Identification of FDA-approved drugs as antivirulence agents targeting the *pqs* quorum sensing system of *Pseudomonas aeruginosa*

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Running Head: New FDA-approved inhibitors of the *pqs* QS system

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ABSTRACT

The long-term use of antibiotics has led to the emergence of multi-drug-resistant bacteria. A promising strategy to combat bacterial infections aims at hampering their adaptability to the host environment without affecting growth. In this context, the intercellular communication system quorum sensing (QS), which controls virulence factor production and biofilm formation in diverse human pathogens, is considered an ideal target.

Here we describe the identification of new inhibitors of the *pqs* QS system of the human pathogen *Pseudomonas aeruginosa*, by screening a library of 1,600 FDA-approved drugs. Phenotypic characterization of *ad hoc* engineered strains and *in silico* molecular docking demonstrated that the antifungal drugs clotrimazole and miconazole, and an antibacterial compound active against Gram-positive pathogens, clofoctol, inhibit the *pqs* system, probably by targeting the transcriptional regulator PqsR. The most active inhibitor, clofoctol, specifically inhibited the expression of *pqs*-controlled virulence traits in *P. aeruginosa*, such as pyocyanin production, swarming motility, biofilm formation, and expression of genes involved in siderophore production. Moreover, clofoctol protected *Galleria mellonella* larvae from *P. aeruginosa* infection and inhibited the *pqs* QS system in *P. aeruginosa* isolates from cystic fibrosis patients. Notably, clofoctol is already approved for clinical treatment of pulmonary infections caused by Gram-positive bacterial pathogens, hence this drug has considerable clinical potential as an antivirulence agent for the treatment of *P. aeruginosa* lung infections.

INTRODUCTION

The discovery and development of new drugs for use in humans is a challenging task that usually requires decade-long laboratory experimentation followed by extensive clinical trials. This process is time consuming and necessitates substantial economic investments with a high-risk of failure mostly due to the poor pharmacological and pharmaceutical properties of newly identified bioactive molecules. This is particularly discouraging for antibiotic discovery as the

investment required cannot be adequately recovered because of the rapid rate at which resistance emerges (1). As a consequence, while spread of multi-resistant pathogens is accelerating at an unprecedented rate, the antibiotic discovery pipeline is running dry, with 15 big pharmaceutical companies out of 18 abandoning antibacterial discovery programmes in the last decade (2,3).

The search for off-target activities in drugs already approved for human use is a promising strategy that could reduce the time and costs generally associated with conventional drug discovery processes, with a high probability of yielding bioavailable and safe compounds which can more easily and swiftly move into clinical trials (4,5).

A number of studies have shown the promise of drug repurposing strategies for the identification of new antibacterial drugs (6,7). Examples are gallium nitrate and 5-fluorouracil, conventionally used for the treatment of hypercalcemia and cancer, respectively, which display growth-inhibitory activities against certain Gram-negative and Gram-positive pathogens (8,9). An alternative approach to the development of new antimicrobials is the inhibition of bacterial virulence, rather than growth (10). Recently, antivirulence activities have been identified in drugs already approved for use in humans (11). As an example, the antifungal compound 5-fluorocytosine inhibits virulence factor production in the Gram-negative human pathogen *Pseudomonas aeruginosa* both *in vitro* and in a mouse model of lung infection (12). Since antivirulence drugs attenuate rather than kill pathogens they should in principle combat bacterial infections without exerting the strong selective pressure for resistance imposed by bactericidal antibiotics (10). Emergence of resistance is less likely to occur for drugs targeting bacterial social behaviours, such as the production of secreted virulence factors. Indeed, resistant mutants expressing extracellular factors that are shared by the members of the entire bacterial population are unlikely to experience a fitness advantage relative to susceptible clones (13). In this context, quorum sensing (QS) is considered to be a promising target for the identification and development of antivirulence drugs, since this intercellular communication system positively controls the expression of virulence factors in a number of different human pathogens including

P. aeruginosa (14,15).

P. aeruginosa is one of the most problematic human pathogens in industrialized countries, since it causes a variety of severe infections, especially among hospitalized and immunocompromised patients (16,17). These infections are difficult to treat due to the intrinsic and acquired antibiotic resistance of *P. aeruginosa* (18) that is further compounded by its ability to form antibiotic tolerant biofilms (19). *P. aeruginosa* is the predominant cause of morbidity and mortality in individuals with cystic fibrosis (CF), as it forms biofilms so establishing chronic lung infections impossible to eradicate with antibiotic treatment (20). The necessity for new therapeutic options for the treatment of *P. aeruginosa* infections was highlighted in a recent World Health Organization report, in which this pathogen is top ranked among pathogens for which new antibiotics are urgently needed (Priority 1 - Critical; www.who.int/mediacentre/news/releases/2017/bacteria-antibiotics-needed/en/).

As a consequence of its importance as a human pathogen, *P. aeruginosa* has been adopted as a model organism for QS inhibition studies. This bacterium is endowed with a complex QS network consisting of four interconnected systems (*i.e. las*, *rhl*, *pqs* and *iqs*), which collectively control social behaviours and the expression of virulence determinants, such as secreted virulence factors, swarming motility and biofilm formation (21,22). Over the last decade, numerous compounds interfering with the *P. aeruginosa* QS circuitry have been identified, and their effectiveness as antivirulence drugs both *in vitro* and *in vivo* has boosted the research in the field (23). Unfortunately, most of the drugs identified so far are cytotoxic or display unfavourable pharmacological properties, thus limiting their transfer to clinical practice (15).

To combine the advantages of drug-repurposing with the antivirulence approach, we previously showed that the anthelmintic drug niclosamide has potent antivirulence activity against *P. aeruginosa* (24). Niclosamide targets the *las* QS system, thereby decreasing the expression of *las*-controlled virulence factors and protecting *Galleria mellonella* larvae from *P. aeruginosa* infection (24).

In the present study we searched for inhibitors of the *pqs* QS system of *P. aeruginosa* among drugs already approved for human use.

The *pqs* QS system of *P. aeruginosa* is based on 2-alkyl-4-quinolones (AQs) as signal molecules, namely 2-heptyl-3-hydroxy-4-quinolone (PQS), and its immediate precursor 2-heptyl-4-hydroxyquinoline (HHQ). Both HHQ and PQS can bind to and activate the transcriptional regulator PqsR (also known as MvfR). The PqsR/HHQ and PqsR/PQS complexes bind the P*pqsA* promoter region and trigger the transcription of the *pqsABCDEphnAB* operon, coding for the enzymes required for the synthesis of HHQ. HHQ is in turn oxidized to PQS by the monooxygenase PqsH. Therefore, in common with other QS systems, HHQ and PQS act as autoinducers by generating an autoinductive feedback loop that accelerates their synthesis (25-28).

While HHQ only activates the expression of the *pqsABCDEphnAB* operon, PQS has additional functionalities; it is an iron chelator, participates in the formation of outer membrane vesicles and controls the expression of virulence genes *via* a PqsR-independent pathway (28-31).

The mechanism of action of the protein coded by the fifth gene of the *pqsABCDEphnAB* operon, PqsE, is still poorly understood. PqsE is a pathway-specific thioesterase, which contributes to the synthesis of HHQ, although loss of its function can be compensated for by other thioesterases in a *pqsE* mutant (27). Notably, PqsE positively controls the expression of multiple virulence factors also in a *P. aeruginosa* genetic background in which it cannot participate to AQs biosynthesis, indicating that this protein has additional functions (29,32,33).

Overall, *P. aeruginosa* mutants defective in AQ synthesis/reception or in PqsE are severely attenuated in different plant and animal experimental models of infection (33-38). Moreover, AQs are detectable in sputum, blood and urine of individuals with CF and their presence correlates with clinical status (39).

In this study, a convenient screening system has been developed and used to select for FDA-approved drugs targeting the *pqs* QS system at multiple levels. This screening campaign led to

the identification of the antifungal drugs clotrimazole and miconazole, and of clofoctol, an antimicrobial compound commonly used to treat lung infections caused by Gram-positive bacteria, as inhibitors of *pqs* signaling, probably targeting the PqsR receptor protein. Phenotypic analyses performed in the laboratory strain PAO1 and in *P. aeruginosa* isolates from CF patients support the antivirulence potential of clofoctol, the most active inhibitor.

RESULTS

Development of a coculture-based system for monitoring *pqs* signaling activity

A reporter system for monitoring the activity of the *pqs* QS system has been developed. This is based on the coculture between wild type *P. aeruginosa* PAO1 (herein referred to as PAO1) and the AQ biosensor strain *P. aeruginosa* Δ*pqsA* P*pqsA*::*luxCDABE* (herein referred to as AQ-Rep; Table S1). AQ-Rep cannot synthesize AQs due to deletion of the *pqsA* biosynthetic gene, and emits light only in response to exogenously provided AQs due to PqsR-dependent activation of the P*pqsA*::*luxCDABE* transcriptional fusion integrated in a neutral chromosomal site (31). Therefore, in the PAO1/AQ-Rep coculture system the AQ signal molecules produced by PAO1 induce bioluminescence, and hence *pqs* inhibitors interfering with each step of the *pqs* signaling circuit, including AQ biosynthesis or response, should reduce bioluminescence (Fig. 1A).

Preliminary experiments directed towards setting-up the screening system revealed that maximal response of AQ-Rep to exogenous PQS was obtained after 5 h incubation in microtiter plates (Fig. S1A), when this biosensor strain was inoculated at an optical density at 600 nm wavelength (OD_{600}) of 0.1 (Fig. S1B). Cocultivation of AQ-Rep and PAO1 at different ratios and in different culture conditions showed that the highest bioluminescence signal was registered when AQ-Rep and PAO1 were inoculated in a ~ 3:1 ratio (OD_{600} of AQ-Rep and PAO1 of 0.1 and 0.03, respectively) (Fig. S1C), and the resulting coculture was incubated at 37°C with shaking (Fig. S1D). Therefore, the screening campaign has been set-up under the above conditions to maximize the biosensor responsiveness to AQs and possibly to drugs interfering

with AQ signaling.

The functionality of the PAO1/AQ-Rep coculture system for the identification of anti-*pqs* drugs was assessed using the commercially available compounds methyl anthranilate and farnesol. Methyl anthranilate inhibits AQs biosynthesis by competing with the HHQ precursor anthranilate for binding to PqsA (40), while farnesol decreases the expression of HHQ biosynthetic genes *via* an unknown mechanism (41). As expected, both methyl anthranilate and farnesol reduced bioluminescence from the PAO1/AQ-Rep coculture in a dose-dependent manner, with an IC_{50} of ca. 1 mM (Fig. 1B), in accordance with literature data (40-41).

Identification of new anti-*pqs* drugs

The PAO1/AQ-Rep coculture system was used to screen a library of 1,600 FDA-approved compounds with known biological activities selected for their high chemical and pharmacological diversity and safety in humans (PHARMAKON). In the primary screening, each drug was tested at two different concentrations, 20 μM and 200 μM, for the ability to reduce bioluminescence in the PAO1/AQ-Rep coculture. Since compounds from the library are dissolved in dimethyl sulfoxide (DMSO), untreated samples containing the same amount of DMSO as the treated samples were used as controls. Cell density and bioluminescence of the untreated samples were considered as 100%, and the criteria for selection of anti-*pqs* drugs were: *i*) inhibition of bioluminescence ≥ 20% at 20 μM; *ii*) inhibition of bioluminescence ≥ 60% at 200 μM; *iii*) reduction of cell density ≤ 10% at both 20 μM and 200 μM. This primary screening led to the selection of seventeen hits meeting these criteria (Fig. S2A) and possibly endowed with *pqs* inhibitory activity.

However, reduced bioluminescence in the samples treated with the selected drugs could be due to their effects on the enzymes involved in light generation or on ATP levels (42,43). Since inhibition of P*pqsA* promoter activity in *P. aeruginosa* should decrease the production of the AQs HHQ and PQS, a secondary screening to test the ability of the seventeen hits to reduce AQ

production in PAO1 was performed. In this case, AQ levels were measured by means of the AQ-Rep biosensor strain in the spent medium from PAO1 cultures grown for 16 h in Luria-Bertani Broth (LB) supplemented with the selected hits at 20 µM or 200 µM concentrations, or with corresponding amounts of DMSO. This analysis revealed that only three drugs specifically reduced the production of AQs in PAO1: clotrimazole, clofoctol and miconazole (I-3, I-9 and I-14 in Fig. S2B, respectively). Two of the drugs identified, clotrimazole and miconazole, are antifungal compounds (44-47), while clofoctol is an antibacterial drug with efficacy in Gram-positive human lung infections (48-50) (Table 1).

