AEM Accepts, published online ahead of print on 6 September 2013
Appl. Environ. Microbiol. doi:10.1128/AEM.02378-13

Resistance to Quorum Quenching Compounds

Rodolfo García-Contreras[1†], Toshinari Maeda[2†], and Thomas K. Wood[3*]

[1]Instituto Nacional de Cardiología, Departamento de Bioquímica, Juan Badiano # 1, Sección XVI, Tlalpan, México DF 14080, México

[2]Department of Biological Functions and Engineering, Kyushu Institute of Technology, Kitakyushu, Japan

[3]Department of Chemical Engineering and Department of Biochemistry and Molecular Biology, Pennsylvania State University, University Park, Pennsylvania, U.S.A.

[†]Contributed equally to this work.

[*]Email: tuw14@psu.edu

Running title: Resistance to quorum quenching compounds

Keywords: quorum sensing, quorum sensing inhibition, anti-virulence, resistance

ABSTRACT

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.

REVIEW

Infectious diseases are the leading cause of death (1) and all antibiotics fail (2); therefore, it is imperative to develop novel ways to fight microbial infections. Here we review the use of chemicals that interfere with cell communication and investigate the likelihood that bacteria will evolve resistance to these compounds.

Quorum sensing and quorum quenching. Bacteria use secreted chemicals as signals for a variety purposes including virulence and biofilm formation. When the compounds build to a threshold concentration and trigger gene expression, the signals are known as quorum sensing (QS) signals. Examples of well-studied QS signals include acylhomoserine lactones, autoinducer 2, and peptide signals, but many other signals exist such as indole (3). In addition to signals, signal synthases, signal receptors, signal response regulators, and regulated genes (QS regulon) are also key components of any QS system (4). For example, LuxI-type enzymes are signal synthases which synthesize acylhomoserine lactones. In addition, LuxR-type regulators are receptor proteins for the autoinducer signals, and signal-receptor binding is responsible for the expression of QS regulons.

Since numerous compounds have been identified that inhibit QS, since QS is linked to virulence, and since inhibition of QS does not usually affect growth (in rich medium), it has been reasoned that inhibition of QS may be an effective means to reduce pathogenicity that is not subject to the usual resistance mechanisms of bacteria (1, 5-8). Inhibition of QS is also known as quorum quenching (QQ)

and is a form of antivirulence.

The well-known examples (9) of QQ compounds include lactonases/acylases that degrade the HSL autoinducers, synthase inhibitors like analogues of anthranilic acid that block synthesis of quinolone signals (10), and receptor inhibitors such as brominated furanones (11). In addition, low concentrations of azithromycin, ceftazidime, and ciprofloxacin (antibiotics) inhibit QS in *P. aeruginosa* (12). Also, among the thousands of drugs approved for clinical use, the anthelmintic drug niclosamide is a QQ compound (13); this drug reduces surface motility, biofilm formation, and production of the secreted virulence factors, elastase, pyocyanin, and rhamnolipids. The rhizosphere bacterium *Stenotrophomonas maltophilia* produces *cis*-9-octadecenoic acid which is a QQ compound that reduces violacein production by *Chromobacterium violaceum* and biofilm formation by *P. aeruginosa* (14). The cyclic dipeptide 2,5-piperazinedione inhibits QS-dependent traits such as protease activity, elastase activity, and the production of pyocyanin and extracellular polymeric substances (15). Further evidence that QQ compounds are readily found in nature include that of 120 bacterial isolates from healthy coral species, up to 24% of the isolates showed anti-QS activity against three QS indicator strains (16); a possible explanation for this behavior is that the interaction of coral-associated bacteria is competitive so QQ compounds are secreted from the dominant communities to diminish undesirable marine biofouling. From this group, a *Favia* sp. coral isolate inhibits the biofilm formation of *P. aeruginosa* and *Acinetobacter baumannii* through the secretion of a low-molecular mass compound which is not inactivated by heat and protease K (16).

