AAC Accepted Manuscript Posted Online 9 September 2019
Antimicrob. Agents Chemother. doi:10.1128/AAC.01095-19

Novel inducers of the expression of multidrug efflux pumps that trigger *Pseudomonas aeruginosa* transient antibiotic resistance

Pablo Laborda[1], Manuel Alcalde-Rico[1], Paula Blanco, José Luis Martínez*, Sara Hernando-Amado*

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

[1]: These authors equally contributed to the work

*Corresponding authors

JLM: jlmtnez@cnb.csic.es

SHA: shernando@cnb.csic.es

ABSTRACT

The study of the acquisition of antibiotic resistance (AR) has mainly focused in inherited processes, namely mutations and acquisition of AR genes. However, inducible, non-inheritable AR has received less attention and most information in this field derives from the study of antibiotics as inducers of their associated resistance mechanisms. Less is known about non-antibiotic compounds or situations that can induce AR during infection. Multidrug resistance efflux pumps are a category of AR determinants characterized by the tight regulation of their expression. Their contribution to acquired AR relies in their overexpression. Herein we analyzed potential inducers of the expression of the chromosomally-encoded *Pseudomonas aeruginosa* clinically-relevant efflux pumps, MexCD-OprJ and MexAB-OprM. For this purpose, we developed a set of *luxCDABE*-based *P. aeruginosa* biosensor strains, which allows the high-throughput analysis of compounds able of modifying the expression of these efflux pumps. Using these strains, we analyzed a set of 240 compounds present in Biolog Phenotype Microarrays. Several inducers of the expression of the genes that encode these efflux pumps were found. The study focused in dequalinium chloride, procaine and atropine, compounds that can be found in clinical settings. Using real-time PCR, we confirmed that these compounds indeed induce the expression of *mexCD-oprJ*. In addition, *P. aeruginosa* presents lower susceptibility to ciprofloxacin (a MexCD-OprJ substrate) when dequalinium chloride, procaine or atropine are present. This work emphasizes the need of studying compounds that can trigger transient AR during antibiotic treatment, a phenotype difficult to discover using classical susceptibility tests.

INTRODUCTION

Pseudomonas aeruginosa is included in the groups of bacteria considered to be of high risk concerning antibiotic resistance (AR) (1, 2). These include *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa* and *Enterobacter* spp., which altogether have been dubbed ESKAPE pathogens (3) and the TOp TEn resistant Microorganisms (TOTEM), as stated by the World Health Organization (1, 2). . This nosocomial pathogen is one of the most prevalent organisms causing infections at hospitals and is the main cause of chronic lung infections in patients with cystic fibrosis and chronic obstructive pulmonary diseases (4, 5). Among the different AR mechanisms of *P. aeruginosa*, multidrug efflux pumps from the Resistance Nodulation and cell-Division (RND) family are relevant elements since they contribute to both intrinsic and acquired resistance (6-10). Among such RND efflux pumps, MexAB-OprM and MexCD-OprJ stand out as significant determinants of multidrug resistance in *P. aeruginosa* (11-13). MexAB-OprM encoding genes are constitutively expressed under regular growing conditions, hence contributing to intrinsic resistance of *P. aeruginosa* to several antibiotics such as quinolones, macrolides, tetracycline, lincomycin, chloramphenicol, novobiocin and β-lactams (14). In addition, *mexAB-oprM* overexpressing mutants have been isolated from patients (9), so that this overexpression is considered as a significant mechanism for acquiring AR under a clinic viewpoint (8). The operon encoding MexCD-OprJ, on its hand, is expressed at very low level under regular growing conditions. Thus, it does not seem to have a relevant contribution in *P. aeruginosa* intrinsic resistance (8). Nevertheless, *mexCD-oprJ* overexpression, usually achieved by loss of function mutations in the gene that encodes its local repressor, *nfxB*, confers resistance to different antibiotics such as quinolones, tetracyclines and chloramphenicol (14, 15).

It is worth mentioning that, besides being AR determinants (16, 17), efflux pumps present other physiological functions important for bacterial behavior, such as modulation of Quorum Sensing (QS) signaling (18-20), response to stress situations (21) and to host defenses (22-24), or plant/bacteria interactions (25, 26). Although the basal level of expression of each efflux

pump can vary, it is well established that their expression may increase in the presence of some compounds or situations (27). In this regard, knowing which compounds are capable of triggering the expression of the genes that encode efflux pumps, and therefore to promote a transient reduction in the susceptibility to antibiotics (28, 29), a situation that is not easily detected using common susceptibility methods (8, 29), is a relevant topic.

It is relevant to highlight that induction of the expression of MDR efflux pumps can be the consequence of the binding of the inducer to the transcriptional regulators of their expression (30, 31) or due to the bacterial response to different injuries (32). While in the first case, a structural similarity of potential effectors is expected, in the second molecules with different structures, but with a similar biological activity, can trigger the expression of MDR efflux pumps.

In the current work, we addressed this issue by analyzing two relevant *P. aeruginosa* efflux pumps that present different levels of basal expression, MexAB-OprM and MexCD-OprJ. Previous studies have shown that *mexAB-oprM* expression is induced by oxidative stress (33), triclosan and pentachlorophenol (34), while envelope stress, benzalkonium chloride, chlorhexidine, tetraphenylphosphonium chloride, ethidium bromide, rhodamine 6G or antimicrobial human peptides IL-37 (35-38) are inducers of *mexCD-oprJ* expression. By the screening of a set of compounds present in Biolog Phenotype Microarrays, an approach that has been previously validated as a useful strategy to discover novel inducers of the expression of the genes that encode efflux pumps (39, 40), we have detected different molecules that induce the expression of *mexAB-oprM* and *mexCD-oprJ* in *P. aeruginosa*. For further analysis, we focused on molecules that this bacterial pathogen can potentially encounter when producing an infection, as procaine, atropine or dequalinium chloride. This work highlights the potential risk associated to the utilization of these compounds in clinical settings, as inducers of transient AR in *P. aeruginosa*, when antibiotic treatments are applied.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions

Bacterial strains and plasmids used in this work are listed in Table 1. Unless otherwise stated, all strains were grown in Lysogeny Broth, Lennox (LB) (Pronadisa) at 37ºC and 250 rpm. *Escherichia coli* strains carrying the mini-CTX-*lux* (Tc^R) or pGEM-T Easy Vector derived plasmids were grown in LB medium with 10 µg/ml of tetracycline or 100 µg/ml of ampicillin, respectively.

