Intraspecies heterogeneity in microbial interactions

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Abstract

Microbial interactions are increasingly recognized as an integral part of microbial physiology. Cell-cell communication mediated by quorum sensing and metabolite exchange is a formative element of microbial interactions. However, loss-of-function mutations in quorum-sensing components are common across diverse species. Furthermore, quorum sensing is modulated by small molecules and environmental conditions that may be altered in the presence of other microbial species. Recent evidence highlights how strain heterogeneity impacts microbial interactions. There is great potential for microbial interactions to act as selective pressures that influence the emergence of common mutations in quorum-sensing genes across the bacterial and fungal domains.

Keywords

quorum sensing; intraspecies; interspecies; microbial interactions; heterogeneity; mutations; evolution; signaling; cross-feeding

Introduction.

Microbial cells are hardly ever alone. Because microbes almost always live adjacent to kin or different microbial species, microbial interactions are intimately linked with microbial viability, activity and community structure. In fact, it has been proposed that obligate mutualism between microbes is a reason why many remain unculturable [1]. The continuous presence of neighbors necessitates control over inter- and intra-species interactions. Quorum sensing is one important mechanism that governs both interaction types. Although the regulatory components vary in composition and specific gene targets, commonalities exist across species (Fig. 1A). For example, quorum sensing frequently controls the production of catabolic enzymes, small inhibitory molecules, and biofilm related factors via signal-responsive transcription factors. Alongside its use in intraspecies signaling, quorum-sensing

systems respond to the signals produced by other species in the local vicinity. Despite the importance of quorum-sensing pathways for intra- and inter-species relationships, loss-of-function mutations in quorum-sensing genes are frequently observed in bacteria and fungi (Fig 1B). At first this appears to present a paradox: since microbial neighbors are present for most microbial life, why might loss-of-function mutations in key mediators of microbial interactions be beneficial? Recent studies provide novel insight into this phenomenon. In this review, we will summarize frequent loss-of-function mutations in quorum sensing-related genes and discuss recent factors that influence the fitness of quorum-sensing mutants. We will review how heterogeneity in quorum sensing affects microbe-microbe interactions and discuss how microbial interactions may influence the rates at which these mutations arise.

Loss-of-function mutations in cell-cell communication genes are common across diverse species.

Numerous studies suggest that the quorum-sensing systems in both gram-positive and gram-negative bacteria are frequently under selection. The *agr* quorum-sensing locus of *Staphylococcus aureus* (*agrBDCA)* exists in several types based on specific single nucleotide polymorphisms in *agrC* and *agrD* encoding a histidine sensor kinase and the precursor peptide of its cognate signal respectively, and loss-of-function mutations have been identified in isolates from persistent and chronic infections [2,3]. LasR, a master regulator of *Pseudomonas aeruginosa* quorum sensing, is selected against *in vivo* [4–6] and a non-functional *lasR* allele is present in one outgroup of the *P. aeruginosa* phylogeny [7]. In a survey of *Vibrio cholerae* clinical and natural isolates, loss-of-function mutations were prevalent in genes that encode the HapR transcriptional regulator or other quorum-sensing components [8]. Furthermore, experimental evolution selects for quorum sensing-defective mutants in *S. aureus* [9], *P. aeruginosa* [10], and the plant pathogen *Burkholderia glumae* [11]. Although frequent, these mutations do not necessarily abolish expression of the genes controlled by quorum sensing as *S. aureus* lacking Agr activity and *P. aeruginosa* lacking LasR function often sustain secondary mutations in other genes that restore downstream activity [3,10,12]. This restorative phenomenon highlights the conditional importance of quorum-sensing regulation.