Mathematical modeling for resistance to QQ. There are several manuscripts describing mathematical models of the QS systems of *P. aeruginosa* and other bacterial species that include planktonic cells (17) and biofilm cells (18) in closed and open spaces such as micro fluidic devices (19). Most of these models describe the effect of classical antibiotics and antivirulence compounds and suggest a narrow therapeutic concentration range for the QQ agent to be effective in biofilms. For example, Anguige et al. (20) found that there is a critical biofilm depth in which the QQ treatment is successful; hence, the biofilm penetrability of QQ drugs is a critical parameter to take into account for the design of antivirulence

74 compounds. Therefore, perhaps resistance against QQ in biofilms may develop by restricting the
75 permeability of the QQ drugs by overproducing extracellular matrix components that ` sequester the QQ
76 agent such as the *ndvB*-encoded glucans that sequester aminoglucosides (21). Other theoretical studies
77 show that for biofilms, the time at which QQ treatment is initiated is critical for effective prevention of
78 QS-mediated virulence (22).

79 Beckmann et al. (23) in 2012 developed what was purported to be the first *in silico* study that showed
80 the possibility of QQ resistance; however, they did not evaluate resistance to QQ compounds since a QQ
81 compound was not introduced but instead signal-blind or signal-deficient mutants were introduced into a
82 wild-type culture which is not the same as developing resistance to QQ compounds. For QQ resistance,
83 the original population would be inhibited from QS by the QQ compound while resistant mutants would
84 be unaffected and continue to QS in the presence of the QQ compound which is a different scenario than
85 that of their simulations. The digital organisms were designed as a type of self-replicating computer
86 program, subject to mutations and natural selection, that exist in a computational environment. These
87 digital organisms communicate with each other by sending messages, and QS is simulated by allowing
88 each organism to receive one message to send six messages to its neighbors creating a positive "signal"
89 feedback loop. In this digital setting, the authors found that wild-type population became resistant to the
90 deleterious effects of the QS mutants (the mutants increased the "energy consumption" in the system,
91 making the system less efficient) by lowering the threshold of the signal necessary to trigger the QS
92 controlled phenotypes. Therefore, the model predicts that wild-type cells would be resistant to take-over
93 by the QS mutants. This interesting theoretical result remains to be tested experimentally.

94 Computational approaches and molecular docking analysis have also been useful for understanding
95 the binding of QQ compounds to receptor proteins to identify potential QQ compounds. Molecular
96 alignment of receptor proteins (e.g., LuxR-type proteins) indicate that there are preserved motifs in the
97 residues of Y53, Y71, W57, D70, and W85 of TraR and Y56, Y64, W60, D73, and W88 of LasR and that
98 the amino-acid residues D70, W57, and Y53 in TraR and D73, W60, Y56, and S129 in LasR are
99 important for interacting with the autoinducer analogs (24). Autoinducer analogs rosmarinic acid,

naringin, chlorogenic acid, morin, and mangiferin have been studied through *in silico* docking analysis and 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 structurally unrelated compounds to autoinducers show potential for attenuating QS in urophatogenic *E. coli* (27). Also, molecular docking was used to identify potential QQ compounds from bark extracts of the mangrove plant *Rhizopora annamalayana* (28). In addition, three compounds which can inhibit the activity of LuxS from *Actinobacillus pleuropneumoniae* (LuxS catalyzes *S*-ribosylhomocysteine into homecysteine and autoinducer-2) were identified computationally (29). In the same manner, possible QQ compounds which can inhibit growth and biofilm formation have been found from 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 variable 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 the 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 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 an absence of a direct effect of the QS inhibitor, the fitness of bacteria during the 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 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 biding site) that altered both the binding of the natural ligand 3-*oxo*-C8-homoserine lactone as well as 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 resistance is possible, the authors concluded the opposite, that resistance to QQ compounds was not likely as they wrote "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*-C8-HSL signaling via TraR in *Agrobacterium tumefaciens* 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. tumefaciens* to detect analogs

of 3-*oxo*-C8-HSL, and "resistance" is not mentioned in the manuscript 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 reducing the growth advantage of cells that are already deficient in QS relative to the wild-type strain. Kholer et al. (38) showed in a hospital setting and in the lab that the administration of azithromycin with *P. aeruginosa* led to an enrichment of the more virulent wild-type strain relative to *lasR* strains.