Construction of mini-CTX::P*mexAB-lux* and mini-CTX::P*mexCD-lux* reporter plasmids

To obtain the mini-CTX::P*mexAB-lux* and mini-CTX::P*mexCD-lux* reporter plasmids, a mini-CTX-*lux* (Tc^R) plasmid (41) was digested with EcoRI and BamHI (New England BioLabs). The promoter region of *mexAB-oprM* operon was amplified with *EcoRI_PmexAB_Fw* and *BamHI_PmexAB_Rv* primers, while the promoter region of *mexCD-oprJ* operon was amplified with *EcoRI_PmexCD_Fw* and *BamHI_PmexCD_Rv* primers (Table 2). The products of PCR were purified from an agarose gel by using a DNA purification kit (GE Healthcare) and were cloned into the pGEM-T Easy Vector following supplier's instructions. Afterwards, *E. coli* OmniMax™ competent cells (Invitrogen) were transformed with these plasmids, which were then purified using the QIAprep Spin miniprep kit 250 (Qiagen), and digested with EcoRI and BamHI. The resulting fragments, corresponding to the efflux pump promoters, and the mini-CTX-*lux* (Tc^R) plasmid linearized using EcoRI and BamHI, were purified from an agarose gel and used to obtain the reporter plasmids, mini-CTX::P*mexAB-lux* and mini-CTX::P*mexCD-lux*, through a ligation reaction with the T4 DNA ligase (New England BioLabs).

Integration of the reporter plasmids, mini-CTX-*lux*, mini-CTX::P*mexAB-lux* and mini-CTX::P*mexCD-lux* in the chromosome of PAO1 wild type strain.

The reporter plasmids, mini-CTX::P*mexAB-lux* and mini-CTX::P*mexCD-lux*, in addition to the mini-CTX-*lux*, used as control plasmid (Table 1), were introduced by transformation in *E. coli* S17-1λ *pir*. Afterwards, these constructions were independently inserted in the chromosome of *P. aeruginosa* PAO1 by conjugation, using as donor strain the *E. coli* S17-1λ *pir* harboring each of the plasmids, and following the protocol previously described (42). The *P. aeruginosa* exconjugants carrying the mini-CTX-*lux* (Tc^R), mini-CTX::P*mexAB-lux* or mini-

CTX::P*mexCD-lux* reporter constructions integrated into their chromosome were selected on petri dishes containing *Pseudomonas* Isolation Agar (PIA) (SIGMA-Aldrich) with 100 µg/ml of tetracycline. The resulting bioreporter strains are PAO1 CTX-*lux*::P*mexAB*, PAO1 CTX-*lux*::P*mexCD* and PAO1 CTX-*lux* (Table 1).

Screening of potential inducers of *mexCD-oprJ* and *mexAB-oprM* expression

The potential ability of 240 compounds to induce *mexAB-oprM* or *mexCD-oprJ* expression was analyzed using the PAO1 CTX-*lux*::P*mexAB*, PAO1 CTX-*lux*::P*mexCD* and PAO1 CTX-*lux* strains. For that purpose, these biosensor strains were grown in the bacterial chemical susceptibility plates (form PM11C to PM20B) of Phenotype MicroArrays™ (BIOLOG) and both absorbance and bioluminescence emitted were monitored along time. 100 µl of LB medium were added to each well of 96-well plates, and the plates were incubated during 2 hours with agitation at room temperature to dissolve the lyophilized compounds. A volume of 10 µl of cell culture was inoculated in each well to a final OD_{600nm} of 0.01. Bacteria were grown at 37ºC for 20 hours, and OD_{600nm} and luminescence was measured every 10 minutes using a Tecan Infinite 200 plate reader (Tecan). Luminescence and OD_{600nm} raw data are shown in Supplementary Material.

Normalization of the results

The ratio luminescence emitted/OD_{600nm} was calculated in those wells in which the growth rate was not impaired, and the resulting values were represented in a graphic against time. The area under the curve for each graphic was calculated using the GraphPad Prism software, giving rise to a collection of numeric values which represent the *luxCDABE* expression in PAO1 CTX-*lux*::P*mexCD*, PAO1 CTX-*lux*::P*mexAB* and PAO1 CTX-*lux* strains when growing in each well. Afterwards, each numeric value obtained in each tested strain (PAO1 CTX-*lux*::P*mexAB* and PAO1 CTX-*lux*::P*mexCD*) was normalized by dividing the value corresponding to the same condition for the control strain (PAO1 CTX-*lux*).

146 The distribution of every normalized luminescence value obtained from each biosensor strain
147 was represented in a box plot in order to determine the threshold from which one value will be
148 consider as indicator of significant induction or repression. This threshold was determined as
149 previously described (40), using the formula Q_3 + 1.5 X IQR for the induction or Q_1 – 1.5 X
150 IQR for the repression, being Q_3 the upper quartile, Q_1 the lower quartile and IQR the
151 interquartile range for each data set.

152 **Analysis of potential inducers of the expression of *mexCD-oprJ* and in the susceptibility of**
153 ***P. aeruginosa* to ciprofloxacin**

154 The growth of *P. aeruginosa* was analyzed by measuring the absorbance OD_{600nm} of bacterial
155 cultures. 10 µl of diluted overnight bacterial cultures were added to 140 µl of LB medium with
156 or without 0.25 µg/ml ciprofloxacin, atropine (ranging 1 to 8 mg/ml), procaine (ranging 1 to 8
157 mg/ml) or dequalinium chloride (ranging 1 to 500 µg/ml) in Flat white 96-well plates with
158 optical bottom (Thermo Scientific Nunc), at a final OD_{600nm} of 0.01. To determine the effect of
159 these potential inducers, the luminescence of either the PAO1 CTX-*lux*::P*mexCD* or the PAO1
160 CTX-*lux* reporter strains was measured in presence and in absence of atropine, procaine or
161 dequalinium chloride. Measures were taken every 10 minutes during 20 or 42 hours in a plate
162 reader (Tecan Infinite 200) at 37ºC. The average of three biological replicates for each strain
163 and condition was used to estimate the values of absorbance and luminescence.

