Spatially dependent alkyl quinolone signaling responses to antibiotics in Pseudomonas aeruginosa swarms

Nydia Morales-Soto, Sage J. B. Dunham, Nameera F. Baig, Joanna F. Ellis, Chinedu S. Madukoma, Paul W. Bohn, Jonathan V. Sweedler, and Joshua D. Shrout

From the Departments of Civil and Environmental Engineering and Earth Sciences, Chemistry and Biochemistry, Chemical and Biomolecular Engineering, and Biological Sciences, University of Notre Dame, Notre Dame, Indiana 46556 and the Department of Chemistry and the Beckman Institute for Advanced Science and Technology, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

There is a general lack of understanding about how communities of bacteria respond to exogenous toxins such as antibiotics. Most of our understanding of community-level stress responses comes from the study of stationary biofilm communities. Although several community behaviors and production of specific biomolecules affecting biofilm development and associated behavior have been described for Pseudomonas aeruginosa and other bacteria, we have little appreciation for the production and dispersal of secreted metabolites within the 2D and 3D spaces they occupy as they colonize, spread, and grow on surfaces. Here we specifically studied the phenotypic responses and spatial variability of alkyl quinolones, including the Pseudomonas quinolone signal (PQS) and members of the alkyl hydroxyquinoline (AQNO) subclass, in P. aeruginosa plate-assay swarming communities. We found that PQS production was not a universal signaling response to antibiotics, as tobramycin elicited an alkyl quinolone response, whereas carbenicillin did not. We also found that PQS and AQNO profiles in response to tobramycin were markedly distinct and influenced these swarms on different spatial scales. At some tobramycin exposures, P. aeruginosa swarms produced alkyl quinolones in the range of 150 μM PQS and 400 μM AQNO that accumulated as aggregates. Our collective findings show that the distribution of alkyl quinolones can vary by several orders of magnitude within the same swarming community. More notably, our results suggest that multiple intercellular signals acting on different spatial scales can be triggered by one common cue.

Bacteria do not respond to stressors such as antibiotics in any standard way. However, most of the detailed research performed to understand the bacterially secreted responses to specific antibiotics has utilized information from homogenized bacterial communities (1–5). Indeed, most biological systems are assumed to exhibit diffusion-limited chemical distributions that are largely homogeneous. Yet, microbial communities, such as biofilms, are composed of individual cells that do not sense stress (or even die) equally throughout their occupied space. Thus, there is an urgent need to spatially map the biochemical profiles of these microbial communities. Here, we exploit multimodal chemical imaging to study spatial heterogeneities within motile “pre-biofilm” swarm communities of the bacterium Pseudomonas aeruginosa. P. aeruginosa is an opportunistic pathogen and one of many bacteria that displays numerous community behaviors, including an ability to readily form surface-attached biofilms. Before establishing stationary biofilm communities, P. aeruginosa is known in vitro to exhibit swarming (1, 6, 7), a group motility behavior employed by some bacteria to explore and expand during surface colonization. Although many studies have addressed biofilm development and the transition to static bacterial biofilms, the community behaviors exhibited by motile bacteria are less understood. In this study, we show that the production of alkyl quinolones (AQs) by P. aeruginosa swarming communities is substantial, and quinolone secretion varies drastically when exposed to the aminoglycoside antibiotic tobramycin as opposed to the β-lactam antibiotic carbenicillin.

Planktonic cells are generally sensitive to antibiotics, whereas surface-attached biofilms and swarming communities display increased survival and resistance (3, 8–11). Therefore, it is imperative to understand how bacterial communities coordinate colonization of new surfaces and how this helps them endure the stress of traditional antibiotics. The apparent invulnerability of biofilms to antimicrobials is generally attributed to physical protection provided by the communalextracellularpolymeric substance layer (12) and a change in metabolic state. However, during the pre-biofilm stage of swarming, introcellu-

The abbreviations used are: AQ, alkyl quinolone; PQS, Pseudomonas quinolone signal; C9-PQS, 2-heptyl-3-nonyl-4(1H)-quinolone; AQNO, alkyl hydroxyquinoline; HHQ, 2-heptyl-4(1H)-quinolone; NHQ, 2-nonyl-4(1H)-quinolone; SIMS, secondary ion mass spectrometry; CRM, confocal Raman microscopy; HQNO, 2-heptyl-4-hydroxyquinoline N-oxide; NONO, 2-nonyl-4-hydroxyquinoline N-oxide; PCA, principal component analysis; PC1 and PC2, principal component 1 and 2, respectively; PI, propidium iodide; FAB, fastidious anaerobe broth.

