J. Indian Inst. Sci.
A Multidisciplinary Reviews Journal
ISSN: 0970-4140 Coden-JIISAD

REVIEW ARTICLE

Physiological Advantage of Phenotypic Heterogeneity in a Quorum-Sensing Population

*E. Rajeshkannan and Supreet Saini**

Abstract | Quorum sensing, or the ability of a population, to respond to an environmental cue in a coordinated manner has made fundamental changes about how we understand bacterial physiology. In this framework, a population, once it exceeds a certain threshold in size, and in the appropriate environmental conditions, coordinates expression of genes across individual members of the population in a fashion so that all individual members are in sync. This ability allows the population to accomplish tasks (like searching for nutrients in the surrounding environment) that would be difficult to perform, if undertaken by a single cell. In this context, quorum sensing is a homogenizing force in a population. However, in recent years, a number of studies have reported that quorum sensing can also lead to a heterogeneous response in terms of gene expression across the population. A discussion of the strategies which explain this heterogeneity in the population at a single-cell resolution is the focus of this review.

1 Introduction: Coordinated Behavior of Bacterial Populations

Bacteria, when thought of with regards to their physiology and life cycle, are often viewed as single cells which make lifestyle decisions independent of their biological neighborhood. The physiological decisions made by this single cell are thought of as being driven solely towards the aim of increasing fitness of that particular individual bacterium. Therefore, bacteria were considered to live asocially with the sole of focus of maximizing chances of proliferation in a given environment.

However, numerous ecological isolates reveal that bacteria rarely reside as a single species[1–4]. Moreover, multiple species coexist and members from each are often interacting with each other. The most well studied niches in this context are intestines of mammals, biofilms on abiotic surfaces, or bacterial communities residing in soil. A study of physiology of bacteria residing in these niches suggests that bacteria residing here cooperate with each other, and this cooperation ensures maximization of fitness of the collective biomass residing in these environments, and not necessarily maximize the fitness of individual bacteria[5–7]. In the above cases, bacterial species cooperate with each and the decisions are taken for the advantage of the community.

Aside from interactions between multiple species, members of a single species too interact with each other. Consider a physiological task, like invading a host. From the perspective of a bacterium, the challenge of taking on the host immune system is unlikely to be met successfully while being undertaken by a single cell by itself[8]. On the other hand, the infection is more likely to be successful if the entire bacterial population "decides" to express virulence factors in a coordinated manner, and consequently overwhelm the host's immune system. Considr another example. In iron-poor environments, bacteria release a complex (siderophore) which tightly sequesters iron present in the environment and presents it to any cell it encounters. But this strategy of producing and releasing siderophores only makes sense if there are several bacteria of the same species. If the population density is too small, a siderophore

[1] *Department of Chemical Engineering, Indian Institute of Technology Bombay, Powai, Mumbai 400076, India.*
**saini@che.iitb.ac.in*

released is likely to diffuse away and not see a host to present its iron molecule to. In this context, production of siderophore only makes sense when cellular densities are high. Several other examples of coordinated behavior leading to enhaced fitness exist[9–11]. These examples highlight the significance of a collective response from bacterial populations and suggest that mechanisms which enable bacterial species to communicate within- and between species must exist. In this context, complexes like siderophores are called "public goods", since they are produced by an individual but contribute towards the fitness of the entire population[12].

The above example suggests that there are certain tasks undertaking which only makes sense when more than a certain number of cells commit to it. If undertaken by a smaller number of cells, the task is likely to result in a physiological failure. Hence, there need to be mechanisms which allow a population to coordinate response in a density-dependent manner, or respond only when their number exceeds a certain critical threshold (Fig. 1). This density-dependent response is referred to as Quorum Sensing (QS), since tasks controlled by this phenomenon are only undertaken if a necessary "quorum" has been met. Understanding this phenomenon has significantly enhanced our understanding of bacterial physiology in the last three decades. The exhibition of this density-dependent response implies that the bacteria must have mechanisms to "count" the number of individuals of their own and other species in their immediate neighborhood. Before we discuss the mechanistic details of the process of "counting" that bacteria employ, we discuss the historical origins of discovery and our understanding of quorum sensing.

Quorum sensing: Cellular response which is triggered in response to the population density of the species in the surrounding environment.

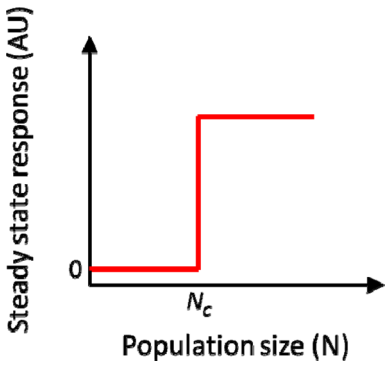

Figure 1: Characteristic response of a quorum-sensing controlled phenomenon. For population size less than a critical size *Nc*, the cellular steady state response is zero. That is, the system is present in the OFF state, for population size below *Nc*. However, as the population size increases beyond *Nc*, the steady state response changes to the ON state.

2 Discovery of Quorum Sensing

The process of quorum sensing was first discovered in the marine living bacterium, *Vibrio fischeri* over 40 years ago[13], although the term was first introduced in the literature in the 1990s[14]. *V. fischeri*, a marine bacterium, is found in the light organ of *Euprymna scolopes*, commonly known as the Hawaiian bobtail squid[15]. The symbiotic relationship between the squid and the bacterium is mediated through quorum sensing. *V. fisheri* produces bioluminescence and therefore, glows in the dark. However, this property is only exhibited when the bacteria are present at a density higher than a critical number. The bacteria were found to reside in the light organ of the squid, which is a nocturnal organism. The existence of this relationship (production of light and the bacteria residing in the squid) was found to be symbiotic because of the following.