Compared to bacteria, less is known about fungal quorum sensing though phenotypic evidence suggests that it is also variable. The levels of farnesol, a quorum sensing molecule detected in *Candida albicans* supernatants, varies between strains and across eight *Candida* species with at least one strain producing undetectable amounts and others producing ~ 60 μM, a concentration well-above that needed to induce a response in individual cells [13–15]. Sensitivity (or responsiveness) to farnesol (as measured by reduced growth, metabolic inhibition, reduced biofilm formation, or morphology changes) is also variable within and across *Candida* species including the emerging multi-drug resistant fungal pathogen *C. auris* [16,17]. Farnesol modulates the Cyr1 adenylate cyclase signaling pathway which influences the activity of the transcription factor Efg1 essential for the control of morphogenesis, and strains frequently lose Efg1 function *in vivo* [18] to suggest interactions may also be altered. Recently, farnesol was shown to induce the activity of two nuclear transcription factors, Tac1 and Znc1, which upregulate the Cdr1 multidrug efflux pump that may mediate farnesol

transport [19]. The Cdr1 transporter sequence or Tac1 transcription factor copy number are often altered in drug resistant isolates suggesting that evolution of drug resistance may also modulate quorum-sensing regulation and vice versa. Repeated loss of quorum-sensing function across species has long been a subject of interest [20], and new findings may provide additional insight into the multitude of ways it both positively and negatively regulates fitness in different contexts.

Small molecules modulate quorum sensing during microbial interactions.

Signal and receptor promiscuity allow for "eavesdropping," or communication between species and across domains of life. This may provide useful information about the local environment, but the ability of other species to modulate quorum sensing regulation may also make it less useful or even a liability. An investigation into the extent of signal promiscuity in the well-studied quorum sensing receptors of *P. aeruginosa* (LasR, RhlR, and QscR), *Vibrio fischeri* (LuxR), *Chromobacterim violaceum* (CviR), and *Burkholderia thailandensis* (BtaR1 and BtaR2) revealed most of these signal-activated transcription factors were strongly responsive to at least one non-cognate signal with structurally similar signals exhibiting more activity [21]. Examples of quorum sensing modulation by other species include autoinducing peptides (AIPs) from coagulase-negative staphylococci that compete for binding with *S. aureus* AgrC signal receptor and inhibit the expression of Agr-regulated targets [22]. Some studies suggest this may also be true across domains of life as *C. albicans*-produced farnesol has chemical characteristics in common with the LasR-specific acyl-homoserine lactone signal 3-oxo-C12-homoserine lactone (3OC12HSL) produced by *P. aeruginosa.* 3OC12HSL was able to modulate two farnesol-responsive phenotypes in *C. albicans*: fluconazole resistance and hyphal formation [23]. Likewise, farnesol has been shown to inhibit the non-acyl homoserine lactone-responsive pathway of the *P. aeruginosa* quorum-sensing system regulated by the transcription factor PqsR (MvfR) [24]. Some quorum-sensing systems detect both broad-spectrum and species-specific (i.e. cognate) quorum-sensing signals. For example, the species-specific cholera autoinducer-1 signal (CAI-1, (*S*)-3-hydroxytridecan-4-one) initiates quorum-sensing activation in wild-type *V. cholerae* but the timing of induction ultimately depends on the production of autoinducer-2, a furanosyl borate diester or tetrahydroxy furan, by neighboring microbes [25]. This coincidence mechanism is possible because of the higher threshold for AI-2-mediated activation compared to the species-specific autoregulatory signal [25]. In another study, exogenous and *C. albicans*-produced farnesol inhibited *S. aureus* production of the golden pigment staphyloxanthin, an important *S. aureus* virulence factor, and this was proposed to be due to competitive inhibition with the pigment precursor farnesyl-diphosphate for an upstream synthase in staphyloxanthin biosynthesis [26]. In turn, farnesol induced a transcriptional response that rendered *S. aureus* more tolerant to H_2O_2 stress and resistant to phagocytic killing [26]. These examples illustrate situations where quorum-sensing mutations may escape control from or avoid the effects on other species.