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3 To whom correspondence should be addressed: Dept. of Civil and Environmental Engineering and Earth Sciences, University of Notre Dame, Notre Dame, IN 46565. Tel.: 574-631-1726; E-mail: joshua.shrout@nd.edu.

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lar cyclic-di-GMP levels are low, extracellular polymeric substance production is down-regulated, and cells are actively growing, resulting in the hypothesis that antimicrobial survival in swarming communities is associated with high cell density (3, 13, 14). Because it is unlikely that survival arises exclusively from high cell density, it is vital to understand whether and how the secretome of swarming communities promotes antimicrobial tolerance.

One critically important class of molecules produced and secreted by P. aeruginosa is the nitrogen-containing heterocyclic AQs (15). We have previously identified members of the AQ family as principal swarm community metabolites (16). Over 50 distinct AQs have been identified in P. aeruginosa, falling into three primary subclasses: (i) 2-alkyl-4(1H)-quinolones, such as 2-heptyl-4(1H)-quinolone (HHQ) and 2-nonyl-4(1H)-quinolone (NHQ), which have the simplest base structure with only one oxygen; (ii) 2-alkyl-3-hydroxy-4(1H)-quinolone (PQS); and (iii) 2-alkyl-3-hydroxy-4(1H)-quinolone (Pseudomonas quinolone signal; PQS) and 2-heptyl-3-nonyl-4(1H)-quinolone (C9-PQS); and (iii) 2-alkyl-4-hydroxyquinoline N-oxides (AQNOs), such as 2-heptyl-4-hydroxyquinoline N-oxide (HQNO) and 2-nonyl-4-hydroxyquinoline N-oxide (NQNO), which are characterized by the presence of an amine oxide bond (17). Of the molecules that belong to these subclasses, the roles of HHQ, PQS, and HQNO are particularly well-documented. The AQs belonging to the PQS pathway participate in processes as diverse as intercellular quorum-sensing signaling, virulence regulation, biofilm development, iron chelation, antimicrobial activity, stress response, and control of cell death (15, 18–27).

In the Pseudomonas literature, AQs are generally presented to contain seven-carbon (C7; UHQ, C7-PQS, and UQNO) side-chain variations, the positive loadings of principal component 1 (PC1; Fig. 2A) closely resembled that of unexposed cells (Fig. 1A), whereas cells exposed to carbenicillin were exceptionally elongated (Fig. 1C). We hypothesized that the P. aeruginosa responses to these two different antibiotic exposures would also be chemically distinct.

To investigate the in situ biochemical response to tobramycin and carbenicillin, we examined intact P. aeruginosa plate-assembly swarms with nondestructive CRM and implemented principal component analysis (PCA) to profile spectral variations following exposure to these antibiotics. Our previous investigations of P. aeruginosa with CRM revealed features of AQs that are distinguishable in the 1338–1376 cm$^{-1}$ window, attributed to the quinoline ring stretch (16). PQS and AQNO subclasses produce specific CRM features (Fig. S1) enabling their differentiation (16, 31).

The distribution of AQs similar to PQS and AQNO varied significantly within the P. aeruginosa swarms examined, and several aspects of the AQ profiles were uniquely linked to the responses to either tobramycin or carbenicillin (Fig. 2). Raman PCA revealed that PQS and its derivatives localized toward the center of the unexposed swarms when compared with the edges, whereas AQNOs were distributed more uniformly throughout the entire swarm (Fig. 2A and Fig. S2 (A and B)). The loading plots showcasing the most significant chemical variations, the positive loadings of principal component 1 (PC1; Fig. 2A), from the edge of the swarms with no exposure to antibiotics, presented features consistent with the HQNO and NQNO standards (Fig. S1), identifying AQNOs as substantial metabolites in expanding swarm colonies.

