The impaired quorum sensing response of MexAB-OprM efflux pump overexpressing mutants is not due to non-physiological efflux of 3-oxo-C12-HSL

Running: MexAB-OprM overexpression impairs AQs production

Manuel Alcalde-Rico[1,2,3], Jorge Olivares-Pacheco[2,3,*], Nigel Halliday[4], Miguel Cámara[4], José Luis Martínez[1,*]

[1] Centro Nacional de Biotecnología. CSIC. Darwin 3. 28049-Madrid. Spain

[2] Grupo de Resistencia Antimicrobiana en Bacterias Patógenas y Ambientales GRABPA, Instituto de Biología, Facultad de Ciencias, Pontificia Universidad Católica de Valparaíso, Chile.

[3] Millennium Nucleus for Collaborative Research on Bacterial Resistance (MICROB-R), Valparaíso, Chile.

[4] National Biofilms Innovation Centre, Nottingham University Biodiscovery Institute, School of Life Sciences, University of Nottingham, Nottingham, United Kingdom.

*Corresponding authors:

JO: jorge.olivares@pucv.cl

JLM: Phone: +34915854542. FAX: +34915854506. jlmtnez@cnb.csic.es,

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Originality-Significance statement

Opportunistic pathogens with environmental origin such as *Pseudomonas aeruginosa* present a complex repertoire of virulence and antibiotic resistance determinants that have evolved to play specific functions in the natural environments they originated from, long before they started to pose a problem to human health. Amongst them, multidrug efflux pumps participate in *P. aeruginosa* resistance/virulence crosstalk since, besides contributing to antibiotic resistance, they can also modulate the quorum sensing (QS)-mediated response. We show that mutants overexpressing the MexAB-OprM efflux pump, present an impaired QS response due to the reduced production of the *Pseudomonas* quinolone signal, not because they extrude, as previously suggested, the QS signal *N*-(3-oxododecanoyl)-L-homoserine lactone. Our results together with previously published work indicate that different MDR efflux pumps can differentially affect the *P. aeruginosa* QS homeostasis, evidencing their ecological role in modulating this cell-to-cell communication system. We propose the term "apparent redundancy" to name this strategy, when different elements produce the same effect, in our case the modulation of QS response driven by the overexpression of different efflux pumps, but the mechanisms involved are specific for each MDR pump.

Summary

Multidrug (MDR) efflux pumps are ancient and conserved molecular machineries with relevant roles in different aspects of the bacterial physiology, besides antibiotic resistance. In the case of the environmental opportunistic pathogen *Pseudomonas aeruginosa*, it has been shown that overexpression of different efflux pumps is linked to the impairment of the quorum sensing (QS) response. Nevertheless, the causes of such impairment are different for each analyzed efflux pump. Herein, we performed an in-depth analysis of the QS-mediated response of a *P. aeruginosa* antibiotic resistant mutant that overexpresses MexAB-OprM. Although previous work claimed that this efflux pump extrudes the QS signal 3-oxo-C12-HSL, we show otherwise. Our results evidence that the observed attenuation in the QS response when overexpressing this pump is related to an impaired production of alkyl quinolone QS signals, likely prompted by the reduced availability of one of their precursors, the octanoate. Together with previous studies, this indicates that, although the consequences of overexpressing efflux pumps are similar (impaired QS response), the underlying mechanisms are different. This 'apparent redundancy' of MDR efflux systems can be understood as a *P. aeruginosa* strategy to keep the robustness of the QS regulatory network and modulate its output in response to different signals.

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen, with an environmental origin, of special concern due to its capability to produce a large variety of serious human infections and to its low susceptibility to different antibiotics (Driscoll et al., 2007; WHO, 2017). As any other opportunistic pathogens with environmental origin, *Pseudomonas aeruginosa* presents a complex repertoire of virulence and antibiotic resistance determinants. These determinants are present in all members of the species and allow *P. aeruginosa* to infect a large variability of hosts, from plants to humans (Rahme et al., 1995; Tan et al., 1999; Rahme et al., 2000; He et al., 2004). This means, that they have evolved to play specific functions in natural environments, long before this bacteria become to be a problem for human health (Alonso et al., 1999; Morales et al., 2004).

It is worth noticing that genes that contribute to *P. aeruginosa* intrinsic antibiotic resistance are, in several occasions, key components of bacterial physiology (Olivares et al., 2013; Blanco et al., 2016b). This is the case of the Resistance, Nodulation and cell-Division (RND) family of efflux pumps. Besides being important mechanisms of antibiotic resistance (Blair et al., 2014; Hernando-Amado et al., 2016), they play relevant roles in non-clinical, environmental ecosystems (Martinez et al., 2009), being involved in the bacterial response to different stresses and their interactions with both human and environmental hosts as plants (Garcia-Leon et al., 2014; Blanco et al., 2016a). In this work, we address the role of the MexAB-OprM efflux system, one of the

most relevant RND systems for intrinsic and acquired antibiotic resistance of *P. aeruginosa* (Morita et al., 2001; Chalhoub et al., 2018), on the modulation of quorum sensing (QS) responses in this bacterium.

The QS response consists in a population-scale cooperative behavior promoted by cell-to-cell communication systems that control the expression of a large set of genes in a cell-density way (Williams and Camara, 2009). This intercellular communication system is based on the synthesis, delivery and progressive accumulation of autoinducer compounds, known as QS signal molecules (QSSMs), which are recognized by specific cell receptors. When QSSMs concentrations reach a threshold, the QS response is triggered in the population. This population-scale response regulates a wide number of diverse physiological processes (Miller and Bassler, 2001), including production of private and public goods (Wilder et al., 2011), biofilm formation (Vidal et al., 2011), host-bacteria interactions (Holm et al., 2016) and virulence factors production (Pearson et al., 1997). The QS responses usually impose a fitness burden at single cell level but with social benefit at population level (Heurlier et al., 2006; Gupta and Schuster, 2013).

The QS regulatory network of *P. aeruginosa* is based on the production of two different kinds of QSSMs: the *N*-acyl-L-homoserine lactones (AHLs) and the 2-alkyl-4(1*H*)-quinolones (AQs) (Williams and Camara, 2009). These signals are synthesized and detected by the Las, Rhl and Pqs regulation systems. The Las system is based on the production of *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) by LasI synthase and the detection of this signal by the LasR transcriptional regulator. The *N*-butanoyl-L-homoserine lactone (C4-HSL) is part of the Rhl system being synthesized

by RhlI and sensed by the RhlR regulator. The synthesis of the Pseudomonas Quinolone Signal (PQS), and its immediate precursor, 2-heptyl-4-hydroxyquinoline (HHQ), requires the enzymes encoded by the *pqsABCDE* operon and *pqsH*. These two QSSMs are detected by the LysR-type transcriptional regulator PqsR (also known as MvfR) and are the main QSSMs associated with the Pqs QS system. The interconnection between these QS systems has been described as hierarchized, with the Las system at the top controlling the activity of the Rhl and Pqs systems, which then modulate both their own activity and the expression of the other elements of the QS system (Williams and Camara, 2009). However, there are studies suggesting that the hierarchy can be affected by other regulators, growth conditions or through the activity of other elements not directly involved in the canonical QS-regulatory network (Choi et al., 2011; Mellbye and Schuster, 2014; Schafhauser et al., 2014; Feltner et al., 2016; Kostylev et al., 2019). Further, there are key elements like PqsE that may function as QS modulators without binding to any QSSM, giving a higher level of complexity to the QS regulatory network (Deziel et al., 2005; Choi et al., 2011; Mellbye and Schuster, 2014; Kang et al., 2017). Another category of modulators of the QS response is formed by multidrug efflux pumps. Indeed, previous works have reported that the RND efflux systems MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexGHI-OpmD are involved in the modulation of the *P. aeruginosa* QS response (Evans et al., 1998; Pearson et al., 1999; Aendekerk et al., 2005; Olivares et al., 2012; Alcalde-Rico et al., 2018). In the case of MexAB-OprM, whose over-expression leads to a lower production of some QS-regulated phenotypes (Evans et al., 1998; Sanchez et al., 2002), it has been described

that this efflux pump is able to extrude 3-oxo-C12-HSL (Pearson et al., 1999). However, our results show that overproduction of MexAB-OprM impairs the QS-mediated response due to the reduction in the AQs production, probably due to a decrease in the availability of their biosynthetic precursor, octanoic acid (Dulcey et al., 2013), and not to a high non-physiological extrusion of 3-oxo-C12-HSL.

Therefore, the results of the present study, together with previously published reports, evidence that the underlying causes of the impaired QS response and the lower production of QSSMs observed in MexAB-OprM, MexCD-OprJ and MexEF-OprN overexpressing mutants are different. We propose that the "apparent redundancy" observed between these RND systems in their modulation of *P. aeruginosa* QS response is not casual and could be used by *P. aeruginosa* to fine tuning its QS regulatory network in response to changes in environmental conditions and/or nutritional requirements. However, more studies are needed to further understand the relationship between environmental signals, RND efflux activity and modulation of the QS response.