The squid, being nocturnal, comes out in the water only at night for foraging. However, swimming near the water surface, it casts a shadow, thereby becoming susceptible to be detected by potential predators. Hence, counter-illumination of the body, using the bioluminescence of the bacteria, to reduce the sillhoute, is used a primary defence mechanism by squid[16]. Hence, luminescence from the bacterial population helps the squid mimic the light falling on its back, and therefore, avoid being detected by the predator. The bacteria on the other hand are provided a nutrient-rich environment in the light organ of the squid, where they flourish and increase in numbers. Upon daybreak, the squid flushes the bacteria out from the light organ, and buries itself in the sand. During this time, the bacteria remaining in the light organ grow and increase in numbers till the squid comes out the following night. This forms a symbiotic relationship between shallow water living squid with *V. fischeri* for the bioluminescence[17]. Here, the need for quorum sensing would be the collective production of bioluminescence proteins that give sufficient bioluminescence from the light organ of squid. Logically, linking physiological manifestations such as bioluminescence to collective decision-making makes sense. For instance, bioluminescence would make little sense if taken up by a single isolated cell in an ecological niche. We

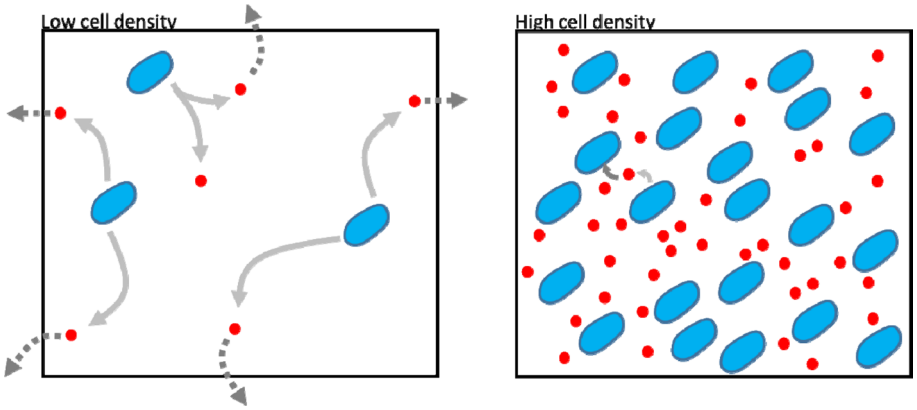

Figure 2: Quorum sensing via signaling molecules. All individuals in the population produce small molecules (called Auto Inducers, AI) into the media (red circles). When cell density is small (Left), these molecules are likely to diffuse away and not be sensed by other bacteria in the environment. However, at high densities (Right), the AI molecule released by one individual is likely to be received by another individual, than diffuse away.

next discuss the mechanistic details of the quorum sensing phenomenon.

3 Mechanistic Details of Quorum Sensing

To communicate presence to members of the same species, cells need to convey information into the environment. This information, then, needs to be received from members of the same species, and analyzed. This is accomplished via several steps in quorum sensing. The information regarding one's presence in the environment is put out in the environment via release of a small molecule. This molecule is synthesized by the cell, and is called the autoinducer (AI). Every member of the population synthesizes and secretes AI in the environment. When the population density is small, the AI molecules so released are more likely to diffuse away than be detected by an individual in the environment[18, 19] (Fig. 2). In this case, the information regarding presence of individuals from the same species (in the form of AI molecules) is not received by any individual in the environment. However, when the cell density is high (beyond a critical number), then the AI molecule is more likely to be received by another individual from the population than diffusing away undetected.

Upon receiving the AI molecule, the transcription of the genes associated with the appropriate cellular response (bioluminescence, nutrition scavenging) gets activated, and cells commit to the associated phenotype. In addition, reception of the freely-diffusing AI molecule also ensures that the cellular commitment to expression of the quorum sensing is frozen. This is done with the help of feedback loops in the QS network, which ensure that upon detection of AI from the environment, the AI production rate increases via this feedback loop. The positive feedback ensures that once committed to expression of QS-controlled genes, an individual cell is locked in the ON state with respect to expression of the appropriate QS-responsive genes.

The precise regulatory network that controls this commitment is as follows (Fig. 3). An enzyme (synthase) controls production of the AI molecule in the cell. The AI molecule diffuses out into the environment. At high cellular density, individuals receive the AI molecule (secreted from them or others in the population), which, upon entry into the cell, binds a regulator molecule, R. The regulator R, upon binding the AI molecule, changes into its activated form and controls transcription of its target genes. To ensure commitment towards QS protein expression, one of the targets of the activated regulator is the synthase enzyme[20].

At a molecular level, for example, In *V. fischeri*, the production of luciferase enzymes (which help exhibit bioluminescence) is regulated by quorum sensing[21]. The lux operon *luxICDABEG* contains the gene that encodes for AI synthase protein, LuxI. LuxI catalyzes the biosynthesis of a diffusible signaling molecule called acylated homoserine lactone (AHL) which is the Autoinducer in Lux QS system[22–24]. The AHL molecule, once synthesized is secreted out and diffuses in the medium.

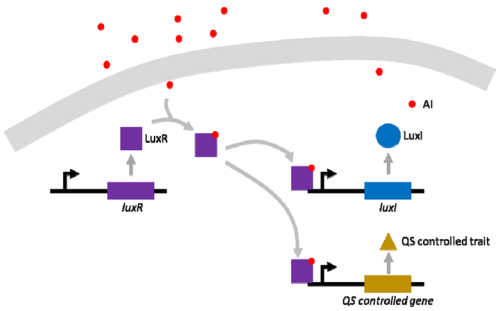

Figure 3: Regulatory network dictating quorum-sensing controlled traits in gram-negative bacteria. The AI molecule (red circle), when received by a cell, binds the LuxR transcription factor. LuxR, when bound to an AI molecule, becomes active and triggers transcription from its target promoters. One of the targets of the LuxR transcription factor is the Synthase (encoded by LuxI) which leads to enhanced production of the AI molecule, and hence, commits the cell and the population towards the quorum-sensing controlled trait.