CRM of swarms exposed to 25 µg of tobramycin revealed spectral features and spatial distributions that differ dramati-

### Results

**Two antibiotic classes elicit universal P. aeruginosa swarm motility responses but distinct chemical responses**

Inhibitory levels of tobramycin and carbenicillin that elicited similar reductions in plate-assay swarming at the macroscopic level (Fig. 1) produced markedly different single-cell phenotypes. Similar to previous single-cell results (34–37), the morphology of cells exposed to tobramycin (Fig. 1B) closely resembled that of unexposed cells (Fig. 1A), whereas cells exposed to carbenicillin were exceptionally elongated (Fig. 1C). We hypothesized that the P. aeruginosa responses to these two different antibiotic exposures would also be chemically distinct.

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![Figure 2](https://example.com/figure2.png)

**Figure 2. P. aeruginosa swarms exhibit chemically distinct responses to specific antibiotics.** Swarm colonies exposed to 0 μg (A) or 25 μg (B) tobramycin (TOB) and 400 μg of carbenicillin (CAR) (C) were analyzed 48 h after exposure to antibiotics by combining CRM (Raman image; includes both PQS and AQNO subclasses) and PCA analysis (loading plots and score images on PC1 and PC2) to identify chemically significant variations within the samples. Shown are representative CRM results (*n* = 3) collected from swarm regions proximal to the antibiotic treatment. Scale bars on Raman images, 30 μm. Loading plots for PC1 and PC2 include features corresponding to Raman spectra from cellular components (black), PQS/C9-PQS (blue), and AQNOs (HQNO/NQNO; red). Score images of PC1 and PC2 show the distribution of each of the principal components.

Physically from the unexposed condition, featuring a prominent presence of PQS (PC1; Fig. 2B). Additionally, the positive loadings of PC2 exhibited strong AQNO features, indicating that both PQS and AQNO subclasses aggregate within the edge regions of swarms exposed to tobramycin. To further test this observation, we examined a moderately inhibitory tobramycin exposure condition of 10 μg that resulted in a diminished swarm response (Fig. S2, C and D). Similar trends in AQ distribution were observed in these swarms, with features corresponding to the PQS subclass dominating the stunted edge of the swarm in close proximity to the added tobramycin and AQNOs dominating the expanding swarm periphery opposite the antibiotic, similar to the unexposed swarm profile. The metabolite profile of swarms exposed to carbenicillin (Fig. 2C) stood in stark contrast to that of swarms exposed to tobramycin (Fig. 2B). Whereas exposure to 400 μg carbenicillin resulted in the characteristic reduction in swarming, PQS was not detected. Whereas the loading plots of PC1 and PC2 closely resembled the profile of the unexposed control condition, the corresponding score images were substantially different (Fig. 2C), not displaying any of the rich spatial structure evident in Fig. 2A.

**PQS promotes cell death and reduced swarming for cells exposed to tobramycin**

Because previous reports have shown various roles for PQS, namely as a stress response, for planktonic *P. aeruginosa* cells exposed to antimicrobials (22, 26, 38–40), we were interested to explore how AQs mediate *P. aeruginosa* behavior in motile, pre-biofilm swarm communities. Additionally, the CRM chemical profile of *P. aeruginosa* swarms provided evidence that tobramycin exposure cues aspects of PQS production or regulation that carbenicillin does not (Fig. 2). Thus, we probed the plate-assay swarming behavior of *P. aeruginosa* WT and PQS-deficient (ΔpqsH) and AQNO-deficient (ΔpqsL) strains in the presence of tobramycin.