Bacteriophage may also play a role in enhancing resistance to QQ compounds. For example, since QS in *E. coli* protects cells against λ 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, bacteriophage 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 on-line 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 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, adenosine inhibits the biofilm formation of *P. aeruginosa* (41), is theorized to be linked to QS to prevent cheating (42), and 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(5*H*)-furanone, known as C-30 (43), that was derived from the natural brominated furanone (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-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 *V. 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 multi-drug resistance operon so as a result of the mutations, the QQ compound was more readily effluxed (a result that was not anticipated). Consistent with the resistance to C-30 of the *mexR* mutant during growth on adenosine, C-30 had diminished ability to reduce several QS controlled virulence factors and phenotypes in the *mexR* muant and the pathogenicity of the *mexR* mutant against the nematode *Caenorhabditis elegans* was not attenuated by the addition of C-30 (40). Critically, this group also used cells from cystic fibrosis patients (Liverpool epidemic strain 12142) with *mexR*/*nalC* mutations to show that even in the absence of the QS inhibitor, cells *naturally* evolve resistance to QQ compounds in the pathogenic state when confronted with the pressures of antibiotic treatment; hence, antibiotic treatment can lead to resistance to QQ compounds. In contrast to the transposon mutants, the spontaneous mutants isolated by Maeda et al. (44) had intact *mexR* and *nalC* genes, indicating resistance can also rise by other uncharacterized mechanisms. Therefore, the authors showed that cells develop resistance to QQ compounds through different mechanisms and that these mutations actually occur in a clinical setting. The fact that the mutations arise in a clinical setting demonstrates that it does not matter whether growth depends on “public” or “private” goods; the crux is that cells were shown definitely to evolve resistance to QQ compounds even in the absence of previous exposure to them.

It may be argued that the Maeda et al. study (44) was predicated on using the QQ compound (C-30) under conditions that it inhibited growth (growth on adenosine requires an active QS system). However, this situation of QQ affecting growth is common since it has been shown that another well-publicized QQ compound, LED229, which inhibits QseC-based signaling in enterohemorrhagic *E. coli* (45), also affects growth (although claimed otherwise) since deletions in *qseC* results in numerous metabolic changes (9). Also, since QS often involves hundreds of genes (46, 47), it is reasonable that inhibiting QS outside of

laboratory conditions (i.e., growth in non-rich medium) may influence growth (9).

Additional clinical evidence of the ability of strains to evolve resistance to QQ compounds was provided by studying the resistance of Mexican clinical isolates from urine, blood, and catheter tips of children to brominated furanone (C-30) and to 5-fluorouracil (5-FU) (48). From a screen of *P. aeruginosa* biofilm mutants, uracil was determined to act as a positive signal for biofilm formation, and 5-FU was shown to be effective in inhibiting this signaling thereby repressing biofilm formation, reducing significantly QS phenotypes (10 μM 5-FU reduced elastase activity by 86%, eliminated pyocyanin production, reduced rhamnolipid production by 87%, eliminated swarming, and eliminated PQS production), and reducing pathogenicity (5-FU increased barley germination) (49). This reduction of *P. aeruginosa* pathogenicity by 5-FU was re-discovered by Imperi et al. (50) four years later when they demonstrated that 5-fluorocytosine, which they showed is converted to 5-FU for its activity, also reduces pyoverdine, PrpL protease, and exotoxin in *P. aeruginosa.* 5-FU has also been used successfully in human trials as a coating for catheters (51) 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, the authors (48) assayed pyocyanin, elastase, and alkaline protease production of eight clinical strains and found two strains 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 (48).

