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ORIGINAL ARTICLE

Quorum quenching quandary: resistance to antivirulence compounds

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Quorum sensing (QS) is the regulation of gene expression in response to the concentration of small signal molecules, and its inactivation has been suggested to have great potential to attenuate microbial virulence. It is assumed that unlike antimicrobials, inhibition of QS should cause less Darwinian selection pressure for bacterial resistance. Using the opportunistic pathogen *Pseudomonas aeruginosa*, we demonstrate here that bacterial resistance arises rapidly to the best-characterized compound that inhibits QS (brominated furanone C-30) due to mutations that increase the efflux of C-30. Critically, the C-30-resistant mutant *mexR* was more pathogenic to *Caenorhabditis elegans* in the presence of C-30, and the same mutation arises in bacteria responsible for chronic cystic fibrosis infections. Therefore, bacteria may evolve resistance to many new pharmaceuticals thought impervious to resistance.

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Introduction

Bacteria have been identified that are resistant to all known antibiotics (Defoirdt *et al.*, 2010), and infectious diseases remain the leading cause of death (Rasko and Sperandio, 2010); hence, it is important to develop new antimicrobials. Indeed, it has been decreed that we live in a post-antibiotic era and heralded that anti-quorum sensing (QS)/anti-virulence methods hold great promise for treating bacterial infections (Rasko and Sperandio, 2010). One of the most attractive features of this approach is that by interrupting cell signaling, these approaches do not impose harsh or direct selective pressure like antibiotics (Bjarnsholt *et al.*, 2010), so there is less evolutionary pressure to develop resistance to antivirulence compounds (Bjarnsholt *et al.*, 2010; Rasko and Sperandio, 2010).

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The best-characterized of the antivirulence compounds are the brominated furanones, which are secreted by the seaweed *Delisea pulchra* to prevent biofilms from inhibiting its photosynthesis; to date, no bacteria have been identified that are resistant to them. The natural compound (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone from *D. pulchra* inhibits both acyl-homoserine lactone-based and autoinducer 2-based QS (Ren *et al.*, 2001), and the synthetic furanone C-30 (Figure 1a inset) has been shown to decrease acyl-homoserine lactone-based signaling as well as decrease the virulence of *P. aeruginosa* in a mouse pulmonary infection model (Hentzer *et al.*, 2003). These brominated furanones interrupt QS by interacting with transcriptional regulators that propagate the QS response (Defoirdt *et al.*, 2007), and they do not affect bacterial growth in rich medium (Gram *et al.*, 1996; Ren *et al.*, 2001; Hentzer *et al.*, 2003). However, growth of pathogens in the host during infections is more likely to involve non-robust carbon sources and may involve compounds whose utilization depends on QS (Defoirdt *et al.*, 2010). Under these conditions, there will be selection pressure to evolve resistance against the compounds

