar represents 2 μm. Microscopy Jan-Philip Schlüter.

in an evolutionary sense—that is, the selective advantage that lead to its emergence in the course of evolution—that has been repeatedly enlivened as autoinducer production has been shown to be modulated by diverse environmental and endogenous factors (Box 1).

The homogeneity assumed for quorum sensing-related processes is a child of its time: 25 years ago, genetically identical bacteria were generally expected to behave alike under identical environmental conditions. However, single-cell analyses have meanwhile demonstrated that this is not always the case. Whereas, for example, housekeeping genes like those encoding DNA maintenance proteins tend to be homogeneously expressed by all cells in a population, numerous other traits are rather heterogeneously activated. Stress response or metabolic genes, for instance, often show cell-to-cell variation in gene expression that occurs independently of genetic or environmental variations; that is, some cells within a clonal population develop a certain phenotype and others do not, or not to the same extent [3–6]. Over the past decade, such phenotypic heterogeneity was also reported for the expression of quorum sensing-related genes in various bacterial species, both at the level of autoinducer production and of target gene activation (Fig. 1) [7].

Not surprisingly, some of the above-mentioned factors modulating quorum sensing systems were also found to modulate phenotypic heterogeneity in these systems, or, rather, to modulate the fraction of cells in a given population contributing either to autoinducer production or to the expression of a quorum sensing-regulated trait; the two aspects are thus intertwined. In this review, we put these recent findings in context with each other in order to carve out possible factors shaping phenotypic heterogeneity in quorum sensing systems, and potential functions this heterogeneity might have. However, covering all reported cases in detail would be beyond the scope of this review. We therefore give a compilation of recent examples in Table 1, look into established and potential molecular mechanisms in a first general section, and exemplify the interconnection between phenotypic heterogeneity and quorum sensing-modulating factors with two Gram-negative model organisms, the α-proteobacteria *Sinorhizobium meliloti* and *Sinorhizobium fredii*. A second general section is dedicated to a discussion of potential functions of phenotypic heterogeneity in quorum sensing-related gene expression. Here, among other examples, we specifically introduce two scenarios from *Bacillus subtilis*, a well-studied Gram-positive model organism

**Table 1.** Recent examples for heterogeneous activation of quorum sensing-related genes or traits

| Gene(s)/trait(s) | Factors modulating heterogeneity | Organisms and corresponding reference(s) |
|---|---|---|
| Autoinducer precursor or AHL synthase genes | Nutrient availability, temperature, external autoinducers, root exudates and plant-derived octopine | *L. monocytogenes* [8], [9]; *P. syringae* [10]; *S. fredii* [11]; *S. meliloti* [12] |
| Biofilm formation | Magnesium | *S. aureus* [13] |
| Toxin production | Magnesium | *S. aureus* [13] |
| Bioluminescence/bioluminescence-related genes | External autoinducers | *V. harveyi* [14–16]; *A. fischeri* [17] |
| Exoprotease gene | External autoinducers | *V. harveyi* [15] |
| Endoglucanase gene | | *X. campestris* [10] |
| Exopolysaccharide genes | | *S. meliloti* [12] |
| Type III secretion system | External autoinducers | *V. harveyi* [15] |
| Biosurfactant production | | *P. putida* [18]; *B. subtilis* [88,89,90] |
| Symbiosis island transfer | | *Mesorhizobium loti* [19] |
| Cell morphology | Homogeneous without AHLs | *Dinoroseobacter shibae* [20] |
| Cell clumping | | *Photorhabdus luminescence* [21] |
| Competence | pH, nutrient availability | *S. mutans* [22–26]; *B. subtilis* [43,44,162] |
| Peptide signal and receptor genes | | *B. subtilis* [27–29] |
| Sporulation/spore revival | | *B. subtilis* [29,30] |

Box 1
The function(s) of autoinducer signaling

According to Redfield, the common view of autoinducer-signaling as a means to establish density-dependent cooperation in bacterial populations and, thus, as a social trait rested on very weak foundations. The author argued that "neither the need for group action nor the selective conditions required for its evolution" had been demonstrated, and instead proposed diffusion sensing as a more direct benefit of autoinducer-mediated responses [2].

Indeed, a variety of physico-chemical factors have been found to influence autoinducer production, accumulation or the reaction to them: for example, flow, spatial distribution or clustering of the cells producing the autoinducers [121−125], pH [24,126−128], temperature [8,70], antibiotic stress [126,129] or the metabolic state of the cell. Glucose and other sugars imported into the cells via the phosphotransferase system, for instance, have been repeatedly shown to affect quorum sensing and quorum sensing-regulated traits via catabolite repression since the early 1980s [130−135], and only recently also directly by binding of the phosphotransferase system−phosphocarrier protein HPr to the autoinducer-2 kinase LsrK [136]. Likewise, opines, low-molecular-weight compounds produced by plant cells transfected with the *Agrobacterium tumefaciens* t-DNA fragment in the course of infection, appear to be a prerequisite for a quorum sensing response in this plant pathogen, as they cause a derepression of the *traR* gene encoding the autoinducer receptor [63,137,138].

