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

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SUPPLEMENTAL MATERIAL

Table S1. Bacterial strains used in this study.

Strains	Characteristics	References
E. coli		
S17.1λ*pir*	conjugative strain for suicide plasmids.	(1)
P. aeruginosa		
PAO1	Nottingham collection wild type strain.	
Δ*pqsR*	PAO1 mutant strain with in frame clear deletion of the *pqsR* gene.	(2)
PAO1 P*pqsA*::*lux*	PAO1 wild type strain carrying chromosomal insertion of the P*pqsA*::*lux* transcriptional fusion; Tc^R.	(3)
PAO1 mini-CTX::*lux*	PAO1 wild type strain carrying chromosomal insertion of the mini-CTX::*lux* empty vector; Tc^R.	(3)
Δ*pqsA* P*pqsA*::*lux*	PAO1 mutant strain deleted in *pqsA* gene carrying chromosomal insertion of the P*pqsA*::*lux* transcriptional fusion; Tc^R (named AQ-Rep).	(4)
Δ*lasI* P*rsaL*::*lux*	PA14 mutant strain deleted in *lasI* gene carrying chromosomal insertion of the P*rsaL*::*lux* transcriptional fusion (named PA14-R3).	(5)
Δ*rhlI* P*rhlA*::*lux*	PAO1 mutant strain deleted in *rhlI* gene carrying chromosomal insertion of the P*rhlA*::*lux* transcriptional fusion; Km^R (named C4-Rep).	(6)
Δ*pqsAH* P*pqsA*::*lux*	PAO1 double mutant strain deleted in *pqsA* and *pqsH* genes carrying chromosomal insertion of the P*pqsA*::*lux* transcriptional fusion; Tc^R.	(3)
Δ*pqsAHR* P*pqsA*::*lux*	PAO1 triple mutant strain deleted in *pqsA, pqsH* and *pqsR* genes carrying chromosomal insertion of the P*pqsA*::*lux* transcriptional fusion; Tc^R.	(7)
PAO1 P*lecA*::*lux*	PAO1 wild type strain carrying chromosomal insertion of the P*lecA*::*lux* transcriptional fusion; Tc^R.	(8)
Δ*pqsR* P*lecA*::*lux*	PAO1 mutant strain deleted in *pqsA* gene carrying chromosomal insertion of the P*lecA*::*lux* transcriptional fusion; Tc^R.	This study

Table S2. Clinical isolates used in this study.

Isolate name[a]	Colonization[b]	Years of colonization	Phenotypic characteristics[c]	Antibiotics susceptibility[d]	Residual AQs production[e]
BG 4	first isolate	-	frayed	R	22
BG 5	chronic early	2	frayed	R	31
BG 6	chronic middle	6	frayed	R	35
BG 7	first isolate	-	circular	S	25
BG 8	chronic early	2	circular	R	26
BG 10	first isolate	-	frayed	S	14
BG 11	chronic early	3	frayed	R	44
BG 12	chronic middle	5	frayed	S	32
BG 13	first isolate	-	frayed	MDR	44
BG 17[e]	chronic early	2	circular	R	24
BG 18[e]	chronic middle	6	circular	R	25
BG 36	chronic early	3	rugose	S	32
BG 56	first isolate	-	small	R	57
BG 76	chronic middle	7	mucoid	R	23
BG 80[e]	chronic middle	5	circular	R	87
BG 92[e]	chronic late	≥15	small	XDR	12
BG 93	chronic late	≥15	rugose	MDR	np
BG 96	chronic late	≥15	circular	R	58
BG 97	chronic late	≥15	frayed	XDR	np
BG 100[e]	chronic late	≥15	small	R	67

[a] CF clinical isolates from the collection of the Bambino Gesú hospital, Rome, Italy.

[b] Different categories depending on the year of infection of the clinical isolates in the lung of individuals with cystic fibrosis: first isolate; chronic early (from 2 to 3 years); chronic middle (from 5 to 7 years); chronic late (equal or more than 15 years).

[c] Characteristics observed when clinical isolates were grown as colony biofilms.

[d] Criteria to define multi-drug resistant (MDR) and extensively-drug resistant (XDR) bacteria have been taken from European Centre for Diseases Prevention and Control (ECDC) web site (http://ecdc.europa.eu/en/Pages/home.aspx): MDR, resistant to one or more antibiotics belonging to at least three different classes; XDR, resistant to one or more antibiotics belonging to all classes except two or less; S, susceptible to all classes of antibiotics; R, resistant to one or more antibiotics belonging to less than three different classes.

