

## SHORT COMMUNICATION

## High variability in quorum quenching and growth inhibition by furanone C-30 in *Pseudomonas aeruginosa* clinical isolates from cystic fibrosis patients

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**One sentence summary:** C-30, a canonical quorum quencher failed to inhibit QS-virulence factors of some *Pseudomonas aeruginosa* isolates from cystic fibrosis due to low permeability; C-30 also severely inhibited growth of some isolates.

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### ABSTRACT

*Pseudomonas aeruginosa* colonizes the lungs of cystic fibrosis patients causing severe damage. This bacterium is intrinsically resistant to antibiotics and shows resistance against new antimicrobials and its virulence is controlled by the quorum-sensing response. Thus, attenuating its virulence by quorum quenching instead of inhibiting its growth has been proposed to minimize resistance; however, resistance against the canonical quorum quencher furanone C-30 can be achieved by mutations leading to increased efflux. In the present work, the effect of C-30 in the attenuation of the QS-controlled virulence factors elastase and pyocyanin was investigated in 50 isolates from cystic fibrosis patients. The results demonstrate that there is a high variability in the expression of both elastase and pyocyanin and that there are many naturally resistant C-30 strains. We report that the main mechanism of C-30 resistance in these strains was not due

to enhanced efflux but a lack of permeability. Moreover, C-30 strongly inhibited the growth of several of the isolates studied, thus imposing high selective pressure for the generation of resistance.

**Keywords:** clinical isolates; *Pseudomonas aeruginosa*; quorum quenching; resistance; virulence factors

## INTRODUCTION

*Pseudomonas aeruginosa* quorum-sensing (QS) coordinates the expression of multiple virulence factors (Antunes et al. 2010); thus, blocking this response is a promising strategy to combat its infections. However, most of the studies about the regulation and the inhibition of QS in *P. aeruginosa* are performed in domesticated strains in rich media at optimal conditions and not with the strains causing infections, growing in the harsh conditions in which they survive (García-Contreras et al. 2015). Indeed, since bacterial growth in optimal conditions is not affected by quorum quenching (QQ), it was speculated that resistance against it was unlikely to be developed (Bjarnsholt et al. 2010; Rasko and Sperandio 2010). Nevertheless, we previously demonstrated that for the canonical quorum quencher furanone C-30, resistance can readily appear by efflux activation, via the multidrug resistance MexAB-OmpR pump (Maeda et al. 2012; García-Contreras, Maeda and Wood 2013). We also showed that this resistance renders C-30 unable to alleviate *P. aeruginosa* experimental infections in *Caenorhabditis elegans* and that some clinical strains that overexpress the pump are also resistant to C-30 (Maeda et al. 2012). Moreover, recently we described the presence of furanone C-30 resistance among eight *P. aeruginosa* strains isolated from catheter tips, blood and urine (García-Contreras et al. 2013). In this work, our analysis was extended to 50 clinical isolates from pediatric patients with cystic fibrosis, for which the effect of C-30 in the expression of the QS-controlled virulence factors elastase and pyocyanin was determined. We found in these clinical strains a high variability in the production of these virulence factors, and in some strains, there is a lack of inhibition and even activation by C-30. Three C-30-resistant isolates with high basal levels of pyocyanin and elastase were characterized, finding that only one strain had a significant C-30 efflux, while the other two had low C-30 permeability that prevents the quencher from entering the cell and exerting QQ. Moreover, we also identified that the growth of some isolates was extremely sensitive to low doses of C-30, which could increase the chances of those isolates to develop C-30 resistance, by their exposure to a very high selective pressure.