Thus, many authors have agreed that the perception of autoinducers as a proxy exclusively for cell density represents an oversimplification [37,120,122,126,139,140]. However, most authors also do not question that cell density does have an influence. Pai and colleagues [141] recently confirmed with a synthetic quorum sensing circuit in *Escherichia coli* that the production of a costly exoenzyme was indeed advantageous only when produced at high cell densities. Moreno-Gámez and colleagues [126] concluded that the environmental stresses they imposed on *Streptococcus pneumoniae* did not override the effect of cell density, but rather modified the rate at which cells produced or sensed the autoinducer and, thus, the relationship between cell density and the quorum sensing response. Hense and colleagues [37,142] incorporated the various influences on quorum sensing in what they called a "hybrid push−pull" model: In this model, cell density, diffusion and spatial clustering act as "push" factors that determine whether or not a certain target behavior would be effective, whereas starvation and other stresses increasing the cells' need for the target behavior are viewed as "pull" factors reducing the threshold for activation. Based on both mathematical modeling and experiments with *Pseudomonas aeruginosa*, Cornforth and colleagues [143] even proposed side-by-side usage of multiple quorum sensing systems—as found in many bacterial species—as a means to distinguish between cell density and environmental factors, in their case diffusion: If diffusion was high, the ratio of different autoinducers in the environment would roughly depend on their production rates, whereas in case of low diffusion, a more stable signal would become relatively more abundant than a less stable one. Combinatorial responses to signal ratios would thus allow quorum sensing cells to differentiate between cell density and diffusion after all. Therefore, and for reasons of practicability, it has been suggested to stick to the well-established term "quorum sensing" [120,126], but to use it "with full appreciation of the many environmental factors that influence it" [120].

in which cell-to-cell communication contributes to bet hedging in fluctuating environments and to division of labor in biofilms [31].

## Possible Origins of Phenotypic Heterogeneity

Quorum sensing systems are almost ubiquitous in bacteria: For most species, at least one such system has been identified, and many even have several. Consequently, the diversity in autoinducer molecules and corresponding regulatory networks is immense [32]. In Gram-negative bacteria, the most common autoinducers for intra-species communication are *N*-acyl homoserine lactones (AHLs). These molecules are usually sensed by cytosolic LuxR-type regulators and produced by likewise cytosolic

LuxI-type synthases, named after the respective molecules controlling bioluminescence in the marine bacterium *Aliivibrio fischeri* [33]. Gram-positive bacteria typically use ribosomally produced oligopeptides sensed either extra- or intracellularly for intra-species communication [34,35], and the so-called autoinducer-2, a by-product of the activated methyl cycle, has been proposed for inter-species communication for both Gram-negative and Gram-positive species [36].

Simplified, all quorum sensing systems consist of a module for autoinducer synthesis, a module for autoinducer perception—usually with very high specificity—and signal transduction, and an output module containing sometimes hundreds of target genes associated with changes in lifestyle [37]. However, the specific architecture of the regulatory network can greatly influence the way in which a particular system is activated. Modes of autoinducer transport, autoinducer modification and site of autoinducer detection could affect how cell density is translated into the autoinducer concentration sensed by the receptor [38]. More importantly, different modes of gene expression regulation can result in vastly different response behaviors: For instance, when Haseltine and Arnold [39] modeled the *A. fischeri* LuxR–LuxI system with varying connectivity of synthase and receptor gene expression, the network without any feedback mechanisms yielded a graded response to increasing autoinducer levels; the network with a strong positive feedback only on autoinducer synthase gene expression and a constitutive receptor gene expression yielded a switch-like response; and the network with a strong positive feedback on both synthase and receptor gene expression produced strong bistability, that is, quorum sensing-responsive and -non-responsive subpopulations over a wide range of cell densities—a special case of phenotypic heterogeneity.

Haseltine and Arnold had assumed a constitutive receptor gene expression for the native LuxR–LuxI system, yielding a switch-like response in their model; however, when they combined the strong canonical positive feedback on *luxI* expression with the weak positive feedback on *luxR* expression proposed by others [40–42], their model also yielded bistability, albeit over a much smaller range of cell densities [39]. Nevertheless, such a positive feedback on *luxR* expression might help explain the phenotypic heterogeneity that Perez and Hagen [17] later observed in the bioluminescence response of individual *A. fischeri* cells to externally added autoinducers.

The role of positive feedback in quorum sensing-related bistability has also been demonstrated, for example, with regard to the autostimulation of the *B. subtilis* competence master regulator ComK [43,44], and more recently with respect to the *Staphylococcus aureus agr* quorum sensing system whose autoregulation seems to be critical for bifurcation of populations into toxin-producing and biofilm-forming subpopulations [13]. However, not only positive feedback loops but also even numbers of negative feedback loops can favor bistability [5,27,45,46]. Such a cross-repressive interaction motif regulates the expression of the *rapA–phrA* signaling system in *B. subtilis* and may contribute to its bimodal activation in a subpopulation of cells that delays the onset of sporulation [28].

However, positive feedback loops and pairs of mutually repressing repressors only work on pre-existing cell-to-cell variations but do not generate them. So where and how do these variations originate? Differences in the microenvironment of the respective cells might play a role in, for example, large colonies or biofilms where cells at the growing edge experience more oxygen and nutrient availability than cells in the center [47]. Likewise, cell-to-cell variability in the activation of target genes in structured settings like two- or three-dimensional colonies might indeed stem from an unequal distribution of autoinducer molecules. Nevertheless, it seems unlikely that the reported cases of phenotypic heterogeneity in quorum sensing-related gene expression are due to microenvironmental differences alone, as many of the respective studies were performed either in microfluidics devices with continuous nutrient supply, or in well-mixed liquid cultures where cells might eventually experience nutrient or oxygen starvation, albeit all to the same extent.

Plener and colleagues [14] attributed the heterogeneity they observed in *Vibrio harveyi* bioluminescence and bioluminescence-related gene expression to the design of the autoinducer receptor network *per se*, with three kinds of bifunctional histidine kinases converging on the same signal transduction cascade, as they observed homogeneous autoinducer responses only when all respective autoinducers were present at high concentrations. Low autoinducer concentrations, on the contrary, yielded cell-to-cell variations in the quorum sensing response. Low autoinducer levels might also figure in other cases of quorum sensing-related heterogeneities, as responses are often triggered at concentrations in the low nanomolar range, which for intracellularly sensed molecules corresponds to less than 10 molecules per cell according to a calculation by Williams and colleagues [42]. However, phenotypic heterogeneity in the *A. fischeri* quorum sensing response, for instance, was observed over a wide range of autoinducer concentrations, and even at concentrations that saturated the output of bulk populations [17].