Subsequent to the first demonstration of resistance to QS compounds using both realistic lab constructs as well as clinical strains by Maeda et al. (40), the Schuster lab (52) published an 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 non-public goods are utilized (i.e., when nutrients are processed by intracellularly by QS enzymes) (52). Hence, their results using QS mimics corroborated those results of Maeda et al. (40) for their laboratory strains grown with adenosine as the intracellular nutrient. For 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 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 which shows that resistance may arise in ways not necessarily related to changes in QS receptors (6).

In addition, moderate resistance against the non-biocidal anti-biofilm group 2 capsule polysaccharide (G2cps), that works by a still unknown mechanisms in *E. coli,* can be achieved by mutations in several *loci* that affect the surface properties of the bacteria (53). This work confirms that resistance against 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, 48) are pioneering and open a whole new emergent research area, that of QQ resistance. In addition the results shown (40, 48) may be significant for the clinic 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 since there is a common resistance mechanism between antibiotics and QQ compounds, treatment with HSL analogues alone could possibly select multiple antibiotic resistance as well. Also it should be taken in to account that QS disruption renders bacteria more sensitive to some antibiotics, like tobramycin particularly in the biofilm mode of growth (54), (43). 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: New QQ resistance mechanisms. In addition to active efflux, other ways to evolve resistance against QQ compounds should exist as suggested (40). This is to be expected since resistance to classical antibiotics can be achieved in many ways, such as a decrease in the permeability of the

compounds, mutation of the target, overexpression of antibiotic targets, as well as degradation/modification of the antibiotics. Along these lines, Maeda et al. found that C-30 can be degraded by PA14 (unpublished results) and are currently investigating if this ability is enhanced in some C-30 resistant clinical isolates.

Further work is also required to determine if resistance against 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) and those that QQ compounds that work extracellularly (e.g., lactonases) since there may be less pressure to evolve resistance for extracellular compounds because greater efflux should not affect the use of these compounds (55). Although to our knowledge no experimental efforts have been devoted to explore this possibility, it can be anticipated that ways in which bacteria could develop resistance against these agents could be an increase in autoinducer production, synthesis of modified autoinducers (less susceptible against the attack of the degrading enzymes), or mutations in the LuxR-like receptors that increase their affinity to the autoinducers (so the necessary threshold of autoinducer concentration will decrease). Examples of the first two possibilities (increase in AI production and presence of different variants of autoinducers) have already been reviewed (2) and for the third possibility, it has been demonstrated that some mutations in *Vibrio fischeri* LuxR, that 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 increase their sensitivity to the endogenous signal (56).

The choice to inhibit QS as a means to inhibit 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 (57). Since QS often involves hundreds of gene targets (46, 47), bacteria may use multiple means to thwart 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, 58); for example, in the gut where hundreds of different species reside.

Complicating matters further in mixed cultures, some pathogenic genes are activated by QS (e.g., *P. aeruginosa*) (47) while others are inactivated (e.g., *Vibrio cholerae*) (59); 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 the clinic and to evolve resistance to QQ compounds even without their use (i.e., when bacteria are confronted with antibiotics and mutation in efflux pump occurs); hence, we should be less sanguine about the possibilities that these novel QQ compounds are as robust as 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 autoinducer binding. Hopefully, even with resistance arising, QQ compounds may be used in combination with other antimicrobials. However, the exaggerated claims by many authors on the benefits of these compounds should be tempered.

ACKNOWLEDGMENTS

This work was supported by the NIH (R01 GM089999). T.K.W. is the Biotechnology Endowed Professor at the Pennsylvania State University. R.G.C. was supported by SEP/CONACyT grant 152794 and by ICyTDF project PICSA 11-78. TM was supported by the Grant-in-Aid for Challenging Exploratory Research (25660062) of the Japan Society for the Promotion of Science.