164 **RNA preparation and real-time PCR**

165 An overnight culture of *P. aeruginosa* PAO1 was used to inoculate Erlenmeyer flasks with 20
166 ml of LB broth to a final OD_{600nm} of 0.01. The flasks were incubated at 37ºC and 250 rpm until
167 exponential phase of growth (OD_{600nm} = 0.6) was reached. Then, the optimal concentrations of
168 each tested compound (10 µg/ml of dequalinium chloride, 2 mg/ml of procaine and 2 mg/ml of
169 atropine) were added to each flask and cultures were incubated for 90 minutes with shaking, as
170 previously described in (40), to perform the induction assay; bacterial cultures without any
171 compound or with ethanol in the case of dequalinium chloride, the solvent of this compound,
172 were used as negative controls, and a *mexCD-oprJ* overexpressing strain (*nfxB**) (Table 1),

grown in the absence of inducer, was used as a control of *mexCD-oprJ* overexpression. When removal of the inducer compounds was necessary, cultures were centrifuged 20 minutes at 7000 rpm and then resuspended in the same volume of LB and grown additional for 30, 60 or 120 minutes. Afterwards, 10 ml of each culture were pelleted by centrifugation at 7000 rpm and 4ºC for 20 minutes.

The RNA extraction from the collected cells was performed as previously described in (43). After a DNase I (Qiagen) treatment, a second treatment using DNase Turbo DNA-free (Ambion) was performed, and the presence of DNA contamination was checked by PCR using primers *rpsL_Fw* and *rpsL_Rv* (Table 2). By using the High-Capacity cDNA reverse transcription kit (Applied Biosystems), cDNA was obtained from 10 µg of RNA.

Real-time PCR was carried out with Power SYBR green PCR master mix (Applied Biosystems) in an ABI Prism 7300 real-time system (Applied Biosystems). 50 ng of cDNA were used in each reaction, except for the wells with no template that were used as negative controls. A first denaturation step at 95°C for 10 minutes was followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute for amplification and quantification. Primers that amplify specific fragments of *mexC* were used at 400 nM (Table 2). Primers *rplU_RTPCR_Fw* and *rplU_RTPCR_Rv* were used to amplify the housekeeping gene *rplU*. All the primers used were designed with Primer3 Input software; their specificity was tested by BLAST alignment against *P. aeruginosa* genome from Pseudomonas Genome Database (44) and their efficiency was analyzed by real-time PCR using serial dilutions of cDNA. Differences in the relative amounts of mRNA were determined according to the $2^{-\Delta\Delta CT}$ method (45). In all cases, the values of relative mRNA expression were determined as the average of three independent biological replicates each one containing three technical replicates.

Search of genetic modifications in *nfxB*

To ascertain the absence of putative mutations in *nfxB* or the intergenic region between this gene and *mexC* after the induction assay (see above), this region was amplified by PCR, using

Antimicrobial Agents and Chemotherapy

the primers included in Table 2. Then, PCR products were purified using the QIAquick PCR purification Kit (QIAGEN) and Sanger-sequenced at GATC Biotech.

Determination of the susceptibility to antibiotics of *P. aeruginosa* in presence of inducers of *mexCD-oprJ* expression

Ciprofloxacin, fosfomycin, tobramycin and ceftazidime susceptibility assays were carried out using MIC-test strips (Liofilchem®) in Mueller Hinton Agar (Pronadisa) containing either 10 µg/ml of dequalinium chloride, 2 mg/ml of procaine, 2 mg/ml of atropine or without any inducer, following supplier's instructions. Overnight bacterial cultures were normalized to an OD_{600nm} of 2.5 and a 1:1000 dilution of each culture was inoculated in the test plates and incubated at 37ºC. Growth curves were also recorded using a Tecan Infinite 200 plate reader, as previously mentioned.

Checkerboard analysis

Standard checkerboard broth microdilution assays were performed using eight serially diluted concentrations of ciprofloxacin and eight of each inducer compound. 140 µl of LB medium with ciprofloxacin (ranging 0.05 µg/ml to 0.2 µg/ml) and dequalinium chloride (ranging 5 µg/ml to 80 µg/ml), procaine (ranging 1 mg/ml to 16 mg/ml) or atropine (ranging 1 mg/ml to 16 mg/ml), were added to each well of three different 96-Uwell plates. A volume of 10 µl of cell culture was inoculated in each well to a final OD_{600nm} of 0.01. Bacteria were grown at 37ºC for 24 hours without sacking. The fraction inhibitory concentration (FIC) of ciprofloxacin, dequalinium chloride, procaine and atropine was calculated as the MIC of the combination of ciprofloxacin with each inducer divided by the MIC of each of the drugs alone. The FIC index is calculated as the addition of the FICs of both drugs. FIC Index value less than 0.5 is considered to indicate synergy and above 2 to indicate antagonism.

RESULTS AND DISCUSSION

Construction and validation of reporter strains

In order to identify potential inducers of the expression of either *mexCD-oprJ* or *mexAB-oprM*, which could trigger *P. aeruginosa* non-heritable resistance to antibiotics, a set of biosensor strains of *P. aeruginosa* PAO1 containing the *mexCD-oprJ* or the *mexAB-oprM* promoter regions controlling the *luxCDABE* operon (PAO1 CTX-*lux*::P*mexCD* and PAO1 CTX-*lux*::P*mexAB* respectively) and the control strain PAO1 CTX-*lux*, were developed as described in Materials and Methods. The proper functioning of the developed reporter strains was analyzed by measuring luminescence and OD_{600nm} of each strain in the presence of known inducers, benzalkonium chloride at 10 µg/ml for *mexCD-oprJ* (37) and H_2O_2 at 0.136 µg/ml for *mexAB-oprM* (33). The luminescence values were normalized with respect to those obtained from the control strain PAO1 CTX-*lux*, as described in Materials and Methods. The presence of the known inducers produced an increase in luminescence emitted by the corresponding biosensor strain of 1.92-fold in the case of benzalkonium chloride, and 1.94-fold in the case of H_2O_2 in comparison to that produced in LB medium without inducers (Figure 1). These results validate the capability of these biosensor strains to detect *mexAB-oprM* and *mexCD-oprJ* inducers.

Screening for inducers of *mexCD-oprJ* and *mexAB-oprM* expression using Biolog Phenotype Microarrays

It has been described that the expression of efflux pumps can be induced by different stressors (32). Consequently, we tested the capability of 240 compounds, several of them with pharmacological interest and present in the PM11C to PM20B bacterial chemical susceptibility arrays of Phenotype MicroArrays™ (BIOLOG), for inducing the expression of either *mexCD-oprJ* or *mexAB-oprM*. Among these compounds, there were antibiotics, heavy metals, antiseptics, fungicides, food preservatives, chelating agents, oxidative stress compounds, amino acids, synthetic organic compounds and different drugs for human and veterinary use. Four different concentrations of each compound are present in each commercial microarray plate;those in which the growth of the biosensor strain was severely impaired were discarded for the analysis.