Last but not least, quorum sensing targets—just like the key elements of the quorum sensing systems (Box 1)—might be subject to overlapping control by

other regulatory networks, or a heterogeneous target gene activation might be due to an immediate reaction of autoinducer-producing cells to the self-produced molecules like recently reported [18,22,48], and thus reflect heterogeneities that already arose upstream in the quorum sensing regulatory network. However, this would only bring us back to the initial question of where and how these variations originate. To this end, evidence from studies specifically on quorum sensing systems is still scarce, but a multitude of mechanisms has been identified in quorum sensing-unrelated studies (Box 2). Whether or not any of these mechanisms play a role in heterogeneity in quorum sensing-related gene expression is not yet established.

## Environmental and endogenous factors modulating quorum sensing in *Sinorhizobium*

All in all, it is very likely that there will not be a single ultimate cause of heterogeneity in quorum sensing, but a specific origin for each of the corresponding regulatory networks. Interestingly, though, in several of the reported cases, environmental and endogenous factors have been identified that modulate the fraction of contributors in a population (Table 1). Based on these modulating factors, one can at least speculate on the processes that might amplify initial cell-to-cell variations and establish distinct phenotypic heterogeneity, as we will illustrate with the two rhizobial model organisms *S. meliloti* and *S. fredii* that can both either be found free-living in the soil or in symbiosis with leguminous plants.

*S. meliloti* has a single quorum sensing system, the so-called Sin system that is a variation of the *A. fischeri* LuxR–LuxI system (Fig. 2a) [66]. Its close relative *S. fredii* has two LuxR–LuxI-type quorum sensing systems operating in parallel: the Ngr system encoded on the chromosome, and the Tra system encoded on a symbiosis-relevant plasmid (Fig. 2b) [60,61]. For both species, heterogeneity in quorum sensing-related gene expression has been reported: Grote and colleagues [11] demonstrated with promoter–*rfp* fusions that in well-mixed liquid cultures of *S. fredii* the promoters of both AHL synthase genes *traI* and *ngrI* are activated heterogeneously. Further work with a double reporter construct showed that some cells transcribe only one or the other autoinducer synthase gene, but not both genes simultaneously [7]. Likewise, heterogeneous expression of the *S. meliloti* AHL synthase gene *sinI*, monitored via a promoter–*mVenus* fusion, was demonstrated in microcolonies grown in microfluidic plates with continuous nutrient supply [12]. Last but not least, heterogeneous activation was shown for a direct target promoter of the receptor-AHL complex involved in exopolysaccharide production and thus biofilm formation in *S. meliloti* [12].

Box 2
Molecular mechanisms underlying phenotypic heterogeneity

Regarding the molecular mechanisms leading to phenotypic heterogeneity, explanations on very different scales have been brought forward. There are examples for various deterministic factors like, for example, cell age [144], a circadian oscillator [145], localization of a key molecule to one cell pole [146] or phase variation and epigenetic modifications [147]. The connectivity of the network components can likewise be critical as oligomerization of a transcription regulator or cooperative binding of a regulator to a promoter can introduce nonlinearity into the system [148]. Nonlinearity can also result from an interplay between activation of a system and a resulting growth retardation [149].

The most prominent explanation for phenotypic heterogeneity in bacteria in the literature, however, is stochasticity or noise [150–152]. This noise can originate from fluctuations in global cellular components, for example, the number of RNA polymerases, ribosomes, or metabolites, and thus affect all corresponding processes in this cell. However, it can also result from fluctuations in components specific to a given trait. If, for instance, transcription factors or mRNA molecules are present at very low copy numbers per cell, stochastic events during cell division that produce daughter cells with unequal molecular composition can be expected to cause cell-to-cell heterogeneity in downstream transcription and translation [153–155]. Likewise, the biochemical reactions underlying gene expression have an intrinsic stochastic element, namely, the probabilistic binding and unbinding of their players, for example, of transcription activators and repressors to their cognate promoters [156,157].

Such initial imbalances can then either be amplified in multiple ways, for instance, by positive feedback loops [5,27,46], pairs of mutually repressing repressors [5,27,46], transcription factor cascades [158] or, simply, strong ribosome binding sites [159,160]. However, they can also get quenched, for instance by uneven numbers of negative feedback loops [159,161].