Although increased concentrations of tobramycin led to a decrease in swarm coverage area for all strains (Fig. 3, A and B), overall expansion of the PQS− (ΔpqsH) swarm was significantly less affected. Cells within swarms of PQS− strains that were not exposed to tobramycin showed cell death localized toward the swarm center as determined by propidium iodide (PI) staining (Fig. 3A). This result agrees with prior reports that show PQS to be involved in cell death and swarm repression (22, 24) and with our CRM results that show the PQS subclass present at the swarm center under these conditions (Fig. S2). Cell death in both WT and AQNO− strains is primarily localized toward the side of the swarm closest to 10 μg of tobramycin and distributed evenly in swarms exposed to 25 μg (Fig. 3A). The ΔpqsL strain, which produces PQS and its derivatives but not the AQNO molecules, does not show a reduced swarm or cell-death phenotype compared with the WT at any of the tested tobramycin exposures (0, 10, and 25 μg) (Fig. 3B). However, swarms of the PQS− strain (ΔpqsH) are less sensitive to tobramycin and present significantly reduced cell death compared with PQS+ strains (Fig. 3, B and C).

Our combined results show that although swarms of WT *P. aeruginosa* exposed to tobramycin undergo a chemical shift that favored PQS (Fig. 2), swarms of the PQS− strain (ΔpqsH) appeared better suited for survival of tobramycin treatment compared with swarms of WT and AQNO-deficient (ΔpqsL)
strains (Fig. 3). As expected, planktonic cells of the PQS− strain (ΔpqsH) were less susceptible to tobramycin than WT and AQNO− strains (Fig. 4A), whereas untreated cells of the three strains behaved similarly (Fig. S3). However, this was not the case for cells exposed to carbenicillin, which did not elicit a PQS response (Fig. 2C). No survival differences were observed between the WT and AQ mutants in planktonic cultures exposed to carbenicillin (Fig. 4B).

**P. aeruginosa independently modulates the PQS and AQNO subclasses in the presence of tobramycin**

Whereas the AQ response of *P. aeruginosa* to tobramycin varied in a dose-dependent manner, the relative AQ levels were also spatially heterogeneous within these plate-assay swarms. In an effort to better understand how swarming communities modulate AQ production in the presence of tobramycin, we employed SIMS product ion imaging to analyze *P. aeruginosa* swarms exposed to 0, 10, or 25 µg of tobramycin (Fig. 4B). SIMS product ion imaging was used to specifically target the seven- and nine-carbon AQS (i.e. PQS, C9-PQS, HQNO, and NQNO). Representative results for PQS and HQNO are shown in Fig. 5, and all replicates for PQS, C9-PQS, HQNO, and NQNO are shown in Fig. S4 (A–D).

SIMS product ion imaging revealed that the overall average intensity of the four targeted AQS increased significantly with the addition of tobramycin, with the largest spatial variation occurring for the PQS subclass (Fig. 5B and Fig. S4E). For swarms not exposed to tobramycin, differences in AQ distributions were indistinguishable by visual inspection of the ion images, but under 10 and 25 µg of exposure, prominent differences in distribution emerged. The most prominent difference is seen at 25-µg exposure, where PQS and C9-PQS were distributed both within the swarm center and in the swarm periphery, whereas HQNO and NQNO were detectable primarily in the swarm center (Fig. 5A and Fig. S4F). We assessed the relative abundance of AQS in swarm regions with less motile and more protected cells (swarm center) with the most motile and exposed cells (swarm edge). In swarms exposed to 0 or 10 µg of tobramycin, the relative abundance of the PQS subclass is significantly higher toward the center of the swarm (Fig. 5C and Fig. S4F). This is in stark contrast to the 25-µg exposure, where the ratio shifts to reveal a significantly greater relative abundance of PQS/HQNO and C9-PQS/NQNO at the edge of the swarm compared with the center (Fig. 5C and Fig. S4F). This edge region is indicative of a less motile phenotype, as evidenced by the lack of swarm expansion. We also assessed the relative abundance of AQS among swarming cells closest to the antibiotic exposure (Near) compared with swarming cells farthest from the antibiotic exposure (Away). Alterations in the PQS/HQNO (Fig. 5D) and C9-PQS/NQNO (Fig. S4G) ratios were observed over 2D space for the 10-µg tobramycin condition, with significantly higher intensities of the PQS subclass relative to HQNO “near” the tobramycin source. A proportional increase in PQS (Fig. 5B) and C9-PQS (Fig. S4E, H and I) as a function of tobramycin exposure was observed on both sides of the swarm. Although increases in multiple AQNO intensities were detected, these increases were not equivalent on both sides. The region near the 10 µg of tobramycin presented reduced levels of HQNO compared with the region “away” from tobramycin (Fig. S4, J and K). Notably, no difference in the ratio of the PQS/AQNO subclasses was observed between the near and away regions of the 0- and 25-µg exposure levels,
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Figure 5. Exposure to tobramycin elicits changes in spatial distribution and relative abundance of PQS and AQNOs. A, representative 2D IMS product ion images for PQS (m/z 260→175) and HQNO (m/z 260→159) ions in P. aeruginosa swarms following exposure to 0, 10, and 25 µg of tobramycin (TOB). Scale bars, 2 mm. Relative quantification of PQS and HQNO determined from the average intensity of each ion normalized to swarm area coverage was compared for entire swarms (B), swarm “edge” and “center” (C), and the near (left) and away (right) tobramycin regions of the swarms (D). For each condition, three replicates were analyzed. Results are mean ± S.D. (error bars). Unpaired t test p values are provided above the comparison brackets. E and F, the PC2 loadings and score images show the separation between PQS (positive loadings) and HQNO (negative loadings) at 10 µg (E) and 25 µg (F) of tobramycin.