To confirm the results of the primary and secondary screening, clotrimazole, clofoctol and miconazole were purchased from an alternative supplier (Sigma-Aldrich). These drugs did not inhibit PAO1 growth in Muller-Hinton Broth or LB even at the highest concentration achievable in solution (*i.e.* MIC clotrimazole > 1.6 mM; MICs for miconazole and clofoctol > 6.4 mM). Moreover, these drugs did not alter the growth profile of wild type PAO1 and of the AQ-Rep biosensor strain up to the maximum concentration used in the primary and secondary screenings (*i.e.* 200 µM; Fig. S3).

The *pqs* inhibitory activity of the drug hits was retested in the PAO1/AQ-Rep coculture assay. Dose-response inhibition of P*pqsA* promoter activity was observed for the three drugs (Fig. 2A). These data generated IC_{50} values of 39 µM, 20 µM and 27 µM for clotrimazole, clofoctol and miconazole, respectively (Table 1). The three hits had no effect on bioluminescence in a *P. aeruginosa* strain in which the expression of the *luxCDABE* operon for light emission is independent on the activity of the *pqs* signaling system (Fig. S4), ruling out the possibility that the inhibitory activity on the PAO1/AQ-Rep coculture was due to no-specific inhibition of bioluminescence. Moreover, the three drugs confirmed their ability to reduce AQ production in PAO1 in a dose-dependent manner (Fig. 2B), in accordance with the repressive effect exerted on the P*pqsA* promoter.

The QS cascade in *P. aeruginosa* is a complex network of interwoven and hierarchical QS

circuits (21,22), and hence the effect of some compounds on the *pqs* QS system may be due to altered activity of the *las* and/or *rhl* QS systems. In particular, the *las* QS system is required for full activation of the *pqs* QS systems (36,51-53), while RhlR has a negative impact on the *pqs* system by repressing PQS signal production through interference with the expression of *pqsR* and *pqsABCDE* (36,54-56). Hence, reduced activity of the *pqs* QS system could be due to a negative or a positive effect of the hits on the *las* or the *rhl* QS systems, respectively. Therefore possible effect of the three hits on these QS systems was investigated by using *las*- and *rhl*-specific biosensor strains. Clotrimazole, clofoctol and miconazole did not decrease light emission in a reporter system in which PAO1 wild type and the *las*-specific biosensor strain PA14 Δ*lasI* P*rsaL*::*luxCDABE* were cocultured (Fig. S5A; 57). Conversely, the three compounds slightly decreased (from 15% to 30% at 200 µM) light emission from a coculture system based on PAO1 wild type and on the *rhl*-specific biosensor strain PAO1 Δ*rhlI* P*rhlA*::*luxCDABE* (Fig. S5B; 24). These data demonstrate that clotrimazole, clofoctol and miconazole do not affect the *las* QS system, while these drugs have a slight negative effect on the *rhl* QS system. Considering that *i)* the repressive effect exerted by the hits on the *pqs* QS system (Fig. 2A) occurs at lower concentration and is more pronounced than the repressive effect exerted by the same molecules on the *rhl* QS system (Fig. S5B), and that *ii)* the *pqs* system exerts a positive effect on the *rhl* system (54,58), these data support a primary activity of the hits on the *pqs* QS system, that consequently reduces *rhl* activity.

Overall, these data confirm that clotrimazole, clofoctol and miconazole exert an anti-*pqs* activity without altering *P. aeruginosa* growth.

Characterization of the mechanism of action of the newly identified *pqs* inhibitors

The inhibition of P*pqsA* activity in the PAO1/AQ-Rep coculture system (Fig. 2A) may be due to inactivation of AQ biosynthesis in the PAO1 strain or of AQ reception in both PAO1 and AQ-Rep strains (Fig. 1A). Similarly, the reduced AQ levels in PAO1 (Fig. 2B) could also be due to

inhibition of either AQ biosynthesis or response, due to the PqsR-dependent regulatory loop governing transcription of the HHQ biosynthetic enzymes (36,55).

To discriminate between these two possibilities, the effect of the three drugs on AQ production was tested in a PAO1 Δ*pqsA*Δ*pqsH* double mutant strain (Δ*pqsAH*; Table S1) carrying the pFD-*pqsABCD* plasmid for constitutive expression of the HHQ biosynthetic enzymes. In this genetic background, in which AQ production does not depend on the ability of AQs to activate P*pqsA* *via* PqsR, the inhibitors did not reduce AQ levels, demonstrating that they do not affect the functionality of the enzymes required for HHQ biosynthesis (Fig. 3A). Moreover, the inhibitors were effective in reducing bioluminescence emission by the AQ-Rep biosensor strain grown in the presence of synthetic PQS (Fig. 3B), suggesting that the inhibitors target the PqsR-dependent AQ response rather than biosynthesis.

To validate this hypothesis, we investigated the effect of the hits on the levels of *pqsR* mRNA and PqsR protein. As shown in Fig. 4A, Real Time RT-PCR analysis revealed that the hits do not affect *pqsR* mRNA levels. Moreover, Western immunoblotting showed that the inhibitors do not reduce PqsR protein levels in a PAO1 Δ*pqsA*Δ*pqsH*Δ*pqsR* triple mutant strain (Δ*pqsAHR*; Table S1) carrying the pPqsR-6H plasmid for IPTG-inducible expression of a 6xHis-tagged variant of PqsR (Fig. 4B; 59). Actually, clotrimazole increased PqsR levels, indicating that this drug has a positive effect on the translation of the *pqsR* mRNA or on PqsR stability. However, clotrimazole decreased P*pqsA* activity (Fig. 2A) and AQs production (Fig. 2B), and reduced the mRNA level of *pqs*-controlled genes, as demonstrated by Real Time RT-PCR analysis performed on total mRNA extracted from PAO1 wild type grown in the absence or in the presence of 100 µM clotrimazole (Fig. S6). Overall, despite increasing PqsR level, clotrimazole seems to hamper the ability of this transcriptional regulator to activate gene expression.

To support PqsR as a target of the hits, we investigated their ability to reduce light emission from the P*pqsA*::*luxCDABE* transcriptional fusion in a PAO1 triple mutant strain unable to synthesize AQs and to produce PqsR (*i.e.* PAO1 Δ*pqsAHR*), carrying the pPqsR-6H plasmid for

IPTG-inducible expression of PqsR. Cultures of this strain were grown in LB supplemented with 10 µM PQS, to induce P*pqsA* activity, with a fixed concentration of the hits (100 µM), and with increasing concentrations of IPTG. The rationale of this experiment is that increased expression of PqsR, due to increased concentration of IPTG, should decrease the repressive effect exerted by the hits on P*pqsA*, if PqsR is the target of the hits. As shown in Fig. 4C, the inhibitory effect exerted by the hits on P*pqsA* activity decreased in parallel to increasing IPTG concentration in the growth medium, thus supporting PqsR as their molecular target. Overall, these data indicate that each of the hits acts downstream of *pqsR* expression, likely hampering PqsR functionality.

To support the hypothesis that the inhibitors directly interact with PqsR, molecular docking simulations were performed based on the crystal structure of the PqsR co-inducer binding domain (CBD) in the apo form (PDB ID: 4JVC) (59). To increase the reliability of the simulations, the docking search space encompassed the entire CBD of PqsR, *i.e.* a "blind" docking procedure was carried out. Amino acid residues previously reported to be involved in the binding of the natural ligand 2-nonyl-4-hydroxy-quinoline (NHQ) to the PqsR CBD (59) were considered flexible (see Materials and Methods for details). This analyses indicated that the three hits bind PqsR with high affinity at the same site as the natural ligand NHQ (Fig. 5) with predicted ΔG values for binding of clotrimazole, clofoctol and miconazole being -8.4, -9.8 and -8.5 kcal/mol, respectively. Interestingly, these values are lower than the predicted ΔG value for binding of NHQ (-7.9 kcal/mol; Table 1). Similar results were obtained when using the PqsR CBD structure bound to NHQ (PDB ID: 4JVD) (59), from which the ligand was removed. In the latter case, ΔG values for binding of clotrimazole, clofoctol, miconazole and NHQ were -9.4, -9.9, -8.1 and -8.1 kcal/mol, respectively. Finally, maintaining all the CBD residues in a fixed position yielded very similar results (data not shown). Interestingly, in each case the predicted affinity of the hits for PqsR parallels their efficacy as *pqs* inhibitors (Table 1).

Overall, these data suggest that the newly identified inhibitors could be endowed with a similar mechanism of action, that is to hamper PqsR functionality by competing with AQ

agonists for PqsR binding. Also the evidence that clotrimazole increases PqsR level (Fig. 4B) while hampering its ability to drive AQ production (Fig. 2B) and to activate *pqs*-controlled genes (Figs. 2A and S6) supports direct interaction of this hit to PqsR.

Notably, both activity assays and *in silico* predictions indicate that clofoctol has greater inhibitory activity relative to miconazole and clotrimazole (Table 1). To support competitive binding of PQS and clofoctol to PqsR, the ability of this drug to repress P*pqsA* activity was evaluated in the AQ-Rep biosensor grown in the presence of a range of concentrations of the native PqsR agonist PQS. This competition assay revealed the reduced ability of clofoctol to inhibit P*pqsA* activity in the presence of increasing concentrations of PQS (Fig. S7), in accordance with the activity of clofoctol as a competitive antagonist of the PQS receptor protein PqsR.

Clofoctol inhibits the expression of *pqs*-controlled virulence phenotypes

By hampering the ability of PqsR to activate the transcription of the *pqsABCDE* operon, clofoctol is expected to reduce the expression of virulence traits controlled by both PQS and PqsE in *P. aeruginosa*. First of all, since the assays previously performed to assess the effect of clofoctol on AQ production did not discriminate between HHQ and PQS, these QS signal molecules were quantified by liquid chromatography - tandem mass spectrometry (LC-MS/MS) analysis of spent media from PAO1 cultures treated with a range of concentrations of clofoctol. As shown in Fig. 6A, this analysis confirmed that clofoctol inhibits AQ production in *P. aeruginosa*, with both HHQ and PQS concentrations being significantly reduced by the drug.

With respect to the effect of clofoctol on PQS- and PqsE-controlled virulence determinants, phenotypic analyses revealed that 100 µM clofoctol leads to > 80% reduction in pyocyanin (Fig. 6B), and considerably reduced swarming motility (Fig. 6C). Moreover, 100 µM clofoctol significantly reduced biofilm formation in a PAO1 strain constitutively expressing GFP *via* the pMRP9-1 plasmid (60) (Fig. 6D). Notably, the effect of clofoctol on the tested phenotypes in

PAO1 mimicked deletion of the *pqsR* gene (Δ*pqsR*; Fig. 6B-D), in accordance with the hypothesis that PqsR is the clofoctol target.

Subsequently, Real Time RT-PCR analyses were performed to examine the effect of clofoctol on the expression of *pqs*-controlled virulence genes (28). The PQS-dependent *pvdS* and *pchR* genes code for the PvdS and PchR regulatory proteins required for the synthesis of the siderophores pyoverdine and pyochelin, respectively (28,61); the PqsE-dependent *lecA* gene codes for the LecA lectin involved in the formation of antibiotic-resistant biofilms (28,62). As a control, the mRNA level of *pqsA* was also measured. Real Time RT-PCR analyses showed that clofoctol significantly decreased the mRNA level of each of the genes tested, in agreement with the down-regulation observed in a PAO1 Δ*pqsR* mutant strain (Fig. 6E). The negative effect exerted by clofoctol on *lecA* transcription was also confirmed by promoter activity assay showing reduced activity of the P*lecA*::*luxCDABE* transcriptional fusion in PAO1 cultures treated with clofoctol (Fig. S8).

Overall, these data support clofoctol as an antivirulence agent active against the *P. aeruginosa* *pqs* QS system.