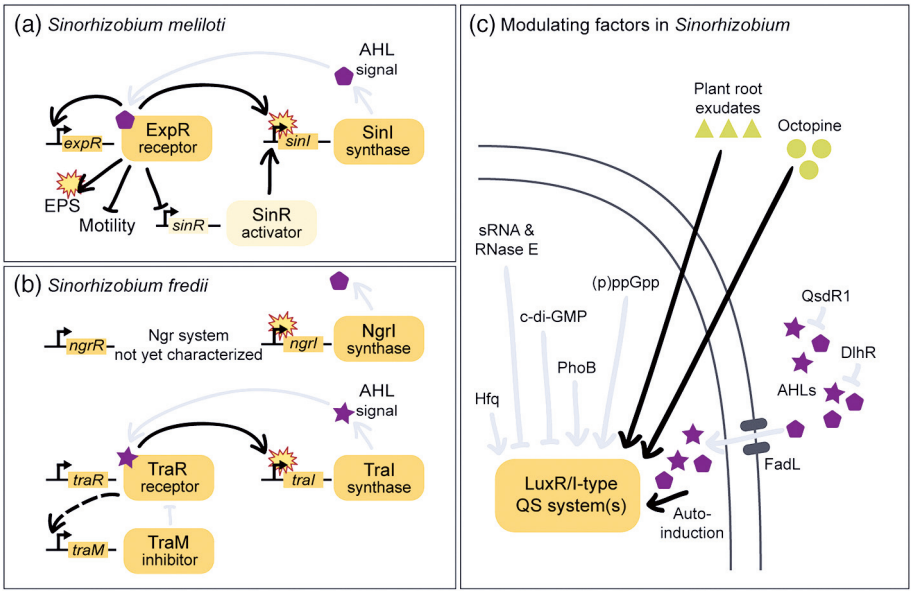


**Fig. 2.** Organization and modulation of quorum sensing systems in two *Sinorhizobium* species. (a, b) AHL synthesis and binding is depicted in gray, and transcriptional regulation is depicted in black. The dashed arrow indicates a regulation described for the homologous system in *A. tumefaciens*. Bright asterisks mark components that display phenotypic heterogeneity. (a) The quorum sensing regulatory network of *S. meliloti*. Expression of the AHL synthase gene *sinI* is strictly dependent on the LuxR-type regulator SinR [49]. SinI produces long-chain AHLs that are bound by another LuxR-type regulator, ExpR, and the ExpR–AHL complex then regulates transcription of the key components of the Sin system in diverse feedback mechanisms [49–51; 53–55]. About 30 more direct and several hundred indirect targets of ExpR–AHL have been identified [51,54,56–58]; most prominently, it affects exopolysaccharide (EPS) production and motility [49,51,52,59]. (b) The two quorum sensing systems of *S. fredii*. The Tra system is homologous to the TraR–TraI system involved in conjugation of the tumor-inducing (Ti) plasmid in *A. tumefaciens* [60,61]. TraI synthesizes 3-oxo-$C_8$-HSL, and TraR–AHL stimulates *traI* expression in a positive feedback loop [60]. The anti-activator TraM binds TraR with nanomolar affinity, thereby interfering with DNA binding of the TraR–AHL complex [62]. In *A. tumefaciens*, TraR–AHL also stimulates expression of *traM* [63]; it is therefore tempting to speculate that this feedback regulation may also occur in *S. fredii*. NgrI appears to synthesize small amounts of an AHL with an 8-carbon acyl chain [60] and a not yet characterized long-chain AHL [60,64]. Genome organization of *ngrR* and *ngrI* is reminiscent of the *sinR-sinI* organization in *S. meliloti* [61], but their regulation and the function of NgrR are mostly unknown. Both *S. fredii* quorum sensing systems negatively affect motility, but several hundred genes seem directly or indirectly regulated by one or the other autoinducer system [64]. Furthermore, there is evidence for crosstalk between and synergistic effects of the two systems [11,60,65]. (c) Factors modulating the quorum sensing systems in *S. meliloti* and/or *S. fredii*. Gray and black arrows indicate data from ensemble and single-cell studies, respectively.

Furthermore, for both species, a number of modulating factors have been described (Fig. 2c). For example, in the above-mentioned studies, the fraction of cells activating the autoinducer synthase promoter was not fixed, but increased considerably over time: In *S. meliloti* wild-type cells, AHL synthase gene expression was observed in only a minority of cells in young microcolonies. In larger microcolonies, on the other hand, fluorescence from the *sinI* promoter fusion was observed in almost every individual [12]. Likewise, in *S. fredii*, about one third of a population of wild-type cells showed fluorescence from P*traI::rfp* and P*ngrI::rfp* in late exponential phase, whereas fluorescing cells made up the majority of the population in stationary phase [11]. This effect could be due to cell density increasing over time and thus the quorum sensing response, as at least for the Sin and the Tra system a positive feedback of the autoinducer molecules on their own production has been described.

Alternatively, or additionally, it could be due to nutrient availability or the growth phase *per se*, since the metabolic state of the cell can also feed into the quorum sensing regulatory network: In *S. meliloti*, expression of *sinR*, the gene encoding the transcription activator crucial for expression of the AHL synthase gene, is stimulated in response to nitrogen downshifts in presence of the *relA* gene encoding a functional (p)ppGpp synthase [67]. Expression of both the transcription activator gene *sinR* and the AHL synthase gene itself is upregulated under phosphate starvation, an increase that has been linked to the phosphate starvation response regulator PhoB [50]. The lifestyle-switch second

messenger c-di-GMP, on the other hand, reduces expression of the synthase gene, and this inhibition is very likely most prominent during exponential growth as intracellular c-di-GMP concentrations in *S. meliloti* are 10- to 30-fold higher in exponential than in stationary growth phase [68]. Moreover, *sinI* mRNA can be bound by a small RNA and subsequently cleaved by RNaseE [69,70], and mRNA levels of the AHL receptor *expR* are modulated by the RNA binding protein Hfq [71].

If AHL-induced positive feedback does play a role in quorum sensing-related heterogeneity, one has to take into account not only the synthesis of the autoinducer molecules but also other processes that can affect autoinducer levels within the responding cells, namely their uptake from the environment and their degradation. For instance, in *S. meliloti*, the outer-membrane protein FadL, a homolog of an *Escherichia coli* fatty acid transporter, has been shown to facilitate uptake of long-chain AHLs and thus increase sensitivity to externally added autoinducers up to 100-fold. Expression of *fadL* itself was not regulated by the ExpR–AHL complex [72]; however, whether it is homogeneously or heterogeneously expressed has not been investigated. Potential cell-to-cell variations in FadL abundance would likely result in heterogeneous quorum sensing responses of the respective cells.

Regarding the stability of autoinducers, a number of enzymes have been described that specifically cleave autoinducer molecules [73,74]. Furthermore, it is very likely that an even larger number of promiscuous enzymes—mainly esterases and oxidoreductases—will also inactivate autoinducers with lower but nevertheless significant turnover rates; especially esterases are well known for their promiscuity [75]. In a recent study on *S. fredii*, five different enzymes were reported that degraded AHLs: Four functionally verified hydrolases and one oxidoreductase [76]. Two of the corresponding genes, *qsdR1* and *dlhR*, encode for lactonases specifically cleaving AHLs and have likewise been shown to be heterogeneously expressed [11]. Thus, it is possible that heterogeneous AHL degradation might generate autoinducer gradients on a microenvironmental scale, or cause variations in intracellular AHL levels, and that these differences are then further enhanced via the positive feedback of the receptor–AHL complex on the expression of the synthase gene, leading to an even higher AHL production in cells that experience above-threshold AHL levels, but not in cells that experience only minute concentrations. Preliminary data with a triple promoter-reporter gene construct carrying the promoters of the two autoinducer synthase genes (*traI* and *ngrI)* and the promoter of one of the quorum quenching lactonases (*qsdR1)* indeed suggest that expression of the quorum quenching gene might very well affect the level of phenotypic heterogeneity in expression of the AHL synthase genes (unpublished data, Duin and Streit).