Figure 5: Exposure to tobramycin elicits changes in spatial distribution and relative abundance of PQS and AQNOs. A, representative 2D IMS product ion images for PQS (m/z 260 → 175) and HQNO (m/z 260 → 159) ions in P. aeruginosa swarms following exposure to 0, 10, and 25 µg of tobramycin (TOB). Scale bars, 2 mm. Relative quantification of PQS and HQNO determined from the average intensity of each ion normalized to swarm area coverage was compared for entire swarms (B), swarm “edge” and “center” (C), and the near (left) and away (right) tobramycin regions of the swarms (D). For each condition, three replicates were analyzed. Results are mean ± S.D. (error bars). Unpaired t test p values are provided above the comparison brackets. E and F, the PC2 loadings and score images show the separation between PQS (positive loadings) and HQNO (negative loadings) at 10 µg (E) and 25 µg (F) of tobramycin.

probably due to the symmetry of these swarm colonies (Fig. 5D and Fig. S4G).

Imaging PCA was performed on the IMS product ion images to account for the complex interactions of the multiple product ions associated with each AQ (Fig. 5E and F) and Fig. S5) (41). The IMS product ion imaging PCA results corroborate the relative quantification results (Fig. 5, C and D) and validate the CRM observations (Fig. 2), indicating that when exposed to the aminoglycoside tobramycin, P. aeruginosa cells undergo a metabolite shift that results in independent modulations of the PQS, C9-PQS, HQNO, and NQNO profiles that are dependent on the proximity to the antibiotic treatment. The PQS response was not uniformly distributed throughout the swarm community at either exposure we analyzed. However, whereas AQNO levels also increased in a dose-dependent manner, the recorded AQNO response was more spatially uniform throughout the entire swarm community of cells.

The amounts of PQS and HQNO produced by these swarming cells were greater than those previously reported for P. aeruginosa planktonic cells using other methods (17). We directly compared the unexposed and tobramycin-exposed swarms with surface density data of analytical standards for both PQS and AQNO subclasses (Fig. S6). Whereas the detailed 3D geometry of these swarms is not known, we estimated the swarm community height to be between 5 and 20 µm (36). Accordingly, the PQS and AQNO concentrations were estimated to be between 60 and 150 µM and between 100 and 600 µM, respectively (Fig. S7). Such concentrations would exceed the solubility of AQs in water (42), which is supported by the visual identification of aggregates using standard light microscopy (Fig. S8). CRM of these aggregates identifies spectral features corresponding to the PQS and AQNO subclasses. These aggregates well exceed the size of single cells or vesicle blebs. Whether these aggregates occur within the CF environment is currently unknown, as are the benefits (if any) of such structures in the biology of P. aeruginosa.