Clofoctol protects *Galleria mellonella* larvae from *P. aeruginosa* infection and inhibits the *pqs* QS system in CF clinical isolates

The antivirulence activity of clofoctol was tested in *G. mellonella* larvae, an insect infection model which correlates well with *P. aeruginosa* mouse infection models (63). Firstly, *G. mellonella* was infected with ca. 10 cells of *P. aeruginosa* PAO1 or of the isogenic Δ*pqsR* mutant and incubated at 37°C for 120 h. As shown in Fig. 7A, mutation of *pqsR* significantly reduced the ability of *P. aeruginosa* to kill the larvae, demonstrating the suitability of this insect model to investigate the antivirulence potential of drugs targeting PqsR.

Since the average weight of *G. mellonella* larvae was ca. 500 mg, and arbitrarily assuming uniform dispersal of injected bacteria and clofoctol in 500 µL of larval volume (64,65), 10 µL of

saline containing 5 mM clofoctol were injected to give 100 µM clofoctol in each larva. Preliminarily, we verified that the injection of 10 µL of saline containing 5 mM clofoctol did not affect the survival of uninfected larvae, and that 2 h incubation of *P. aeruginosa* with 5 mM clofoctol did not affect *P. aeruginosa* growth and viability (data not shown). Then, *G. mellonella* larvae were inoculated with *P. aeruginosa* PAO1 in the absence or in the presence of clofoctol. The treatment with clofoctol led to a survival percentage of 87%, similar to that observed with the Δ*pqsR* mutant (83%), while only 50% of untreated *G. mellonella* larvae survived PAO1 infection (Fig. 7A). Overall, these data demonstrate that clofoctol attenuates *P. aeruginosa* PAO1 lethality in *G. mellonella*.

To verify that clofoctol is active also against clinical *P. aeruginosa* strains, its ability to reduce AQ production was evaluated in a collection of 20 *P. aeruginosa* isolates from the lungs of CF patients, grouped into four categories with respect to the stage of infection (Table S2). A preliminary analysis revealed that only 2 strains isolated from patients with more than 15 years of chronic infection (chronic late group) did not produce detectable levels of AQs (Table S2), hence these strains should be considered resistant to the antivirulence effect of clofoctol. The remaining 18 clinical isolates were grown in LB for 24 h in the absence or presence of 100 µM clofoctol, and the AQ concentration determined in the corresponding spent media by using the AQ-Rep biosensor. Residual AQ production was estimated for each treated isolate relative to the amount of AQ detected in the corresponding untreated sample, considered as 100%. Notably, clofoctol decreased AQ production in each of the clinical isolates tested, with a reduction ranging from 12.7% to 88.4% (Fig. 7B). The median reduction in AQ production in the tested isolates was 68.6%, hence comparable with the reduction in AQ levels measured in PAO1 treated with 100 µM clofoctol under the same conditions (65.7%; Fig. 7B). Differences in the median reduction values among the analysed groups were not statistically significant. Moreover, differences in the median reduction of AQ production were not significant also when grouping the isolates according to their antibiotic resistance profiles (Table S2). Indeed, the median

reduction of AQ levels was 71.6% and 67.4% in 4 antibiotic susceptible and in 12 antibiotic resistant strains, respectively (Fig. S9). Also the 2 multidrug resistant (MDR) or extensively drug resistant (XDR) strains analysed in this study were susceptible to clofoctol, with a reduction of AQs levels of 56.5% and 88.4%, respectively (Fig. S9). Although performed on a limited number of clinical isolates, this analysis indicates that clofoctol is effective in blocking the *pqs* QS system in CF strains, irrespective of their adaptation to the host environment during long lasting chronic lung infection and of their antibiotic resistance profiles.

DISCUSSION

As a consequence of widespread antibiotic resistance, inhibition of virulence rather than growth has become a viable approach for combatting bacterial infections with lower selective pressure for emergence of resistance (10). In particular, *in vitro* evolution experiments suggest that resistant mutants will not emerge for drugs targeting public goods, such as virulence factors that are secreted and shared between individuals (66). Moreover, since antivirulence drugs target specific bacterial functions required for infection, these molecules are not expected to impact on the beneficial resident microbiota relative to that of antibiotics (11,13).

In many bacterial pathogens QS positively controls the expression of multiple secreted virulence factors, hence this communication system is considered a promising target for the development of antivirulence agents (23,67). Since *P. aeruginosa* has four interconnected QS systems that positively control the production of virulence factors and biofilm formation, most of the research on QS inhibition has focused on this bacterium as a model system. Indeed, several molecules inhibiting the *las* QS system of *P. aeruginosa* have been identified (23,67). Recently, a number of studies have described inhibitors of the *pqs* QS system. The *pqs* system positively controls the expression of multiple virulence determinants, including secreted virulence factors and biofilm formation, and *pqs* mutant strains display attenuated virulence in plant and animal models of infection (32-35,38). Moreover, the *pqs* system is active during *P. aeruginosa*

383 infections in humans (39,68,69).

384 Inhibitors of the *pqs* system were previously identified among analogs of anthranilate, the
385 substrate of PqsA in the first step of the biosynthetic route leading to AQ production (37,40).
386 Subsequently, compounds binding to the AQ-biosynthetic enzyme PqsD were shown to act as
387 potent *pqs* inhibitors, with IC_{50} values in the low µM range (from 1 to 14 µM) (70,71). The
388 possibility of interfering with the *pqs* system *via* enzymatic degradation of the AQ signals, rather
389 than *via* small molecules targeting their biosynthesis, was also explored, and PQS degrading
390 activity has been described in *Arthrobacter nitroguajacolicus* and *Achromobacter xylosoxidans*
391 (72,73). However, the majority of anti-*pqs* molecules identified so far are competitive inhibitors
392 of the transcriptional regulator PqsR. Potent PqsR antagonists with IC_{50} values ranging from 0.4
393 to 38.5 µM have been found among analogs of the natural agonists HHQ and PQS (59,74-76).
394 Whole-cell high-throughput screening and structure-activity relationship analyses led to the
395 identification of benzamide-benzimidazole PqsR inhibitors with low IC_{50} values (< 1 µM) some
396 of which also inhibited the PqsBC complex (77-79). Also 2-sulfonylpyrimidines were identified
397 hampering both AQ reception and biosynthesis (80). Overall, a number of reports validated the
398 antivirulence potential of anti-*pqs* molecules, showing their ability to reduce the expression of
399 *pqs*-controlled virulence traits both *in vitro* and in animal models of infection. Despite the
400 promise of anti-*pqs* agents for the treatment of *P. aeruginosa* infections, to the best of our
401 knowledge none of these molecules has so far entered clinical trials. This is probably due to the
402 poor pharmacological properties of the inhibitors, including possible cytotoxicity, and to the lack
403 of ADME-TOX studies required for their evaluation in humans. In this context, searching for
404 off-target activities in drugs already approved for use in humans represents a potential shortcut
405 for developing new anti-*pqs* molecules that could move straight into clinical trials.

406 In this study, a drug-repurposing approach led to the identification of three promising anti-*pqs*
407 drugs already used in humans, by screening a library of 1,600 FDA-approved compounds (Table
408 1; Fig. S2). Data on the acute and chronic toxicity are already available for these drugs, as well

as information on their pharmacokinetics. Clotrimazole and miconazole are antifungal drugs used in humans to treat ring worm, pityriasis versicolor, vaginal and oral candidiasis and skin yeast infections (44,45,81,82). They both alter the permeability of the fungal cell wall by binding to phospholipids and inhibiting the biosynthesis of ergosterol and other sterols required for fungal cell membrane integrity (83,84). Miconazole displays its activity by inhibiting fungal peroxidases, which results in peroxide-mediated cell death (83). Both of these drugs are mainly administered as creams or ointments, thus their current formulations could be particularly suitable for topical treatment of chronic wound infections caused by *P. aeruginosa* (85,86). However, this opportunistic pathogen is a main cause of lung infections especially in individuals with CF, where it establishes chronic infections that can last for decades (87). The use of clotrimazole and miconazole to treat *P. aeruginosa* lung infections would require their reformulation as inhalable nanosuspensions, an approach that has recently demonstrated its value for repurposing the anthelmintic drug niclosamide as an anti-QS agent against *P. aeruginosa* (24,88).

Out of the 1,600 compounds tested in this screening campaign, the most promising anti-*pqs* drug was clofoctol, an antimicrobial used for the treatment of acute and chronic upper respiratory tract infections and for tracheobronchial infections caused by Gram-positive pathogens, especially staphylococci, pneumococci and streptococci (48,50). Clofoctol is also used in preventive and curative treatment of otolaryngology and stomatology (89). The mechanism of action of this drug as an antimicrobial is still poorly understood, but a detrimental effect of clofoctol on membrane and cell wall biosynthesis in Gram-positive bacteria has been reported (49,90). Clofoctol is usually administered as suppositories as it is well absorbed through the rectal mucosa and rapidly spreads through the tissues, reaching the highest concentrations in the respiratory system (91). Since clofoctol mainly acts in the airways, it is potentially valuable as a future treatment of *P. aeruginosa* lung infections. Notably, clofoctol is used to treat infections in infants, and this is another advantageous feature if considering that in CF, *P. aeruginosa* lung

435 infection is established in early life (92).

436 Overall, despite their lower potency compared with other *pqs* inhibitors described so far, the
437 anti-*pqs* drugs identified in this study have considerable potential for human use, and could be
438 directly tested in clinical trials or serve as chemical scaffolds for future drug-optimization
439 programmes.

440 With respect to the mechanism of action of the three FDA-approved drugs, they all affect
441 PqsR functionality, probably by competing with the natural ligands HHQ and PQS for the PqsR
442 ligand-binding site (Figs. 3, 4 and S7). This hypothesis is supported by docking simulations,
443 which predict that all three compounds bind to the PqsR co-inducer binding domain in the same
444 binding site as the natural ligand NHQ (Fig. 5). This result was somehow unexpected, since the
445 PAO1/AQ-Rep coculture used in the screening campaign should primarily identify molecules
446 affecting both AQ biosynthesis and AQs reception (Fig. 1A). Indeed, this coculture-based
447 reporter system was functional in identifying the PqsA-inhibitor methyl anthranilate (Fig. 1B).
448 Intriguingly, the anti-QS activity of the anthelmintic drug niclosamide was discovered using a
449 coculture-based reporter system similar to the one deployed in this work. In common with
450 clofoctol, niclosamide inhibited the QS signal molecule response rather than biosynthesis (24).
451 Therefore, the selection of drugs targeting QS receptors could be a bias intrinsic to the screening
452 system used. In fact, in coculture-based screening systems, drugs interfering with QS signal
453 molecule receptor would have a dual outcome since they would block both QS signal receptor
454 and consequently signal biosynthesis in the wild type, as well as inhibiting the QS receptor in the
455 reporter strain. Conversely, an inhibitor of QS signal molecule biosynthesis would only affect the
456 functionality of the *P. aeruginosa* wild type strain. Hence, the PAO1/AQ-Rep coculture system
457 may offer a more sensitive screen for PqsR inhibitors than for those that inhibit AQ biosynthesis,
458 so that only drugs targeting PqsR will meet the selection criteria for the primary screen.

459 Since each of the hits identified in this study are likely to target PqsR, we focused our
460 attention on the most potent inhibitor, clofoctol (Table 1).

Different elements of the *pqs* QS system have recently been shown to control distinct virulence traits. In particular, the PQS signal molecule drives the expression of genes required for the biosynthesis of siderophores and of genes coding for PrpL and AprX proteases, and exotoxin S, while PqsE is required for the production of pyocyanin, LecA and LecB lectins, hydrogen cyanide, rhamnolipids and ChiC chitinase (28). With regard to pleiotropic virulence phenotypes such as swarming motility and biofilm formation, these appear to be regulated by both PQS and PqsE (33). Consistent with the activity of clofoctol as a PqsR inhibitor, the expression of both PQS-controlled virulence traits, such as the expression of genes required for siderophores biosynthesis (Fig. 6E), and of PqsE-dependent phenotypes, including pyocyanin production (Fig. 6B) and expression of the *lecA* gene (Figs. 6E and S8), were inhibited. Moreover, clofoctol reduced both swarming motility and biofilm formation (Figs. 6C and 6D). Notably, clofoctol exerted an antivirulence effect *in vivo*, since this drug attenuated *P. aeruginosa* infection in *G. mellonella* larvae (Fig. 7A).