Grote and colleagues [11] further demonstrated that the proportion of AHL producers in an *S. fredii* population can be influenced by other organisms, possibly by a sort of inter-kingdom signaling: In their study, almost all bacteria showed activation of the autoinducer synthase promoters when grown in proximity to roots of the model plant *Arabidopsis thaliana*. Similar results were obtained when either root exudates of a leguminous host of *S. fredii* were added to the medium, or 50 μM octopine, a compound produced by plants during infection with *Agrobacterium* to feed free-living bacteria in the rhizosphere. This effect might be mediated by the AHL receptor TraR, which appears to be limiting under laboratory conditions. It has been suggested that *S. fredii traR* expression might be induced in response to plant metabolites [60], analogous to the regulation of *traR* expression in *Agrobacterium tumefaciens* by conjugation-inducing opines released from the tumors of infected plants [63]. Here, interestingly, the same opines also induce expression of an AHL-degrading lactonase [63].

## Potential Functions of Quorum Sensing-Related Phenotypic Heterogeneity

Genome-wide studies on *E. coli* [3,6] have revealed that noise in gene expression varies considerably from gene to gene, and that the degree of noise strongly correlates with gene function: Stress response genes, for example, generally exhibit high levels of heterogeneity, whereas essential genes tend to show low levels of heterogeneity. This correlation, that has likewise been found in the budding yeast *Saccharomyces cerevisiae* [77], is generally interpreted as an indication that natural selection minimizes noise in gene expression whenever it would be harmful [78,79]. According to this rationale, in cases where gene expression has been determined as "noisy", this phenotypic heterogeneity should be at least neutral—that is, as good as a homogeneous expression—for the fitness of the genotype, and maybe even advantageous. What could these potential advantages be? In the following paragraphs, we discuss two established concepts and some variations thereof that we believe might also play a role, either alternatively or additionally. We illustrate them with quorum sensing settings; however, they might just as well apply to other contexts.

### Bet hedging and division of labor

The two potential functions usually proposed for phenotypic heterogeneity in clonal microbial populations are bet hedging and division of labor; both are defined as strategies that increase the fitness of the

genotype while bearing costs for individuals. The term bet hedging describes a risk-spreading strategy in inconstant environments: Sooner or later, conditions will change, but in what way is unforeseeable. The generation of offspring with divers phenotypes is therefore expected to increase chances that at least part of the population will thrive, and thus to increase chances of survival of the genotype [4,5,80]. The sacrifice in terms of individual fitness thereby lies in the presence—phenotypes that might be at an advantage once conditions have changed are probably not as competitive under present conditions—-whereas the potential benefits lie in the future. In the case of division of labor, on the contrary, the population in place more or less immediately benefits from the costs paid by individuals fulfilling a function of public interest. Labor can be divided between two or more different phenotypes, each profiting from the functions contributed by the others; however, it can also be asymmetric, with only one phenotype investing. In any case, a higher cost-efficiency on the population level is expected from the specialization involved [4,81–83]. These two potential functions are also the ones suggested so far with respect to quorum sensing-related phenotypic heterogeneity, as will be introduced with two specific examples from *B. subtilis* and more briefly with examples listed in Table 1.

### *Cell-to-cell signaling contributes to division of labor in B. subtilis*

In *B. subtilis*, pheromone signals are transduced via a classical two-component system (Fig. 3a, left), as reviewed in Ref. [84]. Binding of the ComX pheromone to the extracellular domain of its receptor, the membrane-bound histidine kinase ComP, induces the cytoplasmic domain of ComP to phosphorylate and thereby activate the response regulator ComA, a key transcription factor in *B. subtilis*. One of the direct targets of ComA is the *srfA* operon, which is responsible for production of surfactin, a biosurfactant that facilitates collective motility, displays antimicrobial activities and stimulates plant immunity [85,86]. All cells of the laboratory strain activate the *srfA* promoter in a cell-density-dependent manner [87]. Thus, regulation complies with the classical quorum sensing paradigm. However, as a result of domestication, laboratory strains have lost some of their social abilities. In two undomesticated strains, the *srfA* promoter is indeed activated only in a subpopulation of cells under biofilm-inducing conditions [88–90]. In the biofilm study by López *et al*. [88], *comQXP* was active in all cells and biofilm exudates activated ComA-dependent gene expression. Together this suggests that ComX pheromone is produced by all cells. In addition to signaling peptides, other cell-released factors, including secondary metabolites [88], potassium [91,92] and second messengers [93], affect the organization of biofilms. Some of them coordinate the behaviors of different subpopulations within the biofilm. Specifically, surfactin producers appear to "signal" to induce matrix producers via surfactin-induced potassium leakage, while not producing matrix themselves [88] (see Fig. 3a, right). The exact mechanism that separates matrix producers from surfactin producers remains to be uncovered.

However, it was demonstrated that differentiation into surfactin and matrix producers has clear advantages for colony expansion by facilitating flagella-independent sliding motility [89]: at the edge of an expanding colony, the two subpopulations are spatially separated (Fig. 3b). Filaments of matrix-producing cells align side-by-side and grow in so-called van Gogh bundles, presumably with the help of surfactin, which reduces the friction between cells and their substrate. Van Gogh bundles push themselves away from the colony, thereby facilitating colony migration. It was demonstrated that both extracellular matrix and surfactin are required for the formation of van Gogh bundles. Interestingly, a genetic division of labor by mixing two mutant strains unable to produce either surfactin or matrix in appropriate ratios allowed the biofilm colonies to expand even further than the wild-type strain. This provides further evidence that division of labor between surfactin and matrix-producing cells is beneficial for biofilm colony expansion.