Discussion

Here we have shown that PQS and the associated signaling pathway is not universally invoked in response to all antibiotics. Whereas exposure to the aminoglycoside tobramycin and the β-lactam carbenicillin produced analogous reductions in plate-assay swarming, at the gross cellular morphology and chemical levels, the responses were quite distinct. Although cells exposed to carbenicillin exhibit a dramatic morphology change, we found that exposure to tobramycin, but not carbenicillin, resulted in profile shifts of both PQS and AQNO families during swarming. This is in contrast to reports that have suggested a general PQS response to antimicrobials (22, 26, 27). Our findings are consistent with the hypothesis that AQ modulations following tobramycin exposure during pre-biofilm swarming should contribute to antibiotic tolerance (22, 25, 38); however, other factors must control P. aeruginosa tolerance to carbenicillin.

Surprisingly, we routinely observe PQS and AQNOs in aggregates that are significantly larger than single cells. The behavior of externally applied HQNO on swarm medium suggests that these structures arise from interactions of the AQ molecules with their immediate environment. On their own, these aggregates lack mobility (no spreading visible on swarm media), probably relying on the mobility of the swarm, be it by interactions with the cells or by the action of surfactants such as rhamnolipids (42) for translocation.

Much of what was known regarding the chemical profile of P. aeruginosa communities arises from some combination of homogenized samples and extrapolation of nucleic acid profile data (1, 2, 4, 5). A notable example stems from the work of...
Phelan et al. (44) in which colony biofilms of *P. aeruginosa* strains exposed to increasing concentrations of azithromycin were analyzed by imaging MS. In contrast, there is considerably less information about the spatial distribution of biomolecules associated with *P. aeruginosa* pre-biofilm swarms and how these communities respond both physically and chemically to subinhibitory antibiotic concentrations. Here, we used multimodal chemical imaging to assess biochemical profiles and other aspects of cell behavior preserving the 2D heterogeneity of secreted factors. Employing CRM and SIMS imaging allowed both universal and antibiotic-specific chemical responses to be identified in these communities, as well as localized responses within each swarm colony that depend on the concentration of the antibiotic. These results model and preview the complex biological responses that surface-attached biofilms are likely to exhibit in the clinical setting, where antibiotic gradients may alter the physiochemical profile of some but not all cells in a community, and cell concentrations as well as strain mutations could alter sensitivity to specific antimicrobials.

Our assessment of several metrics at different antibiotic exposure levels leads us to conclude that PQS is a short-range signal compared with AQNOs, both of which increase in response to tobramycin. Clear distinctions in the AQ spatial profile were apparent in 2D space. At 10–μg tobramycin exposure, which still allowed for some *P. aeruginosa* swarming, we observed PQS to be most prevalent in the region nearest the antibiotic, whereas AQNOs localized farthest away from the tobramycin spot on the swarm tendrils. Swarms exposed to 25 μg of tobramycin did not spread over surfaces, and these samples showed a clear distribution bias of PQS toward the swarm edge relative to the center. Our collective evidence indicates that PQS is the AQ signal associated with the most severe stress response and swarming repression upon tobramycin exposure. The spatial partitioning of PQS to the regions proximal to the highest tobramycin concentrations could serve to protect pre-biofilm communities from the deleterious effects of tobramycin or to condition swarms to antimicrobial stress, or it be linked to the role of PQS in cell death and biofilm formation (10, 20–22, 24, 45). In response to tobramycin, AQNOs were distributed throughout the swarm, which leads us to conclude that these molecules act on a community-wide scale.

Aminoglycoside antibiotics are known to primarily bind to the 30S ribosomal subunit, inhibiting protein synthesis (46). As a consequence, aminoglycosides have also been shown to disrupt the cell envelope, leading to cell death. Interestingly, due to its polycationic nature, tobramycin readily binds nucleic acids (47) and is reported to bind to mucin- and DNA-rich fractions of cystic fibrosis sputum (48), reducing effectiveness in the treatment of polymicrobial infections of *P. aeruginosa* and *Staphylococcus aureus* (18). Shifting the balance of PQS and AQNOs may alter cell-membrane permeability and damage, leading to higher concentrations of easily accessible DNA at the edge of the swarm facing the aminoglycoside.