Results

Effect of MexAB-OprM overexpression on the transcriptome of *P. aeruginosa*

To analyze the global effect of MexAB-OprM overexpression on *P. aeruginosa* physiology, the transcriptomes of both wild-type PAO1 strain and a *mexR** mutant, which harbors an inactivating mutation in the *mexAB-oprM* repressor gene, *mexR*, were analyzed by RNAseq and compared. Since it has been described that MexAB-OprM is

involved in the QS response (Evans et al., 1998; Pearson et al., 1999; Maseda et al., 2004) and its expression is growth phase regulated (Evans and Poole, 1999; Maseda et al., 2004), the transcriptomic assays were addressed in exponential (OD_{600} = 0.6) and early stationary phase of growth (OD_{600} = 2.5).

The overexpression of MexAB-OprM in the *mexR** mutant is associated with significant changes in the expression of 182 and 346 genes during exponential and stationary growth phase, respectively (Table S1). The genes affected were grouped based on the functional classes assigned in the Pseudomonas Genome Database (PseudoCAP) (Winsor et al., 2016). The major functional class affected by MexAB-OprM overexpression in both growth phases was the one corresponding to secreted factors, whose genes were mainly expressed at a lower level in the MexAB-OprM overexpressing *mexR** mutant than in the wild-type PAO1 strain (Figure 1). Other functional classes strongly affected in both phases of growth were: i) the biosynthesis of prosthetic groups, cofactors and carriers; ii) antibiotic resistance and susceptibility; iii) those involved in adaptation and protection; iv) genes encoding enzymes involved in carbon metabolism; and v) genes involved in the metabolism of fatty acids and phospholipids. Many of genes, whose expression was altered in the *mexR** mutant in both phases of growth, belong to the QS regulatory network. Among them, key components in AQs biosynthesis such as *pqsA*, *pqsB*, *pqsD*, *pqsE*, *phnA* and *phnB*, as well as genes regulated by Pqs system such as *phzA2*, *phzB1*, *rhlA*, *rhlB* or *lasB* were found (Table 3).

In order to determine which QS regulated genes present an altered expression upon MexAB overexpression, we performed a more in depth analysis of the QS regulon (Table S2), by focusing on those genes whose expression has been shown to be controlled by either PqsE, AQs or the Las and Rhl systems (Hentzer et al., 2003; Schuster et al., 2003; Wagner et al., 2003; Wagner et al., 2004; Schuster and Greenberg, 2007; Gilbert et al., 2009; Lesic et al., 2009; Rampioni et al., 2010; Sana et al., 2012; Rampioni et al., 2016). As shown in Table S1, 82 of the 182 genes affected during the exponential phase of growth, and 209 out of 346 genes with transcriptional variations during the early stationary phase of growth in *mexR** have been associated to QS. These results evidence that overexpression of MexAB-OprM has a strong impact on QS signaling. Taking into account that the production of a large set of virulence factors is QS-regulated, it is not surprising that 76 of the 484 genes, whose expression significantly changed in the *mexR** mutant, are catalogued as virulence factors by PseudoCAP (Winsor et al., 2016) or in previously published works (Folders et al., 2001; Ball et al., 2002; Lesic et al., 2009; Lee et al., 2010; Russell et al., 2011; Pelzer et al., 2014; Chen et al., 2015; Sakhtah et al., 2016) (Table 3 and S1). The expression of most of them, including genes of the HII-T6SS (Hcp secretion island-2 encoded type VI secretion system) and those involved in the synthesis of phenazines, pyoverdine, pyochelin, proteases, hydrogen cyanide, rhamnolipids and even QS autoinducers, was lower in the *mexR** mutant than in the PAO1 strain (Table 3 and S1). However, the opposite was observed for other genes like some belonging to the HI-T6SS, which were expressed at higher level in the stationary growth phase in *mexR** than in PAO1. As

shown below, these results are in line with the impaired production of AQs and C4-HSL signals by the *mexR** mutant and the previously reported Pqs- and Rhl-mediated regulation of these virulence factors (Table S1).

To confirm the transcriptomic results, the expression of a selected set of genes was measured by RT-PCR. Since the main effect of *mexAB-OprM* overexpression was over genes belonging to the QS regulatory network, we focused our analysis in such genes. In exponential growth phase, the genes analyzed were *pqsA*, *pqsE* and *pqsH* (implicated in AQs synthesis); *rhlI* and *lasI* (responsible for C4-HSL and 3-oxo-C12-HSL synthesis, respectively); *pvdQ* (the 3-oxo-C12-HSL acylase). In the case of early stationary phase, the QS-regulated genes analyzed were *antA* (encoding the anthranilate dioxygenase large subunit), *lasB* (coding for elastase), *rhlA* (coding for the rhamnosyl transferase chain A), *lecA* (encoding the PA-I galactophilic lectin), *mexG* (encoding the subunit MexG of the RND efflux system, MexGHI-OpmD) and *phzB1* (coding for a phenazine biosynthesis protein). The RT-PCR analysis showed a similar impairment in *mexR** levels of expression of those genes than those obtained by RNAseq (Figure 2), confirming the results obtained in the transcriptomic assays.

A final aspect to be taken into consideration is that although, to the best of our knowledge, there is no evidence that MexR might directly control the expression of other genes besides *mexAB-oprM*, the possibilty of some of the effects on the transcripome being exclusively due to MexR inactivation, and not to MexAB-OprM overexpression, cannot be fully discarded.

MexAB-OprM overexpression does not produce an increased 3-oxo-C12-HSL efflux.

It has been previously proposed that MexAB-OprM is able to extrude 3-oxo-C12-HSL (Pearson et al., 1999). We thus analyzed by LC-MS/MS the extracellular accumulation of 3-oxo-C12-HSL, C4-HSL, HHQ and PQS in PAO1 and *mexR** cultures grown to late stationary phase. Opposite to previous findings, 3-oxo-C12-HSL accumulation was similar in PAO1 and *mexR**, while levels of C4-HSL and, to a greatert extent, of 2-alkyl-4-quinolones were lower when MexAB-OprM was overexpressed (Figure 3A).

To further analyze if MexAB-OprM is able to extrude 3-oxo-C12-HSL, the extracellular and intracellular amount of 3-oxo-C12-HSL from each strain was also determined by Thin Layer Chromatography (TLC) at late exponential phase of growth (Figure 3B). As shown in Figure 3C, the ratio between supernatant and cell extract (SN/CE) of 3-oxo-C12-HSL in the *mexR** strain was even lower than that of PAO1, which goes against an increased extrusion of this signal by the MexAB-OprM overexpressing mutant. To analyze if this hypothetical extrusion of 3-oxo-C12-HSL through MexAB-OprM could be dependent of a specific growth state, the amount of this signal inside and outside PAO1 and *mexR** cells was determined at different growth stages in a Time-Course Monitoring (TCM) assay. At late exponential growth phase (OD_{600} ≈ 1.2-1.8), the accumulation of 3-oxo-C12-HSL both inside and outside *mexR** cells was slightly lower than that of PAO1 (Figure 3D and 3E). However, once both strains reached early stationary growth phase (OD_{600} ≈ 2.5), the extracellular and intracellular accumulation

of 3-oxo-C12-HSL was even slightly higher in *mexR** than in PAO1. The SN/CE ratio was calculated for each one of the time points analyzed in the TCM assays and just a slightly difference was observed at 6 hours post-inoculation, being the ratio also lower in *mexR** with respect to PAO1 (Figure 3F). Altogether, our results support that, in contrast to what has been previously described (Pearson et al., 1999), the MexAB-OprM efflux pump does not seem to extrude 3-oxo-C12-HSL, at least under our experimental conditions. In this regard, it is relevant to highlight that the work of Pearson *et. al.* was performed using a wild-type strain (Pearson et al., 1999), while our experiments were performed using a MexAB-OprM overexpressing mutant. In any case, our results suggest that the main cause for the impaired QS response associated with the MexAB-OprM overexpression could be an imbalance in the production of PQS, HHQ and likely C4-HSL.

The production of C4-HSL is impaired in the MexAB-OprM overproducer mutant

The C4-HSL extracellular accumulation in PAO1 and *mexR** cultures was measured by TLC and TCM as described in Methods. Since C4-HSL freely diffuses through the plasma membrane and hence should reach an equilibrium between the extracellular and intracellular levels (Pearson et al., 1999), only supernatants were measured. We found that the amount of C4-HSL was lower in *mexR** than in PAO1 supernatants (Figure 4A). To further support the results obtained from these measurements, the activity of the P*rhlI* promoter, which is induced by C4-HSL in a concentration-dependent way, was determined. To do this, we generated the bioreporter strains PAO1_P*rhlI* and

*mexR**_P*rhlI* (Table 1), which harbor the miniCTX::P_{rhlI}-*lux* transcriptional fusion in their chromosomes. Using this combination of tools, we found that C4-HSL accumulation both outside (Figure 4A and 4B) and inside (Figure 4C) the cells was significantly lower in the *mexR** mutant than in the PAO1 wild type strain. Therefore, MexAB-OprM overexpression leads to a significant reduction in C4-HSL production, which could be responsible for the altered expression of some of the Rhl-regulated genes in the *mexR** mutant (Table 3 and S1).