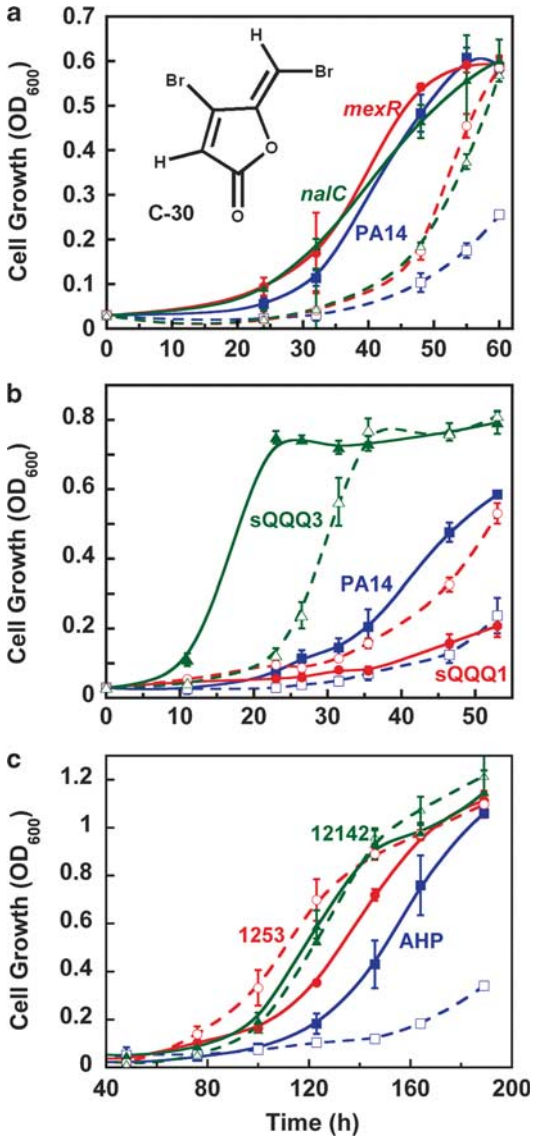

Figure 1 The *mexR* and *nalC* mutations render *P. aeruginosa* less sensitive to QQ compound C-30 during growth in adenosine minimal medium. (**a**) Growth (at 37 °C) of wild-type *P. aeruginosa* PA14 (blue squares), transposon mutant *mexR* (red circles) and transposon mutant *nalC* (green triangles). (**b**) Growth of spontaneous quorum quenching quandary mutants (sQQQ) 1 (red circles) and sQQQ3 (green triangles) vs wild-type PA14 (blue squares). (**c**) Growth of CF clinical isolate 1253 (red circles) and CF Liverpool epidemic strain 12142 (green triangles) with enhanced *mexA* expression vs control strain AHP (blue squares). Open symbols indicate the presence of 50 μM C-30, whereas closed symbols indicate the absence of C-30. Data represent the mean ± s.d.; $n = 3$.

that block QS, and it has been theorized, but not shown, that resistance may arise (Defoirdt *et al.*, 2010). Note that previously mutations to the QS response regulator LuxR were generated in *Escherichia coli*, which altered both the binding of the natural ligand as well as that of quorum quenching (QQ) compounds, but resistance was not investigated and was predicted not to occur (Koch *et al.*, 2005).

To investigate whether QQ-resistant bacterial mutants may arise in the presence of antivirulence compounds, we utilized the best studied bacterium for QS, *P. aeruginosa*, which is an opportunistic pathogen that is responsible for many infections, including those of ventilator-associated pneumonia, urinary and peritoneal dialysis, catheter infections, bacterial keratitis, otitis externa, burns, wound infections and those of the lung (Macé *et al.*, 2008). Wild-type *P. aeruginosa* PA14 was used instead of PAO1 because PA14 is more virulent than PAO1 in diverse infection models (Harrison *et al.*, 2010) and because of the availability of the complete mutant library (Liberati *et al.*, 2006). Our strategy was to utilize a minimal medium (so it resembles more closely clinical situations) using a growth compound whose assimilation requires QS; therefore, QS and growth were inhibited by the antivirulence compound C-30, which has become the gold standard for antivirulence compounds. Growth on adenosine by *P. aeruginosa* depends on the degradative enzyme, nucleoside hydrolase, which is positively controlled by LasR (Heurlier *et al.*, 2005). LasR is the transcriptional regulator that mediates acyl-homoserine lactone-based QS in this strain via *N*-3-oxododecanoyl homoserine lactone ($3OC_{12}$-HSL). In addition, furanone derivatives including C-30 have been predicted to bind LasR at the $3OC_{12}$-HSL binding site to inactivate LasR (Kim *et al.*, 2008; Yang *et al.*, 2009). Hence, growth on adenosine in minimal medium depends on a functional LasR QS pathway (Heurlier *et al.*, 2005), and furanone probably inhibits this growth via LasR. Therefore, we reasoned that mutations that confer resistance to furanone C-30 will disrupt the ability of furanone to inhibit LasR-mediated QS in this strain, and these mutants may be identified easily based on our selection method (faster growth).

It was also advantageous to utilize growth on adenosine as our model system because adenosine is released into the human intestinal tissues and lumen during surgical injury, ischemia and inflammation (Patel *et al.*, 2007; Zaborin *et al.*, 2009), and adenosine induces the *P. aeruginosa* virulence factor PA-I lectin/adhesin, which leads to lethal gut-derived sepsis (Patel *et al.*, 2007). Note the extracellular concentration of adenosine, which is generated by breakdown of secreted ATP (Crane *et al.*, 2002), can reach 5 mM (Kimura *et al.*, 2005) and increase 10^9-fold in human intestinal epithelial cells after exposure to hypoxia (Patel *et al.*, 2007). Because adenosine is secreted by the host as a cytoprotective compound that tightens epithelial cell junctions, *P. aeruginosa* probably metabolizes adenosine to increase its ability to act as a pathogen (Patel *et al.*, 2007). Furthermore, although *P. aeruginosa* is better known as a respiratory pathogen rather than an intestinal pathogen, this strain is one of the most common opportunistic pathogens in the normal gastrointestinal tract (Alverdy *et al.*, 2000), and in critically-ill and immunocompromised

patients where stress levels are high, *Pseudomonas* sp. counts have been shown to increase by as much as 100-fold (Shimizu *et al.*, 2006) leading to the expression of virulence determinants (Alverdy *et al.*, 2000) that are controlled by QS (Hegde *et al.*, 2009) (just the presence of *P. aeruginosa* in the gastrointestinal tract of critically-ill surgical patients has been associated with nearly 70% mortality (Alverdy *et al.*, 2000)). Hence, growth on this carbon source is physiologically relevant and *P. aeruginosa* infections in the gastrointestinal tract are pertinent.