Environmental changes can trigger or repress quorum-sensing activity. *C. albicans* stimulates *S. aureus* Agr-dependent alpha hemolysis as a result of amino acid-dependent alkalinization of the medium [27,28]. For *C. albicans,* acidic conditions lead to transient un-masking of immunogenic beta-glucan in the cell wall, which can be re-masked through a

quorum-sensing dependent process [29]. The effects of pH on quorum sensing highlight how physical environments can influence quorum sensing-mediated interactions between microbes. Quorum-sensing mutations may have added benefit if quorum-sensing regulated genes can be spuriously induced by environmental conditions. Activation of alternate regulatory pathways may enable expression of the quorum-sensing regulon in otherwise non-permissive conditions even in mutants lacking the canonical quorum-sensing systems.

What are the consequences of quorum sensing heterogeneity on microbial interactions?

Strains with deficiencies in quorum sensing can have unexpected intra- and inter-species interactions compared to their wild-type counterparts. Because the *hapR* mutant is often used to study *V. cholerae* biofilm formation due to its strong preference for biofilm over dispersal, the biofilm behaviors of *hapR* loss-of-function mutants are perhaps better understood than their HapR+ counterparts. By investigating the wild-type *V. cholerae* biofilm development over time, it is apparent that *hapR* loss-of-function mutants display some unique biofilm characteristics, including significantly more biomass [25]. The phenotypic differences observed between wild type and quorum-sensing loss-of-function mutants in *V. cholerae* speak to the importance of strain diversity. Intra-strain heterogeneity is particularly relevant in chronic conditions where *in vivo* evolution occurs and closely related isolates can coexist. For example, in mixed colony biofilms with wild-type *P. aeruginosa*, strains lacking the quorum-sensing regulator LasR hyper-produced the LasR-regulated antimicrobial phenazine pyocyanin [30]. This surprising result was mediated by *lasR* mutant siderophore production which was shown to promote citrate release particularly by the LasR+ wild type strain. Ultimately, citrate stimulated quorum-sensing activity in the *lasR* mutant, but not the wild type (Fig. 2). Likewise, *C. albicans*-produced farnesol has been shown to inhibit *P. aeruginosa* quorum sensing through Pseudomonas Quinolone Signal (PQS) in wild type whereas it activates PQS signaling in LasR loss-of-function mutants [24]. The loss of LasR function in *P. aeruginosa* can also lead to reduced rates of oxygen consumption [31], increased expression of microoxic fitness determinants [32], and enhanced catabolism of ethanol [33], which may improve its ability to grow in the presence of oxygen-consuming or ethanol-producing cells. Clinical antibiotic exposure selects for mutations at different rates based on genotype, and this may also reflect differences in susceptibility to antimicrobial compounds produced by other species. As an example, *P. aeruginosa lasR* mutants did not evolve antibiotic resistance as readily as the wild type [34]. Given the frequency with which quorum-sensing mutants are isolated, an understanding of how these types respond to wild-type strains, to other microbes and to the environment is warranted.

Do microbial interactions affect the selection for quorum sensing mutations?

In addition to altering composition and community function through mutualism, direct combat, resource competition, and cross-signaling, interactions between organisms can alter evolutionary trajectories [35]. Nearby microbial life can alter many abiotic factors like pH,

redox, carbon/nitrogen availability, and metal content—all of which are potential selective pressures. In a cellular automation model investigating a mutualistic interspecies interaction, a mutation with a mildly self-detrimental effect but increasingly cooperative interspecies interaction (i.e. net indirect fitness benefit) rose above kin in four out of ten simulations, but only did so in structured, as opposed to well-mixed, environments [35]. Investigation of soil yeast communities revealed strain fitness was largely determined by the other organisms present [36]. Recent evidence suggests that different quorum-sensing signal variants in the *Bacillus cereus* group (known as 'pherotypes') may reflect environment-specific benefits [37]. Loss-of-function mutants in quorum sensing may provide indirect fitness advantages as a result of social cheating or metabolic differences.