The Pqs system is strongly impaired in the *mexR mutant**

Since, both the transcriptomic analysis (Tables 3 and S1) and the LC-MS/MS results (Figure 3A) suggest that the overexpression of MexAB-OprM mainly affects Pqs-dependent regulation, we analyzed the kinetics of AQs accumulation both inside and outside bacterial cells using TLC and TCM assays coupled with the analysis of PqsR-based biosensor strains. In agreement with LC-MS/MS analysis, TLC analysis showed that *mexR** mutant presents a lower extracellular and intracellular accumulation of PQS and HHQ (Figure 5A). To discard that this phenotype was caused by a non-yet reported MexR function beyond the regulation of *mexAB-oprM* expression, we analyzed by TLC the AQs production in presence of the efflux pump inhibitor phenyl-arginine-β-naphthylamide (PAβN) (Lomovskaya et al., 2001). Figure 5B shows that the addition of PAβN to *mexR** cultures restored wild type levels of both intracellular and extracellular AQs, confirming that overproduction of MexAB-OprM is the cause of the QS impairment observed in the *mexR** mutant. Moreover, TCM measurements indicated

that the lower accumulation of AQs occurs throughout growth (Figure 5C and 5D). Altogether, our results indicate that MexAB-OprM overexpression has a strong impact on AQs production in *P. aeruginosa*, affecting their accumulation both inside and outside the cells consequently leading to a disruption of the Pqs-dependent QS response.

The availability of anthranilate is not the bottleneck for the impaired AQs production in *mexR**

Once demonstrated that AQs are the lowest produced QSSMs in *mexR**, we wanted to determine if a non-physiological MexAB-OprM-dependent extrusion of any of their precursors could be acting as a limiting step, a situation previously described for other RND overexpressing mutants in *P. aeruginosa* (Olivares et al., 2012; Alcalde-Rico et al., 2018). For that purpose, *mexR** and PAO1 cultures were supplemented with anthranilate, an immediate precursor of AQs (Calfee et al., 2001; Deziel et al., 2004), and the production of PQS and HHQ was measured at early stationary growth phase. Differing to the situation reported for MexEF-OprN overproducing mutants (Olivares et al., 2012), anthranilate supplementation did not restore the production of PQS and HHQ in *mexR** (Figure 6). Therefore, a reduced availability of anthranilate or its precursors within the PQS/anthranilate biosynthetic pathway is not the cause for the lower PQS and HHQ production by *mexR**.

Supplementation with octanoate partially restores *mexR QS response**

Since PQS and HHQ production was not restored with anthranilate in *mexR**, we analyzed if the availability of octanoate, the other immediate precursor of these QSSMs, could be the limiting step in their production. Since it has been described that the production of both AQs and pyocyanin increase in presence of this compound (Dulcey et al., 2013), the kinetics of extracellular and intracellular AQs accumulation throughout the cell-growth of both PAO1 and *mexR** strains grown in LB supplemented with octanoate 5mM was determined by TCM. In these conditions, production of AQs was delayed in *mexR** when compared with PAO1 (Figure 7A and 7B). However, once the stationary phase of growth was reached (OD_{600} > 2.5), AQs production in the *mexR** and PAO1 wild-type strains were similar, being the accumulation in the culture supernatant of the MexAB-OprM overexpressing mutant slightly higher. Furthermore, the TLC analysis of extracellular and intracellular accumulation of PQS and HHQ at 7 hours post-inoculation (Figure 7C) further supports that supplementation with octanoate restored almost fully PQS/HHQ production in *mexR**.

Once we determined that octanoate supplementation restores AQs production in *mexR**, we analyzed whether or not the presence of octanoate may also allow the recovering of C4-HSL levels, which were also significantly affected in *mexR** (Figure 4). As shown in Figures 7D and 7E, octanoate supplementation affects C4-HSL production by *mexR** in a similar way than for AQs, keeping a delay in C4-HSL accumulation at early growth time, but reaching C4-HSL levels close to those observed in PAO1 once stationary

phase is reached. To get a functional validation of the effect of octanoate supplementation on the recovery of the QS response in *mexR**, the production of pyocyanin by PAO1 and *mexR** growing in presence or absence of octanoate was measured. In absence of octanoate, the *mexR** strain produced less than 15% the amount of pyocyanin produced by the wild type strain, while this production in presence of octanoate reached to 50% of the amount produced by PAO1 (Figure 7F), which is in line with the partial recovery of both AQs and C4-HSL production.

In order to check if MexAB-OprM overexpression leads to a massive extrusion of octanoate, thus affecting biosynthesis of AQs, we analyzed the growth rate of PAO1 and *mexR** strains in minimal medium with octanoate 20 mM as a sole carbon source. As a control of growth rate, we used the same minimal medium but with succinate 20 mM as sole carbon source. In the presence of succinate, the doubling times (min) for PAO1 and *mexR** mutant were 65.1±3.4 and 66.8 ± 3.6 respectively, while with octanoate were 125.5 ± 1.7 and 128.3 ± 2.9 respectively. Therefore, the absence of significant differences of growth between PAO1 and MexAB-OprM overexpressing mutant suggest that the reduced availability of octanoate in the *mexR** mutant is not due to the extrusion of this QS precursor through MexAB-OprM.

Discussion

It has been stated that overexpression of MDR efflux pumps, linked to the acquisition of antibiotic resistance, may strongly modify bacterial physiology, including the expression of QS-regulated virulence determinants (Alcalde-Rico et al., 2016; Blanco et

al., 2016a). Indeed, one of the regulatory processes where efflux pumps may have a role in *P. aeruginosa* is the QS response. Notably, different efflux systems seem to modulate this regulatory network, a feature that at a first sight seems a non-needed redundant function. The first one to be studied was MexAB-OprM and the proposed mechanism of modulating the QS response was the active extrusion of the QS signal 3-oxo-C12-HSL through this efflux system (Pearson et al., 1999). Later on, MexCD-OprJ and MexEF-OprN overexpression was shown to downregulate some QS-controlled phenotypes (Olivares et al., 2012; Alcalde-Rico et al., 2018). Notably, although it has been shown that both efflux systems are able to extrude kynurenine and HHQ (Lamarche and Deziel, 2011; Olivares et al., 2012; Alcalde-Rico et al., 2018), both biosynthetic precursors of PQS (Farrow and Pesci, 2007), there were differences in the underlaying cause behind the impaired QS response associated with MexCD-OprJ overexpression (mainly HHQ extrusion) or with the increased MexEF-OprN expression (mainly kynurenine efflux) (Olivares et al., 2012; Alcalde-Rico et al., 2018). Concerning MexGHI-OpmD, it has been proposed that this efflux system is able to extrude anthranilate and 5-methylphenazine-1-carboxylate (5-Me-PCA), which are immediate biosynthetic precursors of AQs and pyocyanin, respectively, being the latter a known virulence factor regulated by the QS response (Aendekerk et al., 2005; Sakhtah et al., 2016). In addition, it has been shown that *mexGHI-opmD* expression is induced by 5-Me-PCA, whose production is in turn activated by the QS response which is in line with the downregulation of the MexGHI-OpmD efflux pump in mutants with a defective QS network, as is the case of the *mexR** mutant here analyzed (Table 3 and S2).

In the current study we have analyzed the effect of MexAB-OprM overexpression on the *P. aeruginosa* QS response. Although it was earlier stated that MexAB-OprM extrudes 3-oxo-C12-HSL (Pearson et al., 1999), our data do not support that the putative non-physiological extrusion of this AHL by MexAB-OprM is the cause of the impaired QS response of *mexR**. Although a slight imbalance on 3-oxo-C12-HSL kinetics accumulation in *mexR** mutant was observed in our results (Figure 3D and 3E), the absence of an increased ratio SN/CE with respect to the PAO1 wild-type strain in any of the growth time points analyzed (Figure 3C and 3F) discards the possibility of 3-oxo-C12-HSL being highly extruded by MexAB-OprM. However, we showed that production of PQS/HHQ, and to a lesser extent, of C4-HSL were significantly lower in a MexAB-OprM overexpressing mutant that in the wild-type strain. Furthermore, the restoration of wild type PAO1 AQ levels in the *mexR** mutant using an RND efflux pump inhibitor (PAβN) ruled out the possibility of the QS phenotype observed being caused by the loss of MexR function in a MexAB-OprM-independent way. These results are in line with our transcriptomic data, which showed that the expression of the genes responsible for PQS/HHQ biosynthesis and some of the main AQs-regulated genes were the most affected in the *mexR** mutant and that no other RND efflux system was overexpressed. Altogether, we concluded that, differing to what could be expected from previously published work (Pearson et al., 1999), the impaired QS response of the MexAB-OprM overproducing mutant is mainly caused by the low production of PQS and HHQ, rather than by a non-physiological extrusion of 3-oxo-C12-HSL through this multidrug efflux pump.