A major concern with respect to the use of anti-QS drugs for the treatment of CF pulmonary infection originates from evolutionary selection driving *P. aeruginosa* adaptation to the CF lung. Indeed, during chronic infections, CF isolates accumulate mutations that reduce the production of virulence factors, lead to the formation of mucoid biofilms, increase antibiotic resistance mainly as a consequence of efflux pump over-expression, and in some cases inactivated QS systems (93-96). Since *P. aeruginosa* QS-defective mutants should be considered resistant to anti-QS drugs, the suitability of QS-inhibition for CF therapy is under debate. However, most studies have focused on the inactivation of the *las* QS system in chronic CF isolates, while little attention has so far been given to the *pqs* QS system (97-100). The evidence that AQs have been identified in the sputum of CF patients with both intermittent and chronic *P. aeruginosa* infections demonstrate unequivocally that the *pqs* QS system is active in the CF lung (68,69,99,101). In addition, AQs can be detected in the sputum, plasma and urine of ca. 80% of CF patients suffering with *P. aeruginosa* chronic lung infections. Levels of the AQ molecule

NHQ increased at the start of a pulmonary exacerbation and positively correlated with quantitative measures of *P. aeruginosa* cells in the lung (39). This evidence is consistent with the results obtained in this study, since only 2 out of the 20 clinical isolates tested did not produce detectable levels of AQs (Table S2). Notably, clofoctol reduced functionality of the *pqs* QS system in all the *pqs*-proficient CF isolates, irrespective of their antibiotic resistance profiles (Fig. 7B and Fig. S9).

Future analyses performed on a larger panel of *P. aeruginosa* clinical isolates from both CF and chronic wound patients and *in vivo* assays in murine models of infection are required to better assess the suitability of clofoctol, clotrimazole and miconazole for the treatment of *P. aeruginosa* chronic infections. However, the results of this work should encourage further preclinical studies to aid transfer of the newly identified *pqs* inhibitors from the laboratory into clinical practice.

MATERIALS AND METHODS

Bacterial strains, media and chemicals

The bacterial strains, clinical isolates, plasmids and oligonucleotides used in this study are listed in Table S1, S2, S3 and S4, respectively. Bacterial strains were routinely grown at 37°C in Luria-Bertani Broth (LB) with aeration and, when necessary, antibiotics were added at the following concentrations: tetracycline (Tc), 200 μg/mL; carbenicillin (Cb), 150 μg/mL; gentamicin (Gm), 100 μg/mL; kanamycin (Km), 200 μg/mL. When necessary, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at the concentrations indicated in the text. Muller-Hinton Broth (MHB) and M9 minimal medium supplemented with 20 mM glucose as carbon source were used in the MIC assay (Clinical and Laboratory Standards Institute, CLSI) and in the biofilm assay, respectively. Synthetic HHQ and PQS stock solutions were prepared in MeOH. Clotrimazole, clofoctol and miconazole were purchased from Sigma-Aldrich and dissolved in DMSO.

Primary screening for the identification of *pqs* inhibitors

P. aeruginosa PAO1 and the AQ-Rep biosensor strain (PAO1 Δ*pqsA* P*pqsA::luxCDABE*) were grown overnight at 37°C on LB agar plates. Bacteria were scraped from plate surfaces and diluted in LB to an optical density (OD) at 600 nm wavelength (OD_{600}) of 0.1 and 0.03 for the biosensor and PAO1 strains, respectively [procedure modified from (57)]. Two-hundred µL aliquots of the coculture were grown at 37°C in 96-well microtiter plates in LB supplemented with each compound of the PHARMAKON library (20 µM and 200 µM). The OD_{600} and relative light units (RLU) were measured after 5 h incubation by using a Wallac 1420 Victor^{3}V multilabel plate reader (PerkinElmer). Eight samples grown in the presence of DMSO (0.2% or 2%) were used as controls in each microtiter plate. Reporter activity was determined as RLU/OD_{600} for each sample. Residual reported activity was determined in treated sample relative to the control samples grown in the presence of DMSO, considered as 100%.

A similar approach was used to investigate the effect of the hits on the *las* and *rhl* QS systems. In this case, cocultures of the *P. aeruginosa* PAO1 wild type strain and of the PA14-R3 (PA14 Δ*lasI* P*rsaL::luxCDABE*; 57) or the C4-Rep (PAO1 Δ*rhlI* P*rhlA*::*luxCDABE*; 24) biosensor strains were used, respectively.

Quantification of AQs

Levels of AQ signal molecules in treated-*P. aeruginosa* PAO1 culture supernatants were determined by using the reporter strain AQ-Rep, as previously described (102). Bacterial cultures were grown in 96-well microtiter plates at 37°C with shaking. Supernatants were collected after 16 h for the experiments shown in in Figs. 2B, 3A and S2, or after 24 h for experiments shown in Figs. 7B and S9, to allow optimal AQ production in slow-growing clinical isolates. Briefly, 10 µL of culture supernatant was added to 190 µL of LB inoculated with AQ-Rep biosensor (final OD_{600} = 0.1) in 96-well microtiter plates. Microtiter plates were incubated at 37°C with gentle shaking, and the OD_{600} and RLU were measured after 5 h of incubation. A

calibration curve was generated by growing the AQ-Rep biosensor in the presence of increasing concentrations of synthetic HHQ or PQS; the resulting dose-response curve was used to calculate the concentration of the AQ signals in each culture supernatant.

AQs produced by *P. aeruginosa* PAO1 were also quantified in by LC-MS/MS analysis, as previously described (103). Briefly, PAO1 was inoculated into 5 mL of LB in the absence or in the presence of 100 µM clofoctol. After 16 h incubation at 37°C with shaking, cell density of the culture was recorded and the supernatants were filter-sterilized. Supernatants were solvent extracted with ethyl acetate, dried under vacuum and re-dissolved in MeOH prior to quantitative analysis by LC-MS/MS. For each sample, a supernatant concentration of HHQ and PQS was calculated by comparing analytic peak areas with a matched calibration line.

Pyocyanin production, swarming motility and biofilm formation assays

Pyocyanin was extracted and quantified from *P. aeruginosa* PAO1 and Δ*pqsR* grown in LB supplemented with 100 µM clofoctol or with DMSO as a control, as previously described (104). Swarming motility assays were performed on swarming plates [0.8% (wt/vol) nutrient broth N.2, 0.5% (wt/vol) glucose, 0.5% (wt/vol) bacteriological agar]. Plates were supplemented with or without clofoctol (100 µM). After 16 h of growth at 37°C, swarming motility was directly observed at the air-agar interface.

For microscopic visualization of biofilms, *P. aeruginosa* PAO1 or Δ*pqsR* constitutively expressing GFP *via* the pMRP9-1 plasmid (60) were grown in an 8-well chamber slide, as previously described (105), with minor modifications. Briefly, bacterial cells were inoculated at an OD_{600} of 0.02 in 700 µL of M9 minimal medium supplemented with 20 mM glucose as carbon source, in the absence or in the presence of 100 µM clofoctol. Cultures were incubated at 30°C for 24 h to allow the adhesion of the bacterial cells to the glass surface. To maintain bacterial viability, the medium was changed every 24 h. Biofilm formation was examined after 3 days incubation by using the Leica TCS SP5 confocal microscope.

Western immunoblotting

Crude protein extracts were collected from the *P. aeruginosa* PAO1 *pqsA*Δ*pqsH*Δ*pqsR* triple mutant strain carrying the pPqsR-6H plasmid grown in LB supplemented with 10 µM PQS and 20 µM IPTG, in the absence or in the presence of 100 µM clotrimazole, clofoctol or miconazole. The *P. aeruginosa* PAO1 *pqsA*Δ*pqsH*Δ*pqsR* strain carrying the pME6032 empty vector was used as a control. The Bradford assay (106) was used to quantify and normalize total protein content in the samples. Western immunoblotting was performed by using a standard technique (107) with mouse anti-6His antibody (1:5,000; Sigma-Aldrich) and goat anti-mouse IgG HRP-conjugate as secondary antibody (1:6,000; Bio-Rad Laboratories). Final development was performed with Amersham ECL chemiluminescent reagents (Amersham Biosciences). A C-DiGit blot scanner (LI-COR Biosciences) was used for data acquisition.

RNA extraction and Real Time RT-PCR analysis

P. aeruginosa PAO1 and Δ*pqsR* were inoculated at an OD_{600} of 0.02 in 5 mL of LB in the absence or in the presence of 100 µM clotrimazole, clofoctol or miconazole. Cultures were grown at 37°C with vigorous shaking until they reached an OD_{600} of 2.0, and then 1 mL of cells was harvested by centrifugation and resuspended in 2 mL of RNAprotect Bacteria Reagent (Qiagen). Total RNA extraction was performed with the RNeasy Mini Columns Kit (Qiagen) according to the manufacturer's instructions, including the on-column DNase I digestion step. In addition, eluted RNA was treated for 1 h at 37°C with DNase TURBO (0.2 U per µg of RNA; Ambion) and with SUPERase-In (0.4 U per µg of RNA; Ambion). DNase I was removed using the RNeasy Column Purification kit (Qiagen). Purified RNA was quantified using the NanoDrop 2000 spectrophotometer (Thermo-Fisher Scientific). The absence of genomic DNA in the RNA samples was verified by PCR performed with primers FWP*pqsL* and RVP*pqsL* (Table S4). cDNA synthesis was performed with the iScript Reverse Transcription Supermix for RT-qPCR kit (Bio-Rad Laboratories) according to manufacturer's instructions, and quantified with

NanoDrop 2000. Real Time RT-PCRs were performed using iTaq™ Universal SYBR® Green Supermix kit (Bio-Rad Laboratories), according of the manufacturer's instructions, and the Rotor Gene 6000 thermocycler (Corbett Research). Primers employed in Real Time RT-PCR analysis were designed using the Primer-blast software (www.ncbi.nlm.nih.gov/tools/primer-blast) and are listed in Table S4. The reaction procedure involved incubation at 95°C for 1 min and 40 cycles of amplification at 95°C for 10 s and 60°C for 45 s. Fluorescence was registered in the last 15 s of the 60°C step. 16S ribosomal RNA was chosen as an internal control (housekeeping gene) to normalize the Real Time RT-PCR data in each single run, and to calculate the relative fold change in gene expression by using the $2^{-\Delta\Delta Ct}$ method. The average data and standard deviations were calculated from three independent experiments.

Galleria mellonella killing assay

The *G. mellonella* killing assay was performed as previously described (63,65), with minor modifications. Briefly, *G. mellonella* caterpillars in the final instar larval stage (average weight, 486 ± 67 mg) were infected with 10 µL of saline containing about 10 bacterial cells, in the absence or in the presence of 5 mM clofoctol. Although PAO1 cells were incubated in the presence of clofoctol for less than 5 min before injection, preliminary assays showed that 5 mM clofoctol treatment (for up to 24 h) does not significantly affect PAO1 cell or larval viability (data not shown). *G. mellonella* larvae were incubated at 37°C in petri dishes (ten larvae *per* dish) and monitored for 120 h. Larvae were considered dead when they did not respond to gentle prodding. At least 30 larvae *per* condition were used in four independent experiments. Survival curves for the *G. mellonella* killing assay were generated by the Kaplan-Meier method.

Molecular docking simulations

Molecular docking simulations were carried out using DockingApp (108), a user friendly interface for the docking program AutoDock Vina (109). In all simulations, the search space

(docking grid) included the whole PqsR co-inducer binding domain (CBD) structure, in order to carry out "blind" predictions of the 'hit' compound binding sites.

Simulations were carried out on the apo (PDB ID: 4JVC) and holo (PDB ID: 4JVD) forms of the protein (59), both by keeping all protein residues rigid and by allowing flexibility only of the residues previously reported to be involved in PqsR binding to the natural ligand NHQ (*i.e.* ILE 149, ALA 168, VAL 170, ILE 186, LEU 189, LEU 207, LEU 208, PHE 221, ILE 236, TYR 258, ASP 264, THR 265) (52).

Statistical analysis

Statistical analysis was performed with the software GraphPad Prism 5, using one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison tests. Differences having a p value < 0.05 were considered statistically significant.