Luminescence emitted and OD_{600nm} were recorded for each biosensor strain (PAO1 CTX-*lux*::P*mexCD* and PAO1 CTX-*lux*::P*mexAB*) and these values were normalized with those obtained from the control strain (PAO1 CTX-*lux*) as described in Materials and Methods. Luminescence and OD_{600nm} raw data are shown in Supplementary Material. The distribution of normalized values for each promoter was represented in a box plot (Figure 2) and an induction threshold level was calculated for each reporter strain as described in Materials and Methods. These threshold values were 1.73 and 1.53 for *mexCD-oprJ* and *mexAB-oprM* promoter, respectively. Those compounds that produced a fold change in luminescence that exceeded the calculated threshold were considered as potential inducers and are shown in Table 3. For *mexCD-oprJ*, 20 putative inducers were detected, while 10 compounds were found to increase luminescence above the threshold in the case of the *mexAB-oprM* biosensor strain.

Among the latter, we found pentachlorophenol already known to be inducer of *mexAB-oprM* expression (34), as well compounds that may lead to oxidative stress, which is a known inducer condition of the expression of this efflux pump (33), such as flavins or their derivatives (acriflavine, proflavine or 9-aminoacridine) (46) or iodoacetic acid. These results reinforce the robustness of our experimental approach. Novel inducers of *mexAB-oprM* expression detected during the analysis include antibiotics as amikacin and azlocillin (14), sanguinarine, which is a plant-derived compound, ethionamide, which is used as an antibiotic for treating multidrug resistant *Mycobacterium tuberculosis* (47) and sodium cyanate, a neurotoxic compound implicated in neurodegenerative disorders in populations subsisting on the cyanogenic plant cassava (48) (see Table 3).

Concerning MexCD-OprJ, an efflux pump known to be induced by membrane-damaging agents (35, 36), novel putative inducers of its expression were found. Among them, disinfectants (benthezonium chloride, dequalinium chloride, domiphen bromide, alexidine and cetylpyridinium chloride), the chelating agent 2, 2'-dipyridyl, sodium cyanate, methyltrioctylammonium chloride, iodonitro tetrazolium violet, flavin derivatives (acriflavine, proflavine and 9-aminoacridine), the anesthetic agent procaine, the antidepressant drug

amitriptyline, the antihistaminic agent orphenadrine, the β-blocker propranolol, the fungicide dodine and some plant-derived compounds (atropine, harmane and sanguinarine), were identified (see Table 3).

Regarding plant-derived compounds, it is worth mentioning that some of them, as the flavonoids, play important physiological functions in plants as well as in plant-bacteria interactions (49), and it is known that the flavonoid-responsive efflux pump MexAB-OprM of *Pseudomonas syringae* is required for the efficient bacterial colonization of tomato plants (50). Since *P. aeruginosa* is widely distributed in different natural habitats, including plants (51), it seems possible that these efflux pumps could have a role in colonizing such habitats (25, 26).

In addition to the detected inducers of *mexCD-oprJ* and *mexAB-oprM* expression, some compounds (Table 4) were found to reduce the expression of these efflux pumps below the lower threshold (1.1 for *mexCD-oprJ* and 0.7 for *mexAB-oprM*) for each of both promoters calculated as described in Materials and Methods. For *mexCD-oprJ*, those compounds were the antibiotics chloramphenicol, spectinomycin and spiramycin, and the plant derived compounds nordihydroguaiaretic acid and gallic acid (see Table 4). For *mexAB-oprM*, there were antibiotics such as hygromycin and josamycin, chromium chloride, glycine hydroxamate and protamine sulfate (see Table 4). However, these compounds did not seem to be strong inhibitors, since the normalized luminescence values measured in presence of them were close to those of the threshold values and were not further studied.

Among the potential inducers, dequalinium chloride, procaine and atropine, which seemed to induce the expression of *mexCD-oprJ*, were chosen for a deeper analysis because they are used in human therapy and hence *P. aeruginosa* can grow in their presence when causing infections.

Inducers of expression of *mexCD-oprJ* with relevance in clinical settings

Dequalinium chloride is used as a disinfectant (52) that belongs to the group of quaternary amines as benzalkonium chloride, and presents some structural similarities to chlorhexidine. Both compounds are known inducers of *mexCD-oprJ* (37) that promote its expression through

304 the induction of an envelope-stress condition (35, 36). Further, several compounds found as
305 inducers in the analysis are similar to them in terms of biological activity (benthezonium
306 chloride, cetylpyridinium chloride, domiphen bromide or alexidine) and are also used in clinical
307 practice. This finding supports the potential relationship between antibiotics and disinfectants
308 resistance, as well as the possible induction of AR by disinfectants of common use. In
309 particular, the induction of *mexCD-oprJ* by dequalinium chloride could have clinical relevance,
310 as this compound forms part of several antiseptic and disinfection procedures (53) and its use
311 has been considered for the treatment of promyelocytic leukemia (54-57).

312 Procaine is used as a local anesthetic agent (58) in some minor surgeries or in burn injuries,
313 tissues that *P. aeruginosa* frequently colonize (59).. Therefore, the induction of *mexCD-oprJ* by
314 procaine should be taken into consideration in these patients since they are susceptible to *P.*
315 *aeruginosa* infections. Finally, atropine is considered as an essential drug for preoperative
316 medication and sedation by the World Health Organization (47), so it may have an effect on *P.*
317 *aeruginosa* susceptibility to antibiotics when this microorganism infects surgical patients. To
318 note here that it has been earlier described that procaine and atropine have some common
319 pharmacological properties (60), although their structures present some few similarities (Figure
320 3). Whether their mechanism of induction relies in a response to cellular damage as it happens
321 for some inducers of efflux pumps (32) or they are effectors able of binding the regulators of
322 efflux pumps' expression, as it happens for others (30, 31) is an issue that remains to be
323 established.