[e] Residual production of AQs in samples treated with 100 μM clofoctol relative to untreated samples, considered as 100%. np, strains that do not produce detectable levels of AQs.

Table S3. Plasmids used in this study.

Plasmids	Characteristics	References
pME6032	pVS1-p15A shuttle expression (IPTG-inducible) vector; Tc^R.	(9)
pPqsR-6H	pME6032 derivative for IPTG-inducible expression of the PqsR protein fused with a 6xHis tag; Tc^R.	(7)
pBBR1MCS-5	shuttle vector for constitutive expression; Gm^R.	(10)
pBBR-*pqsABCD*	pBBR1MCS-2 derivative for constitutive expression of PqsA, PqsB, PqsC and PqsD proteins in *P. aeruginosa*; Km^R.	(11)
pFD-*pqsABCD*	pBBR1MCS-5 derivative for constitutive expression of PqsA, PqsB, PqsC and PqsD proteins in *P. aeruginosa*; Gm^R. This plasmid was obtained by cloning into pBBR1MCS-5 the SalI-SacI *pqsABCD* fragment extracted from pBBR-*pqsABCD*.	This study
pMRP9-1	pMRP9 derivative for constitutive expression of GFP in *P. aeruginosa*; Ap^R/Cb^R.	(12)

Table S4. Oligonucleotides used in this study.

Name	Sequence (5'-3')
FW*pqsA*	GACCGCGAAGGACACACTAT
FW*pqsA*	TGAACAGATCGTCTTCCCGC
FW*lecA*	CAGGGCAGGTAACGTCGATT
RV*lecA*	CAACCCGGTATTGACCGGAA
FW*pchR*	CTCAGCGCACAGTTCCTTTC
RV*pchR*	CGAACACCTTGCGAAAGCC
FW*pqsR*	AACATGTTCCTCCAGGTCATCG
RV*pqsR*	TGCGCATGTAAGGGATCAGG
FW*pvdS*	GGAACAACTGTCTACCCGCA
RV*pvdS*	GTAGCTGAGCTGTGCCTTGA
FW16S	GAGAGTTTGATCCTGGCTCAG
RV16S	CTACGGCTACCTTGTTACGA
FWP*pqsL*	TCCGCTCGAGGATCGTCACCGTCAACTG
RVP*pqsL*	TAACTGCAGCGTCATGGATGAGTCTCCG