The concentration of AHL molecules increases as the population density increases. At this high concentration, the AI molecules diffuse into the cells and bind with the regulator protein, LuxR which is encoded by the divergently transcribed operon *luxR* to that of *luxICDABEG*[25, 26]. The complex formed between the regulator and AI molecule, LuxR-AI, acts as a transcriptional factor for activation of genes under control of quorum sensing[27]. One of the targets of the LuxR-AI complex is LuxI, which in turn increases the concentration of AI molecule in the surrounding media[28]. This positive feedback facilitates transition from OFF to ON state[18, 29–31]. In gram-negative bacteria, homologues of LuxI/R are present in other QS systems. The AI molecule for a bacterial species is similar to AHL, and varies only in the length of the acyl carbon chain.

Unlike gram-negative bacteria, in gram-positive bacteria, the signalling molecule is a short peptide[32]. The secretion of the peptide is through a ATP-binding cassette (ABC). The extracellular peptide is then received by a membrane protein. On interaction with the peptide, the membrane protein phosphorylates a regulatory protein in the cell.

Thus, even though the functionary components are different in the gram-negative and gram-positive bacteria, the underlining logic dictating QS is the same.

4 How Quorum Sensing is Viewed as a Homogenizing Force

As we discussed in the earlier section, QS is a communication process which enables the population to undertake collective tasks. In this sense, QS is considered as the homogenizing force that regulates the synchronized OFF or ON state of entire population. Therefore, QS facilitates processing of information regarding the cell density of the population, thus, enabling a population to make a decision about expressing a particular phenotype. For example, *Pseudomonas syringae*, a gram-negative bacterium, colonizes a leaf in aggregated cell patches. It has been shown that QS among bacteria in these batches leads to their higher survival ability. The experimental study by Quiñones et.al., showed the concentration AHL molecules increases rapidly after the cell concentration reaches 10^9 (CFU/ml)[33]. However, confocal microscopic images of in vivo experiment from recent studies of the same system demonstrated cell-to-cell variation in the expression of extracellular polysaccharides[34, 35]. Similarly, several recent studies (discussed below) demonstrate that heterogeneity exists in the QS-dependent response of a bacterial population. These deviations from the homogeneity questions the basic assumption of quorum sensing acting as a homogenizing force in the population. In other words, it is not clear how much heterogeneity in the quorum-sensing responding populations can be tolerated.

5 Quorum Sensing and Heterogeneity

Quorum sensing is considered as a homogenizing phenomenon where every cell in the population behaves in unison. Indeed, the phenotype controlled by quorum sensing is only effective when the population reaches a certain threshold population size and/or density[9]. However, a number of recent experimental studies using single-cell analysis demonstrate heterogeneity in cellular behavior, in response to the QS signal[34–39]. For example, in the gram-negative bacterium, *Pseudomonas putida*, the production of putisolvin, a biosurfactant (which aids cellular movement), is regulated through PpuIR QS system. In response to appropriate environmental cues, the production of putisolving in the population, at a single-cell resolution, is heterogeneous. This heterogeneity clearly demonstrated that across a population, a fraction was committed to formation of a biofilm (those that did not produce the surfactant) and another which was committed to movement. However, at high cell-density, cell

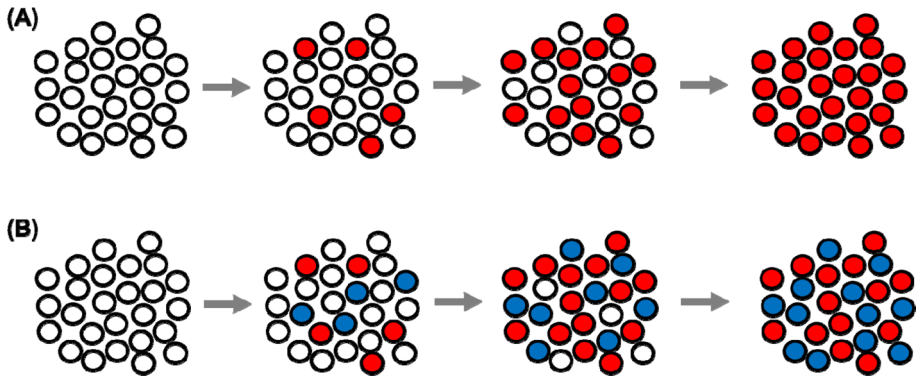

Figure 4: Heterogeneity in quorum-sensing response. **a** Transient heterogeneity. As a population transitions from the OFF (left) to the ON (right) state, cellular transition to ON takes place stochastically. As a result, at intermediate time points, only a fraction of the population is observed to be in the ON state. In this case, the cellular heterogeneity is only observed during the OFF to ON transition time. **b** Steady state heterogeneity. Cell transition from the OFF (left) to the ON (right) state, where a part of the population commits to one steady state (red) and another fraction commits to another steady state (blue). As a result, at steady state, there is heterogeneity in the phenotypes being expressed across the population.

behavior is synchronized and the entire population is committed to movement[38]. This example represents transient heterogeneity in a population, as cell transition from low AI to high AI concentration, in response to cellular density. This transient heterogeneity is observed in a number of biological systems, and is often associated with systems with positive feedbacks. The regulatory structure of QS in bacteria is also under the control of positive feedback. This aspect of QS has been extensively studied earlier[29, 40, 41] (Fig. 4a).

In a few cases, however, at the high population density, even when the system has been allowed sufficient time to attain a dynamic steady state, the phenotypic heterogeneity persists. The RpfFC QS system in *Xanthomonas camestrispv.campestris* (Xcc) regulates the production of endoglucanase, protease, biofilm formation and virulence. At high density, the fluorescence intensity of *gfp*-tagged cells shows a heterogeneous response at the single-cell resolution, even after the addition of excess signalling molecules externally[34]. Similarly, the Las QS system in *Pseudomonas aeruginosa* regulates the formation of biofilm and production of virulence factor displays heterogeneity at high population density[42]. The clinical isolates of *P. aeruginosa* from patients with cystic fibrosis developed heterogeneous biofilm formation and maintained the biofilm structure even after the 6th of inoculation[43] (Fig. 4b).

