Resistance to Quorum-Quenching Compounds

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Bacteria have the remarkable ability to communicate as a group in what has become known as quorum sensing (QS), and this trait has been associated with important bacterial phenotypes, such as virulence and biofilm formation. Bacteria also have an incredible ability to evolve resistance to all known antimicrobials. Hence, although inhibition of QS has been hailed as a means to reduce virulence in a manner that is impervious to bacterial resistance mechanisms, this approach is unlikely to be a panacea. Here we review the evidence that bacteria can evolve resistance to quorum-quenching compounds.
ing resistance to QQ compounds. For QQ resistance, the original population would be inhibited from QS by the QQ compound, while resistant mutants would be unaffected and continue to QS in the presence of the QQ compound, which is a scenario different from that of Beckmann et al.’s simulations. The digital organisms were designed as a type of self-replicating computer program and were subject to the mutations and natural selection that exist in a computational environment. These digital organisms communicate with each other by sending messages, and QS is simulated by allowing each organism to receive one message and to send six messages to its neighbors, creating a positive “signal” feedback loop. In this digital setting, the authors found that the wild-type population became resistant to the deleterious effects of the QS mutants (the mutants increased the “energy consumption” in the system, making the system less efficient) by lowering the threshold of the signal necessary to trigger the QS-controlled phenotypes. Therefore, the model predicts that wild-type cells are resistant to takeover by the QS mutants. This interesting theoretical result remains to be tested experimentally.

Computational approaches and molecular-docking analysis have also been useful for understanding the binding of QQ compounds to receptor proteins to identify potential QQ compounds. Molecular alignment of receptor proteins (e.g., LuxR-type proteins) indicate that there are preserved motifs in the residues of Y53, Y71, W57, D70, and W85 of TraR and Y56, Y64, W60, D73, and W88 of LasR and that the amino acid residues D70, W57, and Y53 in TraR and D73, W60, Y56, and S129 in LasR are important for interacting with the autoinducer analogs (24). The autoinducer analogs rosmarinic acid, naringin, chlorogenic acid, morin, and mangiferin have been studied through in silico docking analysis, and the analyses demonstrated that these compounds can inhibit the production of protease, elastase, and hemolysin (25). In addition, five inducers and three inhibitors which are molecularly distant from the native autoinducer N-3-oxododecanoyl-L-homoserine lactone have been investigated as potential QQ compounds (26). As another example of these modeling approaches, competitive inhibitors of SdiA, a signal receptor of the QS signals of other bacteria in Escherichia coli, have been screened from Melia dubia seed extracts, and 27 compounds structurally unrelated to autoinducers show potential for attenuating QS in uropathogenic E. coli (27). Also, molecular docking was used to identify potential QQ compounds from bark extracts of the mangrove plant Rhizophora annamalayana (28). In addition, three compounds which can inhibit the activity of LuxS from Actinobacillus pleuropneumoniae (LuxS catalyzes S-ribosylhomocysteine into homocysteine and autoinducer 2) were identified computationally (29). In the same manner, possible QQ compounds which can inhibit growth and biofilm formation have been found in various extracts for cariogenic Streptococcus mutans isolates using ligand fit docking protocols (30).

**Early studies on resistance to quorum quenching.** The first suggestion that cells may evolve resistance to QQ compounds was presented as an opinion piece by Defoirdt et al. (2) in 2010. The basis for this supposition was collected from several studies showing that the expression of core QS genes is highly varied between different strains of the same Vibrio species and other pathogenic bacteria, including P. aeruginosa. These core QS genes are involved in the production/detection of autoinducers as well as in QS signal transduction; since their variability is heritable, if this variation confers an advantage in fitness under QQ treatment, the authors concluded that natural selection would favor the spread of QQ resistance. Moreover, this group realized that previous arguments that concluded that resistance to QQ compounds was unlikely had been incorrectly predicated on the growth of pathogens in complex medium. Up to this point, QQ compounds were routinely tested in rich medium, where they were shown to not affect growth and therefore thought not subject to Darwinian selection pressure for resistance. Since pathogens are more likely to encounter conditions more closely resembling minimal media, where they are starved for nutrients and where QQ compounds affect growth, it was reasoned that cells may evolve resistance to QQ compounds. In addition, in mice infection models, the number of viable P. aeruginosa bacteria after QQ treatment in the lungs of infected mice decreases and the ability of this pathogen to disseminate in mice is inhibited; hence, even in the absence of a direct effect of the QS inhibitor, the fitness of bacteria during an infection clearly decreases under QS disruption (2). This is not surprising, since there are numerous studies that show that QS signals as well as QS-controlled virulence factors have a role in protecting bacteria against the immune system and that disruption of QS systems leads to the accelerated death of the bacterial pathogens (31–35).