There are a number of recent examples which illustrate how interspecies interactions may place selective pressure on quorum-sensing systems. A study of 23 clinical *S. aureus* isolates from polymicrobial diabetic ulcers identified one example wherein co-culture with *P. aeruginosa* significantly altered *S. aureus agr* copy number thus illustrating *P. aeruginosa* has the potential to select for differences in *S. aureus agr* quorum sensing components [38]. Experimental co-evolution of *P. aeruginosa* in the presence or absence of dense populations of *S. aureus* revealed differences in the selected outcomes. Specifically, mutations associated with the major outer membrane component, lipopolysaccharide, were only observed when S. *aureus* was present whereas mutations in the Wsp chemosensory system which controls biofilm formation were observed in both lineages to suggest an adaptation to environmental features rather than *S. aureus* itself [39]. Interestingly, *lasR* mutants were only observed in monoculture conditions to suggest they may have been selected against in the presence of *S. aureus* [39]. For *V. cholerae,* quorum sensing directly impacts the acquisition of new genetic material by controlling natural competence [40]. When density is low, the transcription factor AphA, well characterized for its induction of *V. cholerae* virulence and biofilm, inhibits DNA uptake through interactions with the promoter of the gene encoding the master regulator of competence, *tfoX.* When density is high and dispersal is initiated, the quorum-sensing regulator HapR activates the transcriptional cascade to allow competence [40]. In these cases, intra- and inter-species signaling indirectly and directly impact evolutionary trajectories.

Other common genetic changes may influence and be influenced by microbial interactions.

Like quorum-sensing molecules, metabolite exchange mediates microbial interactions. Often the metabolite pool is a direct result of microbial metabolism mediated by bi-directional or uni-directional exchange. Such exchanges can lead to the emergence of metabolic feedback loops that can drive antagonism or mutual growth. While other interactions increase respiration [41,42], *P. aeruginosa* antagonizes *C. albicans* via an antifungal phenazine [43] that inhibits respiration to promote *C. albicans* fermentation. The fermentation product ethanol stimulates phenazine production particularly when phosphate is limiting [44]. On the other hand, the bi-directional exchange of organic acids observed across three bacterial-fungal pairs of Betaproteobacteria and Mucoromycotina stimulated growth [45]. The recently described mutualistic interaction between the fungus *Aspergillus nidulans* and

the bacterium *Bacillus subtilis* exemplify the dynamics of access inherent in microbial exchange [46]. *B. subtilis* supplied nutrition in the form of thiamine to promote *A. nidulans* hyphal growth, and in return *B. subtilis* benefited from the access to space on the growing hyphae [46]. Such metabolic differences are a determining factor in interaction outcomes. For example, in a three-species microcosm, *P. aeruginosa* dominance over *S. aureus* and *Burkholderia cepacia* depended on its ability to metabolize secondary metabolites through uni-directional cross-feeding [47]. Siderophores, a common secondary metabolite, are also mediators of bacterial-bacterial [30,48] and bacterial-fungal interactions [49,50] due to their roles in competition for iron, their contribution to ROS generation, and direct and indirect effects on metabolism. Inter-strain variability greatly influences the nature of microbial interactions, and this is most often the product of altered metabolism, including the secretion and uptake of secondary metabolites.