These observations raise questions regarding the presence of phenotypic heterogeneity in QS-responding population. For instance, what is the importance of heterogeneity in QS-responsive population, and what advantage does heterogeneneity in a QS-reponsive population confer to the individuals and/or population? And are the benefits to the individual and that to the population mutually exculsive? How, mechanistically, is the heterogeneity being achieved? Whether the observed heterogeneity is heritable? We study explanations for the existence of phenotypic heterogeneity in QS-mediated populations and possible mechanisms facilitating this exhibition.

6 Physiological Advantages of Heterogeneity in QS Environments

Phenotypic heterogeneity in an isogenic population is observed in a wide variety of bacteria and is known to confer advantage[44]. However, till recently, heterogeneity in a QS-responsive population was not observed. Recently, phenotypic heterogeneity has been demonstrated in quorum sensing responsive genes, such as the expression of the autoinducers of the system or its target genes[45]. We discuss the likely advantages that heterogeneity in QS environment could confer at a population level.

Phenotypic heterogeneity: Cells with identical genotype (DNA sequence) exhibiting different physical manifestations in an identical environment.

6.1 Bulk Response of Appropriate Strength may lead to Heterogeneous Response

Phenotypes such as bioluminescence and expression of virulence factors are quorum sensing controlled because of the need for bulk production of proteins that serve these functions. This aspect of a population response is best illustrated through

the following example of heterogeneity in the intensity of bioluminescence. Production in bulk of the QS-responsive product allows the presence of cells that underperform the task.

Vibrio fischeri has been the preferred model organism for studying quorum-sensing and cell–cell communication in bacteria[9]. Bioluminescence in *V. fischeri* is regulated by the Lux system, where, LuxI is the autoinducer synthase and LuxR is the regulator. Perez et al., carried out an experiment on *V. fischeri* isolated from the fish *Monocentris japonicus* and studied bioluminescence at single-cell resolution[36]. As part of the experimental design, AI molecules were supplied along with the media that flowed into a specially designed chamber for the study. The concentration of AI molecule was maintained constant through the course of the experiment, and images of the cells were taken for analysis of bioluminescence intensity at a single-cell resolution. Through this experimental layout, the authors demonstrates that, at a single-cell resolution, there is significant heterogeneity (ranging from 10 to 200 photons/min/cell) in the intensity of bioluminescence at higher concentration of autoinducer. The average intensity and the difference between the individual cell intensity increases as the concentration of AI increases from 50 to 1000 nM. Moreover, the time to reach half the maximum intensity shows wide spread in the distribution at higher concentration of AI. The increase in the median values of the intensity of bioluminescence measured over time for different external AI concentration shows the transient nature of the bioluminescence. These results reveal the heterogeneity in the intensity of bioluminescence for all AI concentrations at single-cell resolution[36].

This heterogeneity, in the face of quorum sensing, is not explained by the conventional notion of viewing QS as a homogenizing force. The cellular rationale behind the heterogeneity must be explained in terms of evolutionary advantages it confers to the population in its ecological niche. In the present case, the production of bioluminescence proteins is a collective task. Since the host only needs enough bioluminescence to attract the female, from its point of interest, a bulk production of bioluminescence suffices. Therefore, the internal heterogeneity in the population is likely of little interest to the host organism. Hence, in large populations, if luminescence produced by a fraction of the population suffices in terms of the host's requirements, it makes little evolutionary sense for the rest of the population to invest cellular resources on luminescence. This fraction, could conserve resources, and facilitate a higher growth rate. This could be one of the explanations for heterogeneity being selected for, in an isogenic population. Similarly, in the case of secretion of virulence factor, to be able to cause a successful infection, a critical amount of virulence factor that is sufficient to invade the host is needed. This indicates that there is no need for all the cells in a population to produce virulence factor, provided the threshold of virulence factor is met by a fraction of the population[46]. Such heterogeneity, even if it results in the smallest of fractions of the population freed from production of the public good (bioluminescence, in this case) would eventually result in large energetic savings for the individual and the population.

Cellular memory: Cells exhibiting a phenotype which is dictated by the past environment that the cell has experienced.

6.2 Division of Labour Enabled Through QS

One of the ways to create phenotypic heterogeneity in response to QS, and enhance cellular fitness is to have heterogeneous fractions in the population perform different but complmenting tasks. Such a strategy is known as division of labour. In this strategy, to perform a collective task, a population breaks the entire task into two or more components. Thereafter, the population so divides such that one part of the population perfroms one component of the task, while another performs the other. This strategy is observed in bacteria in many contexts, such as different nutrient utilization in *Bacillus subtilis*, infection process by *Salmonella enterica* in host cells and metabolic functions in cyanobacteria[44, 47, 48]. In cases where completion of the task is essential for survival and growth of the population, the different parts of the population cannot survive without each other. Since QS is the process in which the bacterial population density is the key cue, the heterogeneity in QS response can be used to divide the population to perform various functions. Here, we discuss a few examples that explain the fitness advantage of the bacterial population through the division of labour strategy using QS.

Vibrio harveyi, a close relative to *V. fischeri,* is a marine gram-negative bacterium which also regulates bioluminescence using quorum sensing. However, in *V. harveyi*, there are three signalling molecules that trigger three parallel signalling pathways. All three converge in dephosphorylating of LuxO. The qrr1-5 sRNA is the repressor of the transcription factor, LuxR and dephosphorylation of LuxO results in termination of qrr1-5 sRNA. LuxR positively or

negatively controls expression from multiple genes like those controlling bioluminescence, siderophores production and an extracellular protease. This regulatory design splits the population into two groups and allows the bacterial population to perform tasks collectively with one part performed by the fraction of cells in the QS OFF state and the other part by the fraction of population in the QS ON state. To this effect, analysis at a single-cell resolution carried out by Anetzberger et al., demonstrated heterogeneity in the intensity of bioluminescence in a wild-type population. The cells are differentiated based on the intensity of light and categorized as bright and dark cells. The dead cells are counted using fluorescence images with appropriate filters. The phenotypic heterogeneity is observed in this experimental study shows 25% of dark and 69% of the bright colony of *V.harveyi* in which dark colony is QS OFF state and the bright colony is QS ON state. The bright colony is the one which is in QS ON state and produce bioluminescence. However, on the addition of external AI-2 molecules increases the fraction of bright cells to about 83%. This shows that the AI concentration is maintained below saturation concentration. To find the difference between bright and dark colonies, a constitutive QS-active mutant is used. When compared to the mutant, wildtype shows more aggregated cells and analysis of aggregated cell colony reveals low bioluminescence. This shows that the dark colonies are engaged with the production of biofilm. Therefore, there are two subpopulations in which one of the subpopulations producing protein for bioluminescence and other producing extracellular protein for biofilm. This strategy can be considered as the division of labour to effectivity utilize the resources[49].