324 Since concentrations of each compound in each well of the Biolog Phenotype Microarays are
325 not included in the information provided with the plates, the *mexCD-oprJ* reporter strain was
326 grown during 20 hours in a range of concentrations in order to select one at which an increase in
327 luminescence is observed but bacterial growth is not compromised. These concentrations are 10
328 µg/ml for dequalinium chloride, 2 mg/ml for procaine and 2 mg/ml for atropine, and were the
329 concentrations used for further studies. Luminescence measurements in the presence of inducers
330 at these optimal concentrations were recorded and normalized as previously described. In all

cases, the presence of the tested compounds increased the luminescence produced by the biosensor strain (Figure 4), further supporting they induce the expression of *mexCD-oprJ*.

Dequalinium chloride, procaine and atropine induce *mexCD-oprJ* expression and transient antibiotic resistance

In order to further confirm the inducing capacity of the compounds found in the screening, the expression level of *mexCD-oprJ* was quantified by real-time PCR in the wild type *P. aeruginosa* PAO1, grown in the presence or in the absence of the cognate inducer compounds. The strain overexpressing *mexCD-oprJ* (*nfxB**) (Table 1), which differs from the wild-type strain just by a mutation in *nfxB* (20) was used as efflux pump-overexpressing control strain. The expression of *mexCD-oprJ* increased by 54-fold in the presence of dequalinium chloride, 25-fold in the presence of procaine and 16-fold in the presence of atropine (Figure 5 A). These results confirm that the expression of *mexCD-oprJ* is induced by the compounds selected from the Biolog screening. Although the time for selection is low and bacteria are not growing in the presence of antibiotics, it might be possible that the increased expression of *mexCD-oprJ* could be due to the selection of mutants in the elements that regulate the expression of this efflux pump, namely its promoter region and its local repressor NfxB (15). To ascertain this possibility, *nfxB* and the *nfxB-mexC* intergenic region were amplified by PCR and sequenced in cultures of *P. aeruginosa* after 20 hours of induction. None mutations were detected, neither in the gene coding the regulator nor in the intergenic region between it and the efflux pump encoding operon. This and the results shown below indicate that the increased expression observed is associated to the presence of the inducers.

An interesting issue to determine was whether or not induction of *mexCD-oprJ* remains after removing the inducer. To address this possibility, expression of *mexCD-oprJ* was measured by real time-PCR at different times after removal of the inducers. The expression of *mexCD-oprJ* is reduced to levels close to the untreated cells levels 30 minutes after removal procaine or atropine. However, a 10-fold induction is still observed, as compared with the strain grown in the absence of inducer, after 30 minutes of removal of dequalinium chloride and a 2-fold

induction in *mexCD* experession levels still remains after 120 minutes of removing the inducer (Figure 5 B, C, D). This indicates that the memory of induction is dependent on the inducer. It has been proposed that *mexCD-oprJ* expression is induced as a response to membrane damage (35, 36). The likely reason behind dequalinium chloride durability of induction over time may then be related with the membrane damaging effect of this compound, which may remain for a time after removing the compound and can actually be the cause of its inducer capability. The fact that induction is transient further supports that the cause of the increased *mexCD-oprJ* expression is not the selection of mutants overexpressing this efflux pump.

The *P. aeruginosa* PAO1 MIC of ciprofloxacin, one of the MexCD-OprJ substrates, was analyzed in the presence and in the absence of the aforementioned inducers in order to determine the effect of such induction on the susceptibility of *P. aeruginosa* to this antibiotic. MIC of ciprofloxacin was higher in the presence of the inducers (Table 5). It might be possible that this increased MIC would be the consequence of antagonism between the inducer and antibiotic, in which case the activity of the efflux pump should be of not-relevance for the final outcome. To analyze this possibility, the strain that overexpresses *mexCD-oprJ* (*nfxB**) and a *mexD*-defective mutant (*nfxB**Δ*mexD*) (Table 1) were used as controls. Neither when *mexCD-oprJ* is constitutively overexpressed (*nfxB**) nor when the efflux pump is absent (*nfxB**Δ*mexD*), an antagonism between the inducers and the antibiotic was observed;, the presence of each inducer did not affect the MIC of ciprofloxacin neither for *nfxB** nor for *nfxB**Δ*mexD*. These results indicate that the observed phenotype was caused specifically by *mexCD-oprJ* induction and were not the consequence of non-specific antagonism between the inducers and the antibiotic. Growth curves of PAO1 in presence of ciprofloxacin, with or without these inducers, were also analyzed (Figure 6). The results confirm that atropine, procaine and dequalinium chloride leads to a transient reduction of *P. aeruginosa* ciprofloxacin susceptibility in the wild-type strain, while no change is observed in the *mexD*-defective mutant, further confirming that the phenotype depends on the activity of MexCD-OprJ.

384 Further, checkerboard analyses were performed as described in Materials and Methods for *P.*
385 *aeruginosa* PAO1 wild type strain and the strain lacking the MexCD efflux pump. Inducer
386 compounds showed an antagonistic effect with ciprofloxacin in the case of PAO1 (FIC Index >
387 2.7 in all cases), while neither antagonistic or synergistic effect (FIC Index ≤ 2 and ≥ 0.5 in all
388 cases) was observed for the strain lacking the efflux pump (Figure 7). This observation, in
389 addition to the above-mentioned results (Table 5 and Figure 6), reinforces that dequalinium
390 chloride, procaine and atropine are molecules able to reduce the effect of ciprofloxacin in *P.*
391 *aeruginosa* via a *mexCD-oprJ* induction, while this antagonistic effect is not observed in the
392 strain lacking the gene encoding the mentioned efflux pump (Figure 6 and 7).

393 In order to further confirm the specific induction of *mexCD-oprJ* by these compounds, the MICs
394 of antibiotics which are not substrates of MexCD-OprJ (tobramycin, fosfomycin and
395 ceftazidime (8)), were also measured in presence and absence of the identified inducers. The
396 MICs of those antibiotics did not change in presence of the inducers.

397 Altogether, these results indicate that the three analyzed compounds are able to transiently
398 increase AR through the induction of the expression of the genes that encode MexCD-OprJ.
399 Because of that, the temporal coincidence of any of these compounds with MexCD-OprJ
400 antibiotic substrates for treating patients may be a concern.

401 **MexCD-OprJ efflux pump extrudes procaine**

402 It has been found that, in some occasions, inducers of the expression of efflux pumps are also
403 substrates of these AR determinants (22, 31, 61, 62). One example of this situation is the
404 induction of MexXY-OprM in *P. aeruginosa* by aminoglycosides, which are substrates of this
405 efflux pump (63). However, in other cases, as it happens in the case of *S. maltophilia* SmeVWX
406 and SmeYZ efflux pumps (40), some of their inducers are not substrates of these efflux pumps.