REFERENCES

1. **Rasko DA, Sperandio V.** 2010. Anti-virulence strategies to combat bacteria-mediated disease. Nat Rev Drug Discov **9:**117-128.
2. **Defoirdt T, Boon N, Bossier P.** 2010. Can bacteria evolve resistance to quorum sensing disruption? PLoS Pathog **6:**e1000989.
3. **Jayaraman A, Wood TK.** 2008. Bacterial quorum sensing: signals, circuits, and implications for biofilms and disease. Annu. Rev. Biomed. Eng. **10:**145-167.
4. **Nazzaro F, Fratianni F, Coppola R.** 2013. Quorum sensing and phytochemicals. Int J Mol Sci **14:**12607-12619.
5. **Bjarnsholt T, Tolker-Nielsen T, Hoiby N, Givskov M.** 2010. Interference of *Pseudomonas aeruginosa* signalling and biofilm formation for infection control. Expert Rev Mol Med **12:**e11.
6. **Schuster M, Sexton DJ, Diggle SP, Greenberg EP.** 2013. Acyl-Homoserine Lactone Quorum Sensing: From Evolution to Application. Annu Rev Microbiol. **67**:43–63.
7. **Boyle KE, Heilmann S, van Ditmarsch D, Xavier JB.** 2013. Exploiting social evolution in biofilms. Curr Opin Microbiol **16:**207-212.
8. **Ng WL, Perez L, Cong J, Semmelhack MF, Bassler BL.** 2012. Broad spectrum pro-quorum-sensing molecules as inhibitors of virulence in vibrios. PLoS Pathog **8:**e1002767.
9. **LaSarre B, Federle MJ.** 2013. Exploiting Quorum Sensing To Confuse Bacterial Pathogens. Microbiology and Molecular Biology Reviews **77:**73-111.
10. **Lesic B, Lepine F, Deziel E, Zhang J, Zhang Q, Padfield K, Castonguay MH, Milot S, Stachel S, Tzika AA, Tompkins RG, Rahme LG.** 2007. Inhibitors of pathogen intercellular signals as selective anti-infective compounds. PLoS Pathog **3:**1229-1239.
11. **Defoirdt T, Miyamoto CM, Wood TK, Meighen EA, Sorgeloos P, Verstraete W, Bossier P.** 2007. The natural furanone (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone disrupts quorum sensing-regulated gene expression in *Vibrio harveyi* by decreasing the DNA-binding activity of the transcriptional regulator protein *luxR*. Environ Microbiol **9:**2486-2495.
12. **Skindersoe ME, Alhede M, Phipps R, Yang L, Jensen PO, Rasmussen TB, Bjarnsholt T, Tolker-Nielsen T, Høiby N, Givskov M.** 2008. Effects of Antibiotics on Quorum Sensing in *Pseudomonas aeruginosa*. Antimicrobial Agents and Chemotherapy **52:**3648-3663.
13. **Imperi F, Massai F, Ramachandran Pillai C, Longo F, Zennaro E, Rampioni G, Visca P, Leoni L.** 2012. New life for an old drug: the anthelmintic drug niclosamide inhibits *Pseudomonas aeruginosa* quorum sensing. Antimicrob Agents Chemother **57:**996-1005.