Figure S1

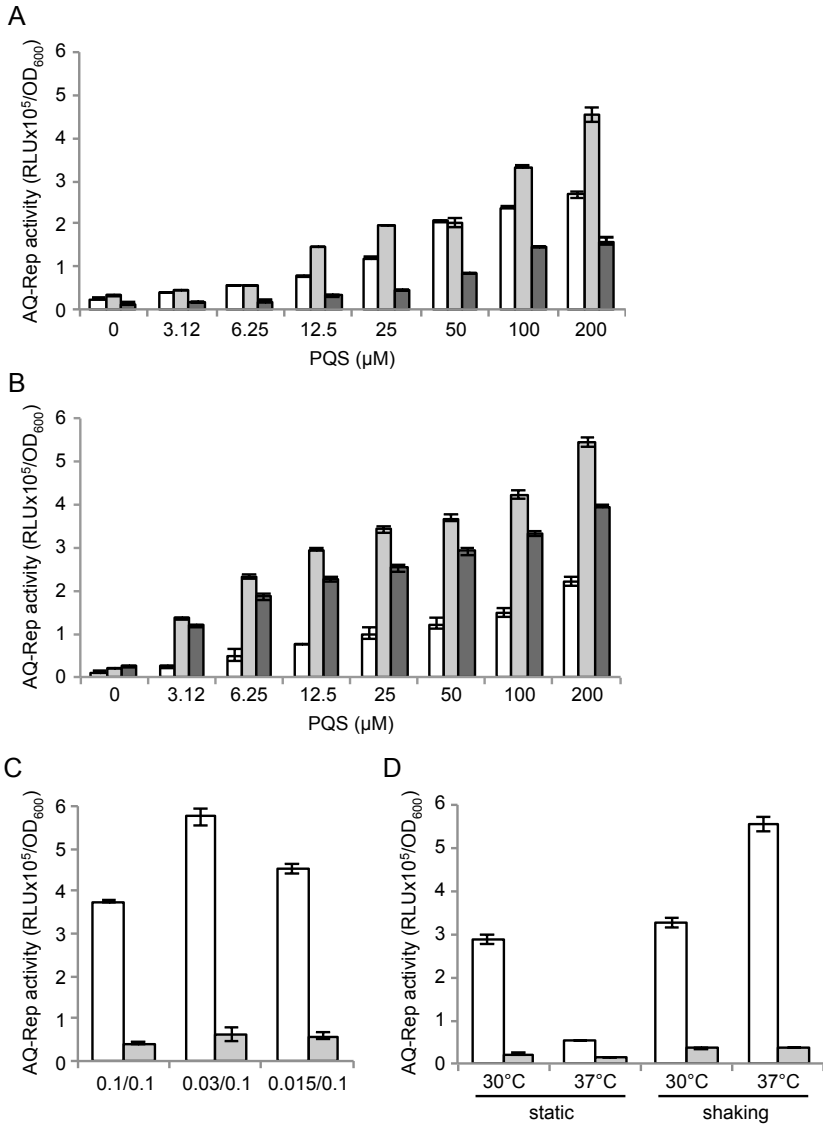

Figure S1. Set up of the PAO1/AQ-Rep coculture system.

(A) Response of the AQ-Rep biosensor after 3 h (white bars), 5 h (light-grey bars) and 7 h (dark-grey bars) incubation in LB supplemented with the indicated concentrations of PQS. **(B)** Activity of the AQ-Rep biosensor inoculated at starting optical density (OD_{600}) of 0.4 (white bars), 0.1 (light-grey bars) and 0.025 (dark-grey bars) after 5 h incubation in LB supplemented with the indicated concentrations of PQS. **(C)** Activity of the AQ-Rep biosensor after 5 h co-incubation with PAO1 (white bars) or Δ*pqsA* (grey bars) strains at the indicated starting optical density (OD_{600}). The first value refers to the PAO1 or Δ*pqsA* strains (OD_{600} from 0.1 to 0.015), the second to the AQ-Rep biosensor (OD_{600} = 0.1). **(D)** Activity of the PAO1/AQ-Rep (white bars) and Δ*pqsA*/AQ-Rep (grey bars) cocultures after 5 h incubation at 30°C or 37°C in static or shaking (200 rpm) conditions. Starting OD_{600} was 0.1 for the AQ-Rep biosensor and 0.03 for the PAO1 and Δ*pqsA* strains. For **(A)-(D)**, biosensor activity is reported as relative light units (RLU) normalized to cell density (OD_{600}); the average of three independent experiments is reported with SD.