Using the novel adenosine screen, we found that *P. aeruginosa* cells may evolve resistance to the QQ compound C-30. The mechanism for this resistance to QQ was determined to be due to *mexR* and *nalC* mutations, which encode repressors of the *mexAB-oprM* multi-drug resistance operon; hence, the mutants have enhanced efflux of C-30. In addition, we demonstrate that the mutants are resistant to C-30 in an animal model. We also show that *P. aeruginosa* isolates with mutations in *mexR* and *nalC* that arise in chronic cystic fibrosis (CF) patients after antibiotic therapy have evolved resistance to the QQ compound C-30, illustrating that these mutants are physiologically relevant.

Materials and methods

Strains and growth conditions

All experiments were conducted at 37 °C and included at least three independent cultures. The bacterial strains used in this study are listed in Table 1. Wild-type *P. aeruginosa* PA14 from Dr Frederick Ausubel (Liberati *et al.*, 2006) was utilized. The brominated furanone C-30 was synthesized (Jones *et al.*, 2005), confirmed by ^{1}H-NMR and GC-MS (Jones *et al.*, 2005) and dissolved in methanol; 50 μM was used for all experiments.

For growth with C-30, overnight cultures in Luria-Bertani (LB) medium were re-inoculated into 20 ml of oxygen-sensing minimal medium containing 0.1% (w/v) adenosine (Heurlier *et al.*, 2005) with or without C-30, and the mixture was incubated with shaking (250 r.p.m.); all cultures had the same initial turbidity at 600 nm. Cell turbidity was measured at 600 nm with a spectrophotometer. The MIC_{50} value for chloramphenicol was determined with an initial inoculum of 5×10^5 cells ml^{-1} using the broth dilution method (Wiegand *et al.*, 2008) with the modifications of using shaking (250 r.p.m.) and LB medium.

Quorum-sensing-related assays

Five quorum-sensing-related assays were performed to investigate the impact of C-30 with *P. aeruginosa*. Pyoverdine production was assayed using overnight cell cultures (100 μl) that were re-inoculated into minimal medium with succinate (Ren *et al.*, 2005) with or without C-30 and incubated for 7 h, then the pyoverdine in the supernatant (20-times diluted) was measured spectrophotometrically at 370 nm (Ren *et al.*, 2005). Pyocyanin production was assayed spectrophotometrically after incubation in LB medium for 9 h and after extracting with chloroform and 0.2 N HCl (Lee *et al.*, 2009) using *phzM* as a negative control (Ueda *et al.*, 2009). Elastase was assayed using elastin-Congo red as a substrate and *lasB* as a negative control (Ueda *et al.*, 2009). Swarming was assayed using BM2-swarming medium after incubation for 24 h (Lee *et al.*, 2009), and *rhlR* as a negative control (Ueda *et al.*, 2009). Biofilm formation was assayed in polystyrene 96-well plates with minimal medium with succinate or glucose by staining with crystal violet (Ueda and Wood, 2009).

Table 1 Bacterial strains used in this study

Strain	*Genotype/relevant characteristics*	*Source*
PA14	*Pseudomonas aeruginosa* wild-type	Liberati *et al.* (2006)
mexR	PA14 *mexR* Ω miniTn5 *luxAB*-Tet, Tc^R	This study
nalC	PA14 *nalC* Ω miniTn5 *luxAB*-Tet, Tc^R	This study
sQQQ1	Spontaneous C-30 resistant PA14 mutant	This study
sQQQ3	Spontaneous C-30 resistant PA14 mutant	This study
phzM	PA14 *phzM* Ω *Mar2xT7*, Gm^R	Liberati *et al.* (2006)
rhlR	PA14 *rhlR* Ω *Mar2xT7*, Gm^R	Liberati *et al.* (2006)
lasB	PA14 *lasB* Ω *Mar2xT7*, Gm^R	Liberati *et al.* (2006)
AHP	*mexR* (V126E), *nalC* (G71E, A78T, S209R)	Tomás *et al.* (2010)
12142[a]	*mexR* (R83C), *nalC* (G71E), *nalD* (D187H)	Tomás *et al.* (2010)
1253[a]	*mexR* (V126E), *nalC* (G71E, A186T), *nalD* (D187H)	Tomás *et al.* (2010)
14169[a]	*mexR* (R83C), *nalC* (G71E), *nalD* (D187H)	Tomás *et al.* (2010)
1681[a]	*mexR* (R83C), *nalC* (G71E), *nalD* (D187H)	Tomás *et al.* (2010)
3149[a]	*nalC* (G71E), *nalD* (D187H, L201P)	Tomás *et al.* (2010)
1248[a]	*mexR* (R83C), *nalC* (G71E), q*nalD* (D187H, L201P)	Tomás *et al.* (2010)
12102[a]	*mexR* (V126E), *nalC* (G71E), *nalD* (D187H)	Tomás *et al.* (2010)
1665[a]	*mexR* (R83C), *nalC* (G71E), *nalD* (D187H, L201P)	Tomás *et al.* (2010)
1712[a]	*mexR* (R83C), *nalC* (G71E, S209R), *nalD* (D187H, L201P)	Tomás *et al.* (2010)
OP50	*Escherichia coli* B strain (uracil auxotroph)	Brenner (1974)