14. **Singh VK, Kavita K, Prabhakaran R, Jha B.** 2013. Cis-9-octadecenoic acid from the rhizospheric bacterium *Stenotrophomonas maltophilia* BJ01 shows quorum quenching and anti-biofilm activities. Biofouling **29:**855-867.
15. **Musthafa KS, Balamurugan K, Pandian SK, Ravi AV.** 2012. 2,5-Piperazinedione inhibits quorum sensing-dependent factor production in *Pseudomonas aeruginosa* PAO1. J Basic Microbiol **52:**679-686.
16. **Golberg K, Pavlov V, Marks RS, Kushmaro A.** 2013. Coral-associated bacteria, quorum sensing disrupters, and the regulation of biofouling. Biofouling **29:**669-682.
17. **Dockery JD, Keener JP.** 2001. A mathematical model for quorum sensing in *Pseudomonas aeruginosa*. Bull Math Biol **63:**95-116.
18. **Anguige K, King JR, Ward JP.** 2006. A multi-phase mathematical model of quorum sensing in a maturing Pseudomonas aeruginosa biofilm. Math Biosci **203:**240-276.
19. **Janakiraman V, Englert D, Jayaraman A, Baskaran H.** 2009. Modeling growth and quorum sensing in biofilms grown in microfluidic chambers. Ann Biomed Eng **37:**1206-1216.
20. **Anguige K, King JR, Ward JP.** 2005. Modelling antibiotic- and anti-quorum sensing treatment of a spatially-structured Pseudomonas aeruginosa population. J Math Biol **51:**557-594.
21. **Sadovskaya I, Vinogradov E, Li J, Hachani A, Kowalska K, Filloux A.** 2010. High-level antibiotic resistance in *Pseudomonas aeruginosa* biofilm: the *ndvB* gene is involved in the production of highly glycerol-phosphorylated beta-(1->3)-glucans, which bind aminoglycosides. Glycobiology **20:**895-904.
22. **Fozard JA, Lees M, King JR, Logan BS.** 2012. Inhibition of quorum sensing in a computational biofilm simulation. Biosystems **109:**105-114.
23. **Beckmann BE, Knoester DB, Connelly BD, Waters CM, McKinley PK.** 2012. Evolution of Resistance to Quorum Quenching in Digital Organisms. Artificial Life **18:**291-310.
24. **Ahumedo M, Diaz A, Vivas-Reyes R.** 2009. Theoretical and structural analysis of the active site of the transcriptional regulators LasR and TraR, using molecular docking methodology for identifying potential analogues of acyl homoserine lactones (AHLs) with anti-quorum sensing activity. Eur J Med Chem **45:**608-615.
25. **Annapoorani A, Umamageswaran V, Parameswari R, Pandian SK, Ravi AV.** 2012. Computational discovery of putative quorum sensing inhibitors against LasR and RhlR receptor proteins of *Pseudomonas aeruginosa*. J Comput Aided Mol Des **26:**1067-1077.
26. **Skovstrup S, Le Quement ST, Hansen T, Jakobsen TH, Harmsen M, Tolker-Nielsen T, Nielsen TE, Givskov M, Taboureau O.** 2013. Identification of LasR ligands through a virtual screening approach. ChemMedChem **8:**157-163.