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FIGURE LEGENDS

Figure 1. Validation of the screening system.

(A) Schematic representation of the coculture-based reporter system. The *P. aeruginosa* PAO1 strain (PAO1) produces AQ signal molecules which activate P*pqsA*::*luxCDABE* transcription, that results in light emission in the biosensor strain AQ-Rep. Drugs interfering with AQ biosynthesis or response are expected to reduce bioluminescence in the PAO1/AQ-Rep coculture, relative to the untreated samples. **(B)** Activity of the PAO1/AQ-Rep coculture system treated with indicated concentrations of the *pqs* inhibitors methyl anthranilate (white bars) or farnesol (grey bars). Bioluminescence of the untreated PAO1/AQ-Rep coculture normalized to cell density is considered as 100%.

Figure 2. Clotrimazole, clofoctol and miconazole inhibit P*pqsA* activity and AQ production.

Effect of clotrimazole (white bars), clofoctol (light-grey bars) and miconazole (dark-grey bars) on the PAO1/AQ-Rep coculture system. Bioluminescence of the untreated PAO1/AQ-Rep coculture normalized to cell density is considered as 100%. **(B)** Effect of clotrimazole (white bars), clofoctol (light-grey bars) and miconazole (dark-grey bars) on AQ production in PAO1. The level of AQs produced by untreated PAO1 is considered as 100%. For both **(A)** and **(B)**, the average of at least three independent experiments is reported with SD.

Figure 3. Clotrimazole, clofoctol and miconazole inhibit AQ reception.

(A) Production of AQs in *P. aeruginosa* PAO1 Δ*pqsAH*(pFD-*pqsABCD*) grown for 16 h in LB in the absence or in the presence of clotrimazole (white bars), clofoctol (light-grey bars) and miconazole (dark-grey bars). The AQ level measured in the untreated sample is considered as 100%. **(B)** Activity of the AQ-Rep biosensor strain grown in LB supplemented with 10 µM synthetic PQS and clotrimazole (white bars), clofoctol (light-grey bars) or miconazole (dark-grey bars). Bioluminescence of the untreated AQ-Rep biosensor normalized to its cell density is considered as 100%. For both **(A)** and **(B)**, the average of at least three independent experiments is reported with SD.

Figure 4. Clotrimazole, clofoctol and miconazole inhibit PqsR functionality.

(A) Real Time RT-PCR analysis showing the mRNA level of *pqsR* in PAO1 cultures treated with 100 µM of the indicated drugs relative to untreated PAO1 cultures. The PAO1 Δ*pqsR* strain was used as a negative control. The average of three independent experiments is reported with SD. ns, non-significant difference; ***, $p < 0.001$ (ANOVA). **(B)** Western immunoblotting performed with anti-6xHis antibody on crude protein extracts of PAO1 Δ*pqsAHR*(pPqsR-6H) grown in LB supplemented with 10 µM PQS and 20 µM IPTG, in the absence (untreated) or in the presence of the indicated drugs (100 µM). The PAO1 Δ*pqsAHR* strain carrying the empty vector pME6032 was used as a control. The data are representative of three independent experiments. **(C)** Effect of 100 µM clotrimazole (white bars), clofoctol (light-grey bars) and miconazole (dark-grey bars) on P*pqsA*::*lux* activity in the PAO1 Δ*pqsA*Δ*pqsH*Δ*pqsR* mutant carrying the pPqsR-6H plasmid, grown in LB supplemented with 10 µM PQS and different concentrations of IPTG as indicated in the graph. The average of three independent experiments is reported with SD.

Figure 5. Putative complexes formed by clotrimazole, clofoctol and miconazole with the

PqsR CBD.

Schematic representation of the complexes formed by clotrimazole **(A)**, clofoctol **(B)** and miconazole **(C)** with the PqsR co-inducer binding domain (CBD), obtained by molecular docking simulations (see Materials and Methods for details). The three drugs are represented in red, while the natural ligand NHQ is represented is green.

Figure 6. Clofoctol inhibits the expression of *pqs*-controlled virulence traits.

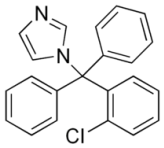
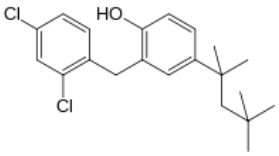
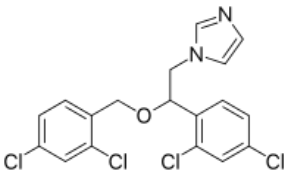
(A) Concentrations of HHQ (white bars) and PQS (grey bars) measured by LC-MS/MS on supernatants of PAO1 cultures grown for 16 h in LB in the absence or in the presence of clofoctol at the indicated concentrations. The average of three independent experiments is reported with SD. **, $p = 0.0062$; ***, $p < 0.001$ (ANOVA). **(B)** Effect of 100 µM clofoctol on pyocyanin production, **(C)** swarming motility, and **(D)** biofilm formation in PAO1. The same phenotypes were evaluated in the Δ*pqsR* mutant as a control. For pyocyanin production **(B)**, the average of three independent experiments is reported with SD and representative supernatants are shown in the inset picture. ***, $p < 0.001$ (ANOVA). For swarming motility **(C)** and biofilm formation **(D)**, representative pictures of three independent experiments are shown. **(E)** Real Time RT-PCR analysis showing mRNA level of the indicated genes in PAO1 treated with 100 µM clofoctol (white bars) and in Δ*pqsR* (grey bars) relative to untreated PAO1. The average of three independent experiments is reported with SD. **, $p = 0.0012$; ***, $p < 0.001$ (ANOVA).

Figure 7. Clofoctol displays an antivirulence effect *in vivo* and inhibits the *pqs* QS system in *P. aeruginosa* CF clinical isolates.

(A) Kaplan-Meier plot showing the percentage survival of *G. mellonella* larvae inoculated with *P. aeruginosa* PAO1 (blue line), with PAO1 and clofoctol at final concentration 100 µM (red line), or with Δ*pqsR* (green line). The mean survival rate calculated from four independent experiments performed on at least 30 larvae *per* condition is reported. **, $p = 0.0033$ for PAO1

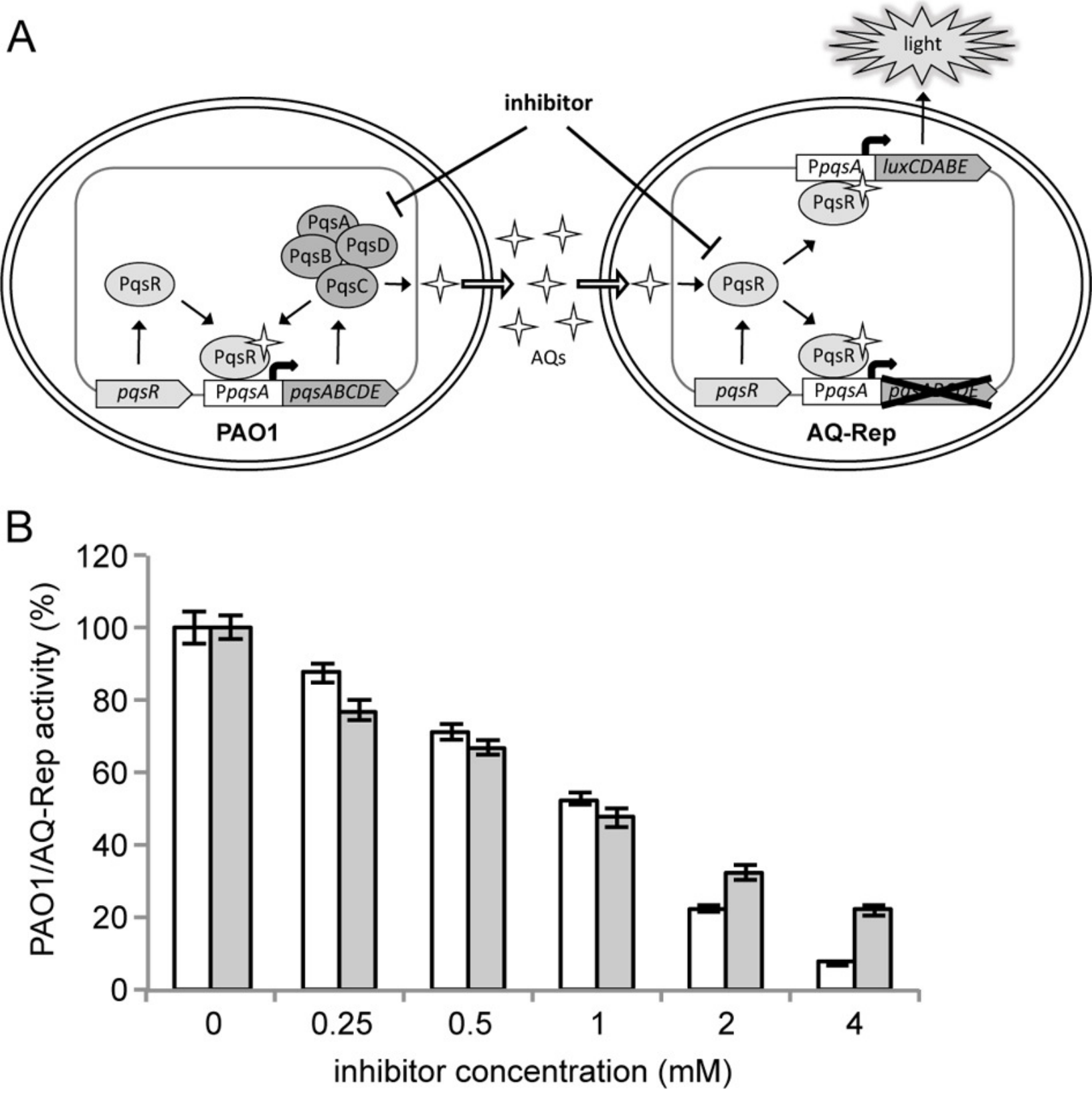
vs. PAO1 plus clofoctol; $p = 0.0016$ for PAO1 vs. Δ*pqsR* (ANOVA). **(B)** Dot plot showing the inhibition of AQ production in *P. aeruginosa* CF isolates (filled symbols) and *P. aeruginosa* PAO1 (open square) treated with 100 µM clofoctol, relative to the untreated samples considered as 100%. Black lines represent the median values: all, 31.4%; first isolate, 25.2%; early chronic, 31.1%; middle chronic, 32.1%; late chronic, 57.8%. AQ production in treated PAO1 cultures was 34.3% relative to untreated PAO1. Differences between the median values are not statistically significant. Mean results of three independent experiments are reported.

Table 1. Anti-*pqs* compounds identified by screening the PHARMAKON library of FDA-approved drugs.