The observed low production of AQs in the MexAB-OprM overexpressing mutant could be due to a reduced availability of their metabolic precursors as it has been previously described in the case of mutants overexpressing either MexEF-OprN or MexCD-OprJ (Olivares et al., 2012; Alcalde-Rico et al., 2018). We have seen that supplementation of *mexR** cultures with anthranilate, one of the immediate precursors of HHQ and PQS (Calfee et al., 2001; Deziel et al., 2004), did not restore the wild-type levels of these QSSMs (Figure 6). However, supplementation with octanoate, the other immediate precursor of PQS and HHQ (Dulcey et al., 2013), resulted in almost full restoration of AQs production in the *mexR** mutant during early stationary growth phase. Furthermore, octanoate supplementation had similar effects on C4-HSL production by *mexR**, resulting in an increase of this AHL in the MexAB-OprM overexpressing mutant to similar levels of those observed in the PAO1 wild type strain. However, the *mexR** mutant did not present a fitness cost with respect to the wild type strain when they grew in minimal medium in the presence of octanoate as the sole carbon source. Altogether, our results suggest that the main cause for the impaired production of PQS/HHQ observed in MexAB-OprM overproducing mutants is the low intracellular availability of octanoate, not necessarily due to its massive extrusion through this efflux pump. In addition, we propose that this defect in AQs synthesis is also responsible for the low production of C4-HSL in *mexR** by decreasing the Pqs-dependent expression of *rhlI* and *acp1* genes, which are implicated in the biosynthesis of this AHL (Table 3) (Raychaudhuri et al., 2005). The possibility that MexAB-OprM could be extruding a fatty acid-related compound other than octanoate, but with an

impact on octanoate availability, should not be discarded. Especially considering that it has been described that this efflux systems is able to extrude cerulenin, thiolactomycin and CHIR-090, which are compounds with a structural similarity to fatty acids able to inhibit important fatty acid synthases enzymes in a binding-dependent way (Schweizer, 1998; Caughlan et al., 2012). In agreement with this hypothesis, we found that MexAB-OprM overexpression led to changes in the expression of many genes involved in fatty acid metabolism such as *desB*, the operon PA0878-83, PA2550 or *gcdH*; in fatty acid biosynthesis such as *fabH2*, *pqsD*, PA3568, *acp1* or *acp3*; and in butanoate metabolism such as *pdxB*, *liuEDB* or the operon PA4152-53 (Díaz-Pérez et al., 2004; Yuan et al., 2012; Winsor et al., 2016; Kanehisa et al., 2019). It should be noted that the expression of these fatty acid-related genes had a markedly tendency to decrease during the exponential phase of growth in the *mexR** strain and/or to increase along the stationary phase of growth (Table 3). The latter could also be in agreement with a reduced production of fatty acids since a common autoregulatory strategy used by metabolic enzymes is based on its own product-dependent inhibition. Based on that assumption, we postulate that overexpression of the MexAB-OprM efflux system leads to an altered fatty acid metabolism that, in the last instance, decreases the intracellular availability of octanoate for AQs biosynthesis. Therefore, we propose that this low intracellular availability of octanoate to be used in AQs biosynthesis is the main reason for the strong decreased production of PQS and HHQ observed in the MexAB-OprM overexpressing mutant, *mexR**. In addition, our data suggest that this octanoate starvation might not be necessarily caused by a massive extrusion of octanoate through

the MexAB-OprM efflux system, but that other fatty acid-related compound could also be extruded. A more in depth analysis should be carried out to gain a better understanding of the relationship between MexAB-OprM activity, octanoate levels and fatty acid metabolism. It is relevant to be noticed that, even in the presence of octanoate, the *mexR** mutant presented a delay in AQs and C4-HSL biosynthesis with respect to PAO1. Therefore, a slight extrusion of PQS and/or HHQ through MexAB-OprM might be involved in the observed phenotype as well, since the overproducer mutant, in the presence of octanoate, accumulates more AQs outside the cells with respect to the parental PAO1 (Figure 7A and 7C), whereas the opposite trend was observed in cellular extracts (Figure 7B and 7C).

This work, together with previous studies (Aendekerk et al., 2005; Lamarche and Deziel, 2011; Olivares et al., 2012; Sakhtah et al., 2016; Alcalde-Rico et al., 2018) show that, although the consequences of overexpressing each multidrug efflux pump are similar (an impaired QS response), the underlying mechanism by which the overexpression of these RND efflux systems interferes with the *P. aeruginosa* QS response is different in every case (Figure 8). However, the potential functional role of these multidrug efflux pumps on the modulation of the QS response, considering the environmental and nutritional bacterial requirements remains largely unknown, particularly if we take into consideration that the physiological signals that trigger their expression are largely ignored. For example, besides their role in modulating the QS response in clonal populations, the activity of these RND systems could have unknown social implications in heterogeneous populations of *P. aeruginosa*. In this context, the

expression levels of each of the QS-related RND systems could determine the ability of *P. aeruginosa* to act, in function of the needs, either as social cooperators or as social cheaters (Wilder et al., 2011; Feltner et al., 2016).

One of the key features for the successful adaptation of bacteria to continuous changing environment lies in the plasticity of their physiology and in the presence of global regulation networks able to translate environmental signals to the cells (Williams and Camara, 2009; Moradali et al., 2017). Although bacteria present a wide variety of classical master regulators, modulation of the activity of such regulatory networks can be also achieved through the action of other elements involved in fundamental aspects of bacterial physiology (Wang and Levin, 2009; Venkataraman et al., 2014; Blair et al., 2015). One of the ways to achieve such modulation is by interfering with the available concentrations of the signals that control the regulatory networks. For this purpose, multidrug efflux pumps can be particularly well suited, because the activity of these antibiotic resistance determinants can affect the bacterial physiology through the extrusion of endogenous/exogenous molecular compounds with relevance for bacterial physiology (Alcalde-Rico et al., 2016; Blanco et al., 2016b).

Whereas redundancy may help in keeping the robustness of living systems, it also bears a cost and it is sometimes difficult to foresee the reason of this redundancy. In the case of potential modulation of QS-signaling by *P. aeruginosa* MDR efflux pumps, it is worth mentioning that signal molecules or precursors of the QS have similar physicochemical properties and their extrusion by RND systems would not be

surprising per se. However, there is not an evolutionary reason for keeping such kind of redundant systems, unless this type of redundancy is just apparent. Indeed, the fact that the molecular basis of such modulation is different in each case supports that the role of these QS-related efflux systems should not be considered strictly redundant. For that reason, we propose the term 'apparent redundancy' to define this kind of situation in which similar phenotypes, produced by proteins belonging to the same family (in our case RND efflux pumps) are prompted by different mechanisms. This situation may allow keeping both the homeostasis and the plasticity of physiological networks with a fundamental role in bacterial adaptation to habitat changes as the QS response. One example of that RND-mediated re-accommodation of the QS response in *P. aeruginosa* is the work published by Oshri *et al.* (2018) (Oshri et al., 2018), in which the authors shown that a *lasR*-null mutant, with a defective QS response, is able to regain a partial QS-dependent cooperative behavior when grown in casein medium (QS-favored conditions). The study revealed that this partial restoration of casein growth was associated with an increased activation of the RhlIR system mediated by the reduced activity of MexEF-OprN, which was in turn caused by selection of a non-functional mutation in *mexT* that encodes a master regulator needed for *mexEF-oprN* expression (Tian et al., 2009). These evidences further reinforce the role that efflux pumps have in the non-canonical modulation of the *P. aeruginosa* QS response.

Altogether, this work, along with others previously published, evidences the complexity of the relationship between several RND efflux systems and the QS regulatory network. Furthermore, we propose that the "apparent redundancy" observed among different

efflux systems belonging to a particular bacterial species could have an adaptive role on its successful colonization of the range of niches where it can be present.

Experimental procedures

Bacterial strains, plasmids and primers

All the bacterial strains and plasmids used in this work are shown in Table 1. Primers used are listed in Table 2.

Growth media and culture conditions

The experiments were carried out in 100 ml glass-flasks containing 25 ml of broth, which were inoculated at the beginning of the experiments at OD_{600} = 0.01, unless other conditions are stated. All strains were routinely cultured in Lysogeny Broth (LB) Lennox (Pronadisa) at 37 °C with shaking (250 rpm). For the growth of *E. coli* strains containing plasmids with an ampicillin (Amp) resistance marker, LB medium containing Amp 100 μg/ml was used. For the growth of *E. coli* or *P. aeruginosa* strains harboring the miniCTX::P_{rhlI}-*lux* constructions, growth media containing tetracycline (Tc) 10 μg/ml or 100 μg/ml were used, respectively. For efflux pump inhibition assay, PAβN (Lomovskaya et al., 2001) was added to the LB medium at a final concentration of 25 μg/ml. LB medium containing anthranilate 1 mM (Across organics, Thermo Fisher Scientific) was obtained using a stock solution of anthranilate 100 mM adjusted to pH = 7.2. For the supplementation of the LB medium with octanoate 5 mM, the corresponding amount of sodium octanoate (Sigma, Aldrich) was directly dissolved in

LB medium and sterilized by filtration through a membrane with a 0.22 μm pore size. For detecting of the QSSMs by TLC (see below), two different semi-solid media were used according to Yates *et al.* (2002) (Yates et al., 2002) and Fletcher *et al.* (2007) (Fletcher et al., 2007a). For determining the growth kinetics in minimal medium, the defined medium M63 (US Biological) was supplemented with 1 mM MgSO4 and with either succinate 20 mM (Sigma, Aldrich) or octanoate 20 mM (Sigma, Aldrich) as sole carbon sources. The experiments were performed in triplicate using flat 96-well plates and 150 μl of the different media. The growth (OD_{600}) was monitored along 24 hours by using a multi-plate reader (TECAN infinite 200).

