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Perspective

Quorum sensing going wild

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SUMMARY

The first discovered and well-characterized bacterial quorum sensing (QS) system belongs to *Vibrio fischeri*, which uses *N*-acyl homo-serine lactones (AHLs) for cell-cell signaling. AHL QS cell-cell communication is often regarded as a cell density–dependent regulatory switch. Since the discovery of QS, it has been known that AHL concentration (which correlates imperfectly with cell density) is not necessarily the only QS trigger. Additionally, not all cells respond to a QS signal. Bacteria could, via QS, exhibit phenotypic heterogeneity, resulting in sub-populations with unique phenotypes. It is time to ascribe greater importance to QS-dependent phenotypic heterogeneity, and its potential purpose *in natura*, with emphasis on the division of labor, specialization, and "bet-hedging". We hope that this perspective article will stimulate the awareness that QS can be more than just a cell-density switch. This basic mechanism could result in "bacterial civilizations", thus forcing us to reconsider the way bacterial communities are envisioned *in natura*.

INTRODUCTION

Canonical bacterial cell-cell signaling systems consist of a "signaling" and "sensing" module, where the first produces a chemical signal, and the second interacts with it. Many species undergo cell-cell communication, each with its own variety of cell-cell signals that regulate several adaptive phenotypes.[1] Cell-cell signaling systems are usually referred to by using the broad and well-established term "Quorum Sensing" (QS).[2,3] In this perspective article, we will discuss the common proteobacterial LuxI-family N-acyl homoserine lactone (AHL) synthases and the LuxR-family AHL-dependent transcriptional regulators[2] as a model example of "signaling" and "sensing", respectively. However, we think that the insights provided here can be generalized and applied to bacteria that possess other types of QS mechanisms.

AHLs interact directly with the LuxR-family transcriptional regulators located in the cytoplasm. The LuxR-AHL complexes then bind to specific DNA motifs upstream of target genes and modulate gene transcription. The AHL synthase gene is usually one of the targets, thus generating a positive feedback loop.[1,4] The purpose of QS is usually considered to be cell density sensing (which correlates imperfectly with AHL concentration); at "quorum" (threshold of cell density) target gene expression is modulated. A "quorum", however, is often only one of the many conditions that must be met to trigger QS-regulated phenotypes. Supra-regulation of QS has been already reported at the discovery of the first QS in *Vibrio fischeri*,[5] where the QS response was shown to be dependent upon catabolite repression and cyclic adenosine monophosphate (cAMP).[6–9] Supra-regulation is also present in many other AHL QS systems.[3,10–14]; for example, the very well-studied AHL QS response of the plant pathogen *Agrobacterium tumefaciens* is strongly influenced by the plant.[15] Similarly, the two extensively studied hierarchically organized AHL QS systems of *Pseudomonas aeruginosa* are part of a complex regulatory network involving many other regulators (e.g., RpoS, RpoN, RsaL, …) which affect their expression.[13,16] QS systems of other bacteria like *Vibrio harveyi, Ralstonia solanacearum*, and *Rhizobium leguminosarum,* have also been evidenced to be under the control of other regulators, small RNAs and environmental cues.[11,12,17–19] Cyclic-di-guanosine monophosphate (cyclic-di-GMP) has been shown to play a role in triggering the QS response in *Sinorhizobium meliloti*[20,21] and *P. aeruginosa*.[22] Some AHL QS systems feature an intergenic element between the *luxI* and *luxR* genes that most often negatively and stringently affects the quantity of produced AHLs via regulation of the *luxI* family AHL synthase.[23] The production of signaling molecules is therefore not always turned on by default, as one could assume if considering QS as a mere cell-density switch. *Pseudomonas fuscovaginae* is one such example, where both of its AHL QS systems, while functional, do not produce AHLs in laboratory conditions.[24] Additionally, even when a QS signal is present, bacteria can often modulate the response. For example, Smith and Schuster[25] described an anti-activator system in *P. aeruginosa*, which dampens the cellular response to the signal and with it prevents self-activation. Elucidating the biological role of this supra-regulation of AHL QS systems and how it affects the cell-cell communication response of a bacterial community therefore still represents a major future challenge.

THE ARGUMENT FOR INHERENT PHENOTYPIC HETEROGENEITY OF QS REGULATION

Bacterial populations, where only a subset of clonal cells exhibits a biologically meaningful trait are considered to be phenotypically heterogeneous.[26,27] If the entire population exhibits the trait, it will be considered phenotypically homogeneous. We consider a trait to be biologically meaningful when it significantly increases the fitness of a species under the selective pressure of its natural habitat (*in natura*). Phenotypic

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heterogeneity might arise in bacteria due to stochastic processes[28] or due to active cellular mechanisms which makes it an evolvable trait.[29] Here we will focus on phenotypic heterogeneity that arises due to QS. Furthermore, we will be presenting an argument for phenotypic heterogeneity as an inherent property of QS regulation *in natura*.