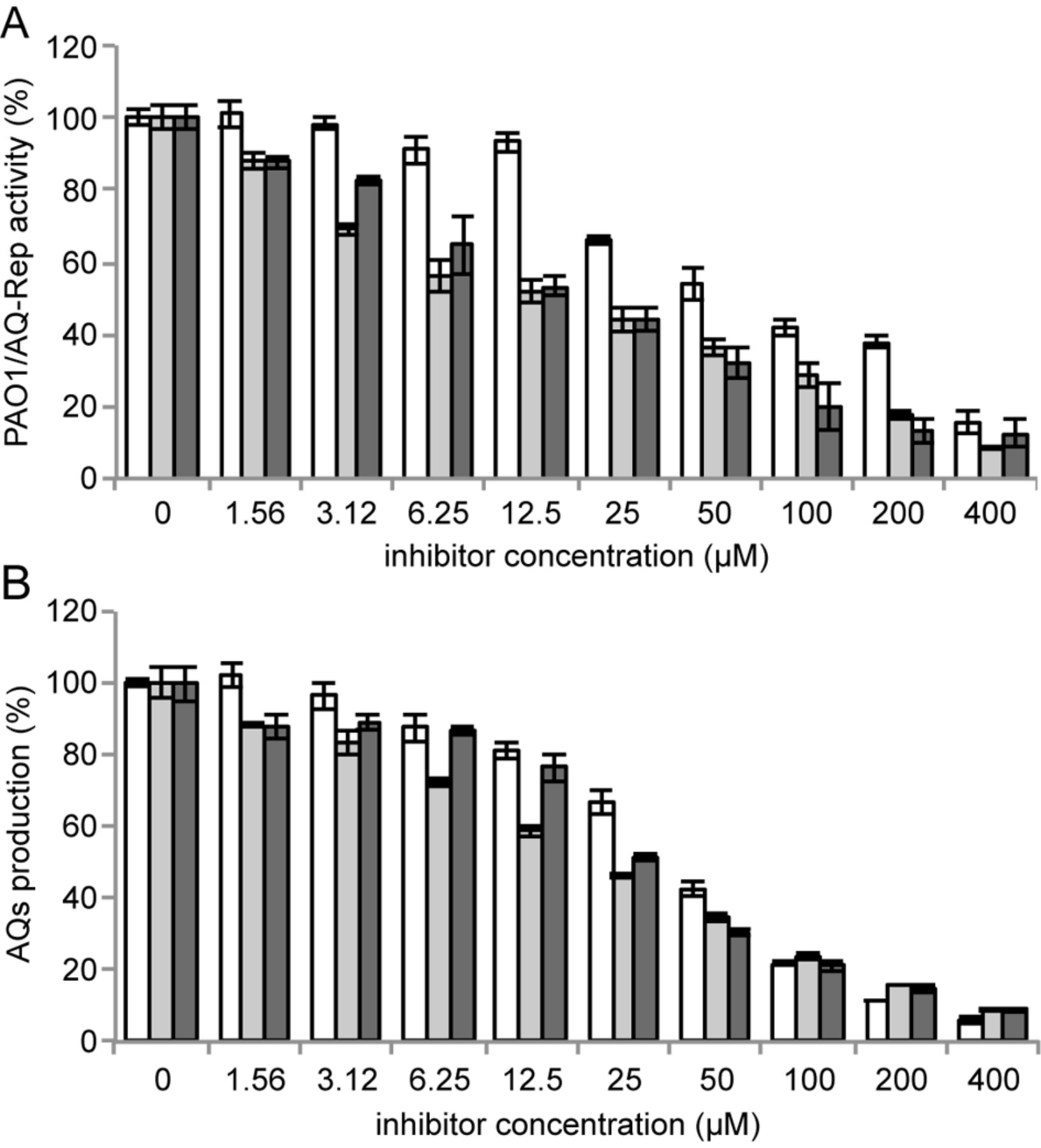
Drug name	Property	Structure	IC_{50}[a]	ΔG
Clotrimazole	Antifungal		39	-8.4
Clofoctol	Antibacterial		20	-9.8
Miconazole	Antifungal		27	-8.5

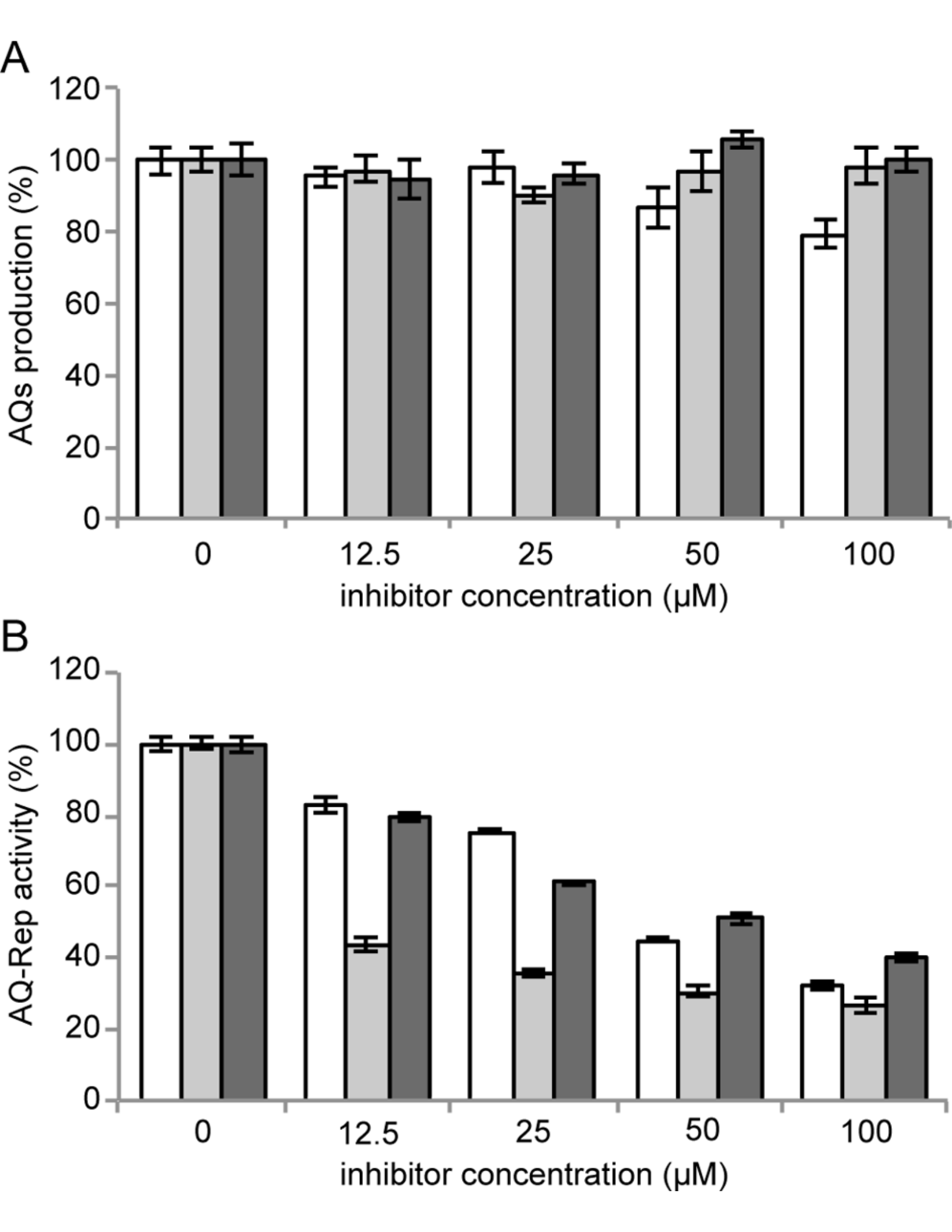
[a] The IC_{50} values (μM) were determined by using the PAO1/AQ-Rep coculture system.

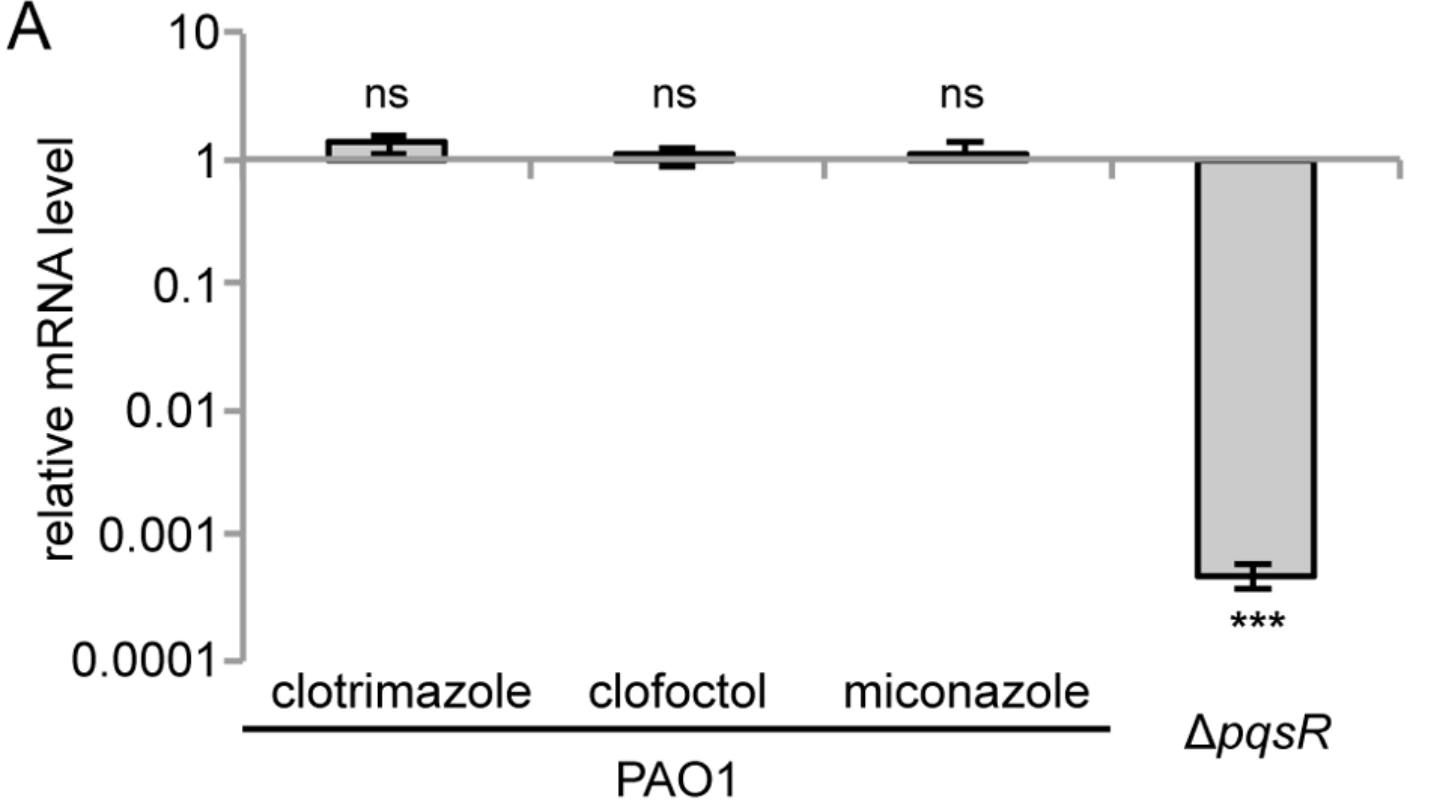
[b] ΔG values (kcal/mol) for drugs binding to the PqsR CBD apo form (PDB ID: 4JVC) (59) predicted by molecular docking simulations.

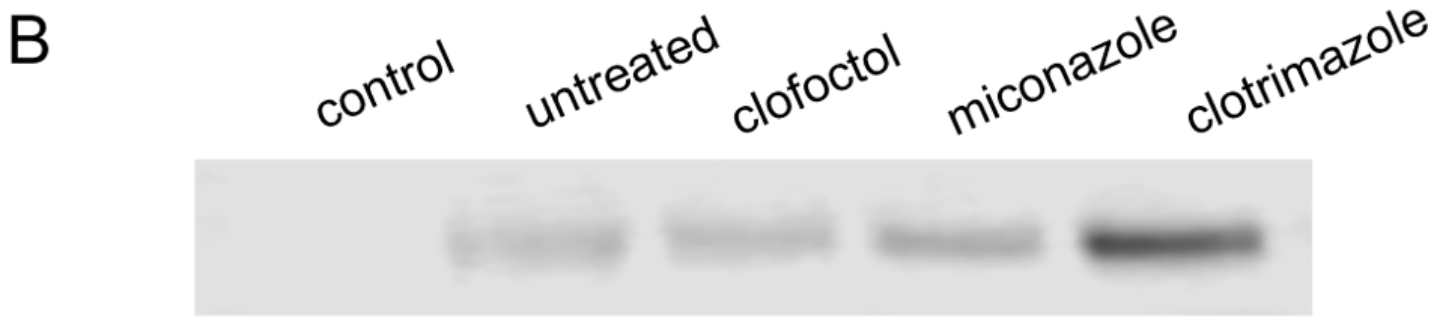

A
light
inhibitor
PqsA
PqsB
PqsD
PqsC
PqsR
PqsR
pqsR
PpqsA
pqsABCDE
PAO1
AQs
PpqsA
luxCDABE
PqsR
PqsR
PqsR
pqsR
PpqsA
AQ-Rep