## RESULTS AND DISCUSSION

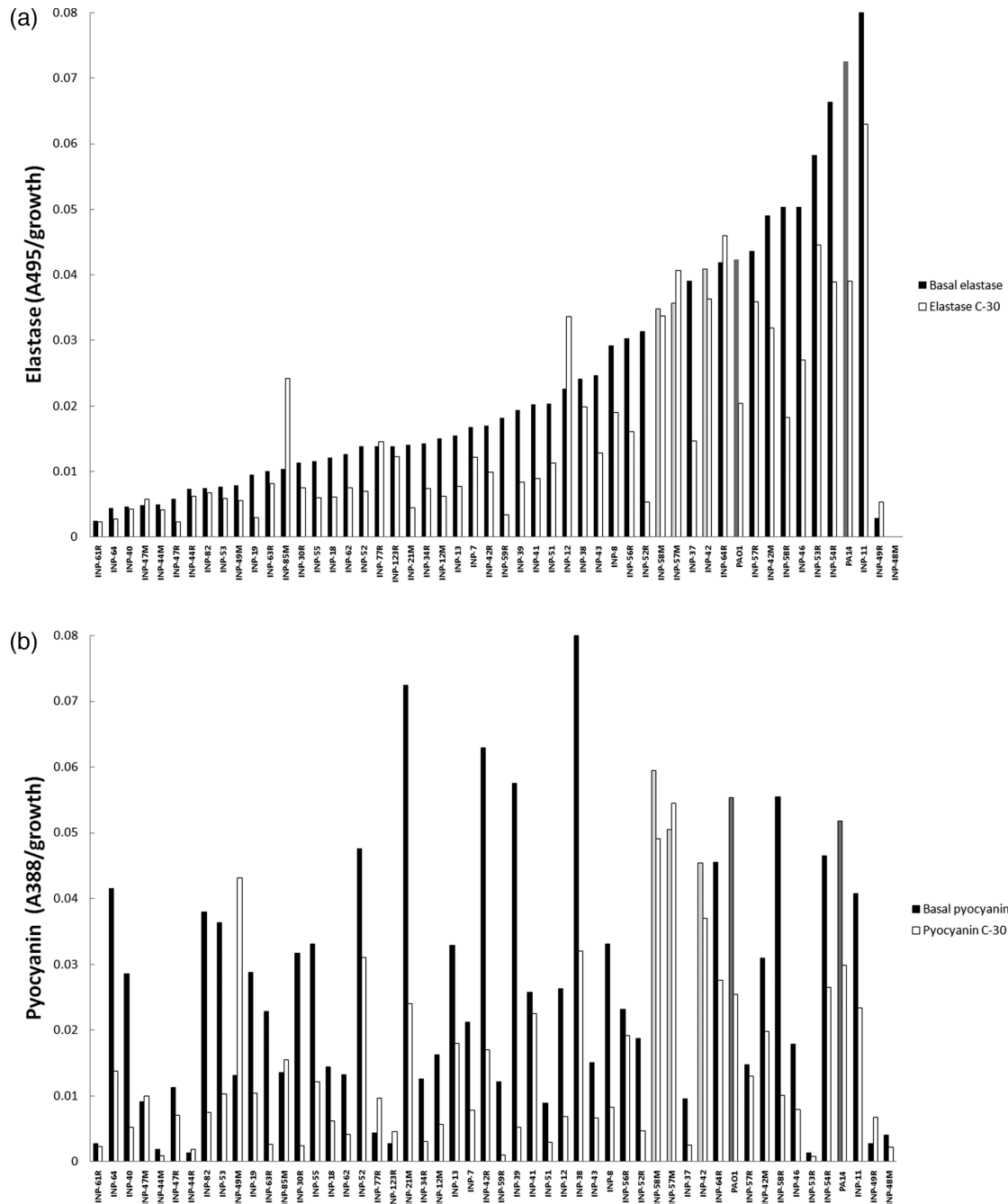
The basal levels of elastase activity and pyocyanin production varied considerably among the 50 clinical isolates studied (Fig. 1A and B), similarly to what was found for isolates from ventilator-associated pneumonia (Le Berre et al. 2008). To evaluate the state of QS in these strains, the production of the main homoserine lactone (HSL) autoinducer (3OC12-HSL) which is sensed by LasR was determined using the cross-feeding bioassay with *Agrobacterium tumefaciens* NTL4 (Schaber et al. 2004), and we found detectable levels of the autoinducer for all the strains except the strain INP-43 (the lack of 3OC12-HSL production by this strain was corroborated by the  $\beta$ -gal assay with strains car-