Amino acid replacements encoded by the mutations in the CF isolates and their control are shown in parenthesis (note there may be other mutations in these strains).
Tc^R and Gm^R indicate tetracycline and gentamicin resistance, respectively.
[a]Indicates clinical CF isolate.

Isolation of mutants less sensitive to C-30
A *P. aeruginosa* PA14 transposon mutant library was generated (Ueda and Wood, 2009) using conjugation with *E. coli* S17-1 (λpir)/pUT-miniTn*5 luxAB*-Tc^R, which delivered the Tn*5-luxAB* transposon. After conjugation, cells (100 μl) were added to 10 ml of oxygen-sensing minimal medium containing 0.1% (w/v) adenosine (Heurlier *et al.*, 2005) supplemented with C-30, 75 μg ml^{-1} of tetracycline (to select *P. aeruginosa* transposon mutants) and 20 μg ml^{-1} of nalidixic acid (to kill the donor *E. coli* cells because *P. aeruginosa* is naturally resistant to this antibiotic). The mixture was incubated with shaking for 2 days, then the cell culture (100 μl) was re-inoculated into the same medium and incubation continued for four passages. The most abundant transposon mutants were selected by streaking an aliquot of the cell culture on LB agar plates containing 75 μg ml^{-1} of tetracycline and 20 μg ml^{-1} of nalidixic acid. Single colonies were picked, and their resistance to C-30 on growth was confirmed. To identify the position of the insertion of the transposon, genomic DNA from the transposon mutants was sequenced using two-step PCR (Ueda and Wood, 2009). The *mexR* mutant with the insertion position at bp 387 (Ala129) from the start of the coding region and the *nalC* mutant with the insertion at bp 156 (Tyr52) were used for further experiments. Spontaneous mutants that were less sensitive to C-30 were obtained using the same sequential dilution method (without tetracycline).

C. elegans *fast-killing assay*
To investigate the virulence effect of PA14 strains, the *C. elegans* fast-killing assay was performed (Mahajan-Miklos *et al.*, 1999). An overnight LB culture was used to spread each of the bacterial cultures (roughly 5×10^7 cells in 5–10 μl) on 3.5-cm diameter peptone-glucose-sorbitol (PGS) agar plates and incubated at 37 °C for 24 h. C-30 was included in both the overnight culture and in the agar plates. After 6–8 h at room temperature, bacterial lawns were heat killed by incubating at 65 °C for 30 min. After cooling to room temperature, each plate was seeded with 20 early to mid-L4 stage hermaphrodite worms (wild-type Bristol N2 strain, *Caenorhabditis* Genetics Center), and three to four replicates were used for each independent culture. Plates were incubated at 25 °C and scored for live worms. *E. coli* OP50 was used as a negative control.

Efflux assay
Efflux of C-30 was assayed by measuring the concentration of C-30 exported from cells in minimal succinate medium using high-performance liquid chromatography (4 × 50 mm Inertisil ODS column, Shimadzu SPD-10AVP instrument (Kyoto, Japan) with a UV–Vis detector at 285 nm, and 40% acetonitrile as the mobile phase). Overnight cultures were resuspended in minimal succinate medium to a turbidity of 1 at 600 nm and were allowed to import C-30 for 30 min; the uptake activity of C-30 was determined by measuring the C-30 concentration in the supernatant. The cell pellets were resuspended in minimal succinate medium to a turbidity of 1 at 600 nm and the amount of C-30 effluxed to the supernatant was detected both at 0 h and after 15 h. M9 buffer instead of the minimal succinate medium was used as a negative control.

Whole-transcriptome analysis
The *P. aeruginosa* genome array (Affymetrix, Santa Clara, CA, USA; P/N 510596) was used to investigate differential gene expression in planktonic cells between PA14 and the *mexR* mutant in the presence of C-30 (Ueda and Wood, 2009). When the culture grew to the mid-exponential phase (turbidity of 0.25), C-30 was added for 2 h. Total RNA was isolated as described previously (Ren *et al.*, 2004) using a bead beater (Biospec, Bartlesville, OK, USA) and RNA*later* buffer (Applied Biosystems, Foster City, CA, USA) to stabilize the RNA. cDNA synthesis, fragmentation, hybridizations and data analysis were as described previously (Lee *et al.*, 2009). To ensure the reliability of the induced/repressed gene list, genes were identified as differentially expressed if the *P*-value was <0.05 and if the expression ratio was >2 (as the s.d. for the expression ratio for all the genes was 1.7 (Ueda and Wood, 2009)). The whole-transcriptome data were deposited in the NCBI Gene Expression Omnibus (GSE24262).