407 To address the capacity of MexCD-OprJ to extrude its described inducers, the growth of the
408 wild-type *P. aeruginosa* PAO1 and the *mexCD* deficient *nfxB**Δ*mexD* strain were compared in
409 the presence of a toxic concentration of each inducer (4 mg/ml of atropine, 4 mg/ml of procaine

or 20 µg/ml of dequalinium chloride). No relevant differences in growth were observed in presence of dequalinium chloride or atropine in the MexD-deficient strain compared to the wild type. However, growth of the MexD-deficient strain was reduced in the presence of procaine (Figure 8) indicating that this compound, besides being an inducer, might also be a substrate of the MexCD-OprJ efflux pump.

In summary, the combination of Biolog Phenotype Microarrays and luminescent biosensor strains has allowed us to describe novel inducers of *P. aeruginosa* efflux pumps, some of which must be carefully taken into consideration in the clinic field; in particular, when these compounds and antibiotics are simultaneously applied.

Acknowledgments

Work has been supported by grants from the Instituto de Salud Carlos III (Spanish Network for Research on Infectious Diseases [RD16/0016/0011]), from the Spanish Ministry of Economy, Industry and Competitivity (BIO2017-83128-R), from JPI Water StARE JPIW2013-089-C02-01) and from the Autonomous Community of Madrid (B2017/BMD-3691). The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication. PL is the recipient of a FPU fellowship, PB is the recipient of a FPI fellowship.

REFERENCES

1. Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D, Rice LB, Scheld M, Spellberg B, Bartlett J. 2009. Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. Clin Infect Dis 48:1-12.
2. Rello J, Kalwaje Eshwara V, Lagunes L, Alves J, Wunderink RG, Conway-Morris A, Rojas JN, Alp E, Zhang Z. 2019. A global priority list of the TOp TEn resistant Microorganisms (TOTEM) study at intensive care: a prioritization exercise based on multi-criteria decision analysis. Eur J Clin Microbiol Infect Dis 38:319-323.
3. Pendleton JN, Gorman SP, Gilmore BF. 2013. Clinical relevance of the ESKAPE pathogens. Expert Rev Anti Infect Ther 11:297-308.
4. Tummler B, Wiehlmann L, Klockgether J, Cramer N. 2014. Advances in understanding Pseudomonas. F1000Prime Rep 6:9.
5. Martinez-Solano L, Macia MD, Fajardo A, Oliver A, Martinez JL. 2008. Chronic *Pseudomonas aeruginosa* infection in chronic obstructive pulmonary disease. Clinical Infectious Diseases 47:1526-33.

6. Nikaido H. 2011. Structure and mechanism of RND-type multidrug efflux pumps. Adv Enzymol Relat Areas Mol Biol 77:1-60.
7. Breidenstein EB, de la Fuente-Nunez C, Hancock RE. 2011. Pseudomonas aeruginosa: all roads lead to resistance. Trends Microbiol 19:419-26.
8. Hernando-Amado S, Blanco P, Alcalde-Rico M, Corona F, Reales-Calderon JA, Sanchez MB, Martinez JL. 2016. Multidrug efflux pumps as main players in intrinsic and acquired resistance to antimicrobials. Drug Resist Updat 28:13-27.
9. Ziha-Zarifi I, Llanes C, Kohler T, Pechere JC, Plesiat P. 1999. In vivo emergence of multidrug-resistant mutants of Pseudomonas aeruginosa overexpressing the active efflux system MexA-MexB-OprM. Antimicrob Agents Chemother 43:287-91.
10. Garcia-Leon G, Salgado F, Oliveros JC, Sanchez MB, Martinez JL. 2014. Interplay between intrinsic and acquired resistance to quinolones in Stenotrophomonas maltophilia. Environ Microbiol 16:1282-96.
11. Poole K. 2001. Multidrug resistance in Gram-negative bacteria. Curr Opin Microbiol 4:500-8.
12. Rodriguez-Martinez JM, Poirel L, Nordmann P. 2009. Molecular epidemiology and mechanisms of carbapenem resistance in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 53:4783-8.
13. De Kievit TR, Parkins MD, Gillis RJ, Srikumar R, Ceri H, Poole K, Iglewski BH, Storey DG. 2001. Multidrug efflux pumps: expression patterns and contribution to antibiotic resistance in Pseudomonas aeruginosa biofilms. Antimicrob Agents Chemother 45:1761-70.
14. Masuda N, Sakagawa E, Ohya S, Gotoh N, Tsujimoto H, Nishino T. 2000. Substrate specificities of MexAB-OprM, MexCD-OprJ, and MexXY-oprM efflux pumps in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 44:3322-7.
15. Poole K, Gotoh N, Tsujimoto H, Zhao Q, Wada A, Yamasaki T, Neshat S, Yamagishi J, Li XZ, Nishino T. 1996. Overexpression of the *mexC-mexD-oprJ* efflux operon in *nfxB*-type multidrug-resistant strains of *Pseudomonas aeruginosa*. Mol Microbiol 21:713-24.
16. Piddock LJ. 2006. Multidrug-resistance efflux pumps - not just for resistance. Nat Rev Microbiol 4:629-36.
17. Alvarez-Ortega C, Olivares J, Martinez JL. 2013. RND multidrug efflux pumps: what are they good for? Front Microbiol 4:7.
18. Evans K, Passador L, Srikumar R, Tsang E, Nezezon J, Poole K. 1998. Influence of the MexAB-OprM multidrug efflux system on quorum sensing in *Pseudomonas aeruginosa*. J Bacteriol 180:5443-7.
19. Olivares J, Alvarez-Ortega C, Linares JF, Rojo F, Kohler T, Martinez JL. 2012. Overproduction of the multidrug efflux pump MexEF-OprN does not impair *Pseudomonas aeruginosa* fitness in competition tests, but produces specific changes in bacterial regulatory networks. Environ Microbiol 14:1968-81.
20. Alcalde-Rico M, Olivares-Pacheco J, Alvarez-Ortega C, Camara M, Martinez JL. 2018. Role of the Multidrug Resistance Efflux Pump MexCD-OprJ in the Pseudomonas aeruginosa Quorum Sensing Response. Front Microbiol 9:2752.
21. Poole K. 2005. Efflux-mediated antimicrobial resistance. J Antimicrob Chemother 56:20-51.
22. Lin J, Sahin O, Michel LO, Zhang Q. 2003. Critical role of multidrug efflux pump CmeABC in bile resistance and *in vivo* colonization of *Campylobacter jejuni*. Infect Immun 71:4250-9.
23. Alcalde-Rico M, Hernando-Amado S, Blanco P, Martinez JL. 2016. Multidrug Efflux Pumps at the Crossroad between Antibiotic Resistance and Bacterial Virulence. Front Microbiol 7:1483.
24. Thanassi DG, Cheng LW, Nikaido H. 1997. Active efflux of bile salts by *Escherichia coli*. J Bacteriol 179:2512-8.