Figure S2

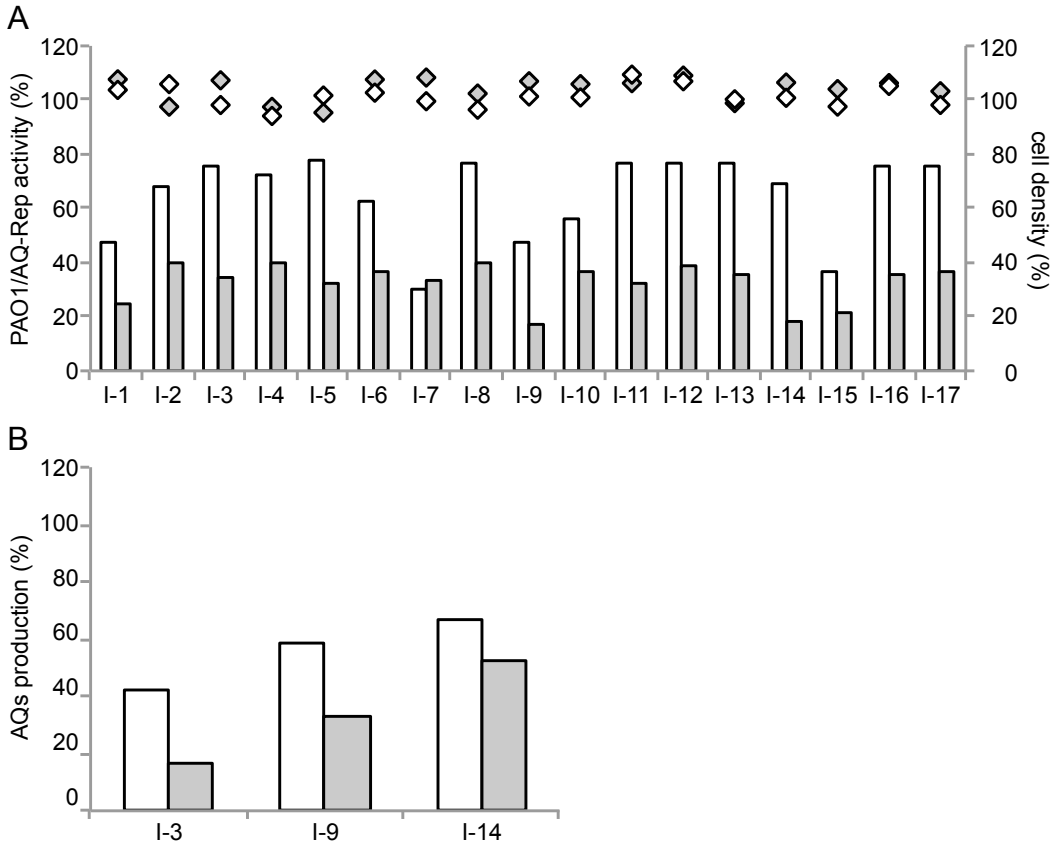

Figure S2. Primary and secondary screens of the PHARMAKON library.

(A) PAO1/AQ-Rep activity (bars) and cell density (diamonds) measured after 5 h incubation at 37°C in shaking conditions in LB supplemented with molecules of the PHARMAKON library, indicated with codes from inhibitor 1 (I-1) to inhibitor 17 (I-17), at 20 µM (white bars and diamonds) and 200 µM (grey bars and diamonds) concentration. PAO1/AQ-Rep activity and cell density measured in the presence of 0.2% and 2% DMSO were considered as 100%. **(B)** AQ production measured in supernatants of the PAO1 strain treated with PHARMAKON library compounds, clotrimazole (I-3), clofoctol (I-9) and miconazole (I-14) at 20 µM (white bars) and 200 µM (grey bars) concentration. AQs were quantified using the AQ-Rep biosensor strain.

Figure S3

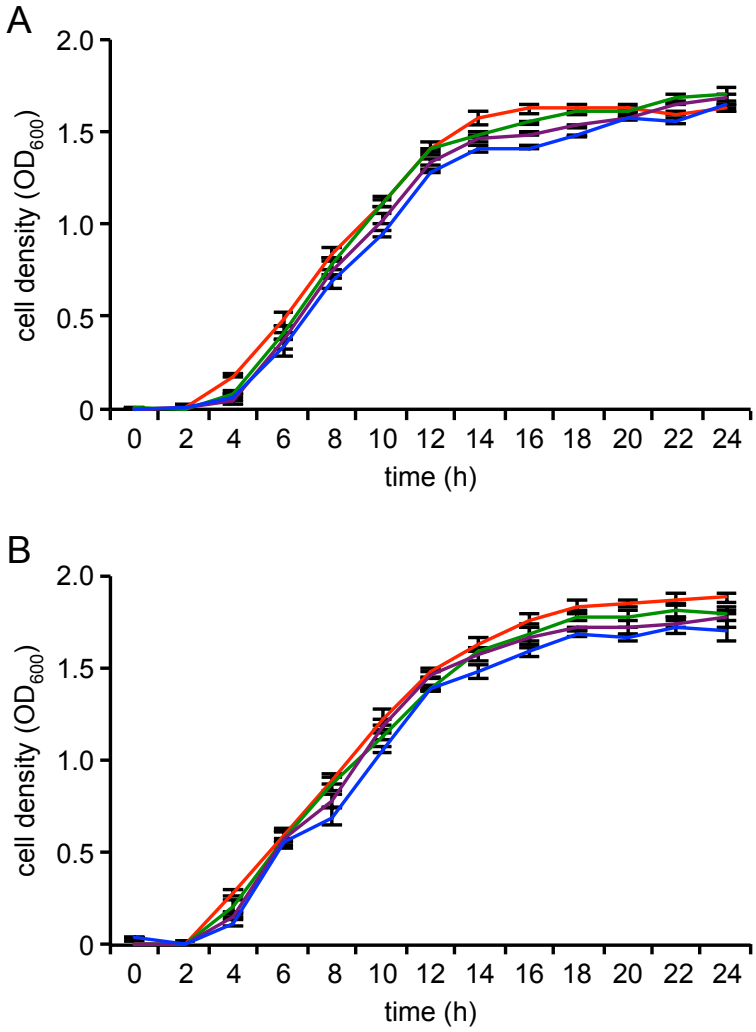

Figure S3. Effect of the *pqs* inhibitors on PAO1 growth.