Pyocyanin extraction

The *P. aeruginosa* cultures were incubated along 20 hours at 37 °C with shaking and the accumulation of pyocyanin in cell-free supernatants was determined following the method described by Essar et al. (Essar et al., 1990). The concentration of pyocyanin was calculated based on its molar extinction coefficient.

Total RNA extraction, sequencing and analysis of transcriptomes.

Overnight cultures of *P. aeruginosa* were washed and diluted in LB to an OD_{600} of 0.01. They were incubated up to exponential phase of growth (OD_{600} = 0.6) and then were diluted again to an OD_{600} of 0.01. The cultures were grown to reach the exponential (OD_{600} = 0.6) or early stationary phase of growth (OD_{600} = 2.5), time points in which total RNA was obtained as described Schuster *et al*. (2003) (Schuster et al., 2003) using the RNeasy mini kit (QIAGEN). Before RNA extraction, the samples for RNA

sequencing were treated with “RNAProtect Bacteria Reagent” (QIAGEN) following the manufacturer's instructions. For Real-Time reverse transcription PCR (RT-PCR) assays, the RNA extractions were carried out in triplicate. To discard possible DNA contamination, a PCR reaction using the PCR Master Mix (Promega) and the primers *rplU*_Fwd and *rplU*_Rev (Table 2) was carried out. RNA samples were sequenced at the "Centro Nacional de Análisis Genómico" (CNAG), Barcelona (Spain). Ribosomal RNA was removed using "RiboZero rRNA Removal kit for Bacteria". To generate the libraries, 2 μg of RNA were treated with "TruSeq RNA sample preparation kit" (Illumina) combined with a specific strand labeling using dUTPs (Sultan et al., 2012). The sequencing in 2 x 75 pair-end format with Illumina technology was performed. The sequences were aligned against the PAO1 reference genome NC_002516 available in the "Pseudomonas Genome Database" (PseudoCAP) (Winsor et al., 2016) and gene expression was quantified using the "CLC Genomics Workbench" software (QIAGEN). The numeric value of gene expression was normalized to Reads Per Kilobase of gene per million Mapped reads (RPKM). Subsequently, a cut-off value of 1 was added to each RPKM (RPKM + 1) in order to minimize the misleading fold change values caused by RPKMs close to 0 (Charles D. Warden, 2013). The LogRatio parameter of each gene was calculated using the formula LogRatio = Log_2 ($RPKM_{mexR*}/RPKM_{PAO1}$). Genes with LogRatio values higher than 1 or lower than -1 were considered to be affected in their expression and were grouped in the functional classes established in PseudoCAP (Winsor et al., 2016).

Real-time Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

cDNA synthesis was carried out using the High Capacity cDNA reverse transcription kit (Applied Biosystems). The RT-PCR reactions were performed in an ABI prism 7500 system" (Applied Biosystems) using the Power SYBR green kit (Applied Biosystems) following manufacturer`s instructions, and adding to each reaction 50 ng of cDNA and 400 nM of paired-primers (Table 2). The housekeeping gene *rpsL* was used for normalization and gene expression was quantified using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

LC-MS/MS Analysis of QSSMs in Supernatants

The extraction of QSSMs from supernatants of *P. aeruginosa* cultures grown in LB to reach the late stationary phase (16 hours of incubation) and their identification and quantification by LC-MS/MS was performed as previously described (Ortori et al., 2011). In essence, the efficiency and reproducibility of QSSMs extraction were monitored by the addition of a deuterated AHL-internal standard, d9-C5-HSL, and a deuterated AQs-internal standard, d4-PQS, to each sample prior to the extraction protocol. The analyte peak areas obtained for each one of the QSSMs analyzed in a specific sample were normalized with respect to those obtained for the corresponding internal standard in the same sample.

Thin Layer Chromatography (TLC) and Time-Course Monitoring (TCM) of QSSMs accumulation.

The extraction of QSSMs was carried out following the methodology detailed by Alcalde-Rico *et al.* (2018) (Alcalde-Rico et al., 2018). The TLC-based detection of AHLs and AQs were performed following the instructions described by Yates *et al.* (2002) (Yates et al., 2002) and Fletcher *et al.* (2007) (Fletcher et al., 2007a), respectively. The TCM-based detection of QSSMs were carried out following the methodology described by Alcalde-Rico *et al.* (2018) (Alcalde-Rico et al., 2018). For TLC-spots quantifications by densitometry, three independent measurement for each sample and TLC were obtained using the "ImageJ" software. For TCM-based calculation of the SN/CE ratio, the bioluminescence emitted was normalized to the OD_{600} and the coefficient between SN and CE values were calculated for each one of the bacterial strains in all of the time of growth analyzed.

Site-specific insertion of the miniCTX::P*rhlI*-*lux* reporter in the chromosome of *P. aeruginosa* strains and real-time analysis of P*rhlI* activation

The transfer of the miniCTX::P_{rhlI}-*lux* plasmid and its integration into the chromosome of *P. aeruginosa* was carried out following the methodology described by Hoang *et al.* (2000) (Hoang et al., 2000). Overnight cultures of donor (*E. coli* S17-1λ *pir* (miniCTX::P_{rhlI}-*lux*)) and recipient strains (*P. aeruginosa* PAO1 and *mexR**) were washed with LB medium and mixed prior to be poured over LB plates, which were incubated for 8 hours. *P. aeruginosa* transconjugants were selected using Petri dishes

with *Pseudomonas* Isolation Agar (PIA, Fluka) containing Tc 100 µg/ml. The chromosomal insertion of miniCTX::P$_{rhlI}$-*lux* was checked by PCR using the CTX-Fwd and CTX-Rev primers (Table 2). The analysis of the P*rhlI* promoter activity was carried out in Flat white 96-well plates with clear bottom (Thermo Scientific Nunc) and each reporter strain was inoculated to an OD_{600} of 0.01 in 200 µl of LB. The luminescence and OD_{600} were monitored every 10 minutes for 20 hours using a multi-plate reader (TECAN infinite 200). The mean values correspond to the average of three biological replicates and the error bars represent their standard deviation.

Statistical Analysis

All the experiments, in which Student's two-tailed test with a confidence interval of 95% were applied to analyze statistical significance, were performed at least in triplicate. Variations with a P-value lower than < 0.05 were considered significant (* represent P-values < 0.05; ** P-values < 0.01; *** P-values < 0.001).

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Table 1. Bacterial strains and plasmids used in the present work.

Bacterial Strains/Plasmids	Description	Reference/origin
Escherichia coli		
S17-1λ *pir*	Strain used as donor strain in conjugation assays: F^- *thi pro hsdR* $hsdM^+$ *recA* RP42-Tc::Mu-Km::Tn7	(Simon et al., 1986)
S17-1λ *pir* (miniCTX::P_{rhlI}-*lux*)	Strain used to transfer miniCTX::P_{rhlI}-*lux* reporter plasmid to *P. aeruginosa* strains by conjugation assays	Strain provided by Stephan Heeb´s laboratory (pending for publication)
JM109-pSB1142 (LasR-based Biosensor)	Strain used for detecting 3-oxo-C12-HSL, one of the QS signal produced by *P. aeruginosa* strains	Miguel Cámara´s lab collection
JM109-pSB536 (AhyR-	Strain used for detecting C4-HSL, one	(Swift et al., 1997;

based Biosensor)	of the QS signal produced by *P. aeruginosa* strains	Kirke et al., 2004)
Pseudomonas aeruginosa		
PAO1	Wild type PAO1-V clinic strain	(Linares et al., 2005)
PAO1 CTX-*lux*::P*rhlI* (PAO1_P*rhlI*)	PAO1-V strain with the reporter plasmid miniCTX::P_{rhlI}-*lux* integrated in the chromosome through specific insertion in *attB* region	Present work
JFL30 (*mexR**)	Spontaneous resistant mutant obtained from PAO1-V strain, which harbours a punctual inactivating mutation in *mexR* gene, leading to an overproduction of the MexAB-OprM efflux system	(Linares et al., 2005)
JFL30 CTX-*lux*::P*rhlI*	JFL30 strain with the reporter plasmid miniCTX::P_{rhlI}-*lux* integrated in the	Present work

(*mexR**_P*rhlI*)	chromosome through specific insertion in *attB* region	
PAO1 CTX::P$_{pqsA}$-*lux::pqsA* (PqsR-based Biosensor)	Strain used for detecting AQs QSSMs produced by *P. aeruginosa* strains	(Fletcher et al., 2007a; Fletcher et al., 2007b)
Plasmids		
miniCTX::P$_{rhlI}$-*lux*	Reporter plasmid derived from mini-CTX-*lux* (Becher and Schweizer, 2000). The expression of the *luxCDABE* operon is controlled by the *rhlI* promoter region of *P. aeruginosa*. Tc^R	Reporter plasmid ceded by Stephan Heeb´s laboratory (pending for publication)
pSB1142	Reporter plasmid that carries the LasR-based Biosensor for detecting 3-oxo-C12-HSL. Tc^R	Miguel Cámara´s lab collection

pSB536	Reporter plasmid that carries the AhyR-based Biosensor for detecting C4-HSL. Amp^R	(Swift et al., 1997; Kirke et al., 2004)

Table 2. Primers used in the present work.