493 25. Blanco P, Hernando-Amado S, Reales-Calderon JA, Corona F, Lira F, Alcalde-Rico M,
494 Bernardini A, Sanchez MB, Martinez JL. 2016. Bacterial Multidrug Efflux Pumps: Much More
495 Than Antibiotic Resistance Determinants. Microorganisms 4.
496 26. Martinez JL, Sanchez MB, Martinez-Solano L, Hernandez A, Garmendia L, Fajardo A,
497 Alvarez-Ortega C. 2009. Functional role of bacterial multidrug efflux pumps in microbial natural
498 ecosystems. FEMS Microbiol Rev 33:430-49.
499 27. Sun J, Deng Z, Yan A. 2014. Bacterial multidrug efflux pumps: mechanisms, physiology
500 and pharmacological exploitations. Biochem Biophys Res Commun 453:254-67.
501 28. Levin BR, Rozen DE. 2006. Non-inherited antibiotic resistance. Nat Rev Microbiol
502 4:556-62.
503 29. Corona F, Martinez JL. 2013. Phenotypic Resistance to Antibiotics. Antibiotics (Basel)
504 2:237-55.
505 30. Sanchez MB, Decorosi F, Viti C, Oggioni MR, Martinez JL, Hernandez A. 2015. Predictive
506 Studies Suggest that the Risk for the Selection of Antibiotic Resistance by Biocides Is Likely Low
507 in *Stenotrophomonas maltophilia*. PLoS ONE 10:e0132816.
508 31. Hernandez A, Ruiz FM, Romero A, Martinez JL. 2011. The binding of triclosan to SmeT,
509 the repressor of the multidrug efflux pump SmeDEF, induces antibiotic resistance in
510 *Stenotrophomonas maltophilia*. PLoS Pathog 7:e1002103.
511 32. Poole K. 2014. Stress responses as determinants of antimicrobial resistance in
512 Pseudomonas aeruginosa: multidrug efflux and more. Can J Microbiol 60:783-91.
513 33. Chen H, Hu J, Chen PR, Lan L, Li Z, Hicks LM, Dinner AR, He C. 2008. The Pseudomonas
514 aeruginosa multidrug efflux regulator MexR uses an oxidation-sensing mechanism. Proc Natl
515 Acad Sci U S A 105:13586-91.
516 34. Ghosh S, Cremers CM, Jakob U, Love NG. 2011. Chlorinated phenols control the
517 expression of the multidrug resistance efflux pump MexAB-OprM in *Pseudomonas aeruginosa*
518 by interacting with NalC. Mol Microbiol 79:1547-56.
519 35. Purssell A, Fruci M, Mikalauskas A, Gilmour C, Poole K. 2015. EsrC, an envelope stress-
520 regulated repressor of the mexCD-oprJ multidrug efflux operon in Pseudomonas aeruginosa.
521 Environ Microbiol 17:186-98.
522 36. Fraud S, Campigotto AJ, Chen Z, Poole K. 2008. MexCD-OprJ Multidrug Efflux System of
523 *Pseudomonas aeruginosa*: Involvement in Chlorhexidine Resistance and Induction by
524 Membrane-Damaging Agents Dependent upon the AlgU Stress Response Sigma Factor.
525 Antimicrob Agents Chemother 52:4478-4482.
526 37. Morita Y, Murata T, Mima T, Shiota S, Kuroda T, Mizushima T, Gotoh N, Nishino T,
527 Tsuchiya T. 2003. Induction of mexCD-oprJ operon for a multidrug efflux pump by disinfectants
528 in wild-type Pseudomonas aeruginosa PAO1. J Antimicrob Chemother 51:991-4.
529 38. Strempel N, Neidig A, Nusser M, Geffers R, Vieillard J, Lesouhaitier O, Brenner-Weiss G,
530 Overhage J. 2013. Human host defense peptide LL-37 stimulates virulence factor production
531 and adaptive resistance in Pseudomonas aeruginosa. PLoS One 8:e82240.
532 39. Bochner BR, Gadzinski P, Panomitros E. 2001. Phenotype microarrays for high-
533 throughput phenotypic testing and assay of gene function. Genome Res 11:1246-55.
534 40. Blanco P, Corona F, Martinez JL. 2018. Biolog Phenotype Microarray Is a Tool for the
535 Identification of Multidrug Resistance Efflux Pump Inducers. Antimicrob Agents Chemother 62.
536 41. Becher A, Schweizer HP. 2000. Integration-proficient Pseudomonas aeruginosa vectors
537 for isolation of single-copy chromosomal lacZ and lux gene fusions. Biotechniques 29:948-50,
538 952.
539 42. Hoang TT, Karkhoff-Schweizer RR, Kutchma AJ, Schweizer HP. 1998. A broad-host-
540 range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA
541 sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene
542 212:77-86.