27. **Ravichandiran V, Karthi S, Princy SA.** 2013. Screening of SdiA Inhibitors from *Melia dubia* Seed Extracts towards the Hold Back of Uropathogenic *E.coli* Quorum Sensing-Regulated Factors. Med Chem. **9:**819-827.
28. **Musthafa KS, Sahu SK, Ravi AV, Kathiresan K.** 2013. Anti-quorum sensing potential of the mangrove *Rhizophora annamalayana*. World J Microbiol Biotechnol.
29. **Li L, Sun L, Song Y, Wu X, Zhou X, Liu Z, Zhou R.** 2013. Screening of *Actinobacillus pleuropneumoniae* LuxS Inhibitors. Curr Microbiol.
30. **Al-Sohaibani S, Murugan K.** 2012. Anti-biofilm activity of *Salvadora persica* on cariogenic isolates of *Streptococcus mutans*: in vitro and molecular docking studies. Biofouling **28:**29-38.
31. **Telford G, Wheeler D, Williams P, Tomkins PT, Appleby P, Sewell H, Stewart GS, Bycroft BW, Pritchard DI.** 1998. The *Pseudomonas aeruginosa* quorum-sensing signal molecule N-(3-oxododecanoyl)-L-homoserine lactone has immunomodulatory activity. Infect Immun **66:**36-42.
32. **Bjarnsholt T, Jensen PO, Burmolle M, Hentzer M, Haagensen JA, Hougen HP, Calum H, Madsen KG, Moser C, Molin S, Hoiby N, Givskov M.** 2005. *Pseudomonas aeruginosa* tolerance to tobramycin, hydrogen peroxide and polymorphonuclear leukocytes is quorum-sensing dependent. Microbiology **151:**373-383.
33. **Kuang Z, Hao Y, Walling BE, Jeffries JL, Ohman DE, Lau GW.** 2011. *Pseudomonas aeruginosa* elastase provides an escape from phagocytosis by degrading the pulmonary surfactant protein-A. PLoS One **6:**e27091.
34. **Dössel J, Meyer-Hoffert U, Schroder JM, Gerstel U.** 2012. *Pseudomonas aeruginosa*-derived rhamnolipids subvert the host innate immune response through manipulation of the human beta-defensin-2 expression. Cell Microbiol **14:**1364-1375.
35. **Leid JG, Willson CJ, Shirtliff ME, Hassett DJ, Parsek MR, Jeffers AK.** 2005. The exopolysaccharide alginate protects *Pseudomonas aeruginosa* biofilm bacteria from IFN-gamma-mediated macrophage killing. J Immunol **175:**7512-7518.
36. **Koch B, Liljefors T, Persson T, Nielsen J, Kjelleberg S, Givskov M.** 2005. The LuxR receptor: the sites of interaction with quorum-sensing signals and inhibitors. Microbiology **151:**3589-3602.
37. **Zhu J, Beaber JW, More MI, Fuqua C, Eberhard A, Winans SC.** 1998. Analogs of the autoinducer 3-oxooctanoyl-homoserine lactone strongly inhibit activity of the TraR protein of Agrobacterium tumefaciens. Journal of bacteriology **180:**5398-5405.
38. **Kohler T, Perron GG, Buckling A, van Delden C.** 2010. Quorum sensing inhibition selects for virulence and cooperation in *Pseudomonas aeruginosa*. PLoS Pathog **6:**e1000883.
39. **Hoyland-Kroghsbo NM, Maerkedahl RB, Svenningsen SL.** 2013. A quorum-sensing-induced bacteriophage defense mechanism. MBio **4:**e00362-00312.