Youk and Lim[30] have observed that when autoinducing systems feature a positive feedback loop mechanism, like the one present in the majority of AHL QS systems,[4] a homogeneous QS response will be observed. This would suggest that QS, in specific circumstances, promotes phenotypic homogeneity (e.g., in a laboratory setting where the signal is rather stable). However, in the same article,[30] when an active mechanism that degrades the signal was introduced, the auto-induced response exhibited phenotypic heterogeneity. QS signals *in natura* are subject to many factors affecting their stability and presence,[31] as opposed to a laboratory environment. Additionally, many bacteria feature mechanisms with which QS signals are actively degraded.[32] Therefore, we speculate, that if an autoinducing signaling mechanism with a positive feedback loop evolved *in natura*, where signal stability is most likely low,[31] it evolved to promote phenotypic heterogeneity, because it is a biologically meaningful trait.

By taking all the above into account, we argue, that QS regulation will inherently result in phenotypic heterogeneity *in natura*. This aspect of QS should therefore be considered, and we propose that phenotypic heterogeneity represents the "default" state of QS regulation. In most environments where signal stability is low and QS is under additional supra-regulation, this is likely to serve as a bacterial differentiation mechanism. Phenotypic homogeneity, on the other hand, occurs in bacteria, in which their QS system evolved to minimize its inherent phenotypic heterogeneity.

CHRONOLOGICAL CONSIDERATIONS OF QS RESEARCH

The LuxI/R system of *V. fischeri* was the first AHL QS system to be discovered[33] and therefore one of the best characterized. This symbiotic bacterium can be found within the bobtail squid's light organ.[34] The squid recruits and grows the bacterium[35]; AHL levels increase with bacterial cell density, and at "quorum" bacteria will switch on bioluminescence.[36] The term QS is now used for bacterial AHL signaling and for cell-to-cell communication in general,[2,3] although additional conditions other than the "quorum" must also be met in *V. fischeri* for luminescence induction.[5–9] This is most commonly not emphasized in favor of simplified explanations that mostly consider cell density as the predominant factor in bacterial QS regulation. The additional regulatory controls, however, have strong implications for the biological significance of QS. The additional trigger conditions are met in the squid's light organ or in the laboratory environment, but it might not always be so *in natura*. QS might therefore have other modes of function on top of being a cell-density switch (e.g., phenotypic heterogeneity, oscillative QS response patterns, or a combination of both). QS regulation is shaped by selective pressure that is present in the specific environment bacteria inhabits (e.g., soil) which is often significantly different than the ones that are present in the bobtail squid's light organ.

We speculate, that natural selection most probably maximizes luciferase production by *V. fischeri* as experiments by Bose et al.[37] support. The squid purges 95% of its symbiotic population on a daily basis,[34] so we assume that the selective pressure exerted on the bacterium is strong. QS regulation can be greatly affected by selecting for phenotypes under QS regulation,[38] and the squid's light organ could be doing just that. Squid with light organs that fail to properly select for luciferase-producing *V. fischeri* could struggle surviving and passing on their genes due to their dim light organ. Therefore, we speculate that the squid's light organ has evolved to "domesticate" the bacterium, and any QS heterogenic response that might arise is probably minimized in order to maximize luminescence. Therefore, we presume, that if the AHL QS system of *V. fischeri* synchronizes luminescence in its populations toward homogeneity, it is because of the environment the bacterium finds itself in. Controversially, even if the average QS response of *V. fischeri* appears to approach homogeneity, recent research has indicated that in an artificial setting, *V. fischeri* will still exhibit a certain degree of phenotypic heterogeneity[39,40] We argue, that this is so, because *V. fischeri's* QS system is actually inherently heterogenic; the squid's light organ merely minimizes this heterogeneity (possibly by selecting the strain and manipulating the growth conditions in its light organ). This would further support the idea that QS regulated phenotypes are inherently heterogeneous. Therefore, if the speculation we provide above turns out to be true, a QS-driven phenotypically homogeneous response should not be considered an inherent property of QS systems; phenotypic heterogeneity, on the other hand should be. We argue that a homogenized QS response (to various degrees) should be considered as one of the extreme possible outcomes of inherently heterogeneous QS regulation. Consequently, other bacteria, that possess a QS system, might not necessarily use it to homogenize a populational response, because they find themselves occupying a different environment, with different selective pressures compared to the squid's light organ.