In *P. aeruginosa*, the PQS molecule and associated regulatory pathway are linked to numerous cellular processes including swarm repression, stress response, cell lysis, DNA release, outer membrane vesicle biogenesis, and biofilm development (20–22, 24, 29, 45). The AQNOs, HQNO and NQNO, are both effective anti-staphylococcal molecules that modulate the interaction between *P. aeruginosa* and *S. aureus* (18, 25). Because both PQS and AQNOs serve critical roles, we expect that the ubiquitous nature of AQNOs may serve as protection or interspecies community-wide communication for these pre-biofilm explorers, whereas the abundance of PQS at the center of the swarm may promote a biofilm state. Although the PQS− strain (ΔpqsH) shows a significant swarm reduction in response to tobramycin, it also shows significantly less cell death than either the WT or the AQNO mutant (ΔpqsL). The overall reduction of swarm expansion in response to tobramycin and carbenicillin for all strains suggests that other mechanisms that control the physical behavior of the swarm may come into play (36). Because neither the ΔpqsH nor ΔpqsL strains show significant increases in AQNO or PQS, respectively, upon exposure to tobramycin, we conclude that AQs in general, and the PQS pathway specifically, are not regulated by a classical mechanism of transcriptional induction or feedback inhibition.

WT (and ΔpqsL) swarms that produce PQS exhibited increased cell death response to tobramycin exposure. Our research showed greater levels of expressed DNA in the swarm region closer to tobramycin (Fig. 3). This barrier of highly accessible DNA or damaged cells could temporarily prevent swarm expansion to the region closer to the antibiotic, cue biofilm development, or serve as cellular decoys to bind and sequester tobramycin. Thus, DNA may protect swarming cells from the action of aminoglycosides, as has been found within stationary biofilms (10, 47).

Our multiplexing approach allowed us to obtain a comprehensive picture of *P. aeruginosa* behavior in bacterial communities that could not be observed in most laboratory-scale systems. The results argue against PQS serving as a generalized cellular “preconditioning” cue to stress. Instead, they are more consistent with PQS serving as a specific and short-range signal. We find dramatic spatial heterogeneity of PQS and other AQ molecules in these high-density *P. aeruginosa* swarm communities. Clearly, our current understanding of *P. aeruginosa* quinolone signaling is limited, because the AQ distributions within these swarming communities cannot be explained solely by population-dependent quorum sensing or stress response dogma. Our findings indicate that PQS and AQNO are independently regulated signals intended to communicate differing messages. After the closest *P. aeruginosa* cells sense tobramycin exposure, PQS signals reach only a nearby subset of the community, whereas AQNOs propagate community-wide. Thus, before developing attached biofilms, *P. aeruginosa* may be capable of relaying a complex hierarchy of chemical messages to community members in response to a single environmental stimulus.

The spatial range of alkyl quinolone/quinoline signaling in vivo is not yet clear. Indeed, translation of in vitro laboratory-based assays that enable biofilm development, swarming, or other surface motility phenotypes to the colonization and infection of eukaryotic cells is difficult. However, our findings clearly show that spatial heterogeneity in signaling is possible within a *P. aeruginosa* community. Accordingly, subpopulations of *P. aeruginosa* can be predicted to exhibit differing responses and pathogenic behavior within any given infection.
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### Table 1: Bacterial strains, plasmids, and specialized materials

| Strain/Plasmid | Description | Source or reference |
|----------------|-------------|---------------------|
| **P. aeruginosa PA14** Strain | Wildtype | Wildtype strain | Ref. 52 |
| | Wildtype GFP | PA14 mini-Tn7-gfp2, Gm′ | This study |
| | ΔpqsH | ΔpqsH in PA14 wildtype | Ref. 53 |
| | ΔpqsL | ΔpqsL in PA14 wildtype | Ref. 17 |
| | ΔpqsA | ΔpqsA (U479) in PA14 wildtype | PA14 Transposon Insertion Mutant Library (54) |