6.3 Quorum-Sensing Heterogeneity Facilitates Bet-Hedging Strategy

In a fluctuating environment, bacteria need to adapt rapidly in response to the environment to survive. One such survival strategy is bet-hedging, which is adopted from trade investments, where instead of putting all the resources (and risk) in one trade, investment of resources is spread over a number of avenues (thus, spreading risk). Similarly, in biology, a species with phenotypic heterogeneity in a population has the probability of surviving a fluctuating environment, since under given physical environment, there is a chance that some of the individuals (exhibiting a particular phenotype) are able to survive and reproduce. This is compared against a population where no heterogeneity is exhibited.

Exhibition of this phenotypic heterogeneity decreases the overall growth rate of the population in certain, favorable environments. Nevertheless, favorable conditions conducive for growth are likely only a laboratory artifact, and are unlikely to be present in ecological niches for any length of time. Hence, maintainence of phenotypic heterogeneity is likely an important property being selected for in ecological niches. Developmental choice between sporulation and competence in *B. subtilis* and persister cells which confer resistance to antibiotics are two well-known examples of bet-hedging strategy observed in the bacterial population[50–52]. A few examples where observed heterogeneity in quorum-sensing population is explained as a bet-hedging strategy are discussed below.

In an isogenic population, phenotypic heterogeneity may provide a bet-hedging strategy for the population in adverse environmental conditions. To reason out the presence of heterogeneity in QS bacterial population, Pradhan et al., carried out an experimental study on *Pseudomonas syringae pv.syringae* (Pss) wildtype and QS-deficient mutants. The single-cell analysis with FACS is carried out on the *gfp* (Green Fluorescence Protein)-tagged cells. Depends on the fluorescence intensity, two subpopulations named GFP^+ (bright) and GFP^- (dark) are classified. FACS analysis shows heterogeneity in Pss wildtype population. Moreover, the progeny of either of the colony also shows heterogeneity in the population with a similar fraction of dark and bright fraction of population, indicating that the heterogeneity is heritable. AhlI/R is the quorum-sensing system in Pss which positively regulates the production of extracellular polysaccharides and negatively regulates motility. The extra-cellular polysaccharide forms an external matrix which leads to biofilm formation. The experimental work carried out in the swarm plates to investigate the effect QS on motility shows GFP^+ dendrites and dark patches of cell colonies. Unlike in *V. harveyi* where the heterogeneity facilitates the division of labour, in Pss the heterogeneity provides different lifestyle such as planktonic and biofilm, for the bacteria. Thus the heterogeneous population which consists of two subpopulations with either motility or aggregation gives an evolutionary advantage during dynamic environmental conditions as a bet-hedging strategy[34].

A bet-hedging strategy has also been observed in *Dinoroseobacter shibae*, a gram-negative bacterium. Patzelt et al., carried out an experiment

with *D.shibae* wild-type and QS-mutant to study the effect of quorum sensing. In this work, a biosensor *E. coli* is used to monitor the production of AHL molecule. The cell count and the fluorescence are obtained using flow cytometry. The results from this work show the growth advantage of QS-mutant cells with minimum production of AHL molecule. Moreover, the authors demonstrate that two different modes of cell division are observed at high concentrations of AHL in the wildtype. The time-lapse microscopic images show cell division via both budding (where the buds bulging out from the parent) and binary fission (where the parent cell divided into two daughter cells). The images of the cell division process show there is a mix of unequal size division due to budding and almost equal division due to binary division. These results demonstrate a heterogeneous cell division in an isogenic wildtype population. Moreover, a QS mutant was able to divide only through binary fission. The QS mutant also exhibited a higher growth rate, when compared to wildtype. Even though the cost of QS reduces the growth rate, the presence of heterogeneity in cell division gives survival advantage for wildtype during fluctuating environment when size-selective pressure is imposed on the population[37].

6.4 A Subpopulation Provides Education About the Environmental Fluctuation

In the works discussed above, the definition of quorum sensing is consistent with sensing of AI concentration for a particular threshold, beyond which the system switches to the ON state. However, there are two scenarios through which quorum sensing can be manipulated. First, if the cells are in a closed environment and leakage of AI molecule to the external environment is minimum, then even with the low cell-density, AI molecule concentration will reach the threshold level[53]. Second, if the diffusivity of the medium is very high, then even at high population there may not be enough accumulation of AI concentration to trigger the QS ON state. These scenarios lead to two different manifestations of quorum sensing: Cell Density (CD) sensing and Diffusion (DF) sensing. However, both these scenarios could not explain the response observed in the experimental study by Chu et al. In this study, using microfluidics, the size of the 720 microchambers remains constant, however, the number of connecting tubes are varied which in turn varies the nutrient supplies and diffusivity among the chambers. In such a setting, response to QS was studied using a genetically engineered *E.coli* strain which carries the Lux QS system. Phase contrast and fluorescence images from the experimental geometry revealed that the QS response varies between the microchambers, despite them having the same cell density. The authors speculated if this manifestation could be because of the variation in the supply of nutrients or the diffusion of the AI molecules through the connecting tubes. However, the relation between the fluorescence (proxy of QS responsiveness) and mean growth rate exhibited a biphasic nature which suggested that availability of nutrients or diffusivity may not be the only factor that holds control over QS response. Moreover, the addition of exogenous AI which would be expected to induce homogenous QS behaviour had no effect on the biphasic nature. These results suggest that the interpretation of QS as CD and DF is not sufficient to explain this behaviour[39].