Name	Sequence	Description
*rplU*_Fwd	5'-CGCAGTGATTGTTACCGGTG-3'	To check DNA contamination of RNA samples
*rplU*_Rev	5'-AGGCCTGAATGCCGGTGATC-3'	
*rpsL*_Fwd	5'-GCAAGCGCATGGTCGACAAGA-3'	Real-time RT-PCR (Housekeeping)
*rpsL*_Rev	5'-CGCTGTGCTCTTGCAGGTTGTGA-3'	
*pqsA*_Fwd	5'-CAATACACCTCGGGTTCCAC-3'	Real-time RT-PCR
*pqsA*_Rev	5'-TGAACCAGGGAAAGAACAGG-3'	
*pqsE*_Fwd	5'-GACATGGAGGCTTACCTGGA-3'	Real-time RT-PCR
*pqsE*_Rev	5'-CTCAGTTCGTCGAGGGATTC-3'	

*pqsH*_Fwd	5'-ATGTCTACGCGACCCTGAAG-3'	
		Real-time RT-PCR
*pqsH*_Rev	5'-AACTCCTCGAGGTCGTTGTG-3'	
*lasI*_Fwd	5'-CTACAGCCTGCAGAACGACA-3'	
		Real-time RT-PCR
*lasI*_Rev	5'-ATCTGGGTCTTGGCATTGAG-3'	
*pvdQ*_Fwd	5'-ACATCCAGCTGGTGACCTTC-3'	
		Real-time RT-PCR
*pvdQ*_Rev	5'-AATGCTTAGCCGTTGCAGTT-3'	
*rhlI*_Fwd	5'-CTCTCTGAATCGCTGGAAGG-3'	
		Real-time RT-PCR
*rhlI*_Rev	5'-GACGTCCTTGAGCAGGTAGG-3'	
*antA*_Fwd	5'-GCGCAACCTCAATCTCTACC-3'	
		Real-time RT-PCR
*antA*_Rev	5'-CGGAGACGTTGAAGAAGTCC-3'	
*lasB*_Fwd	5'-ATCGGCAAGTACACCTACGG-3'	Real-time RT-PCR

*lasB*_Rev	5'-ACCAGTCCCGGTACAGTTTG-3'	
*rhlA*_Fwd	5'-CGAGGTCAATCACCTGGTCT-3'	Real-time RT-PCR
*rhlA*_Rev	5'-GACGGTCTCGTTGAGCAGAT-3'	
*lecA*_Fwd	5'-ATAACGAAGCAGGGCAGGTA-3'	Real-time RT-PCR
*lecA*_Rev	5'-TTGCCAATCTTCATGACCAG-3'	
*mexG*_Fwd	5'-GGCGAAGCTGTTCGACTATC-3'	Real-time RT-PCR
*mexG*_Rev	5'-AGAAGGTGTGGACGATGAGG-3'	
*phzB1*_Fwd	5'-AACGAACTTCGCGAAAAGAA-3'	Real-time RT-PCR
*phzB1*_Rev	5'-TTTGTCTTTGCCACGAATGA-3'	
CTX-Fwd	5'-GTCATGCTCTTCTCTAATGCGTG-3'	To check the insertion of miniCTX::P_{rhlI}-*lux* in the chromosome of *P. aeruginosa* strains
CTX-Rev	5'-GCGTAATACGACTCACTATAGGGC-	

3'

Table 3. Selected list of the genes whose expression is affected by MexAB-OprM overexpression

Gene ID[a]	Gene name[b]	Product description[c]	LogRatio (Exponential growth phase)[d]	LogRatio (Stationary growth phase)[e]	PqsE Regulated[f]	AQs Regulated[g]	LasIR Regulated[h]	RhlIR Regulated[i]	LasIR/RhlIR Regulated[j]
		Quorum sensing modulators[k]							
PA2385	*pvdQ*	3-oxo-C12-homoserine lactone acylase PvdQ	-1.1			+			
PA2593	*qteE*	quorum threshold expression element, QteE	-1.69					+	
PA0996	*pqsA*	PqsA	-5.04	-1	-	+	+		
PA0997	*pqsB*	PqsB	-4.67	-1.13	-	+	+		+
PA0998	*pqsC*	PqsC	-4.46		-	+	+		+
PA0999	*pqsD*	3-oxoacyl-[acyl-carrier-protein] synthase III	-3.25	-1.1	-	+	+		+
PA1000	*pqsE*	Quinolone signal response protein	-3.11	-1.04	+	+	+		+
PA1001	*phnA*	anthranilate synthase component I	-2.74	-1.15	+	+	+		+
PA1002	*phnB*	anthranilate synthase	-3.09	-1.15	+	+	+		+

		component II							
PA3476	*rhlI*	autoinducer synthesis protein RhlI	-1.29				+	+	+
PA1869	*acp1*	acyl carrier protein, Acp1	-1.37	-1.12	+		+	+	+
		Virulence Factors[l]							
PA0051	*phzH*	potential phenazine-modifying enzyme		-1.48	+				
PA1899	*phzA2*	probable phenazine biosynthesis protein	-1.43	-2.43		+			
PA1900	*phzB2*	probable phenazine biosynthesis protein		-2.07		+			
PA1901	*phzC2*	phenazine biosynthesis protein PhzC		-1.95	+	+	+	+	+
PA1902	*phzD2*	phenazine biosynthesis protein PhzD		-1.74	+		+	+	+
PA1903	*phzE2*	phenazine biosynthesis protein PhzE		-1.7	+	+	+	+	+
PA1904	*phzF2*	probable phenazine biosynthesis protein		-1.71	+		+	+	+

PA1905	*phzG2*	probable pyridoxamine 5'-phosphate oxidase		-1.74	+		+	+	+
PA4205	*mexG*	hypothetical protein		-6.17	+	+	+		+
PA4206	*mexH*	RND efflux membrane fusion protein precursor		-5.65	+	+	+		+
PA4207	*mexI*	RND efflux transporter		-4.9	+	+		+	+
PA4208	*opmD*	probable outer membrane protein precursor		-4.28	+	+	+		+
PA4210	*phzA1*	probable phenazine biosynthesis protein		-1.39	+	+		+	
PA4211	*phzB1*	probable phenazine biosynthesis protein	-2.08	-1.1	+		+	+	+
PA4212	*phzC1*	phenazine biosynthesis protein PhzC	-1.33			+	+		
PA4213	*phzD1*	phenazine biosynthesis protein PhzD		-1.66			+		
PA4214	*phzE1*	phenazine biosynthesis protein PhzE		-1.69	+	+	+		
PA4215	*phzF1*	probable phenazine		-1.68			+		

		biosynthesis protein							
PA4216	*phzG1*	probable pyridoxamine 5'-phosphate oxidase		-2.05			+		
PA2193	*hcnA*	hydrogen cyanide synthase HcnA	-2.07		+		+	+	+
PA2194	*hcnB*	hydrogen cyanide synthase HcnB	-2.19		+		+	+	+
PA2195	*hcnC*	hydrogen cyanide synthase HcnC	-2.32		+		+	+	+
PA0070	*tagQ1*	TagQ1		1.28					
PA0072	*tagS1*	TagS1	-1.08			-	-		
PA0073	*tagT1*	TagT1	-1.28			-	-		
PA0076	*tagF1*	TagF1	-1.07	1.4		-	-		
PA0077	*icmF1*	IcmF1		1.16		-	-		
PA0078	*tssL1*	TssL1		1.52		-	-		
PA0084	*tssC1*	TssC1		1.1	-	-	-		
PA0085	*hcp1*	Hcp1		1.41		-	-		
PA0088	*tssF1*	TssF1		1.36		-	-		
PA0089	*tssG1*	TssG1		1.03		-	-		
PA00	*clpV*	ClpV1		1.45		-	-		

90	*1*								
PA0091	*vgrG1*	VgrG1		1.44		-	-		
PA1844	*tse1*	Tse1	-1.01						
PA1845	*tsi1*	Tsi1	-1.01						
PA3485	*tsi3*	Tsi3	-1.1						
PA1658	*hsiC2*	HsiC2	-1.13			+	+	+	+
PA1659	*hsiF2*	HsiF2	-1.38			+	+	+	+
PA1660	*hsiG2*	HsiG2	-1.31			+	+	+	+
PA1661	*hsiH2*	HsiH2	-1.2			+	+	+	+
PA1662	*clpV2*	clpV2	-1.36			+	+	+	+
PA1663	*sfa2*	Sfa2	-1.08			+	+	+	+
PA1665	*fha2*	Fha2	-1.29			+	+	+	+
PA1666	*lip2*	Lip2	-1.26			+	+	+	+
PA1668	*dotU2*	DotU2	-1.4			+	+	+	+
PA1669	*icmF2*	IcmF2	-1.05			+	+	+	+
PA1671	*stk1*	Stk1	-1.53			+	+	+	+
PA5266	*vgrG6*	VgrG6		-1.01					