The selective pressure, applied by the squid to *V. fischeri* inhabiting its light organ is similar to the selective pressure applied by humans to other bacteria when exploited for industrial purposes. In this case, it is wanted that most bacteria exhibit the desired uniform community phenotype at a maximum level.[41] An ideally uniform and homogeneous bacterial population is a highly desired scenario in such settings. Another well-studied AHL QS system is the one of *P. aeruginosa*, which is mostly considered in the context of human pathogenicity. The habitat of *P. aeruginosa* clinical isolates, most commonly the lung of a patient with cystic fibrosis,[42] is significantly different from other environmental conditions where one might find similar strains of the same genus and with similar QS systems. Additionally, Smith and Schuster[25] have recently shown that *P. aeruginosa* possesses an active mechanism that synchronizes QS-regulated phenotypes by preventing self-sensing. Maybe such an active mechanism evolved because in CF lungs a phenotypically homogeneous QS response is more optimal than a heterogeneous one. However, while a homogeneous response could be advantageous during pathogenesis, it might not be so in a different environmental setting where wild type strains use the QS system for a different purpose.

QS-REGULATED PHENOTYPES *IN NATURA*

The scientific community is aware of AHL QS phenotypic heterogeneity, as several research articles and other endeavors employing single cell techniques have evidenced, and even more so because sometimes QS heterogeneity is apparent even when strains are grown in a classic laboratory setting.[26,27,29] However, we think that there is still a strong general tendency to study QS in the context of homogeneity via a cell-density-dependent response in rather stable environments and/or in bacteria that colonize specific environments as described above for *V. fischeri* and *P. aeruginosa*. This might also be due to the methodology traditionally used when studying the QS response. Very often target gene expression is quantified using laboratory techniques that yield an estimate for the expression of the entire population, which is then normalized per cell number. Such methods can often fail to detect small groups of individual cells with higher-than-average gene expression. Additionally, when determining QS regulons, an approach that compares differential transcription of QS "active" and "inactive" (e.g., mutants in QS genes) cells is most often employed. To determine the QS regulon a cut-off fold value in expression will be chosen. Phenotypically heterogeneous QS responses will more likely fall below this threshold and remain undetected or disregarded as artifacts, unlike a phenotypically homogeneous response. Nevertheless, phenotypic heterogeneity is sometimes considered, acknowledged, and given importance by hypothesizing its biological significance. For example, observed phenotypic heterogeneity was hypothesized to play a role in "bet hedging" in *Listeria monocytogenes*,[43] *Pseudomonas syringae*[44] and *Xanthomonas campestris*.[44] Observed phenotypic heterogeneity in *V. harveyi*[45,46] *and Bacillus subtilis*[47] was postulated to play a role in division of labor. Bettenworth et al. discussed the above examples of phenotypic heterogeneity in length.[27] Collective decision making was hypothesized to explain observed heterogeneities in *B. subtilis* and *S. meliloti*[20,48] and the latter function of phenotypic heterogeneity was further corroborated by mathematical modeling.[49] One could, most likely, find even more examples in the scientific literature, where observed phenotypic heterogeneity was properly addressed. We, nevertheless, propose that an even more dramatic shift away from the homogenized QS response dogma is warranted. This is mostly because, as already presented before, many wild type bacterial isolates are not necessarily under selective pressures that shaped them to exhibit increasingly phenotypically homogeneous responses; therefore, a synchronized and phenotypically homogeneous response should not be considered a default feature of QS. We therefore speculate that most wild type strains, unless "domesticated", will probably tend to exhibit a higher degree of QS-driven phenotypic heterogeneity. We also argue that the importance of QS-driven phenotypic heterogeneity and other unusual QS configurations have been overlooked and need to be given more attention while also considering their appropriate ecological context. Additionally, the potential alternative roles of QS communication need to be explored even further.

THE PURPOSE OF PHENOTYPIC HETEROGENEITY *IN NATURA*

There are several known examples of phenotypic heterogeneity in bacteria.[27] Specific environments shaping QS regulation *in natura* will often feature several different selective pressures. A group of specialized and cooperating bacteria is likely to be more successful in such environments; division of labor allows specialization and optimization for a particular task (e.g., optimizing its metabolomic flux). Some phenotypes benefit the entire community or are necessary to guard from competitors. They might also however have non-specific, detrimental effects on the individual bacterium producing them, so it would make sense to designate a "warrior caste" of bacteria that produces those for the greater good of the community. Consequently, it might not always be necessary that all cells of a population engage a specific adaptive phenotype, thereby also saving on the metabolic cost. Especially when the environmental pressure to which the adaptive phenotype is needed displays instability. QS-regulated phenotypes might also regulate individualistic behavior; Cárcamo-Oyarce et al.,[50] have shown that QS triggers the exit from biofilms in *Pseudomonas putida*. In this case, bacteria could be viewed as cells that are designated to further explore and colonize other environments. Such "bacterial pioneers" might then find suitable ecological niches, potentially saving their species from extinction. Even if the potential benefit is high, going "all-in" and opting for an exodus is likely suboptimal. On the other hand, sacrificing a few bacteria has no significant consequences for the population. QS could therefore enable the allocation of a sub-population of bacteria that will be able to respond to a non-constant, unpredictable, and/or chaotic environmental selective pressure[27] thus enabling bet-hedging. Future research endeavors should be encouraged to place bacteria in an appropriate ecological context, identify the relevant selective pressure, and include the testing of hypotheses that mechanistically explain if and how QS architectures, which deviate from the cell-density switch dogma, present a fitness benefit to the bacterial population. Generalizing QS should be avoided, even if the system is mechanistically similar, it might serve completely different purpose according to the ecological niche of the strain.