The authors argue that integration of quorum sensing with dilution rates (with are a proxy for growth rates and protein degradation) allows for a much more complicated processing of environmental signals than merely the threshold mechanism as thought by quorum sensing. More specifically, the integration of the QS network with cellular growth rates allowed the cells to respond to stimulus such as space, transient signals, and couple that with the population size and density. This, the authors argue, allows the population to respond better to a fluctuating environment.

7 Further Examples

In addition to the above examples, other evidence for heterogeneity in quorum-sensing systems exists. However, the physiological role in these manifestations remains unknown. For example, in *Streptococcus mutans*, ComX, which controls cellular commitment to competence, is quorum sensing regulated[54]. In addition, comCDE, which are responsible for production of antibacterial peptides, bacteriocins, are also regulated through quorum sensing. CSP (competence stimulating peptide) is an autoinducer for ComDE quorum-sensing system. In this system, the two feedback loops exist to each other. However, the exact mechanism(s) of this feedback are not known. An experimental and simulation study on *S. mutans* shows the heterogeneous phenotype of competence behaviour. In this study, the authors proposed two configurations for the activation of comX. First, the extracellular feedback loop

through which the XIP, autoinducer molecule for comRS QS system which regulates comX, is imported. To import the extracellular XIP, Opp, an oligopeptide permease, is needed. Thereafter, XIP binds ComR to form transcriptional factor for comX gene. XIP molecules are synthesized by ComS. The second mechanism proposed for activation of comX involved an intracellular feedback loop in which ComRS forms transcription factor for comX gene. In this work, XIP and CSP were supplied externally and the system exhibited heterogeneity only when CSP was supplied externally. The authors explain this manifestation via the intrinsic noise in ComS production[55].

The TraI/R and NgrL/R quorum-sensing system in *Sinorhizobium fredii* regulates about 186 genes that include flagellar biosynthesis genes and exopolysaccharide (EPS) biosynthesis[56]. To quantify the gene expression of *tra* and *ngr* genes, a promoter fusion with *rfp* (Red Fluorescence Protein) is used. The phase contrast and fluorescence images of the promoter fusion strain show heterogeneity in fluorescence intensity. However, the experimental studies show that the plant-derived molecule octopine drives to homogenous response to have a symbiotic relationship. Moreover, in addition to external AHL molecule also decrease the heterogeneity observed in *S. fredii* wildtype population. However, in both of the above examples, the evolutionary context in which these mechanisms could evolve and provide a fitness advantate are yet unknown[57].

8 Understanding Heterogeneity in QS Systems

A number of attempts have been made to quantitatively represent QS systems in bacteria[58–62] [reviewed in reference[62]]. However, these attempts have largely modeled the effects of QS as a homogenizing force in a population. How, given the mechanistic details of the regulatory interactions in and between cells, could quorum sensing lead to heterogeneity in a population has been largely ignored. The heterogeneity discussed in QS systems can be understood in two different contexts. The first, where a population with a QS system, depending on the biochemical parameters defining the interactions in the network exhibits heterogeneity. That is, at any given time, a fraction of the population is in QS ON state, while the remaining remain in the QS OFF state. This state between the two groups of populations is maintained in the given regime, such that the number of cells transitioning from OFF to ON is the same as the cells transitioning from ON to OFF. This dynamics equilibrium is maintained at the steady state of the system.

A number of bacterial species have more than one QS systems (see reference[63]). The multiplicity of the QS systems in a single cell could lead to a number of fallouts. First, the two (or more) QS systems could be used to encode hierarchical activation of QS-responsive systems. Second, the two QS systems could have antagonistic interactions, where activation of one leads to the other one switched OFF. Such a scenario could easily have so evolved such that in a given environment, a part of the population switches on one QS system, and another switches on the other QS system. Each system could activate target proteins which either encode a bet-hedging or a division of labor strategy as discussed earlier in the article[64].

Mechanistically, one of the important variables largely not looked at in QS studies is how does the space interact with the cells to lead to QS response. A recent work analyzed this in the context of multiple antagonistic QS systems in a cell, and demonstrated that given the precise geometry of the space and its interplay with diffusivity of the AI molecule, the most likely outcome of the QS controlled system was heterogeneous activation of the QS systems, and consequently the genes controlled by these QS systems. However, experimental manifestations of this phenomenon need to explored in further studies.

9 Conclusions

At a larger level, the question remains: why do cells exhibit heterogeneity (Fig. 5). Recent studies in cell–cell variability among individuals of an isogenic population indicate that phenotypic heterogeneity is a trait which is observed much more widely than thought previously. This evidence has forced us to review the context in which we view heterogeneity, and the underlying noise in cellular processes which leads to these manifestations. While a number of studies focus on analysis of noise from a variety of contexts: its relation with cell-cycle, expression levels, effects on fitness; there has been little emphasis on experimental design where enhanced noise in expression levels of a gene has been selected for[65, 66]. Till we design experiments which help us explore this aspect of cellular physiology, our understanding of noise and its implications in dictating fate of populations (and their evolutionary trajectories) remains poorly understood and merely empirical. Recent evidence suggests that phenotypic heterogeneity is

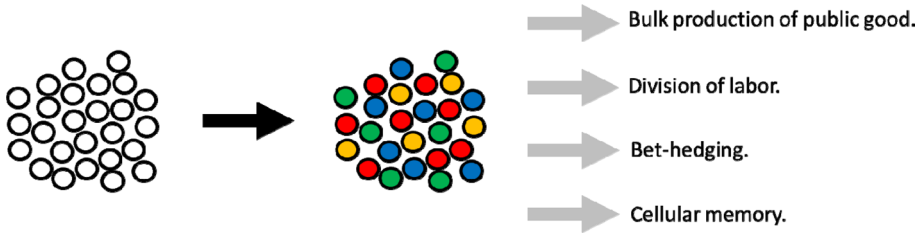

Figure 5: Heterogeneity in quorum-sensing population helps accomplish one/more of a number of cellular strategies.

ubiquituous in biological systems[67]. The underlying logic in all these cases seems to be an increase in phenotypic heterogeneity in the population. From a Darwinian context, this heterogeneity is important for driving forward all evolutionary processes. Heterogeneity in quorum sensing in bacteria is likely only one such manifestation of this process.