PA5267	*hcpB*	secreted protein Hcp		-2.53					
PA1871	*lasA*	LasA protease precursor		-1			+		+
PA2300	*chiC*	chitinase		-2.35	+	+	+	+	+
PA2570	*lecA*	LecA		-2.16	+	+	+	+	+
PA3361	*lecB*	fucose-binding lectin PA-IIL		-2.47	+		+		+
PA3478	*rhlB*	rhamnosyltransferase chain B	-1.92	-1.13	+		+	+	+
PA3479	*rhlA*	rhamnosyltransferase chain A	-1.85	-1.39	+		+	+	+
PA3724	*lasB*	elastase LasB	-1.39	-1.11			+	+	+
PA4175	*piv*	protease IV		-1.36		+	+		+
PA4295	*fppA*	Flp prepilin peptidase A, FppA		-1.24					
		Related to fatty acids metabolism[m]							
PA0447	*gcdH*	glutaryl-CoA dehydrogenase		1.13					+
PA0878		hypothetical protein		1.17					
PA0879		probable acyl-CoA dehydrogenase		1.35					
PA0880		probable ring-cleaving		1.35					

		dioxygenase							
PA0881		hypothetical protein		1.75					
PA0882		hypothetical protein		1.07					
PA0883		probable acyl-CoA lyase beta chain		1.13					
PA0884		probable C4-dicarboxylate-binding periplasmic protein		1.16					
PA1375	*pdxB*	erythronate-4-phosphate dehydrogenase	-1.02						
PA2011	*liuE*	3-hydroxy-3-methylglutaryl-CoA lyase		1.04					
PA2012	*liuD*	methylcrotonyl-CoA carboxylase, alpha-subunit (biotin-containing)		1.06					
PA2014	*liuB*	methylcrotonyl-CoA carboxylase, beta-subunit		1.11	-				+
PA2550		probable acyl-CoA dehydrogenase		1.23					
PA3327		probable non-ribosomal peptide	-2.15	1.2	-			+	

		synthetase							
PA3328		probable FAD-dependent monooxygenase	-2.57	1.4	-			+	+
PA3329		hypothetical protein	-2.27	1.18				+	+
PA3330		probable short chain dehydrogenase	-2.44	1.34				+	+
PA3331		cytochrome P450	-2.56	1.46			+	+	+
PA3332		conserved hypothetical protein	-2.51	1.7			+	+	+
PA3333	*fabH2*	3-oxoacyl-[acyl-carrier-protein] synthase III	-2.43	1.69				+	+
PA3334	*acp3*	probable acyl carrier protein	-2.17	1.49			+	+	+
PA3568		probable acetyl-coa synthetase		1.02	-				
PA4152		probable hydrolase		1.11					
PA4153		2,3-butanediol dehydrogenase		1.32					
PA4888	*desB*	acyl-CoA delta-9-desaturase, DesB		1.18					

[a] Identification code, [b] name and [c] product description of the genes following the established in the "Pseudomonas Genome DataBase" (PseudoCAP) (Winsor et al., 2016).

[d] LogRatio values of the genes that presented at least two-fold transcriptional variations in the *mexR** strain with respect to the wild type PAO1 strain (LogRatio > 1 and LogRatio < -1) during the exponential phase of growth (OD_{600} = 0.6) or [e] during the stationary phase of growth (OD_{600} = 2.5).

[f] Genes previously identified as positively or negatively regulated by the *pqsE* induction in AQs-independent way (Lesic et al., 2009; Rampioni et al., 2010; Rampioni et al., 2016).

[g] Genes previously identified as positively or negatively regulated either by the exogenous addition of PQS/HHQ or by the loss of function of the *pqsA* gene, which abolish the AQs production (Lesic et al., 2009; Rampioni et al., 2010; Rampioni et al., 2016).

[h] Genes previously identified as positively or negatively regulated by the Las-QS system, either by the exogenous addition of 3-oxo-C12-HSL, by the loss of function of *lasI* and/or *lasR* genes, or by the direct identification of LasR-binding sequences (Hentzer et al., 2003; Schuster et al., 2003; Wagner et al., 2003; Wagner et al., 2004; Schuster and Greenberg, 2007; Gilbert et al., 2009; Lesic et al., 2009; Sana et al., 2012).

[i] Genes previously identified as positively or negatively regulated by the Rhl-QS system, either by the exogenous addition of C4-HSL or by the loss of function of *rhlI*

and/or *rhlR* genes (Hentzer et al., 2003; Schuster et al., 2003; Wagner et al., 2003; Wagner et al., 2004; Schuster and Greenberg, 2007; Sana et al., 2012).

[j] Genes that, either, have been demonstrated to be regulated by both the Las- and Rhl-QS systems, or it has not be possible to exactly determine if they are regulated by one system or another (Hentzer et al., 2003; Schuster et al., 2003; Wagner et al., 2003; Wagner et al., 2004; Schuster and Greenberg, 2007; Gilbert et al., 2009; Lesic et al., 2009; Sana et al., 2012).

[k] Genes that could be function as QS modulators (Raychaudhuri et al., 2005; Venkataraman et al., 2014; Winsor et al., 2016).

[l] Genes classified as virulence factor either by PseudoCAP (Winsor et al., 2016) or in this work based on previous publications (Folders et al., 2001; Ball et al., 2002; Lesic et al., 2009; Lee et al., 2010; Russell et al., 2011; Pelzer et al., 2014; Chen et al., 2015; Sakhtah et al., 2016).

[m] Genes with potential or corroborated implications in fatty acid metabolism according to KEGG, PseudoCAP or other publications (Díaz-Pérez et al., 2004; Yuan et al., 2012; Winsor et al., 2016; Kanehisa et al., 2019).

Figure Legends

Figure 1. Genes affected in the MexAB-OprM overproducer mutant along both the exponential and early stationary phase of growth. The total RNA was extracted from PAO1 and *mexR** cultures at both (A) exponential (OD_{600} = 0.6) or (B) early stationary phase of growth (OD_{600} = 2.5) as is described in Methods. The expression value for each gene was calculated based on its RPKM (Reads Per Kilobase of gene per million Mapped reads) and only RPKM changes over or below two-fold with respect the control condition were considered as significant variation of the gene expression. The genes affected were grouped in the corresponding functional class stablished in PseudoCAP and the number of the genes affected in each functional class is represented near to the corresponding bar. The percentage of genes whose expression is affected in each functional class was calculated over the total genes grouped in the same category. The results showed that overexpression of the MexAB-OprM system has a strong impact over the transcriptome of *P. aeruginosa* in the both growth phases analyzed and that the most affected functional class is "Secreted Factors (toxins, enzymes, alginate)".

Figure 2. Validation of the results obtained from the transcriptomic assay in PAO1 and *mexR cultures.** Total RNA were extracted in triplicate for each strain at both (A) exponential (OD_{600} = 0.6) and (B) early stationary phase of growth (OD_{600} = 2.5). The expression of the genes selected for each growth phase was determined by real-time RT-PCR and compared with results obtained by RNAseq, validating the transcriptomic assays.

Figure 3. Determination of QSSMs production by PAO1 and *mexR strains growing in LB medium.** (A) The extracellular accumulation of C4-HSL, 3-oxo-C12-HSL, PQS and HHQ from PAO1 and *mexR** cultures were analyzed at late stationary phase of growth (16 hours post inoculation) by LC-MS/MS. (B) Thin-Layer Chromatography (TLC) of both supernatant and cellular extracts of PAO1 and *mexR** cultures grown to late exponential phase (OD_{600} = 1.7) coupled to the growth of LasR-based Biosensor strain. (C) Determination of the Ratio Supernatant/Cell Extract (SN/CE) of 3-oxo-C12-HSL through densitometry analysis of the light spots derived from TLC assay. (D) Time-course Monitoring (TCM) of 3-oxo-C12-HSL accumulation both in the supernatant and (E) inside the cells of PAO1 and *mexR** cultures after 4, 5, 6 and 7 hours of inoculation. (F) The ratio SN/CE for each one of the time point from TCM curves of both PAO1 and *mexR** cultures was calculated in order to analyze along the entire incubation time the ability of MexAB-OprM to extrude the 3-oxo-C12-HSL. Altogether, these results demonstrate that the impaired QS response observed in the MexAB-OprM overproducer mutant, *mexR**, is not caused by a non-physiological extrusion of 3-oxo-C12-HSL through this efflux system.

Figure 4. Analysis of the accumulation of C4-HSL both outside and inside the cell of PAO1 and *mexR cultures grown in LB medium.** (A) TLC assay of the supernatant extracts from PAO1 and *mexR** cultures grown to late exponential phase of growth (OD_{600} = 1.7), in which the presence of C4-HSL was revealed using the AhyR-based biosensor strain (Swift et al., 1997; Kirke et al., 2004). (B) Analysis of the accumulation kinetics of C4-HSL in the supernatant extracts of PAO1 and *mexR**

cultures at 4, 5, 6 and 7 hours post-inoculation. (C) Analysis of the C4-HSL-dependent activation of the P*rhlI* promoter in PAO1-P*rhlI* and *mexR**-P*rhlI* reporter strains along 20 hours of growth. The results showed that the production of C4-HSL is impaired in the MexAB-OprM overproducing mutant.