Growth curves of PAO1 wild type **(A)** and of the biosensor strain AQ-Rep **(B)** incubated at 37°C in shaking conditions in LB supplemented with 200 µM clotrimazole (blue), clofoctol (green), miconazole (purple) or with the corresponding amount of DMSO (red). The average of three independent experiments is reported with SD.

Figure S4

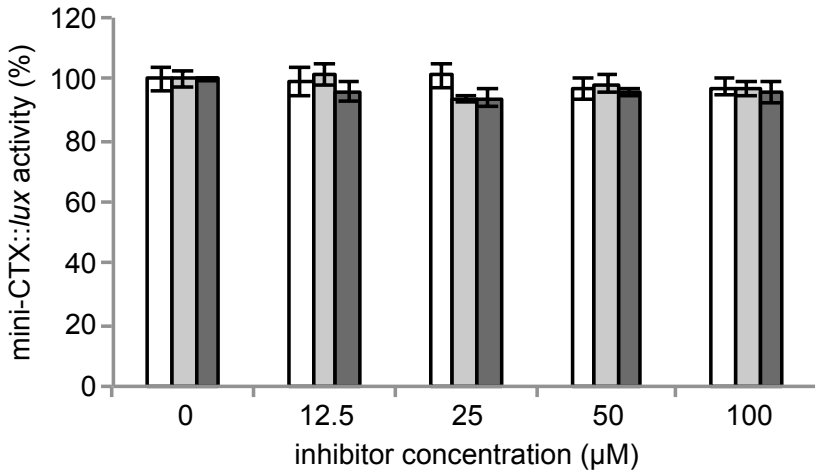

Figure S4. Effect of the *pqs* inhibitors on constitutive bioluminescence.

Percentage of light emitted by the PAO1 strain carrying the mini-CTX::*lux* empty vector grown at 37°C in shaking conditions in LB supplements with 200 µM clotrimazole (white bars), clofoctol (light-grey bars), or miconazole (dark-grey bars). Bioluminescence of the same strain grown in the presence of DMSO was considered as 100%. The average of three independent experiments is reported with SD.

Figure S5

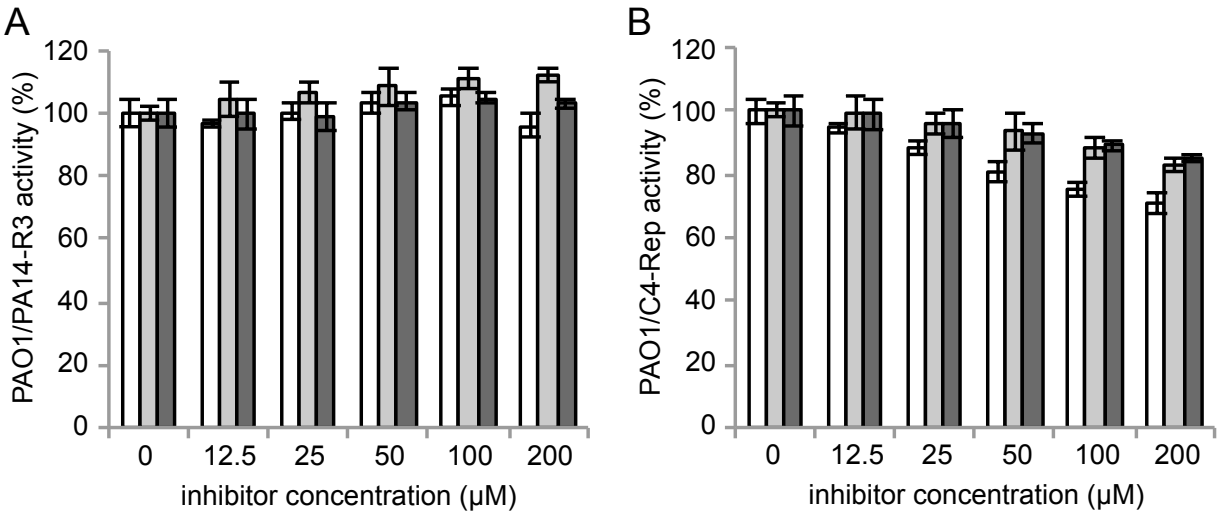

Figure S5. Effect of the *pqs* inhibitors on the *las* and *rhl* QS systems.