The impact of metabolism on microbial interactions highlights the importance of recognizing the variability of respiration and general metabolism phenotypes among strains within a species. Strain-dependent microbial interactions are reported between *S. aureus* and *P. aeruginosa* in which some pairings are capable of antagonism and others are not depending on their secretion profiles [51]. In fungi, mitochondria composition and function are diverse across fungal clades [52,53] and mitochondrial DNA has been proposed to display more heterogeneity than nuclear DNA [54]. Iron availability is a major driver of metabolism in many species, thus it is also important to recognize the variability in iron-acquisition strategies among strains. Iron-scavenging siderophores are the result of diverse biosynthetic gene clusters. The specific gene content and regulation can vary across isolates [55]. *Pseudomonads* make diverse siderophores, enabling the practice of siderophore typing among *Pseudomonas* isolates [56]. In a study looking at over 50 clinical isolates of *P. aeruginosa,* pyoverdine production was found to be very heterogenous [57] and isolates are often observed to lose pyoverdine production altogether [57,58]. The variation observed in siderophore type may be a result of the concurrent gain and loss of functionally redundant biosynthetic clusters through horizontal gene transfer [59] given presence on mobile elements [60]. The nutrient and metabolite spatiotemporal gradients [61] created during microbial interactions increase the opportunity for selection by generating more niches. However, one of the factors that may restrict the rise of metabolic and regulatory mutants from dominance is the dynamic environment itself.

Conclusions.

The diversity in functional genotype, especially with respect to quorum sensing, makes it important to consider the specific physiology of the interacting strains and genotypes when studying an interaction. Given the probability that a microbe is in the presence of others (either non-identical kin or distinct species), the impact of microbial interactions on evolution may be extensive. We posit that microbe-microbe interactions have the potential to drive and prevent selection of many of the mutations that commonly arise. The metabolic capacity of a community can depend on the relative initial ratios of each organism [62] and this likely extends to genotype heterogeneity in cases where the interacting variants have different metabolic properties. Continued investigation of commonly mutated genes or pathways and their roles in microbial interactions may reveal interesting commonalities.

This will merit additional studies to understand further the consequences of these mutations on microbial interactions and to assess the contribution of microbial interactions for their selection.

Acknowledgements

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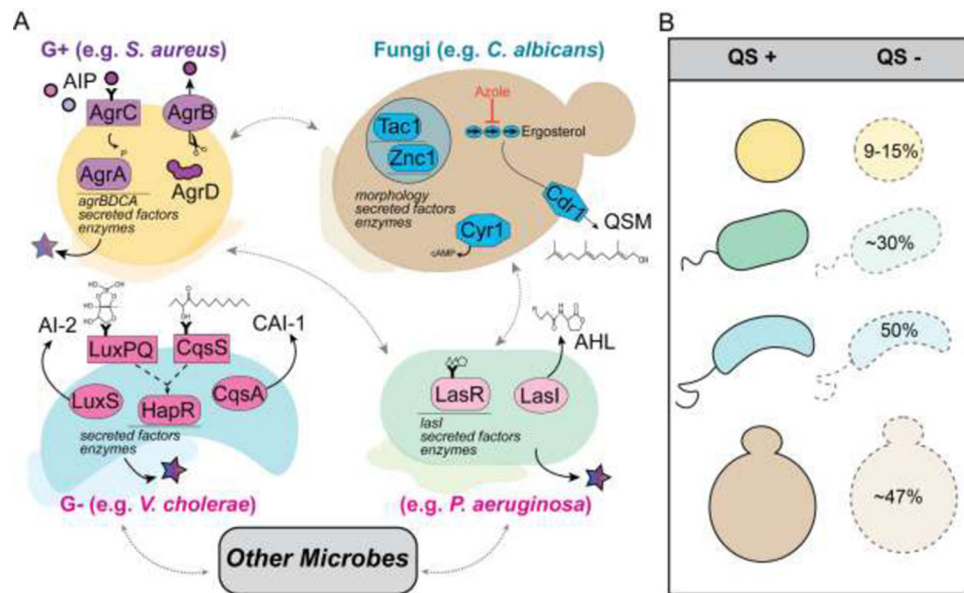**Figure 1. Quorum sensing (QS) mediates microbial interactions between gram positives (G+), gram negatives (G−), and fungi.**