rying plasmid pECP64 which was used as a bioassay; Pearson, Pesci and Iglewski 1997), and low levels produced by the isolates INP-82, INP-47M and INP-47R (Fig. S1, Supporting Information). In agreement with the lower level of 3OC12-HSL detected, the elastase and pyocyanin levels of these three strains were much lower than those of wild-type PAO1 (Figs 1A, B and S1, Supporting Information). However, despite that the strain INP-43 produced undetectable levels of 3OC12-HSL and that it produced lower levels of elastase and pyocyanin, the fact that it produced some pyocyanin and elastase suggests that the expression of these factors could be triggered in a LasR-independent manner in this isolate (Figs 1A, B and S1, Supporting Information). Remarkably, the production of 3OC12-HSL by the strains had a very high variation (Fig. S1, Supporting Information) and there is an apparent lack of correlation between the signal production and the basal expression of elastase and pyocyanin (Fig. S1, Supporting Information). This lack of correlation was previously found for pyocyanin and autoinducers in isolates from critically ill ventilator-associated pneumonia patients. In contrast, in these isolates a good correlation was found between autoinducer production and elastase (Le Berre et al. 2008). Indeed, for some of the strains tested in this work, high or medium levels of both virulence factors were found in the absence of higher amounts of 3OC12-HSL, suggesting a QS-independent expression of both factors in these strains (INP-43R). Moreover, a high production of autoinducers without the production of virulence factors was also observed for some strains (INP-61R) (Fig. S1, Supporting Information).

For the 50 clinical isolates, although C-30 was generally effective in reducing pyocyanin levels and elastase activity, a high variability among its effects was found, since it reduced the production of both factors of the reference strains (PAO1 and PA14) by ~50%, but on average, it reduced these two virulence factors by only  $35 \pm 61\%$  for pyocyanin and  $27 \pm 38\%$  for elastase in the clinical isolates, this high variation in the C-30 inhibition of both factors is noteworthy since some strains are not affected by C-30 (INP-61R, INP-42, INP-57M, INP-58M, etc.) and in some cases C-30 activates the virulence factors rather than inhibits them (elastase of INP-85M and INP-12, pyocyanin of INP-49M and INP-49R, etc.) (Fig. 1A and B).

Our results reveal that some high producers of elastase and pyocyanin (INP-42, INP-57M and INP-58M) are still able to produce high levels of both compounds in the presence of C-30; that other isolates with medium-low basal levels are also insensitive (INP-85M and INP-77R) and that C-30 is able to inhibit the expression of one of the virulence factors but not both in some strains (INP-12, INP-49M, etc.). These results illustrate the enormous variation of QQ effects shown by C-30 in CF clinical isolates.

Furthermore, for the three isolates resistant to C-30, INP-42, INP-57M and INP-58M, that produced high basal levels of pyocyanin and elastase, the production of alkaline protease was not affected by C-30, and rhamnolipid production and growth in



**Figure 1.** (A) Basal levels and the effect of 50  $\mu$ M of C-30 in the expression of the QS-controlled virulence factors elastase in the clinical strains. For the basal level expression of virulence factors, the strains were grown in LB medium to stationary phase (after  $\sim$ 9 h of culture). To test the effect of C-30, it was added at 50  $\mu$ M to cultures when they reached the middle logarithmic growth phase (O.D. 600 nm  $\sim$  1.0) and were incubated under the same conditions for 5 h. The virulence factors were determined for at least two independent cultures, and the averages are shown. The basal levels of the reference strains (PAO1 and PA14) are shown in dark gray, and the basal levels of the identified C-30-resistant clinical isolates (INP-42, INP-57M and INP-58M) are shown in light gray. (B) Basal levels and the effect of 50  $\mu$ M of C-30 in the expression of the QS-controlled virulence factors pyocyanin in the clinical strains. (C) C-30 uptake and efflux determined by gas chromatography in selected strains: PA14 (reference strain), mexR mutant (strain that effluxes C-30), INP-37 (clinical isolate sensitive to C-30), INP-42, INP-57M and INP-58M (clinical isolates resistant to C-30). The amount of C-30 initially added, the amount that did not enter the cell after 1 h of incubation, the amount found in the washing step and the amount effluxed after 15 h of incubation were determined. Significant differences (in a one-tailed t-test  $P < 0.05$ ) relative to the values observed for the reference strain PA14 are shown by asterisk. Experiments were done in four independent cultures per strain and the average  $\pm$  SEM for each condition is shown.

minimal medium using adenosine as carbon and energy source, which is LasR dependent (Heurlier et al. 2005), was also not affected by C-30 (Fig. S2, Supporting Information).