There persists in the literature the misperception that some early QS work demonstrated resistance to QQ compounds. For example, there is an excellent paper by Koch et al. (36) based on a lock-and-key relationship between the receptor and autoinducers that identified substitutions in LuxR (L42A, a point mutation in the LuxR signal binding site) that altered both the binding of the natural ligand 3-oxo-C_8-HSL and homoserine lactone and that of QQ compounds. However, resistance to QQ compounds was not investigated (36), as has been suggested (6). Koch et al. did not check the substituted LuxR protein in the original Vibrio host but instead did their work in E. coli, so there were no studies of resistance to a QS system (36). Also, far from conducting experiments on resistance and deducing that resistance is possible, the authors concluded the opposite, that resistance to QQ compounds was not likely, as they wrote the following:

Although there is no selective pressure imposed by the inhibitors per se, it is conceivable that pathogenic bacteria in the long run might develop resistance to quorum-sensing inhibitors that are based on agonist structure. In contrast, our furanone analysis suggests that through time inhibitors have been selected in nature where single amino acid changes in a separated receptor site leading to resistance are less likely to occur (36).

Similarly, Zhu et al. (37) studied the ability of AHL analogs to disrupt 3-oxo-C_8-HSL signaling via TraR in Agrobacterium tumefacien by investigating the ability of these compounds to activate expression of a TraR-regulated promoter. Although claimed otherwise (6), resistance to these compounds was not explored, since growth in the presence of these QQ compounds was not studied. Instead, the intent of the authors was to determine if differences in TraR levels affect the ability of A. tumefacien to detect analogs of 3-oxo-C_8-HSL, and “resistance” is not mentioned in the paper, nor was it explored.

Although distinct from demonstrating the development of resistance to QQ compounds, it has also been demonstrated that QQ compounds can select for a more virulent population by re-
ducing the growth advantage of cells that are already deficient in QS relative to that of the wild-type strain. Kohler et al. (38) showed in a hospital setting and in the lab that the administration of azithromycin in cases with P. aeruginosa infection led to an enrichment of the more virulent wild-type strain relative to lasR strains. Bacteriophages may also play a role in enhancing resistance to QQ compounds. For example, since QS in E. coli protects cells against phage attack (39), in the presence of bacteriophages and a QQ compound, QQ-resistant bacteria would have a competitive advantage relative to QQ-sensitive individuals, since the QQ-resistant bacteria would have an active QS system that would make them less susceptible to phage attack. Therefore, bacteriophages may select for QQ-resistant clones.