### *Rap–Phr signaling contributes to bet hedging in fluctuating environments*

Another important class of quorum sensing systems in *B. subtilis* are the Rap–Phr systems, as reviewed in Ref. [35]. The Rap proteins inhibit activity of diverse response regulators either by complex formation or by dephosphorylation. Inhibition is released by binding of cognate Phr signaling peptides, which are produced by an export/import circuit. The RapA–PhrA system regulates the activity of the sporulation pathway in response to starvation stress (Fig. 3c). Clearly, Phr signaling does not globally synchronize the onset of sporulation, as sporulation is heterochronic [28,94–96]. In starving microcolonies, sporulation timing strongly correlates with expression of *rapA*: in early forming spores, *rapA* is inhibited, while cells delaying sporulation highly express it. Since cells keep dividing, a delay in sporulation serves to increase the quantity of spores. However, the capacity of the spores to revive, that is, their quality, decreases over time [30]. Therefore, the RapA–PhrA signaling pathway determines collective traits of the *B. subtilis* starvation response, including overall spore yield and the composition of the final spore

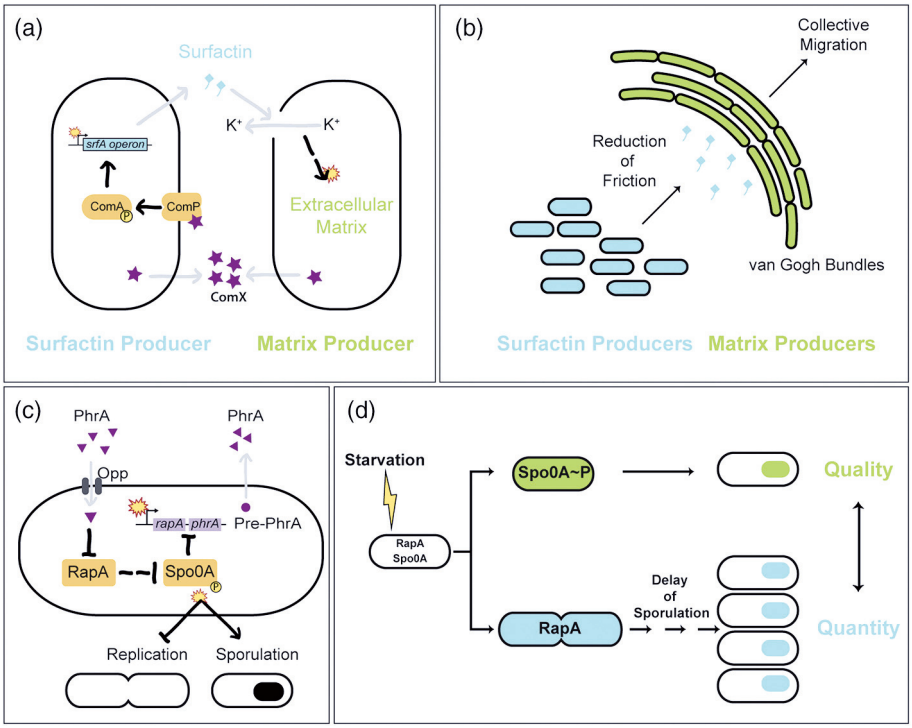


**Fig. 3.** Signaling coordinates complex population-level behavior in *B. subtilis*. Ovals denote cells or spores. Release/uptake of signals or exo-factors is depicted by gray lines, direct regulatory interactions by solid and indirect regulatory interactions by dashed black lines. Bright asterisks mark components that display phenotypic heterogeneity. (a) Cell-to-cell communication in biofilms. All cells produce the ComX pheromone. Left cell: ComX stimulates the ComP histidine kinase, which phosphorylates ComA. ComA~P binds to $P_{srfA}$ and activates production of surfactin. Right cell: Surfactin induces potassium leakage, which stimulates the production of extracellular matrix components via a complex signaling pathway (not shown). (b) Collective migration at the leading edge of a biofilm: Surfactin producers (cyan) provide the surfactant that enables matrix producers (green)—which grow in "van Gogh" bundles—to push the boundary of the colony forward. (c) PhrA–RapA signaling: The cell produces a pre-PhrA peptide (circle) that is cleaved into the PhrA signal (triangle). PhrA is pumped into the cytoplasm by the oligopeptide permease Opp and is sensed by the RapA receptor. RapA is a sporulation phosphatase that inhibits the accumulation of Spo0A~P. PhrA inhibits RapA. High levels of Spo0A~P induce sporulation and down-regulate the transcription of the highly regulated *rapA–phrA* operon. (d) In response to starvation stress, cells can adopt two strategies. Top: A cell that quickly accumulates Spo0A~P (green) sporulates and generates a high-quality spore. Bottom: A cell that expresses RapA (cyan) to high levels delays sporulation, continues to replicate and thus, ultimately generates more spores, albeit of lower quality.

population (Fig. 3d). Mutlu *et al*. [30] suggested that this might be a bet-hedging strategy to cope with uncertainty in the future environmental conditions, specifically with the amount of available nutrients. Sporulation strategies optimizing for spore yield are beneficial under nutrient-rich revival conditions, while high-quality spores with a high capacity to revive are favored in poor environments.