Publisher's Note

Received: 4 May 2020 Accepted: 8 May 2020
Published online: 26 May 2020

References

1. Roder HL, Olsen NMC, Whiteley M, Burmolle M (2019) Unravelling interspecies interactions across heterogeneities in complex biofilm communities. Environ Microbiol 22:5
2. Shang L et al (2018) Multi-species oral biofilm promotes reconstructed human gingiva epithelial barrier function. Sci Rep 8:16061
3. Lohse MB, Gulati M, Johnson AD, Nobile CJ (2018) Development and regulation of single- and multi-species *Candida albicans* biofilms. Nat Rev Microbiol 16:19–31
4. Elias S, Banin E (2012) Multi-species biofilms: living with friendly neighbors. FEMS Microbiol Rev 36:990–1004
5. Popat R et al (2012) Quorum-sensing and cheating in bacterial biofilms. Proc Biol Sci 279:4765–4771
6. Quigley EM (2013) Gut bacteria in health and disease. Gastroenterol Hepatol (NY) 9:560–569
7. Claessen D, Rozen DE, Kuipers OP, Sogaard-Andersen L, van Wezel GP (2014) Bacterial solutions to multicellularity: a tale of biofilms, filaments and fruiting bodies. Nat Rev Microbiol 12:115–124
8. Vendeville A, Winzer K, Heurlier K, Tang CM, Hardie KR (2005) Making 'sense' of metabolism: autoinducer-2, LuxS and pathogenic bacteria. Nat Rev Microbiol 3:383–396
9. Miller MB, Bassler BL (2001) Quorum sensing in bacteria. Annu Rev Microbiol 55:165–199
10. Taga ME, Bassler BL (2003) Chemical communication among bacteria. Proc Natl Acad Sci USA 100(2):14549–14554
11. Dandekar AA, Chugani S, Greenberg EP (2012) Bacterial quorum sensing and metabolic incentives to cooperate. Science 338:264–266
12. Schuster M, Sexton DJ, Hense BA (2017) Why quorum sensing controls private goods. Front Microbiol 8:885
13. Nealson KH, Platt T, Hastings JW (1970) Cellular control of the synthesis and activity of the bacterial luminescent system. J Bacteriol 104:313–322
14. Fuqua WC, Winans SC, Greenberg EP (1994) Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. J Bacteriol 176(2):269–275
15. Wei SL, Young RE (1989) Development of symbiotic bacterial bioluminescence in a nearshore cephalopod Euprymna scolopes. Marine Biol 103:541–546
16. McFall-Ngai MJ (1990) Crypsis in the pelagic environment. Am Zool 30:175–188
17. Lee KH, Ruby EG (1994) Effect of the squid host on the abundance and distribution of symbiotic *Vibrio fischeri* in nature. Appl Environ Microbiol 60:1565–1571
18. Papenfort K, Bassler BL (2016) Quorum sensing signal-response systems in Gram-negative bacteria. Nat Rev Microbiol 14:576–588
19. Mukherjee S, Bassler BL (2019) Bacterial quorum sensing in complex and dynamically changing environments. Nat Rev Microbiol 17:371–382
20. Rai N et al (2012) Prediction by promoter logic in bacterial quorum sensing. PLoS Comput Biol 8:e1002361
21. Schaefer AL, Hanzelka BL, Eberhard A, Greenberg EP (1996) Quorum sensing in *Vibrio fischeri*: probing autoinducer-LuxR interactions with autoinducer analogs. J Bacteriol 178:2897–2901
22. Eberhard A (1972) Inhibition and activation of bacterial luciferase synthesis. J Bacteriol 109:1101–1105
23. Eberhard A et al (1981) Structural identification of autoinducer of *Photobacterium fischeri* luciferase. Biochemistry 20:2444–2449
24. Friedrich WF, Greenberg EP (1983) Glucose repression of luminescence and luciferase in *Vibrio fischeri*. Arch Microbiol 134:87–91