Resistance to QS inhibition. The first demonstration that cells evolve resistance to QQ techniques was that of Maeda et al. (40) (published ahead of print in 2011). The opportunistic pathogen P. aeruginosa was used as the reference bacterium since it is notorious for causing severe infections and since it is one of the main QS bacterial model systems. A novel screen was developed to test if cells could evolve resistance to a QQ compound by using adenosine as the sole carbon source; growth on adenosine requires an active LasI/LasR N-3-oxododecanoyl homoserine lactone QS system, since the expression of the nucleoside hydrolase (nuh) gene is under its control. Hence, if QQ compounds inhibit the LasI/LasR system, the cells grow more slowly on adenosine (40), and if cells evolve resistance to the QQ compound, they will grow more rapidly on adenosine. In addition, the finding that adenosine inhibits the biofilm formation of P. aeruginosa (41) is theorized to be linked to QS to prevent cheating (42), and adenosine is produced from ATP at high levels in the human host (up to 5 mM) during surgical injury, ischemia, and inflammation, so it is a relevant carbon source for this pathogen and one that affects its physiology significantly. The gold standard of QQ compounds, the synthetic brominated furanone 4-bromo-5-(bromomethylene)-2(5H)-furanone, known as C-30 (43), which was derived from the natural brominated furanone 5Z-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone of the algae Delisea pulchra, was used since it is by far the best-characterized QQ compound. For example, this family of compounds inhibits all three QS systems of Vibrio harveyi (11). Maeda et al. (40) used a concentration of brominated furanone (C-30) that did not affect growth in rich medium (so it did not inhibit growth as a toxin) and used both transposon mutagenesis and spontaneous mutants to identify resistant bacteria. The mechanism for this resistance in the transposon mutants was that the bacteria developed mexR and nalC mutations (40); these genes encode repressors of the MexAB-OprM multidrug resistance operon, so as a result of the mutations, the QQ compound was more readily effluxed (a result that was not anticipated). C-30 had a diminished ability to reduce significantly reducing QS phenotypes (10 μM 5-FU reduced elastase activity by 86%, eliminated pyocyanin production, reduced rhamnolipid production by 87%, eliminated swelling, and eliminated Pseudomonas quinolone signal production), and reducing pathogenicity (5-FU increased barley germination) (48). This reduction of P. aeruginosa pathogenicity by 5-FU was rediscovered by Imperi et al. (49) 4 years later, when they demonstrated that 5-fluorocytosine, which they showed is converted to 5-FU for its activity, also reduces pyoverdin, PrP protease, and exotoxin in P. aeruginosa. 5-FU has also been used successfully in human trials as a coating for catheters (50), making it the first QQ compound to be used in medicine and the first QQ compound to have undergone large-scale human trials.

To identify strains resistant to 5-FU, García-Contreras et al. (47) assayed pyocyanin, elastase, and alkaline protease production of eight clinical strains and found two strains to be resistant to the brominated furanone C-30. One of the resistant strains was not sensitive to antibiotics, indicating that the C-30 resistance mechanism of this strain is likely not related to active efflux. Also, some clinical isolates showed resistance for at least one phenotype with 5-FU (47).

Subsequent to the first demonstration of resistance to QQ compounds by Maeda et al. using both realistic lab constructs and clinical strains (40), Mellbye and Schuster (51) published a hypothesis/opinion report in which QS mimic approaches were used rather than realistic ones and in which no QS inhibitor was utilized. They utilized a P. aeruginosa lasR rhlR strain as a mimic of a QQ-sensitive strain and the wild-type strain as a QQ-resistant mimic. In this artificial system, they determined that cells resistant to QQ compounds should not have a growth advantage when
public goods are utilized (i.e., when nutrients are processed extracellularly by QS-related enzymes) and that cells resistant to QQ compounds should have a growth advantage when nonpublic goods are utilized (i.e., when nutrients are processed intracellularly by QS enzymes) (51). Hence, their results using QS mimics corroborated the results of Maeda et al. (40) for their laboratory strains grown with adenosine as the intracellular nutrient. With regard to the more complex case of growth in the lungs of cystic fibrosis patients and the QQ-resistant mutants that were isolated from this real environment by Maeda et al. (40), the relevance of the Mellbye and Schuster study is not clear. Also, the result that the QQ-resistant mutations that were identified by Maeda et al. (40) had enhanced efflux rather than the predicted changes in QS receptors (6) shows that resistance may arise in ways not necessarily related to changes in QS receptors.

In addition, moderate resistance to the nonbiocidal antibiofilm group 2 capsule polysaccharide (G2cps), which works by a still-unknown mechanism in *E. coli*, can be achieved by creating mutations in several loci that affect the surface properties of the bacteria (52). This work confirms the idea that resistance to compounds that do not impair growth is possible, although multiple mutations were required in this case, and so it was reasoned that such resistance would be rare.