### *Further examples*

Bet hedging (Fig. 4a) was also proposed by both Garmyn and colleagues [8] and Pradhan and Chatterjee [10] as a potential explanation for the heterogeneity they observed in *Listeria monocytogenes, Pseudomonas syringae* and *Xanthomonas campestris* quorum sensing systems. This interpretation is immediately plausible with respect to quorum sensing-controlled traits or lifestyle switches, as, for example, flagellated cells and matrix-producing cells obviously gain different benefits under varying conditions, and Lowery and colleagues [97] indeed suggested that slow-growing, but stress-resistant biofilm subpopulations might serve as a precaution against environmental uncertainty. A selective advantage for an autoinducer-producing cell, on the contrary, is difficult to deduce as long as autoinducers are viewed solely as signaling molecules that take effect only after accumulating in the environment. However, chelating, antimicrobial and structuring properties have been described for several autoinducers *per se*, independent of a signaling role [98]. Besides, a few recent studies reported an immediate quorum sensing response within autoinducer-producing cells

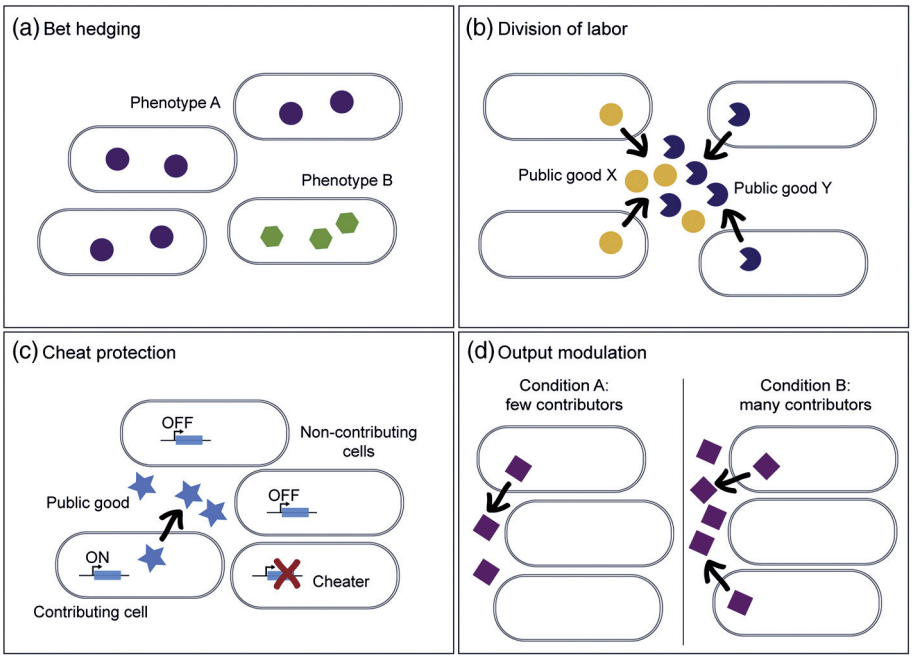


**Fig. 4.** Potential roles of phenotypic heterogeneity in quorum sensing-related gene expression. (a) Different phenotypes in a population might serve as a preparation for environmental fluctuations and thus represent bet hedging. (b) Alternatively, the contribution of functions of public interest only by subpopulations might represent a case of division of labor. (c) In an isogenic, but phenotypically heterogeneous population, non-contributing individuals should be able to directly compete with non-contributing mutants (cheaters) for reproductive success, for example, at the leading edge of growing colonies. However, in contrast to the latter, they would transmit the cooperative genotype to their offspring and might thus help to stabilize cooperation in the population. (d) Varying the number of cells contributing a certain public good might serve as an alternative or additional means to fine-tune the output of the population as a whole.

themselves, thus connecting heterogeneous autoinducer production with a likewise heterogeneous (and potentially advantageous) phenotype: Cárcamo-Oyarce and colleagues [18] proposed initially self-regulatory signals as an explanation for the heterogeneous—albeit autoinducer-regulated—expression of biosurfactants in *Pseudomonas putida* biofilms, ultimately causing the signal-producing cells to be the first to leave their microcolonies, and, possibly, the first to reach new resources. Likewise, such intracellular signaling or self-sensing has been reported for *Streptococcus mutans* affecting the alternative sigma factor controlling expression of late competence genes [22], and for *B. subtilis* affecting persistence to antibiotic treatment [48].

Division of labor (Fig. 4b) has, for example, been suggested for *V. harveyi*, where Anetzberger and colleagues [15] observed that more than half of the cells in a population showed a clear bias for either bioluminescence or exoprotease production, although both phenotypes are quorum sensing regulated. However, not only expression of quorum sensing target genes but also autoinducer production itself might be subject to division of labor: Keller and Surette [99] estimated the cost of one AgrD autoinducer oligopeptide in *S. aureus* to correspond to 184 ATP molecules, and that of one butyryl-homoserine lactone, a simple short-chain AHL produced by *Pseudomonas aeruginosa*, to eight ATP. Furthermore, Ruparell and colleagues [100] reported that production of AHLs can impose a significant fitness disadvantage and attributed this disadvantage to the drain AHL production imposes on the levels of the AHL precursor, S-adenosyl-L-methionine, which is the major methyl donor in bacteria, and whose availability significantly impacts on central metabolism. In this context, one could speculate that autoinducer production by only a subset of cells as observed in, for example, *S. meliloti* and *S. fredii*, might represent an asymmetric—or asynchronous—form of division of labor, as all cells in a population profit from the information conferred by the autoinducer molecules, while only the producers bear the costs.

## Further possible functions: cheat protection and output modulation

Such an asymmetric division of labor might even serve as a form of cheat protection: Evolutionary theory predicts that cooperation—like the quorum sensing-regulated production of public goods, or the production of autoinducers as the trigger for this cooperation—is subject to exploitation by non-

contributing mutants, so-called cheaters [81,101]. These cheaters benefit from the public goods produced by others without carrying any of the costs and thus have a fitness advantage over the contributors and eventually outcompete them—a situation that might ultimately lead to the elimination of the cooperative genotype from the population [101,102]. As cooperation in general and quorum sensing more specifically persist in nature, a number of solutions to this dilemma have been proposed: for example, the physical exclusion of cheaters by biofilm matrix [103,104], the limitation of the cooperative phenotype to situations when its impact on fitness is low [105], the activation of quorum sensing and quorum sensing-regulated traits only when the respective cells are surrounded by close relatives [106] or other contributors [107], the coupling of important private goods to the quorum sensing response [108,109], and even active "policing" strategies like the quorum sensing-regulated production of toxic substances coupled with likewise quorum sensing-regulated resistance or immunity mechanisms [110,111].