25. Stevens AM, Greenberg EP (1997) Quorum sensing in *Vibrio fischeri*: essential elements for activation of the luminescence genes. J Bacteriol 179:557–562
26. Engebrecht J, Nealson K, Silverman M (1983) Bacterial bioluminescence: isolation and genetic analysis of functions from *Vibrio fischeri*. Cell 32:773–781
27. Sitnikov DM, Schineller JB, Baldwin TO (1995) Transcriptional regulation of bioluminesence genes from *Vibrio fischeri*. Mol Microbiol 17:801–812
28. Fuqua WC, Winans SC, Greenberg EP (1994) Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. J Bacteriol 176:269–275
29. Shadel GS, Baldwin TO (1992) Positive autoregulation of the *Vibrio fischeri* luxR gene. LuxR and autoinducer activate cAMP-catabolite gene activator protein complex-independent and -dependent luxR transcription. J Biol Chem 267:7696–7702
30. Dunlap PV, Kuo A (1992) Cell density-dependent modulation of the *Vibrio fischeri* luminescence system in the absence of autoinducer and LuxR protein. J Bacteriol 174:2440–2448
31. Shadel GS, Baldwin TO (1991) The *Vibrio fischeri* LuxR protein is capable of bidirectional stimulation of transcription and both positive and negative regulation of the luxR gene. J Bacteriol 173:568–574
32. Banerjee G, Ray AK (2017) Quorum-sensing network-associated gene regulation in Gram-positive bacteria. Acta Microbiol Immunol Hung 64:439–453
33. Quinones B, Pujol CJ, Lindow SE (2004) Regulation of AHL production and its contribution to epiphytic fitness in *Pseudomonas syringae*. Mol Plant Microbe Interact 17:521–531
34. Pradhan BB, Chatterjee S (2014) Reversible non-genetic phenotypic heterogeneity in bacterial quorum sensing. Mol Microbiol 92:557–569
35. Samal B, Chatterjee S (2019) New insight into bacterial social communication in natural host: evidence for interplay of heterogeneous and unison quorum response. PLoS Genet 15:e1008395
36. Perez PD, Hagen SJ (2010) Heterogeneous response to a quorum-sensing signal in the luminescence of individual *Vibrio fischeri*. PLoS ONE 5:e15473
37. Patzelt D et al (2013) You are what you talk: quorum sensing induces individual morphologies and cell division modes in *Dinoroseobacter shibae*. ISME J 7:2274–2286
38. Carcamo-Oyarce G, Lumjiaktase P, Kummerli R, Eberl L (2015) Quorum sensing triggers the stochastic escape of individual cells from *Pseudomonas putida* biofilms. Nat Commun 6:5945
39. Chu EK, Groisman A, Levchenko A (2019) Environmental sensing in dynamic quorum responses. BioRxiv 2019:745091
40. Haseltine EL, Arnold FH (2008) Implications of rewiring bacterial quorum sensing. Appl Environ Microbiol 74:437–445
41. Williams JW, Cui X, Levchenko A, Stevens AM (2008) Robust and sensitive control of a quorum-sensing circuit by two interlocked feedback loops. Mol Syst Biol 4:234
42. Whiteley M, Lee KM, Greenberg EP (1999) Identification of genes controlled by quorum sensing in *Pseudomonas aeruginosa*. Proc Natl Acad Sci USA 96:13904–13909
43. Lee B et al (2005) Heterogeneity of biofilms formed by nonmucoid *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. J Clin Microbiol 43:5247–5255
44. Ackermann M et al (2008) Self-destructive cooperation mediated by phenotypic noise. Nature 454:987–990
45. Grote J, Krysciak D, Streit WR (2015) Phenotypic heterogeneity, a phenomenon that may explain why quorum sensing does not always result in truly homogenous cell behavior. Appl Environ Microbiol 81:5280–5289
46. Weigel WA, Dersch P (2018) Phenotypic heterogeneity: a bacterial virulence strategy. Microbes Infect 20:570–577
47. Adams DG (2000) Heterocyst formation in cyanobacteria. Curr Opin Microbiol 3:618–624
48. Veening JW et al (2008) Transient heterogeneity in extracellular protease production by *Bacillus subtilis*. Mol Syst Biol 4:184
49. Anetzberger C, Pirch T, Jung K (2009) Heterogeneity in quorum sensing-regulated bioluminescence of *Vibrio harveyi*. Mol Microbiol 73:267–277
50. Hutchison EA, Miller DA, Angert ER (2014) Sporulation in bacteria: beyond the standard model. Microbiol Spectr 2014:2
51. Dubnau D (1991) Genetic competence in *Bacillus subtilis*. Microbiol Rev 55:395–424
52. Lewis K (2010) Persister cells. Annu Rev Microbiol 64:357–372
53. Boedicker JQ, Vincent ME, Ismagilov RF (2009) Microfluidic confinement of single cells of bacteria in small volumes initiates high-density behavior of quorum sensing and growth and reveals its variability. Angew Chem Int Ed Engl 48:5908–5911
54. Shanker E, Federle MJ (2017) Quorum sensing regulation of competence and Bacteriocins in *Streptococcus pneumoniae* and mutans. Genes (Basel) 2017:8
55. Hagen SJ, Son M (2017) Origins of heterogeneity in *Streptococcus mutans* competence: interpreting an environment-sensitive signaling pathway. Phys Biol 14:015001
56. Krysciak D et al (2014) RNA sequencing analysis of the broad-host-range strain Sinorhizobium fredii NGR234 identifies a large set of genes linked to quorum sensing-dependent regulation in the background of a traI and ngrI deletion mutant. Appl Environ Microbiol 80:5655–5671
57. Grote J et al (2014) Evidence of autoinducer-dependent and -independent heterogeneous gene expression in

Sinorhizobium fredii NGR234. Appl Environ Microbiol 80:5572–5582
58. Torres-Cerna CE, Morales JA, Hernandez-Vargas EA (2019) Modeling quorum sensing dynamics and interference on *Escherichia coli*. Front Microbiol 10:1835
59. Ueda H, Stephens K, Trivisa K, Bentley WE (2019) bacteria floc, but do they flock? Insights from population interaction models of quorum sensing. MBio 2019:10
60. Gilbert D, Heiner M, Ghanbar L, Chodak J (2019) Spatial quorum sensing modelling using coloured hybrid Petri nets and simulative model checking. BMC Bioinform 20:173
61. Zhao K et al (2019) Behavioral heterogeneity in quorum sensing can stabilize social cooperation in microbial populations. BMC Biol 17:20
62. Perez-Velazquez J, Golgeli M, Garcia-Contreras R (2016) Mathematical modelling of bacterial quorum sensing: a review. Bull Math Biol 78:1585–1639
63. Mattiuzzo M et al (2011) The plant pathogen *Pseudomonas fuscovaginae* contains two conserved quorum sensing systems involved in virulence and negatively regulated by RsaL and the novel regulator RsaM. Environ Microbiol 13:145–162
64. Prajapat MK, Saini S (2018) Logic of two antagonizing intra-species quorum sensing systems in bacteria. Biosystems 165:88–98
65. Beaumont HJ, Gallie J, Kost C, Ferguson GC, Rainey PB (2009) Experimental evolution of bet hedging. Nature 462:90–93
66. Van den Bergh B, Michiels JE, Michiels J (2016) Experimental evolution of *Escherichia coli* persister levels using cyclic antibiotic treatments. Methods Mol Biol 1333:131–143
67. Tawfik DS (2010) Messy biology and the origins of evolutionary innovations. Nat Chem Biol 6:692–696

E. Rajeshkannan is a PhD student in the Department of Chemical Engineering at IIT Bombay. His research includes theoretical analysis of regulatory networks.

Supreet Saini is an Associate Professor in the Department of Chemical Engineering at IIT Bombay. His group works in understanding speciation events and evolutionary processes.