Quantitative real-time reverse transcription PCR
Quantitative real-time reverse transcription PCR was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems). Expression of *nuh* gene was determined using the total RNA isolated from two independent cultures of PA14 and the *mexR* mutant. When the culture reached the mid-exponential phase (turbidity of 0.25), C-30 was added for 30 min. Total RNA was isolated in the same manner as described above for the whole-transcriptome analysis. The primers for quantitative real-time reverse transcription PCR are 5′-GGAC-TACCTGATCCGTACC-3′ and 5′-GATCTTGTGGGT-CACGTC-3′. The housekeeping gene *rplU* (Kuchma *et al.*, 2007) was used to normalize the gene expression data.

Results and discussion

Growth of *P. aeruginosa* on adenosine in minimal medium was determined in the presence of the QQ compound C-30. As expected, the growth yield at 48 h of *P. aeruginosa* in adenosine (0.1% w/v) minimal medium was decreased by 5 ± 1-fold with 50 μM C-30 (Figure 1a), whereas there was no growth inhibition due to C-30 in LB medium

($\mu = 1.4 \pm 0.1\ h^{-1}$) as found previously (Hentzer *et al.*, 2003). Hence, we successfully created a method by which there is selective pressure to evolve resistance to the antivirulence compound C-30 as it represses QS.

To confirm that C-30 represses QS phenotypes with *P. aeruginosa*; that is, it acts as a QQ compound, we chose five phenotypes that have been linked to QS with this strain: pyocyanin production (Ueda *et al.*, 2009), pyoverdine production (Hentzer *et al.*, 2003), elastase activity (Hentzer *et al.*, 2003), swarming (Ueda *et al.*, 2009) and biofilm formation (Ueda *et al.*, 2009). Swarming was abolished by the addition of C-30 (Figure 2a), and biofilm formation was reduced by 3.7 ± 0.7-fold with C-30 in glucose minimal medium and 2.3 ± 0.5-fold in succinate minimal medium. Also, pyocyanin production was reduced by 1.49 ± 0.04-fold upon addition of C-30 (Figure 2b), and as shown previously with C-30 (Hentzer *et al.*, 2003), pyoverdine production was decreased by 2.4 ± 0.4-fold and elastase was decreased by 8 ± 2-fold. Hence, the synthesized C-30 was active and inhibited QS-related phenotypes with *P. aeruginosa*.

To facilitate the evolution of *P. aeruginosa* for resistance to C-30, random transposon mutagenesis was performed. In this way, cells with mutations that allow for faster growth with C-30 could be identified rapidly by DNA sequencing from the transposon. Using minimal medium containing adenosine and C-30, mutants less sensitive to C-30 were identified by their faster growth in sequential shake flask cultures. After four sequential dilutions, the difference in the growth rates allowed putative C-30 mutants to be purified. After performing this procedure several times, for eight mutants, the insertion position of Tn5-*luxAB* transposon was determined to be in *mexR*, the gene for the repressor of the locus that encodes the MexAB-OprM multidrug resistance efflux pump (Chen *et al.*, 2010). Corroborating these results, four mutants had the insertion in *nalC*, which also encodes a negative regulator of *mexAB-oprM*. Note *mexR* was previously found to be induced by C-30 (Hentzer *et al.*, 2003).