Effect of clotrimazole (white bars), clofoctol (light-grey bars) and miconazole (dark-grey bars) on the PAO1/PA14-R3 **(A)** and PAO1/C4-Rep **(B)** coculture systems. PA14-R3: *las*-specific biosensor strain PA14 Δ*lasI* P*rsaL*::*luxCDABE* (5); C4-Rep: *rhl*-specific biosensor strain PAO1 Δ*rhlI* P*rhlA*::*luxCDABE* (6). Bioluminescence of untreated cocultures normalized to cell density is considered as 100%.

Figure S6

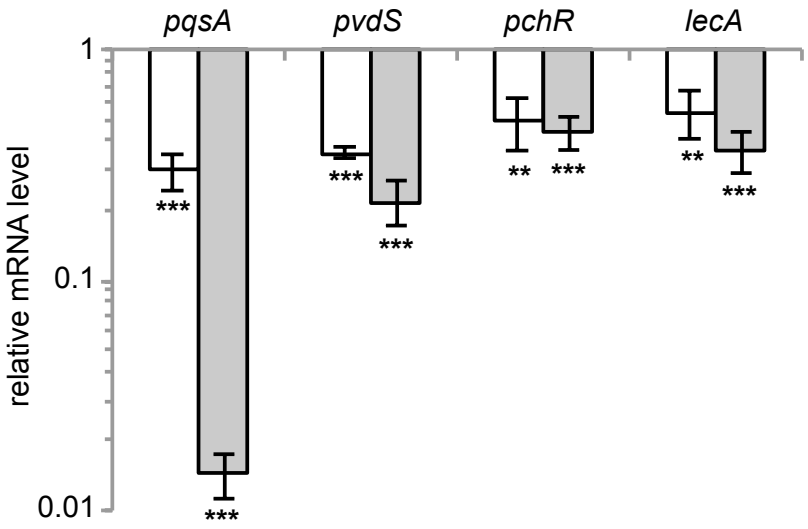

Figure S6. Effect of clotrimazole on the mRNA level of *pqs*-controlled genes.

Real Time RT-PCR analysis showing mRNA level of the indicated genes in PAO1 treated with 100 μM clotrimazole (white bars) and in Δ*pqsR* (grey bars) relative to untreated PAO1. The average of three independent experiments is reported with SD. **, $p < 0.01$; ***, $p < 0.001$ (ANOVA).

Figure S7

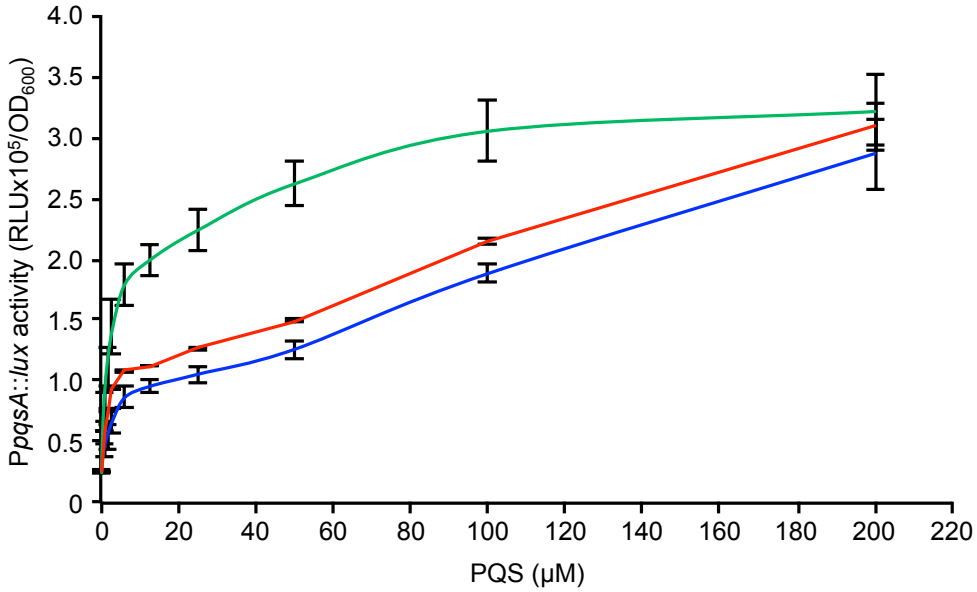

Figure S7. Competition assay between clofoctol and PQS for binding to PqsR.