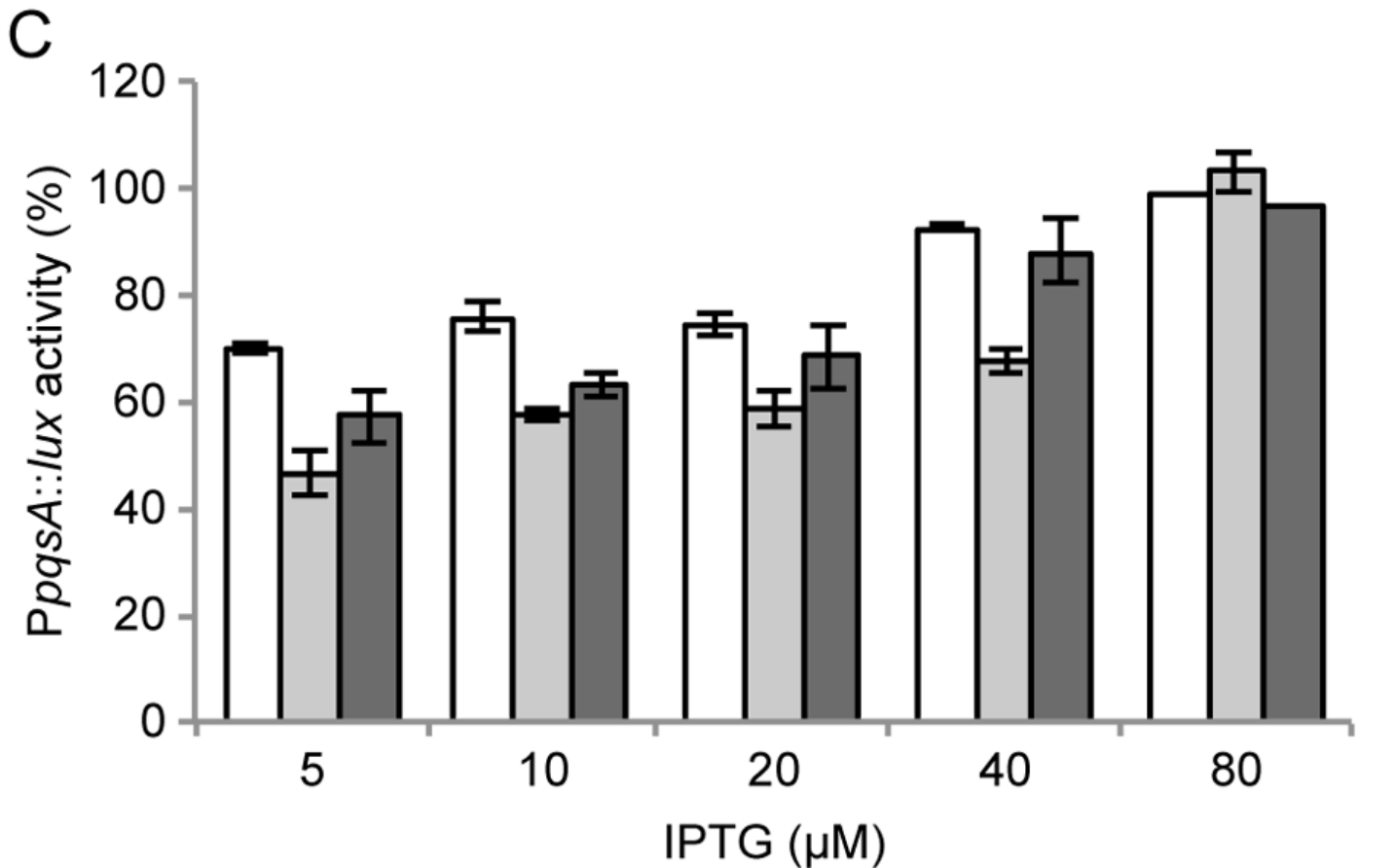
B
PAO1/AQ-Rep activity (%)
120
100
80
60
40
20
0
0
0.25
0.5
1
2
4
inhibitor concentration (mM)

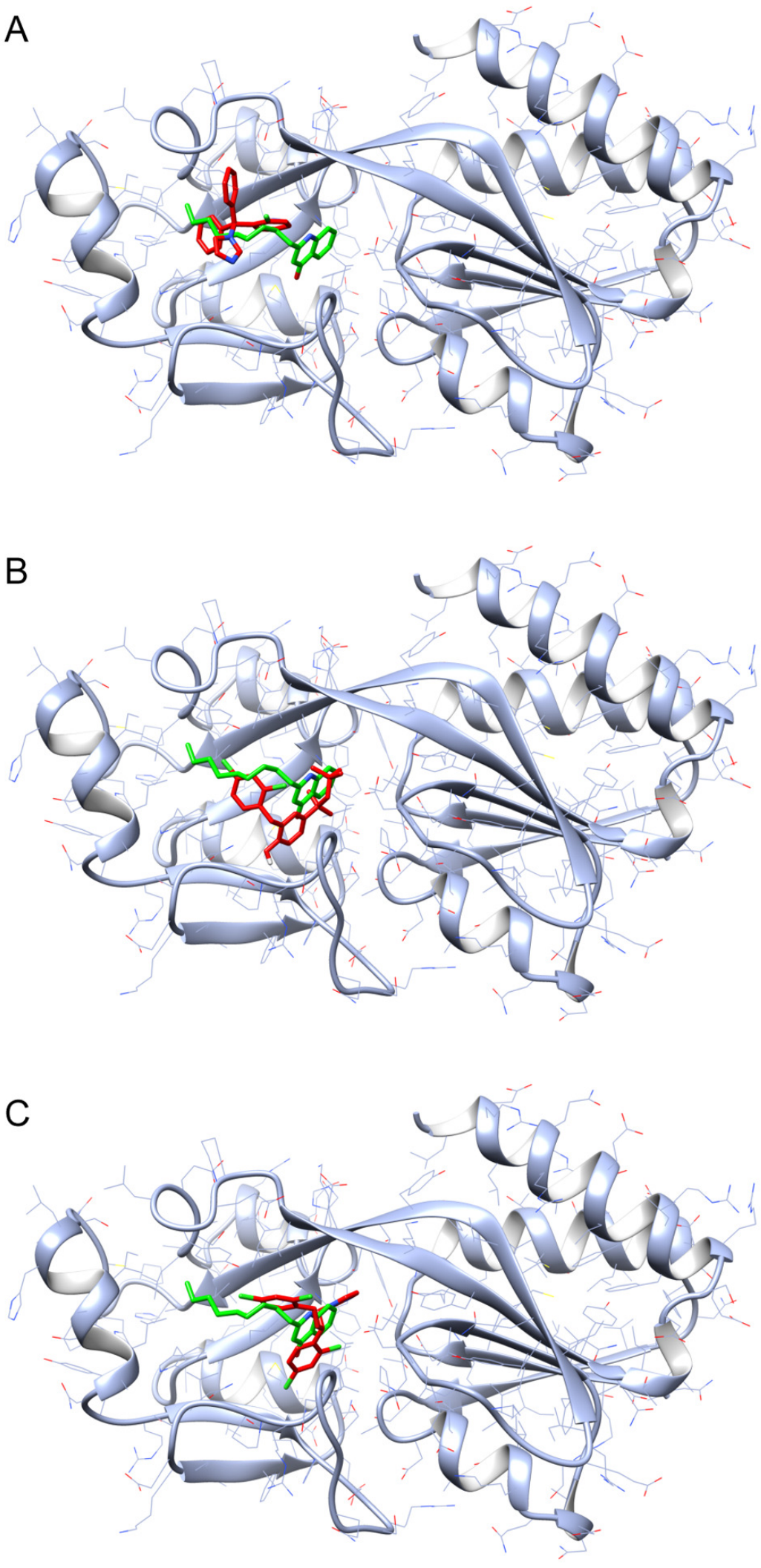

A
PAO1/AQ-Rep activity (%)
120
100
80
60
40
20
0
0
1.56
3.12
6.25
12.5
25
50
100
200
400
inhibitor concentration (μM)
B
AQs production (%)
120
100
80
60
40
20
0
0
1.56
3.12
6.25
12.5
25
50
100
200
400
inhibitor concentration (μM)

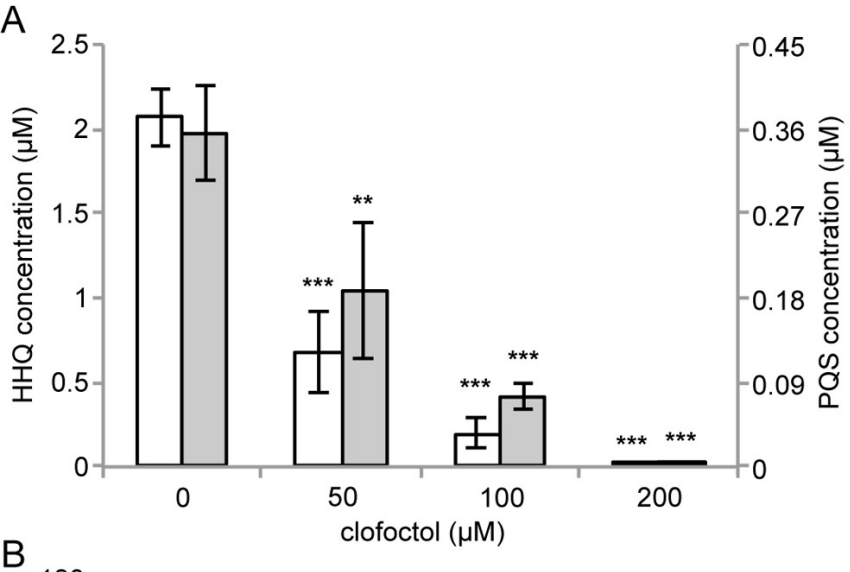

A
120
100
80
60
40
20
0
AQs production (%)
0
12.5
25
50
100
inhibitor concentration (μM)
B
120
100
80
60
40
20
0
AQ-Rep activity (%)
0
12.5
25
50
100
inhibitor concentration (μM)

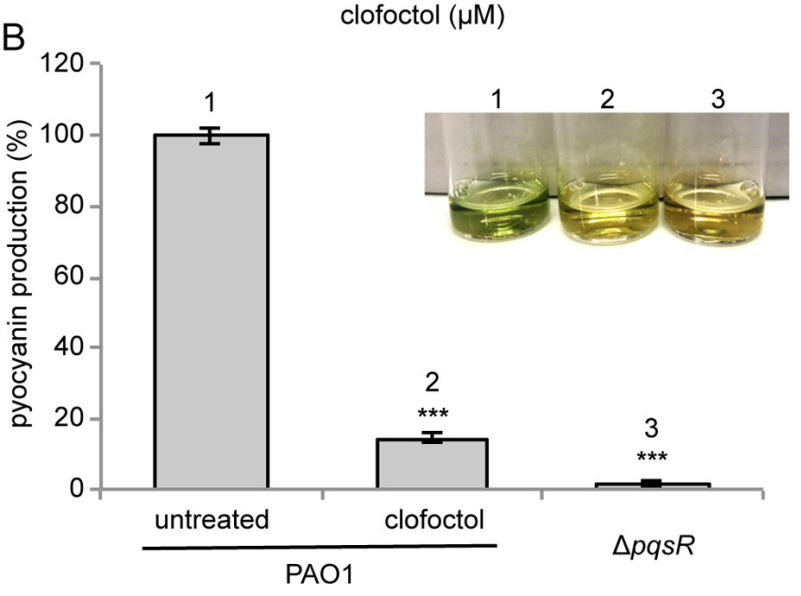

A
relative mRNA level
10
1
0.1
0.01
0.001
0.0001
ns
ns
ns
***
clotrimazole
clofoctol
miconazole
ΔpqsR
PAO1