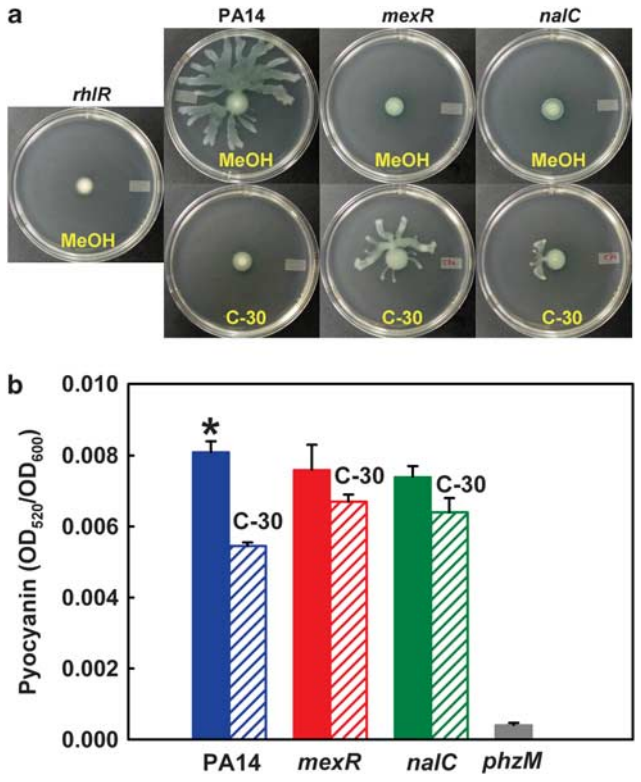

Figure 2 The *mexR* and *nalC* mutations decrease C-30 inhibition of *P. aeruginosa* QS phenotypes. (**a**) Swarming motility at 37 °C after 24 h using *rhlR* as a negative control. Three independent cultures were used for each strain, and two swarming plates were used for each culture. Representative plates are shown. (**b**) Pyocyanin production after incubation in LB medium for 9 h. Mutant *phzM* was used as a negative control. C-30 was used at 50 μM. Data represent the mean ± s.d.; $n = 3$. *$P < 0.01$ by Student's *t*-test compared with the value in the presence of C-30.

We also obtained two types of spontaneous mutants resistant to C-30 that did not rely on transposon mutagenesis using the same selection method. After 13 sequential dilutions, two types of spontaneous QQ quandary mutants (sQQQ) were obtained (Figure 1b): C-30 enhances growth with sQQQ1, and sQQQ3 grows more rapidly with and without C-30 than the PA14 wild-type strain.

To confirm that the *mexR* and *nalC* mutants were more resistant to furanone C-30, growth was checked in minimal adenosine medium. Although C-30 still inhibited growth of the *mexR* and *nalC* strains, the growth yield of these two strains was two- to three-fold higher than the wild-type strain after 50–60 h (Figure 1a). In addition, C-30 had little effect on the QS-related phenotypes of the *mexR* and *nalC* strains because its addition did not alter pyocyanin formation (Figure 2b). Also, the addition of C-30 enhanced the swarming motility of *mexR* and *nalC* mutants rather than inhibiting it as for the wild-type strain (Figure 2a). Hence, the *mexR* and *nalC* mutations render the cells less sensitive to the anti-QS effects of the brominated furanone C-30.

To compare the impact of C-30 on PA14 and *mexR*, a whole-transcriptome analysis was performed with planktonic cells of PA14 and *mexR* grown in adenosine minimal medium with C-30. C-30 was previously shown to repress the majority of QS-induced genes in *P. aeruginosa* (Hentzer *et al.*, 2003). Addition of C-30 to *mexR*-induced 23 QS genes (Hentzer *et al.*, 2003; Schuster *et al.*, 2003; Wagner *et al.*, 2003) relative to the wild-type strain with C-30 (Table 2); hence, the ability of C-30 to repress QS was reduced in the *mexR* mutant. These QS genes include *lasR* and *rhlR* that encode the two transcriptional regulators of the acyl-homoserine lactone system of *P. aeruginosa* (Schuster *et al.*, 2003). In our whole-transcriptome analysis, the *nuh* gene, which encodes the nucleoside hydrolase that is required to assimilate adenosine (Heurlier *et al.*, 2005), was slightly induced (1.2-fold). Because *mexR* strain grows better in adenosine medium than

Table 2 List of induced quorum-sensing genes for planktonic cells of *mexR* vs PA14 wild-type