P*pqsA*::*lux* activity measured in the AQ-Rep biosensor grown in LB supplemented with different concentrations of PQS in the absence (green) or in the presence of 12.5 µM (red) or 50 µM (blue) clofoctol. Promoter activity is reported as relative light units (RLU) normalized to cell density (OD_{600}).

Figure S8

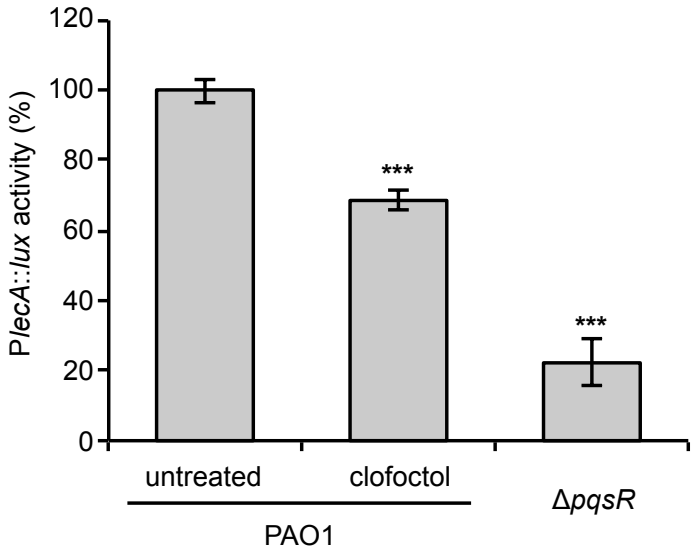

Figure S8. Effect of clofoctol on P*lecA* activity.

Activity of the P*lecA* promoter in PAO1 cultures grown in LB supplemented with DMSO (untreated) or with 100 µM clofoctol, and in the Δ*pqsR* culture grown in LB supplements with DMSO. P*lecA* activity in untreated PAO1 is considered as 100%. The average of three independent experiments is reported with SD. ***, $p < 0.001$ (ANOVA).

Figure S9

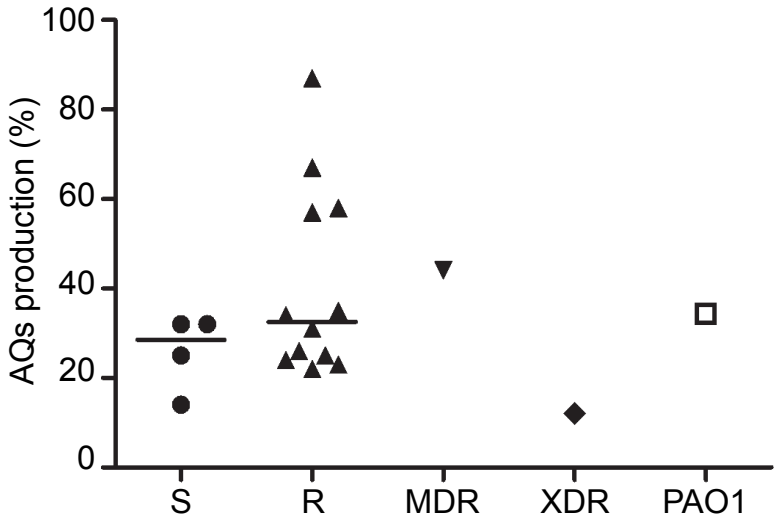

Figure S9. Effect of clofoctol on AQ production in *P. aeruginosa* CF isolates grouped according to their antibiotic resistance profile.

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: S, 28.4%; R, 32.6%. AQ production in the MDR, XDR and PAO1 strains were 43.5%, 11.6% and 34.3%, respectively. Differences between the median values are not statistically significant. Mean results of three independent experiments are reported.

References of the Supplemental Material

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