The above-discussed articles (2, 40, 47) are pioneering and open a whole new emergent research area, that of QQ resistance. In addition, the results shown (40, 47) may be significant for clinicians since they indicate that the treatment of multiple-antibiotic-resistant strains with active efflux pumps with HSL analogues, such as C-30, may be futile and suggest that there is a common resistance mechanism between antibiotics and QQ compounds, treatment with HSL analogues alone may select for multiple-antibiotic resistance as well. Also, it should be taken in account that QS disruption renders bacteria more sensitive to some antimicrobials and antibiotics, like tobramycin, particularly in the biofilm mode of growth (43, 53). Therefore, for concomitant treatment of QQ and classical antibiotics, even if QQ compounds do not exert selective pressure by themselves, they will exert it indirectly by making cells more sensitive to antibiotics. **Perspectives on new QQ resistance mechanisms.** Ways of evolving resistance to QQ compounds other than active efflux should exist, as suggested previously (40). This is to be expected, since resistance to classical antibiotics can be achieved in many ways, such as by decreasing the permeability of the compounds, mutating the target, overexpressing antibiotic targets, and degrading/modifying the antibiotics. Along these lines, Maeda et al. found that C-30 can be degraded by PA14 (unpublished results), and they are currently investigating if this ability is enhanced in some C-30-resistant clinical isolates.

Further work is also required to determine if resistance to other kinds of quorum quenchers, such as signal-degrading enzymes, like lactonases or acylases for HSL autoinducers, is possible. Hence, it is important to distinguish those QQ compounds that must enter the cell to be effective (e.g., brominated furanones) from QQ compounds that work extracellularly (e.g., lactonases), since there may be less pressure to evolve resistance to extracellular compounds because greater efflux should not affect the use of these compounds (54). Although, to our knowledge, no experimental efforts have been devoted to explore this possibility, it can be anticipated that ways in which bacteria develop resistance to these agents may be to (i) increase autoinducer production, (ii) synthesize modified autoinducers (which are less susceptible to the attack of the degrading enzymes), or (iii) evolve mutations in the LuxR-like receptors that increase their affinity to the autoinducers (so that the necessary threshold of autoinducer concentration will decrease). Examples of the first two possibilities (an increase in autoinducer production and the presence of different variants of autoinducers) have already been reviewed (2), and for the third possibility, it has been demonstrated that some mutations in *Vibrion fischeri* LuxR, which normally recognizes the 3-oxo-C6-HSL signal, make it able to respond to different autoinducers, like octanoyl-HSL, pentanoyl-HSL, and tetradecanoyl-HSL, and moreover, some subset of these mutations also increases their sensitivity to the endogenous signal (55).

The choice to inhibit QS as a means of inhibiting pathogens (6) is also a questionable goal, since it violates one of the main postulates of preventing resistance, namely, that it is far better to make antivirulence drugs that are specific rather than to target general agents (56). Since QS often involves hundreds of gene targets (45, 46), bacteria may use multiple means of thwarting this approach. Additional complications for this approach are that since QS is used by many bacteria, beneficial microorganisms may also be affected by any general approach (9, 57), for example, in the gut, where hundreds of different species reside. Complicating matters further in mixed cultures is the fact that some pathogenic genes are activated by QS (e.g., *P. aeruginosa*) (46), while others are inactivated (e.g., *Vibrio cholerae*) (58); hence, QQ approaches may have unintended consequences in communities with many bacteria.

**Conclusions.** As outlined here, bacteria have been shown to evolve resistance to QQ compounds both in lab studies and in clinics and to evolve resistance to QQ compounds even without their use (i.e., when bacteria are confronted with antibiotics and mutation in the efflux pump occurs); hence, we should be less
sanguine about the possibilities that these novel QQ compounds are as robust as has been frequently indicated in the current literature (6). One actual mechanism of QQ resistance involving enhanced efflux (40) is shown in Fig. 1A, whereas Fig. 1B shows the predicted mechanism of QQ resistance of LasR receptor insensitivity based on the lock-and-key relationship through the amino acid change L42A, which led to an inability of the autoinducer to bind. Hopefully, even with resistance arising, QQ compounds may be used in combination with other antimicrobials. However, the exaggerated claims by many authors about the benefits of these compounds should be tempered.

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