PAO1 ID	*PA14 ID*	*Gene name*	*Fold change*	*Description*	*References*
Multidrug efflux operon mexAB-oprM					
PA0425	PA14_05530	*mexA*	1.7	Resistance-nodulation-cell division (RND) multidrug efflux membrane fusion protein	
PA0426	PA14_05540	*mexB*	1.5	RND multidrug efflux transporter	
PA0427	PA14_05550	*oprM*	1.6	Multiple antibiotic resistance efflux outer membrane protein	
Quorum-induced genes identified by previous studies					
PA1430	PA14_45960	*lasR*	2.0	Transcriptional regulator LasR	
PA3477	PA14_19120	*rhlR*	3.2	Transcriptional regulator RhlR	a, b, c
PA0105	pA14_01290	*coxB*	2.0	Cytochrome c oxidase, subunit II	b
PA0106	pA14_01300	*coxA*	3.0	Cytochrome c oxidase, subunit I	b
PA0107	pA14_01310		2.1	Probable cytochrome c assembly protein	b, c
PA0108	pA14_01320	*coIII*	2.3	Cytochrome c oxidase, subunit III	b
PA1174	PA14_49250	*napA*	2.0	Periplasmic nitrate reductase protein NapA	
PA1175	PA14_49230	*napD*	2.0	Protein of periplasmic nitrate reductase	b
PA1176	PA14_49220	*napF*	2.0	Ferredoxin protein NapF	b
PA1245	PA14_48140		2.0	Hypothetical protein	b
PA1247	PA14_48100	*aprE*	2.1	Alkaline protease secretion protein AprE	a, b
PA1887	PA14_40110		2.0	Hypothetical protein	
PA1888	PA14_40100		2.3	Hypothetical protein	b
PA2303	PA14_34830		2.6	Putative regulatory protein	a, b, c
PA2306	PA14_34800		2.1	Putative transporter, LysE family	c
PA2591	PA14_30580		2.0	Probable transcriptional regulator, LuxR family	a, b, c
PA2747	PA14_28600		2.0	Hypothetical protein	a, b
PA3032	PA14_24860	*snr1*	2.1	Cytochrome c	b, c
PA3904	PA14_13390		2.8	Hypothetical protein	a, b, c
PA4139	PA14_10380		2.0	Hypothetical protein	a, b
PA4496	PA14_58350		2.1	Probable binding protein component of ABC transporter	c
PA4677	PA14_61870		2.3	Hypothetical protein	a, b, c
PA4738	PA14_62680		2.0	Hypothetical protein	a, b
PA4739	PA14_62690		2.6	Hypothetical protein	a, b
PA5482	PA14_72370		2.1	Hypothetical protein	a, b

Cells were incubated in adenosine minimal medium containing 50 μM C-30. Raw data for the two DNA microarrays are available using GEO series accession number GSE24262.
a, (Hentzer *et al*., 2003); b, (Schuster *et al*., 2003); c, (Wagner *et al*., 2003).

the wild-type in the presence of C-30, *nuh* should show elevated expression. To explore this further, we conducted quantitative real-time reverse transcription PCR and found that *nuh* was induced 6 ± 3-fold in the *mexR* mutant relative to wild-type PA14 after addition of C-30 to minimal adenosine medium for 30 min. Therefore, these whole-transcriptome results are consistent with our hypothesis that the *mexR* mutation renders *P. aeruginosa* less sensitive to the QQ compound C-30.

To further characterize the *mexR* mutant, we investigated its pathogenicity using *Caenorhabditis elegans* (Mahajan-Miklos *et al*., 1999) as the animal model. It has been shown that the *C. elegans* fast-killing model is mediated by pyocyanin and other toxins, which are also required for pathogenesis in plants and mice (Mahajan-Miklos *et al*., 1999). In addition, because pyocyanin is QS controlled and inhibited by C-30 in wild-type PA14, this model is related to QS. Our goals were to determine if the *mexR* mutant could still be pathogenic as well as whether it was more virulent, compared with the wild-type strain, in the presence of the QS inhibitor C-30. L4 stage hermaphrodite worms were exposed to lawns of PA14 and *mexR* grown on PGS agar plates with or without C-30. A *mexA* mutant with reduced MexAB-OprM efflux pump activity was found previously to be less virulent to *C. elegans* in a similar assay (Mahajan-Miklos *et al*., 1999), and our results indicate that the *mexR* mutant with enhanced MexAB-OprM efflux pump activity is as virulent as the wild-type strain: 50∼60% of the worms were killed within 10 h for both strains (Figure 3). Critically, C-30 reduced wild-type PA14 virulence by 2.8 ± 0.5-fold whereas it had little effect on the *mexR* mutant. In contrast, worms exposed to *E. coli* OP50 on the same medium plates showed 100% viability. These results indicate that the *mexR* mutant remains pathogenic under the conditions we tested and that it is much more virulent in the presence of the QS inhibitor than the wild-type strain.

To corroborate these *C. elegans* results with the C-30-resistant *mexR* and *nalC* mutants obtained from growth on adenosine, clinical isolates from patients with chronic CF infections (Tomás *et al*.,

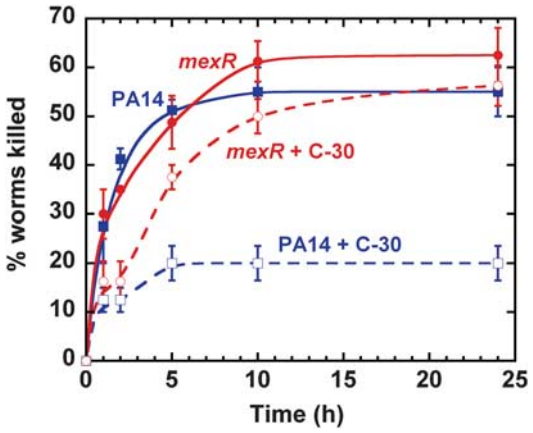

Figure 3 The *mexR* mutation decreases C-30 inhibition of killing of *C. elegans* by *P. aeruginosa*. L4 stage hermaphrodite worms were exposed to wild-type *P. aeruginosa* (blue squares) and *mexR* (red circles) grown on PGS medium with (open symbols) or without (closed symbols) 50 μM C-30. Worms exposed to *E. coli* OP50 on the same medium plates were used as the negative control, which showed 100% viability. Data represent the mean ± s.d.; $n = 4$ plates, 20 worms per plate.