However, in structured environments like expanding colonies where growth and cell division primarily take place in a relatively small outer ring of cells and only few genotypes dominate the newly colonized area [112–115], phenotypic heterogeneity might represent yet another way of solving this dilemma, or at least contribute to its resolution (Fig. 4c): Here, competition is limited both in space and in time, and it is not the mean reproduction time of a genotype that matters, but the individual reproduction times of the cells located at the moving front—they determine whether or not the respective cells and their offspring are among the reproducing pioneers or whether they loose contact to the moving front and are then left behind. Should a non-contributing mutant arise at this front, it might outcompete equally contributing cooperators in phenotypically homogeneous populations, and it might just as well outcompete the individuals paying the costs for public goods production in phenotypically heterogeneous populations. However, the phenotypic non-contributors in such heterogeneous populations should grow just as fast as the cheaters and should thus be able to compete with them for predominance in the pioneering front. However, in contrast to cheaters, phenotypic non-contributors maintain the ability to switch to public good production, transmit the cooperative genotype to their offspring and might thus help to stabilize cooperation in the population.

Such an effect of phenotypic heterogeneity has already been proposed in the context of *Salmonella enterica* serovar Typhimurium infections of mice. Here, virulence is a phenotypically heterogeneous, cooperative trait, and the fraction of the isogenic population that expresses the virulence genes grows only slowly compared to the phenotypically avirulent subpopulation. During the infection process fast-growing avirulent mutants emerge, and the phenotypically avirulent subpopulation was reported to slow down the cheaters' rise in frequency [116]. In a *B. subtilis* strain, a reduction in the fraction of surfactin producers by about two thirds compared to closely related strains likewise rendered populations much less vulnerable to cheater invasion. Here, however, it remained unclear whether the observed effect was due to the increase in phenotypic non-contributors, or rather to the resulting overall reduction in surfactin production, which did not leave much to exploit [117].

Last, but not least, phenotypic heterogeneity might—simply—serve as a means to calibrate the output of the population as a whole (Fig. 4d): As discussed above, several recent studies showed that the fraction of cells contributing to autoinducer-related gene expression indeed varied depending on growth conditions [8,9,11,13,17,23,24]. Therefore, when adapting to environmental changes, certain bacteria might modulate the fraction of contributors in a population instead of—or in addition to—fine-tuning the production rate in all cells. Increasing the fraction of cells in a population engaging in autoinducer production, for example, under stress conditions, might thus trigger a quorum sensing response at lower cell numbers compared to those required under relaxed conditions. In order to fulfill this function, a heterogeneous autoinducer production would not necessarily have to be advantageous in terms of production costs, but merely neutral—the latter should be enough for it not to be selected against in the course of evolution. Furthermore, if applied in addition to the usual modes of gene expression regulation, it might pay off in another respect: The additional layer of control—the fraction of contributing cells that is variable—might enlarge the dynamic range of the output, and this, *per se*, might represent an advantage for the population and favor spreading of the genotype in the course of evolution.

## Conclusions

Twenty-five years after the introduction of the term "quorum sensing" [1] and more than 50 years after the first report on a "hormone-like cell product" as a new regulatory mechanism in bacteria [118], there is still much to explore about autoinducer-related processes. Not only are there still new autoinducer molecules, new regulatory components and new network designs discovered [32,33,35,87,119], there are also novel insights into the principles of the process: for example, the common model of quorum sensing implies that the autoinducer molecules first accumulate in the environment before triggering a response; however, a few recent studies have instead reported, or suggested, an immediate

response within autoinducer-producing cells themselves, a phenomenon the authors termed "self-regulatory" or "self-directed" [18], "intracellular signaling" [22] or "self-sensing" [48].

Likewise, the discovery of phenotypic heterogeneity, both in autoinducer production and in target gene activation, was unexpected. Here, the hitherto published accounts only provide a first glimpse of the situation and leave us with many open questions: How prevalent is phenotypic heterogeneity in quorum sensing systems? What are the molecular mechanisms behind this phenomenon? Are there general features distinguishing quorum sensing systems that show heterogeneity from those that do not, or are the mechanisms unique for each system? Is quorum sensing-related phenotypic heterogeneity always connected with a benefit, as suggested, for example, for the differentiating *B. subtilis* [30,89], or are there also cases where it is merely neutral? And, concerning the phenotypic heterogeneity observed in quorum sensing-controlled traits, can also "non-differentiating" bacteria show phenotypic specialization, and if so, to which degree? Where does heterogeneity end and differentiation begin?

Compared to these more recent developments, the influence of environmental factors on quorum sensing systems is already well established, and it has been proposed that it should be integrated into the model [37,120]. Thus, sticking with the analogy of the quorum, the numerous reports on environmental factors influencing autoinducer production and sensing would mean that this "minimum number of members of a group that are necessary to transact a business for this group" is not necessarily a fixed number. Instead, it seems that it can vary depending on growth conditions. Furthermore, determination of this "legal minimum" is only the beginning of decision-making and followed by actual voting. Here, the recent reports of phenotypic heterogeneity in autoinducer production suggest that decisions do not necessarily have to be reached in unison; instead, some of the voters might abstain from voting (the phenotypic non-contributors), or even cast dissenting votes (the cells engaging in quorum quenching). Finally, once the voting is over, some members might refuse to obey the decided orders, or might interpret them differently (i.e., not activate the specific target genes at all, or only a subset of them). How and why these complexities might be advantageous for individuals or clonal populations are questions that attract increasing attention.

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