We also determined the presence of the core QS genes *lasR*, *rhlR* (HSL receptors) and *lasI*, *rhlI* (HSL synthases) genes in these

strains by PCR and the production of the autoinducers C4-HSL (Fig. S3, Supporting Information) and PQS (data not shown) by thin layer chromatography (Grosso-Becerra et al. 2014) and found that they produce similar levels to those produced by PAO1 type strain.

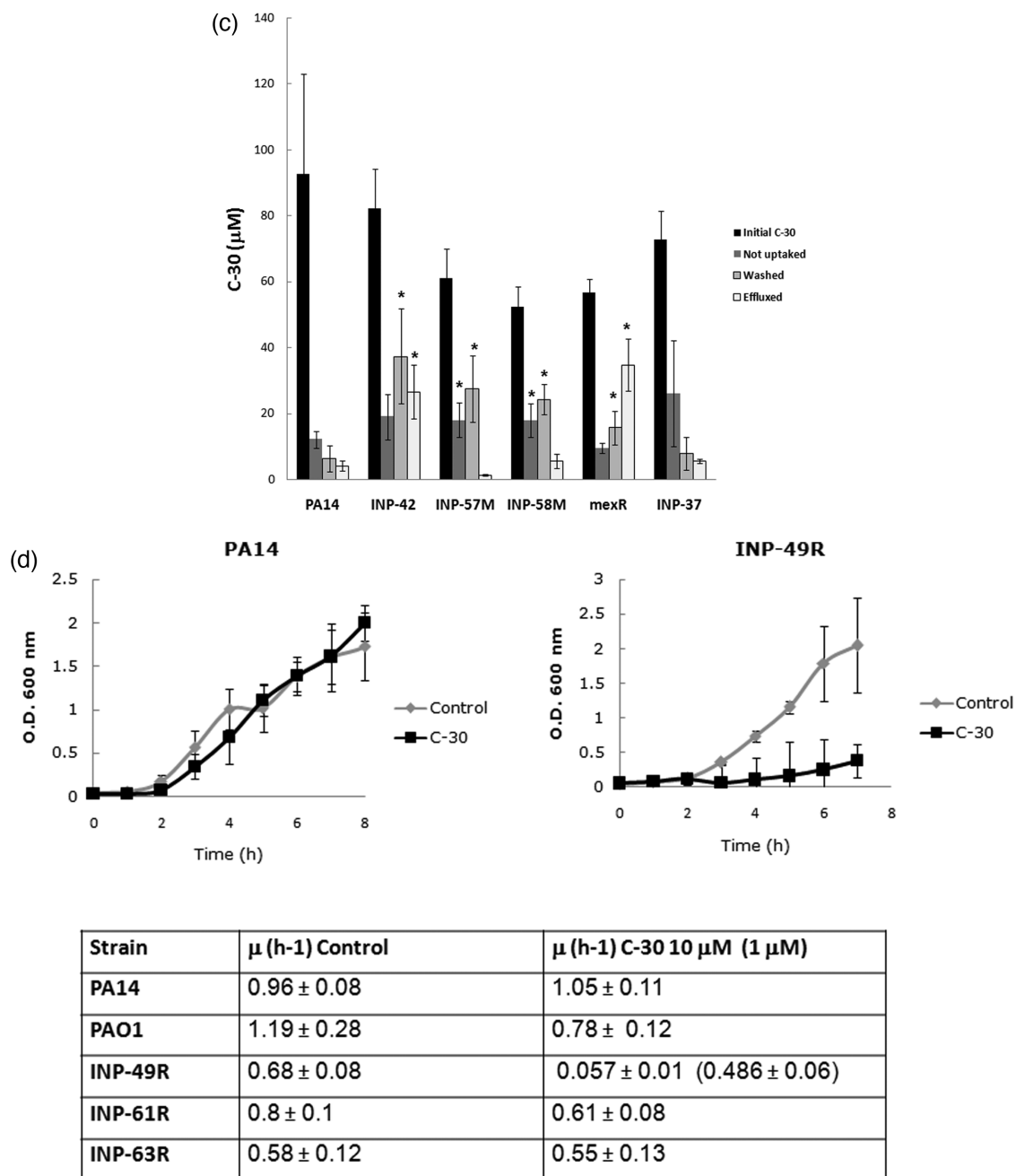


Figure 1. (Continued)