Figure 5. Analysis of the extracellular and cell-associated accumulation of PQS and HHQ into PAO1 and *mexR cultures when grow in LB medium with or without PAβN.** (A) TLC assays from both supernatant and cellular extracts of PAO1 and *mexR** cultures grown to early stationary phase (OD_{600} = 2.5) in LB medium, in which the presence of PQS and HHQ were revealed using the PqsR-based biosensor strain (Fletcher et al., 2007a; Fletcher et al., 2007b). (B) The MexAB-OprM-dependent phenotype was analyzed by TLC assay, using supernatant and cellular extracts from PAO1 and *mexR** cultures grown to early stationary phase in LB medium with or without 20 µg/mL of the efflux pump inhibitor, PAβN. Analysis of the accumulation kinetics of AQs both (C) in the supernatant and (D) in cellular extracts of PAO1 and *mexR** cultures at 4, 5, 6 and 7 hours post-inoculation. These results showed that the production of PQS and HHQ is impaired in the antibiotic resistant *mexR** mutant in a MexAB-OprM-dependent way, ruling out the possibility that loss of MexR-dependent regulation of other than *mexAB-oprM* operon being the cause of the observed phenotype.

Figure 6. Analysis of the outside and inside accumulation of PQS and HHQ in PAO1 and *mexR cells when grow in presence of anthranilate 1 mM.** TLC assays

from both supernatant and cellular extracts of PAO1 and *mexR** cultures grown to early stationary phase in LB medium and LB supplemented with anthranilate 1 mM, respectively. The presence of PQS and HHQ were revealed using the PqsR-based biosensor strain (Fletcher et al., 2007a; Fletcher et al., 2007b). This result confirms that availability of anthranilate and/or its precursors is not the bottleneck for PQS and HHQ production in *mexR** mutant.

Figure 7. Analysis of the production of PQS, HHQ, C4-HSL and pyocyanin by PAO1 and *mexR cultures grown in LB medium supplemented with octanoate 5 mM.** Analysis by TCM of (A and B) AQs and (D) C4-HSL accumulation both (A and D) outside and (B) inside the cells of PAO1 and *mexR** cultures after 4, 5, 6 and 7 hours of incubation. (C) TLC of supernatant and cellular extracts from cultures incubated along 7 hours in LB medium supplemented with octanoate 5 mM, which was revealed using PqsR-based biosensor strain (Fletcher et al., 2007a; Fletcher et al., 2007b). (E) Real-time monitoring of the C4-HSL-dependent activation of the P*rhlI* promoter in PAO1 and *mexR** cultures grown along 20 hours in presence of octanoate 5 mM. (F) Quantification of pyocyanin production by PAO1 and *mexR** strains grown along 20 hours in LB medium supplemented with octanoate 5 mM. The results showed that LB supplementation with octanoate 5 mM partially restore the production of AQs, C4-HSL and pyocyanin in MexAB-OprM overproducer mutant, suggesting that a decreased availability of octanoate or any of its precursors should be the cause of the QS impairment observed in *mexR**.

Figure 8. RND-dependent production of the QS signals in *P. aeruginosa*. The main autoinducer signals involve in the QS response in *P. aeruginosa* are 3-oxo-C12-HSL, C4-HSL, HHQ and PQS (Williams and Camara, 2009). The synthesis of the two homoserine lactones, 3-oxo-C12-HSL and C4-HSL, are carried out by the synthases enzyme LasI and RhlI, respectively, which use both acyl carrier proteins and *S*-adenosyl-L-methionine as precursors (Gould et al., 2004; Raychaudhuri et al., 2005). The synthesis of HHQ is mediated by the synthetic enzymes PqsA, PqsB, PqsC and PqsD using anthranilate and octanoic acid as immediate precursors, while PQS is a chemical modification of HHQ carried out by PqsH (Dulcey et al., 2013). Pearson *et al.* (1999) (Pearson et al., 1999) described that C4-HSL is an autoinducer signal able to freely diffuse through the plasma membrane and proposed that 3-oxo-C12-HSL could be actively extruded through MexAB-OprM efflux system. However, in this work we demonstrate that MexAB-OprM is not able to extrude this QS signal. At the same time, we show that the overexpression of MexAB-OprM leads to a decreased PQS/HHQ production and, in consequence, to an impaired QS response. We suggest that the low intracellular availability of one of their immediate precursor, the octanoic acid, probably due to an imbalance in the fatty acid metabolism, could be triggering the QS deficiencies observed in the *mexR** mutant. In addition, the MexCD-OprJ and MexEF-OprN efflux systems are able to extrude both kynurenine and HHQ (Lamarche and Deziel, 2011; Olivares et al., 2012; Alcalde-Rico et al., 2018), which are both precursors of PQS (Farrow and Pesci, 2007). However, the affinity by one or another substrate seems to be different, since the bottleneck for the impaired PQS production

observed in the MexCD-OprJ overproducer mutants is the massive extrusion of HHQ (Alcalde-Rico et al., 2018), while in MexEF-OprN overproducer mutants is the massive extrusion of kynurenine (Olivares et al., 2012). Furthermore, it has been suggested that MexAB-OprM and MexCD-OprJ could be also extruding, to a lesser extent, PQS, since it has been observed a slight increase on outside/cell-associated PQS accumulating ratio respect to PAO1 wild type strain (This work and (Alcalde-Rico et al., 2018)). Finally, it has been described that MexGHI-OpmD is able to extrude anthranilate and 5-methylphenazine-1-carboxylate (5-Me-PCA), which are either precursors of PQS and of the QS-regulated virulence factor, pyocyanin, respectively (Aendekerk et al., 2005; Sakhtah et al., 2016).

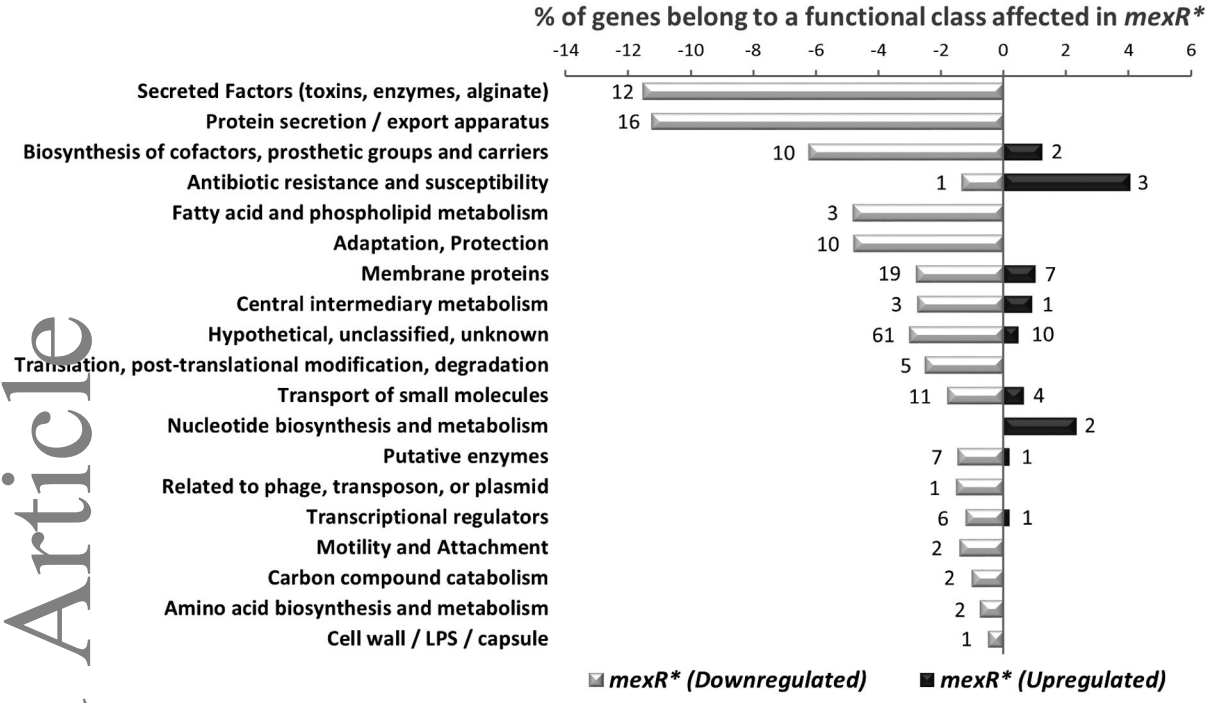
Supplementary material

Table S1. Complete list of genes whose expression presented at least two-fold change in the *mexR** strain with respect to the wild type PAO1 strain (LogRatio > 1 and LogRatio < -1) during the exponential (OD_{600} = 0.6) or stationary phase of growth (OD_{600} = 2.5)

Table S2. Review of the genes whose expression is regulated in a QS-dependent way, stating the differences between each one of the QS system implicated in the control of their expression level.

A

Exponential phase of growth



B

Early stationary phase of growth