A. Diffusible autoinducers signal within and between species to regulate biofilm formation (colored base) and the production of secreted factors and enzymes (stars) which can modulate the extracellular milieu. Key elements of QS systems in *Staphylococcus aureus*, *Candida albicans*, *Vibrio cholerae* and *Pseudomonas aeruginosa* whose function is implicated in microbial interactions are shown. Shapes indicate protein activity: histidine kinases, rectangles; transcription factors, rounded rectangles bound to thin line of DNA; and enzymes involved in the synthesis or modification of the autoinducer signal, ovals. A small "Y" amended to a protein structure indicates that it binds the autoinducer. Structures for the autoinducers AI-2, CAI-1, acyl homoserine lactones such as 3OC12HSL, and the QS molecule (QSM) farnesol are shown. Autoinducing peptides (AIPs) from *S. aureus* and other species are shown as circles with color indicating species specificity. Dashed black lines indicate indirect activity, and dotted grey lines depict microbes with characterized interactions. B. In the species shown in A, strains with non-functional components of the QS system are frequently identified. In *S. aureus* (yellow) 9–15% of strains have dysfunctional Agr [63,64], in *P. aeruginosa* (green) roughly 30% have *lasR* loss-of-function alleles [65], in *V. cholerae* (blue) as high as 50% of isolates show defective quorum sensing [8], and although less clear in *C. albicans* (tan), roughly 47% of isolates show reduced farnesol production [66].
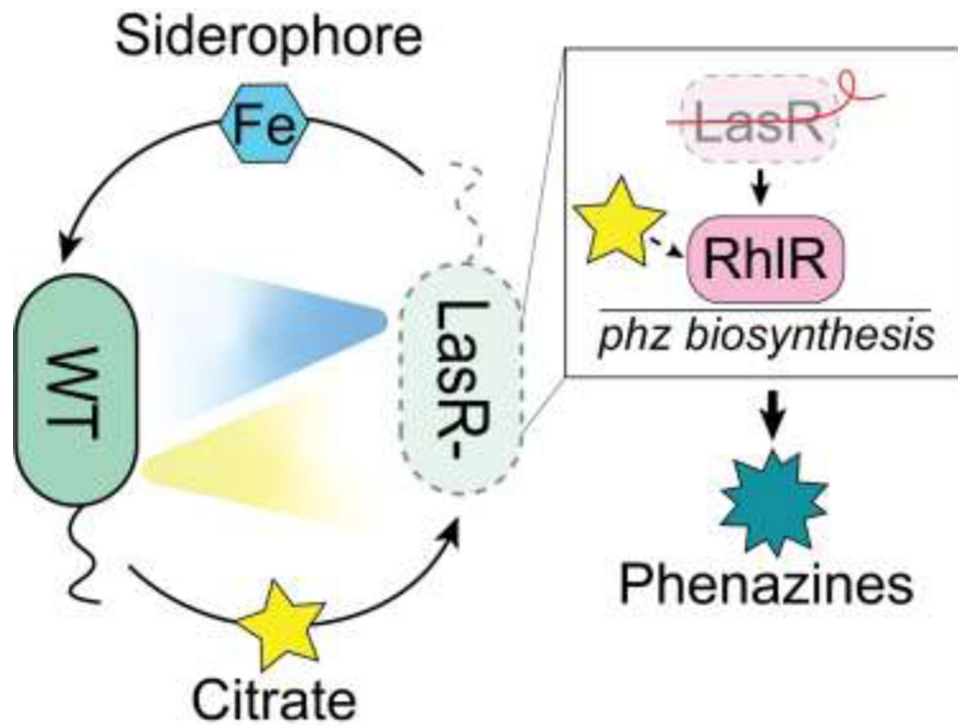
Figure 2. Model of intraspecies interactions between *P. aeruginosa* wild type (WT) and frequently observed *lasR* loss-of-function mutants (LasR−).

In response to the siderophore pyochelin (blue hexagon) produced by LasR− strains, WT (LasR+) *P. aeruginosa* releases citrate (star). Citrate preferentially stimulates quorum sensing in LasR− strains (and not WT) by activating the downstream quorum-sensing regulator RhlR resulting in the increased production of phenazines (decagram) and other secreted factors [30].