40. **Maeda T, García-Contreras R, Pu M, Sheng L, Garcia LR, Tomas M, Wood TK.** 2012. Quorum quenching quandary: resistance to antivirulence compounds. ISME J **6:**493-501.
41. **Sheng L, Pu M, Hegde M, Zhang Y, Jayaraman A, Wood TK.** 2012. Interkingdom adenosine signal reduces *Pseudomonas aeruginosa* pathogenicity. Microb Biotechnol **5:**560-572.
42. **Dandekar AA, Chugani S, Greenberg EP.** 2013. Bacterial quorum sensing and metabolic incentives to cooperate. Science **338:**264-266.
43. **Hentzer M, Wu H, Andersen JB, Riedel K, Rasmussen TB, Bagge N, Kumar N, Schembri MA, Song Z, Kristoffersen P, Manefield M, Costerton JW, Molin S, Eberl L, Steinberg P, Kjelleberg S, Hoiby N, Givskov M.** 2003. Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. The EMBO journal **22:**3803-3815.
44. **Maeda T, Garcia-Contreras R, Pu M, Sheng L, Garcia LR, Tomas M, Wood TK.** 2012. Quorum quenching quandary: resistance to antivirulence compounds. ISME J **6:**493-501.
45. **Rasko DA, Moreira CG, Li DR, Reading NC, Ritchie JM, Waldor MK, Williams N, Taussig R, Wei S, Roth M, Hughes DT, Huntley JF, Fina MW, Falck JR, Sperandio V.** 2008. Targeting QseC Signaling and Virulence for Antibiotic Development. Science **321:**1078-1080.
46. **Schuster M, Lostroh CP, Ogi T, Greenberg EP.** 2003. Identification, Timing, and Signal Specificity of *Pseudomonas aeruginosa* Quorum-Controlled Genes: a Transcriptome Analysis. Journal of Bacteriology **185:**2066-2079.
47. **Wagner VE, Bushnell D, Passador L, Brooks AI, Iglewski BH.** 2003. Microarray Analysis of *Pseudomonas aeruginosa* Quorum-Sensing Regulons: Effects of Growth Phase and Environment. Journal of Bacteriology **185:**2080-2095.
48. **García-Contreras R, Martinez-Vazquez M, Velazquez Guadarrama N, Villegas Paneda AG, Hashimoto T, Maeda T, Quezada H, Wood TK.** 2013. Resistance to the quorum-quenching compounds brominated furanone C-30 and 5-fluorouracil in *Pseudomonas aeruginosa* clinical isolates. Pathog Dis **68:**8-11.
49. **Ueda A, Attila C, Whiteley M, Wood TK.** 2009. Uracil influences quorum sensing and biofilm formation in *Pseudomonas aeruginosa* and fluorouracil is an antagonist. Microb Biotechnol **2:**62-74.
50. **Imperi F, Massai F, Facchini M, Frangipani E, Visaggio D, Leoni L, Bragonzi A, Visca P.** 2013. Repurposing the antimycotic drug flucytosine for suppression of *Pseudomonas aeruginosa* pathogenicity. Proceedings of the National Academy of Sciences **110:**7458-7463.
51. **Walz JM, Avelar RL, Longtine KJ, Carter KL, Mermel LA, Heard SO.** 2010. Anti-infective external coating of central venous catheters: A randomized, noninferiority trial comparing 5-fluorouracil with chlorhexidine/silver sulfadiazine in preventing catheter colonization. Critical Care Medicine **38:**2095-2102.

52. **Mellbye B, Schuster M.** 2011. The sociomicrobiology of antivirulence drug resistance: a proof of concept. MBio **2:** e00131-11.
53. **Travier L, Rendueles O, Ferrières L, Herry J-M, Ghigo J-M.** 2013. *Escherichia col*i Resistance to Nonbiocidal Antibiofilm Polysaccharides Is Rare and Mediated by Multiple Mutations Leading to Surface Physicochemical Modifications. Antimicrobial Agents and Chemotherapy **57:**3960-3968.
54. **Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW, Greenberg EP.** 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science **280:**295-298.
55. **Defoirdt T.** 2013. Antivirulence therapy for animal production: filling an arsenal with novel weapons for sustainable disease control. PLoS Pathog **in press**.
56. **Collins CH, Arnold FH, Leadbetter JR.** 2005. Directed evolution of Vibrio fischeri LuxR for increased sensitivity to a broad spectrum of acyl-homoserine lactones. Mol Microbiol **55:**712-723.
57. **Greene SE, Reid A.** 2013. Moving Targets: Fighting Resistance in Infections, Cancers, Pests. Microbe **8:**279-285.
58. **Hong K-W, Koh C-L, Sam C-K, Yin W-F, Chan K-G.** 2012. Quorum Quenching Revisited—From Signal Decays to Signalling Confusion. Sensors **12:**4661-4696.
59. **Ng W-L, Perez L, Cong J, Semmelhack MF, Bassler BL.** 2012. Broad Spectrum Pro-Quorum-Sensing Molecules as Inhibitors of Virulence in Vibrios. PLoS Pathog **8:**e1002767.

FIGURE CAPTION

Fig. 1. Mechanism of actual and predicted inhibition to quorum quenching compounds. (A) Actual quorum quenching resistance in *P. aeruginosa* based on enhanced efflux of furanone C-30 due to mutations in the genes that encode efflux repressors MexR and NalC (40). **(B)** Predicted LasR receptor insensitivity to C-30 based on the lock-and-key concept (36). AI: autoinducer, QS: quorum sensing, OM: outer membrane, CM: cytosolic membrane.