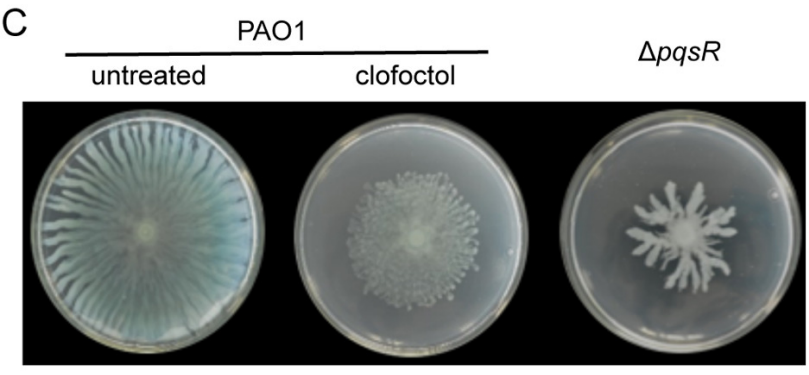

B
control
untreated
clofoctol
miconazole
clotrimazole

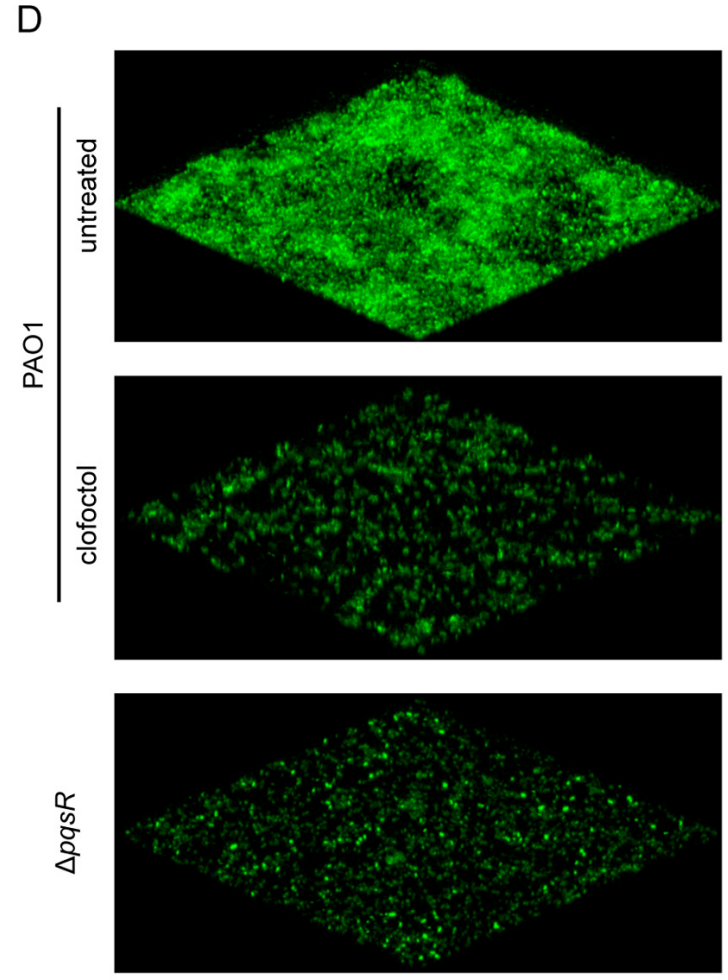

C
PpqsA::lux activity (%)
120
100
80
60
40
20
0
5
10
20
40
80
IPTG (μM)

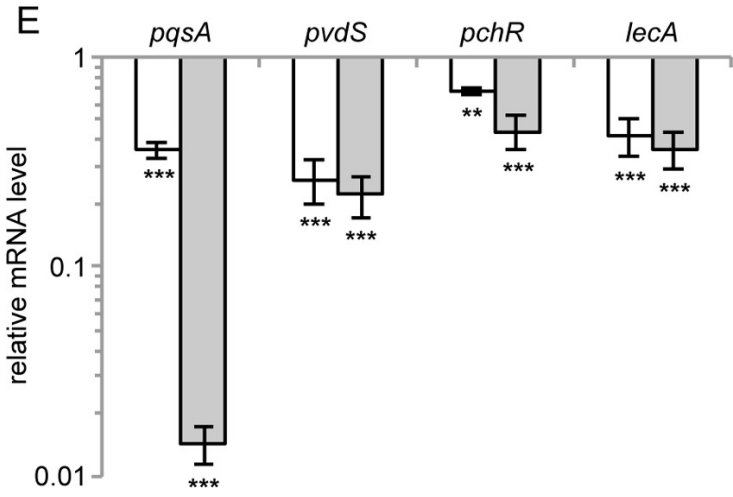

A
B
C

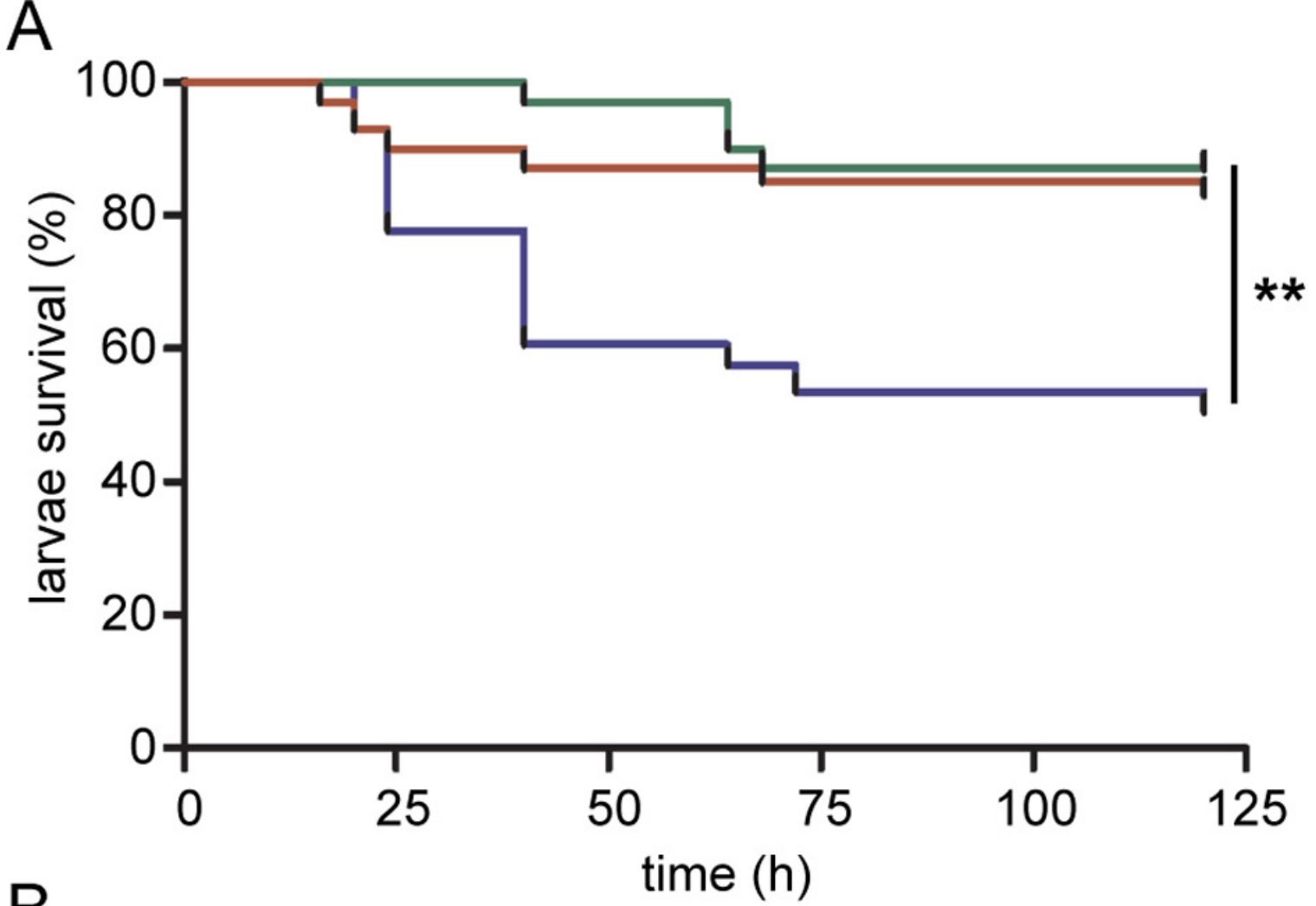

A
HHQ concentration (μM)
PQS concentration (μM)
clofoctol (μM)
0
50
100
200
2.5
2
1.5
1
0.5
0
0.45
0.36
0.27
0.18
0.09
0
**
***

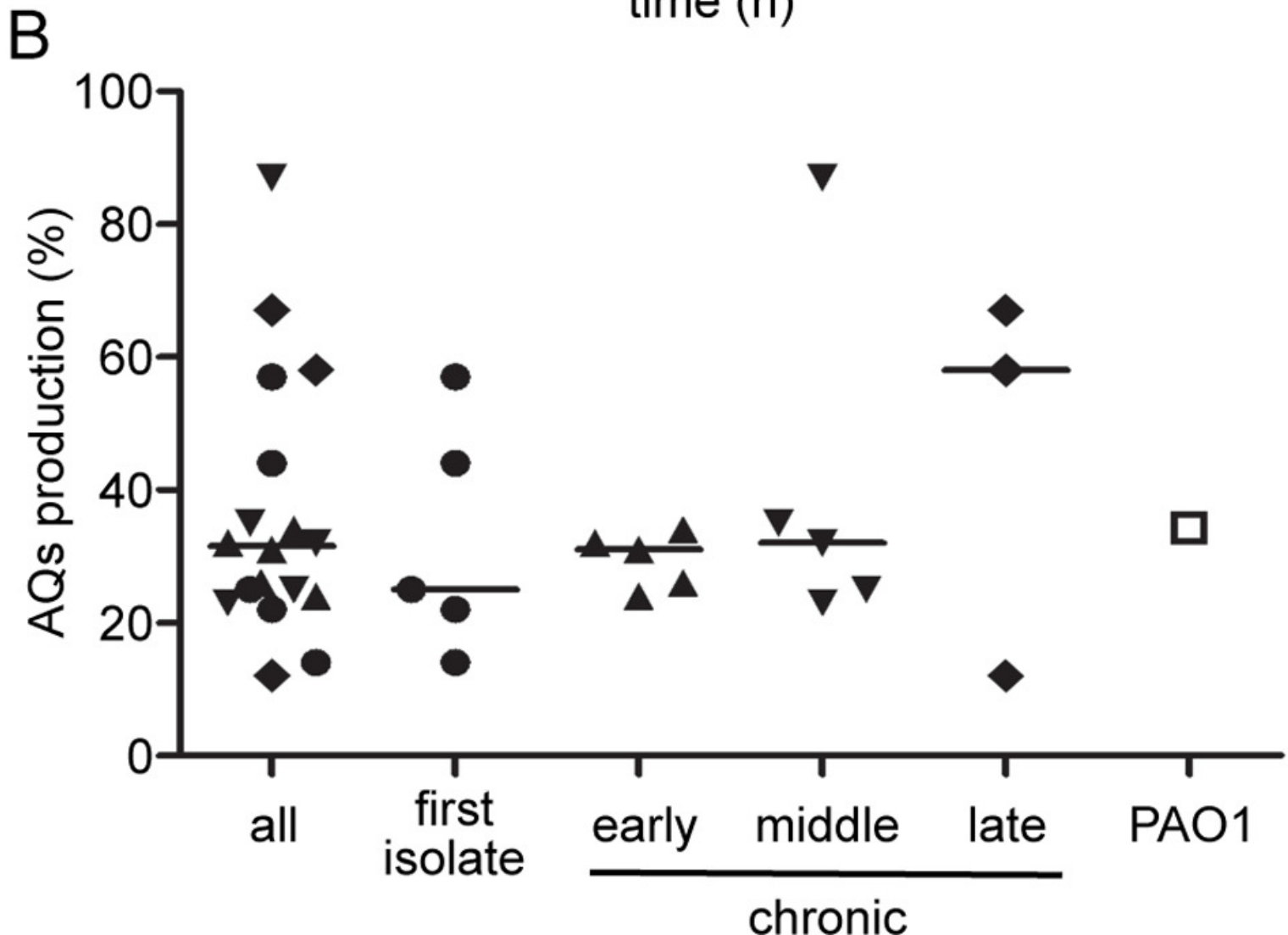

B
pyocyanin production (%)
120
100
80
60
40
20
0
1
2
3
***
untreated
clofoctol
PAO1
ΔpqsR


C
PAO1
untreated
clofoctol
ΔpqsR


D
PAO1
untreated
clofoctol
ΔpqsR


E
pqsA
pvdS
pchR
lecA
relative mRNA level
1
0.1
0.01
***
**


A
100
80
60
40
20
0
larvae survival (%)
**
0
25
50
75
100
125
time (h)


B
100
80
60
40
20
0
AQs production (%)
all
first isolate
early
middle
late
PAO1
chronic