To investigate the resistance mechanisms of C-30 in these three isolates (INP-42, INP-57M and INP-58M), their C-30 uptake and efflux were determined. As shown in Fig. 1C, the reference strain PA14 and a C-30-sensitive clinical strain (INP-37) uptake most of C-30 and are unable to efflux it, as expected. In comparison, the PA14 *mexR* mutant that is resistant to C-30 (Maeda *et al.* 2012) effluxed ~75% of C-30 in 15 h. Interestingly, for two of the C-30-resistant CF isolates, INP-57M and INP-58M, only ~10% of the C-30 entered the cells, while for the INP-42 isolate, also a substantial amount of C-30 was found extracellularly. However, the INP-42 isolate also poses a significant C-30 efflux activity, which correlates with the tolerance of this strain to some antibiotics (data not shown). The lack

of permeability for C-30 in these strains seems not to be related with the overproduction of surface substances such as polysaccharides, since isolates either resistant or sensitive to C-30 had higher levels of surface carbohydrates than the reference strains as evidenced by Congo red binding and capsule staining (data not shown). In addition, alginate was overproduced also by both C-30-sensitive and -resistant CF isolates and surface hydrophobicity did not correlate with C-30 resistance (Fig. S4, Supporting Information). An alternative explanation for the lack of C-30 permeability of the INP-57M and INP-58M strains is that they may have mutations in genes codifying C-30 specific transporters; however, those transporters have not yet been identified.

Furanone C-30 inhibits the expression of PA14 QS-regulated virulence factors without affecting its growth in LB medium unless concentrations of 100  $\mu\text{M}$  or higher are used (Hentzer et al. 2003; Maeda et al. 2012), while the toxicity of C-30 in clinical strains is not well documented. We found, while testing the C-30 effects in LB medium, that strain INP-49R was unable to grow after the addition of 50  $\mu\text{M}$  C-30; so the effect of 10  $\mu\text{M}$  C-30 on the growth of all CF isolates was determined, using a culture medium based in the patient's sputum composition (Palmer, Aye and Whiteley 2007). In agreement with the results with LB medium, INP-49R growth was severely inhibited by C-30 in sputum medium (~90%). Similarly, the growth of other three strains (INP-58R, INP-61R and INP-63R) was inhibited ~50% and the growth curves in the same medium confirmed these results (Figs 1D and S5). The toxicity effect of C-30 was likely not related to QS inhibition since a PA14 *lasR rhlR* double mutant grew only slightly lower than the wild-type strain. Also, the potent growth inhibition effect of C-30 in INP-49R is likely not related to impairment in its general ability to withstand stress as evidenced by phenotypic microarrays (Fig. S6, Supporting Information). Studies to determine the mechanism of C-30 toxic effect are underway.

Our results demonstrate that there exists high variation in QS in CF isolates which is a condition that promotes resistance to QQ compounds (Defoirdt, Boon and Bossier 2010). Additionally, they demonstrate that QQ may be effective against laboratory strains but it is unable to attenuate QS in many clinical isolates. Moreover, our results show that C-30-resistant strains are common among strains infecting CF pediatric patients that have never been treated with a QS inhibitor. Also, C-30 exhibited a high variation in the repression of elastase and pyocyanin among the strains, despite that the target of C-30 is the LasR protein that controls the expression of both traits. Noteworthy, this fact that was previously observed with C-30 and another quorum quencher, 5-fluorouracil, in a collection of eight clinical isolates from urine, blood and catheters (García-Contreras et al. 2013), suggesting the existence of complex QS-virulence regulatory networks in clinical strains. So far, the current known mechanisms of C-30 resistance among clinical isolates are active efflux and a lack of C-30 permeability, but other mechanisms may also exist among *P. aeruginosa* clinical isolates. In addition, we found that the QQ treatment with C-30 severely affects the growth of some isolates, which may strongly promote the generation of resistance.

Overall, our work suggests that using conventional QQ methods that were originally developed using laboratory strains may be in many cases useless (or perhaps even make the infections worse) when used to treat clinical strains; this hypothesis should be further explored using animal infection models.

## SUPPLEMENTARY DATA

Supplementary data are available at FEMSPD online.

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**Conflict of interest.** None declared.

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