2010) were investigated for their resistance to C-30. These *P. aeruginosa* strains have mutations that include *mexR* and *nalC* (Tomás *et al.*, 2010); such mutations arise frequently in chronic but not in acute infections (Oliver and Mena, 2010). For the nine strains we checked (Table 1), strain 12142 with the highest *mexA* induction (15.9-fold) showed no growth defect in the presence of C-30 (Figure 1c), and strain 1253 had increased growth in the presence of C-30. In contrast, growth yield of the control strain AHP (Tomás *et al.*, 2010) with reduced *mexA* expression (8.3-fold) was reduced 4.2 ± 0.7-fold by C-30. Hence, natural isolates in CF patients have obtained mutations, which include *mexR* and *nalC*, and these natural CF isolates are also resistant to the QQ compound C-30.

MexR is the primary negative regulator of the MexAB–OprM complex, which exports diverse antibiotics, including quinolones, β-lactams, tetracycline, chloramphenicol, novobiocin, macrolides and biocides (Chen *et al.*, 2010); this pump also exports the $3OC_{12}$-HSL of the *P. aeruginosa* Las QS system (Pearson *et al.*, 1999). As a possible mechanism for the resistance of the *mexR* mutant to the QS quenching C-30, we hypothesized that inactivation of the *mexR* gene led to increased efflux of C-30 by the *P. aeruginosa* cells; thereby, the inefficiency of C-30 as an antivirulence compound in the *mexR* mutant may be because of it being exported, which limits its effect on the QS pathways. Indeed, the three genes in *mexAB-oprM* operon were induced slightly in the *mexR* mutant upon addition of C-30 (Table 2). More significantly, the minimum inhibitory concentration of chloramphenicol, an antibiotic known to be effluxed by the MexAB–OprM complex (Chen *et al.*, 2010), was increased 3.5 ± 0.4-fold for the C-30-resistant, *mexR* mutant. Furthermore, both the *mexR* transposon mutant and the wild-type strain had similar uptake activities of C-30 (229 ± 7 nmol mg^{-1} and 250 ± 13 nmol mg^{-1} protein, respectively) after 30 min; however, only the *mexR* mutant effluxed C-30 in minimal succinate medium (3 ± 2 nmol h^{-1} mg^{-1} protein) as there was no efflux found with the wild-type strain (20 ± 10% of the C-30 imported into the cells was exported by the *mexR* mutant after 15 h). To avoid the possibility of nonspecific binding of C-30 to the cells, the concentration of C-30 was measured after washing and no C-30 was detected for either strain. In addition, there was no measurable efflux by either strain in buffer, which served as a negative control for non-active release. Therefore, the *mexR* mutation renders the cells more resistant to C-30 because of its increased export.

It was not anticipated that the antibiotic efflux pump MexAB-OprM may export a QQ compound. Although the MexAB-OprM pump is able to export the $3OC_{12}$-HSL signal (Pearson *et al.*, 1999), it does not export the C_4-HSL signal (Pearson *et al.*, 1999), which has a shorter *N*-acyl side chain than $3OC_{12}$-HSL, and the shorter C_4-HSL signal resembles to C-30 more than $3OC_{12}$-HSL. Hence, the discovery of the QQ-resistance mechanism found here because of increased efflux activity is novel.

In conclusion, our results show that bacteria can obtain resistance to antivirulence compounds such as the best-studied brominated furanones. The overlap of the resistance to the antivirulence compounds and the resistance to antibiotics for the C-30-resistant mutants indicates that these kinds of mutants are easily obtained. These mutants with increased MexAB-OprM efflux activities would evolve not only under adenosine-usage selective pressure, but also under antibiotic treatment, which constitute a very strong selective pressure. Hence, treatments with antibiotics may induce resistance to antivirulence compounds (and perhaps *vice versa*). Corroborating this hypothesis, we also demonstrated that natural isolates from chronic infections also are resistant to the QQ compound C-30 as we showed that some of the isolates that arise in CF patients (those with the *mexR* and *nalC* mutations) are resistant to C-30. Hence, these results are important for the development of rational strategies for the utilization of antivirulence compounds for the clinical treatment of infectious diseases in that clinicians should take into account the possibility of a rapid selection and dissemination of resistant bacteria by diverse resistance mechanisms.

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