| Plasmids | Description | Source or reference |
|-----------|-------------|---------------------|
| AKN66 | Mini-Tn7-gfp2; Gm′, Cm′ | Ref. 55 |
| pUX-BF13 | Conjugation helper plasmid, Ap′ | Ref. 50 |
| pRK600 | Mobilization plasmid, Cm′ | Ref. 43 |

| Specialized materials | Description | Source or reference |
|-----------------------|-------------|---------------------|
| HHQ | 2-Heptyl-4-quinolone | Sigma |
| NHQ | 2-Nonyl-4-quinolone | Cayman Chemicals |
| PQS | 2-Heptyl-3-hydroxy-4 (1H)-quinolone | Cayman Chemicals |
| C9-PQS | 2-Nonyl-3-hydroxy-4 (1H)-quinolone | Cayman Chemicals |
| HQNO | 2-Heptyl-4-hydroxyquinoline N-oxide | Cayman Chemicals |
| NQNO | 2-Nonyl-4-hydroxyquinoline N-oxide | Cayman Chemicals |
| Copper tape | 0.088-mm-thick double-sided conductive copper tape, 3M 1182 | Ted Pella |

Such variations in behavior would be underestimated or entirely missed when assessing the proteomic, genomic, or other phenotype using a homogeneous analytical approach.

### Experimental procedures

**Bacterial strains and culturing conditions**

Strains, plasmids, and specialized materials used in this study are listed in Table 1. Planktonic cultures were grown by inoculation of isolated bacterial colonies from lysogeny broth agar into 6 ml of modified FAB minimal medium supplemented with 30 mM glucose. Cultures were grown overnight (<16–20 h) at 37 °C, 240 rpm.

A GFP-expressing version of *P. aeruginosa* PA14 was chromosomally labeled with GFP using a mini-Tn7-gfp2 cassette introduced by four-parental mating (49). GFP expression was confirmed via microscopy.

**Swarm assays**

Swarm motility plate assays were performed in 60-mm diameter Petri dishes containing 7.5 ml of modified FAB culture medium supplemented with 12 mM glucose and solidified with 0.45% Noble agar (Sigma) (16). Response to antibiotics was tested by adding 5 µl of diluted antibiotics to 10-mm diameter filter discs (Sigma) placed on top of the swarm medium. Normalized cultures (*A*<sub>600 nm</sub> = 0.5) were inoculated with a platinum needle 10 mm away from the edge of the filter disc containing the antibiotic treatment. For assays in which the extent of cell damage or DNA release was determined by microscopy, 20 µM PI was added to the medium before solidification. Swarm assays were incubated inverted at 30 °C, 85% relative humidity, for 48 h and removed for analysis. Swarms were analyzed directly without further preparation by confocal laser-scanning microscopy and CRM or processed for SIMS imaging.

**Microtiter dish antibiotic susceptibility assays**

Assays were performed in a 96-well microtiter dish containing 199 µl of normalized undiluted or 1,200-fold diluted *P. aeruginosa* cultures in FAB supplemented with 12 mM glucose. Three replicates containing 1 µl of antibiotic dilutions in sterile H<sub>2</sub>O for final antibiotic concentrations of 0 µg/ml, 1.0 µg/ml tobramycin, and 100 µg/ml carbenicillin were included. Cultures were incubated in a Biotek H1 Synergy microplate reader at 37 °C, and *A*<sub>600 nm</sub> readings were collected every 1 h. Data analysis was performed with GraphPad Prism.

**Microscopy, CRM imaging, SIMS imaging, product ion imaging, relative quantification, and image analysis**

Optical images of cells within swarms were acquired using fluorescence microscopy or confocal laser-scanning microscopy as described previously (6, 36) and as detailed in supporting Experimental procedures. CRM imaging was performed as described previously (16) and as detailed in supporting Experimental procedures. Swarm samples were prepared for examination via TOF SIMS imaging using a custom instrument described in detail elsewhere (51) and subsequently analyzed as detailed in supporting Experimental procedures.

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