SOCIOMICROBIOLOGY OF PHENOTYPIC HETEROGENEITY

A small subpopulation of cells that exhibits a QS-regulated phenotype via the production of "public goods" that are beneficial to the entire bacterial population (as opposed to being beneficial only to the cell producing it) can be considered "altruistic" because it invests resources to maximize the chances of survival of the entire bacterial population. This could be construed as paradoxical according to the "Prisoner's Dilemma/The Tragedy of The Commons" thought experiment. "Selfish" cells should, in the long run, overgrow cooperating ones, because they are better off in terms of fitness by not paying the price of cooperation,[51] and this can lead to a populational collapse.[52,53] The "The Tragedy of The Commons"' could therefore be used as an argument against extreme societal division of labor in bacteria. There are several examples, where QS-regulated adaptive phenotypes are exploitable by cheaters in a laboratory environment. Important experimental work on *P. aeruginosa* for example makes it evident that a large population of cooperating individuals that produces a secreted proteases is required for the population to sustain itself.[54–57] Such experiments, we argue, should still not be interpreted as counterevidence for the possibility of cooperation and division of labor *in natura*. In the case of *P. aeruginosa* specifically[55,57] where protein is the only food source, and where

bacteria proliferate to high cell densities, QS systems which regulate cooperative behavior needed for survival, will indeed be selected to function in a way that ensures a sufficiently high population of cooperating individuals, otherwise, the population collapses and becomes extinct. In such cases, a sufficiently high population of cooperating individuals is ensured by the introduction of policing, metabolic prudency, and group selection.[52] Some examples of policing include cheater cyanide susceptibility in *P. aeruginosa*[58] or the QS control of type VI secretion systems in *Burkholderia thailandensis*.[59] However, some AHL QS systems, especially the ones that are found in wild type bacterial isolates, might still function differently, and exhibit a high degree of phenotypic heterogeneity without conflicting with the "Prisoner's Dilemma" since they could perform a dramatically different function. *In natura*, if e.g., *P. aeruginosa* were to occupy an ecological niche where protein is not the only food source, its presence sporadic, and not as essential for growth, it could be wasteful to over-commit to the energy investment of protease production. Not degrading protein in such environments might not necessarily result in a populational collapse. A small subpopulation of cells that produces proteases could be enough to degrade protein into available food and provide the population with a small energy boost that would nevertheless help the bacterial population. If it does not, the upkeep energy is still met some other way and nothing dramatic happens to the bacterial population. Admittedly, determining a base upkeep cost *in natura* is not straightforward. In theory, QS could regulate phenotypic differentiation to a degree, where the regulated phenotype can nevertheless still sustain or be of benefit to a sub-population. Other sub-populations would then reciprocate, by manifesting some other phenotype, again beneficial to the species as a whole.

Conclusion

There is a strong need to shift the study of QS to a more appropriate ecological context where the conditions under which we propagate, and study QS resemble more closely those *in natura*. This perspective aims to re-envision the way we understand QS cell-cell communication in bacteria and revive research in this field by redirecting it toward the exploration of its ecological and socio-microbiological relevance, as already envisioned by Whiteley et al.[60] QS is under considerable underemphasized additional regulation which in their natural habitat, could enable other behaviors, including bacterial phenotypic differentiation and specialization, ultimately allowing a higher degree of societal organization and collective decision-making. QS could be considered as a molecular mechanism that helps determine and establish the "bacterial societal vocation". By understanding more about signaling mechanisms that do not function as we would expect and their genetic/molecular configurations, we can also assume more about the environmental selective pressures, which shaped them in the first place, because we argue that QS systems *in natura* have a purpose in overcoming them. A teleological understanding of QS systems will provide us with testable hypotheses, will guide us toward a deeper understanding of cell-cell signaling, and will provide a strong basis for further translational research enabling easier exploitation and, most of all, understanding of bacteria *in natura*.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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