43. Blanco P, Corona F, Sanchez MB, Martinez JL. 2017. Vitamin K3 Induces the Expression of the Stenotrophomonas maltophilia SmeVWX Multidrug Efflux Pump. Antimicrob Agents Chemother 61.
44. Winsor GL, Griffiths EJ, Lo R, Dhillon BK, Shay JA, Brinkman FS. 2016. Enhanced annotations and features for comparing thousands of Pseudomonas genomes in the Pseudomonas genome database. Nucleic Acids Res 44:D646-53.
45. Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-8.
46. Imlay JA. 2013. The molecular mechanisms and physiological consequences of oxidative stress: lessons from a model bacterium. Nat Rev Microbiol 11:443-54.
47. Anonymous. April 2015. WHO Model List of Essential Medicines (19th List). World Health Organization.
48. Tor-Agbidye J, Palmer VS, Spencer PS, Craig AM, Blythe LL, Sabri MI. 1999. Sodium cyanate alters glutathione homeostasis in rodent brain: relationship to neurodegenerative diseases in protein-deficient malnourished populations in Africa. Brain Res 820:12-9.
49. Vargas P, Farias GA, Nogales J, Prada H, Carvajal V, Baron M, Rivilla R, Martin M, Olmedilla A, Gallegos MT. 2013. Plant flavonoids target Pseudomonas syringae pv. tomato DC3000 flagella and type III secretion system. Environ Microbiol Rep 5:841-50.
50. Vargas P, Felipe A, Michan C, Gallegos MT. 2011. Induction of Pseudomonas syringae pv. tomato DC3000 MexAB-OprM multidrug efflux pump by flavonoids is mediated by the repressor PmeR. Mol Plant Microbe Interact 24:1207-19.
51. Silby MW, Winstanley C, Godfrey SA, Levy SB, Jackson RW. 2011. Pseudomonas genomes: diverse and adaptable. FEMS Microbiol Rev 35:652-80.
52. Babbs M, Collier HO, Austin WC, Potter MD, Taylor EP. 1956. Salts of decamethylene-bis-4-aminoquinaldinium (dequadin); a new antimicrobial agent. J Pharm Pharmacol 8:110-9.
53. Mendling W, Weissenbacher ER, Gerber S, Prasauskas V, Grob P. 2016. Use of locally delivered dequalinium chloride in the treatment of vaginal infections: a review. Arch Gynecol Obstet 293:469-84.
54. Galeano E, Nieto E, Garcia-Perez AI, Delgado MD, Pinilla M, Sancho P. 2005. Effects of the antitumoural dequalinium on NB4 and K562 human leukemia cell lines. Mitochondrial implication in cell death. Leuk Res 29:1201-11.
55. Helige C, Smolle J, Zellnig G, Fink-Puches R, Kerl H, Tritthart HA. 1992. Effect of dequalinium on K1735-M2 melanoma cell growth, directional migration and invasion in vitro. Eur J Cancer 29A:124-8.
56. Abdul M, Hoosein N. 2002. Expression and activity of potassium ion channels in human prostate cancer. Cancer Lett 186:99-105.
57. Chan CF, Lin-Shiau SY. 2001. Suramin prevents cerebellar granule cell-death induced by dequalinium. Neurochem Int 38:135-43.
58. Shah J, Votta-Velis EG, Borgeat A. 2018. New local anesthetics. Best Pract Res Clin Anaesthesiol 32:179-185.
59. Lachiewicz AM, Hauck CG, Weber DJ, Cairns BA, van Duin D. 2017. Bacterial Infections After Burn Injuries: Impact of Multidrug Resistance. Clin Infect Dis 65:2130-2136.
60. Dutta NK. 1949. Some pharmacological properties common to atropine, pethidine, procaine, and quinidine. Br J Pharmacol Chemother 4:197-201.
61. Duque E, Segura A, Mosqueda G, Ramos JL. 2001. Global and cognate regulators control the expression of the organic solvent efflux pumps TtgABC and TtgDEF of *Pseudomonas putida*. Mol Microbiol 39:1100-6.
62. Rosenberg EY, Bertenthal D, Nilles ML, Bertrand KP, Nikaido H. 2003. Bile salts and fatty acids induce the expression of *Escherichia coli* AcrAB multidrug efflux pump through their interaction with Rob regulatory protein. Mol Microbiol 48:1609-19.

593 63. Masuda N, Sakagawa E, Ohya S, Gotoh N, Tsujimoto H, Nishino T. 2000. Contribution of
594 the MexX-MexY-oprM efflux system to intrinsic resistance in *Pseudomonas aeruginosa*.
595 Antimicrob Agents Chemother 44:2242-6.
596 64. Simon R, O'Connell M, Labes M, Puhler A. 1986. Plasmid vectors for the genetic
597 analysis and manipulation of rhizobia and other gram-negative bacteria. Methods Enzymol
598 118:640-59.
599 65. Linares JF, Lopez JA, Camafeita E, Albar JP, Rojo F, Martinez JL. 2005. Overexpression of
600 the multidrug efflux pumps MexCD-OprJ and MexEF-OprN is associated with a reduction of
601 type III secretion in *Pseudomonas aeruginosa*. J Bacteriol 187:1384-91.

602

603

604 **Table 1 | Bacterial strains and plasmids used in this work**

Bacterial strain/ plasmid	Description	Reference/origin
Bacterial strains		
Escherichia coli		
One Shot OmniMax™	Host strain used for the maintenance of cloning plasmids	Invitrogen
S17-1λ *pir*	Conjugative donor strain used for transferring plasmids to *P. aeruginosa* strains by conjugation	(64)
Pseudomonas aeruginosa		
PAO1-V	PAO1 strain stored at the laboratory of Victor de Lorenzo	(20, 65)
nfxB*	PAO1 spontaneous mutant in *nfxB* gene that overexpresses *mexCD-oprJ*	(20, 65)
***nfxB** ΔmexD*	Loss of function of MexCD-OprJ system mutant, obtained from *nfxB** by *mexD* deletion by homologous recombination	(20)
PAO1 CTX-*lux*	PAO1 strain which contains the mini-CTX-*lux*	This study

	construction inserted in a neutral zone of the chromosome	
PAO1 CTX-*lux*::P*mexCD*	PAO1 strain which contains mini-CTX::P*mexCD-lux* construction inserted in a neutral zone of the chromosome	This study
PAO1 CTX-*lux*::P*mexAB*	PAO1 strain which contains mini-CTX::P*mexAB-lux* construction inserted in a neutral zone of the chromosome	This study
Plasmids		
pGEM-T Easy	Commercial plasmid used for cloning optimization of PCR products. Amp^R	Promega
mini-CTX-*lux*	*luxCDABE* operon inserted into the mini-CTX-1 plasmid. Tc^R	(41)
mini-CTX::P*mexCD-lux*	Plasmid derived from mini-CTX-*lux* in which the expression of the *luxCDABE* operon is under control of *mexCD-oprJ* promoter region of *P. aeruginosa*. Tc^R	This study
mini-CTX::P*mexAB-lux*	Plasmid derived from mini-CTX-*lux* in which the expression of the *luxCDABE* operon is under control of *mexAB-oprM* promoter region of *P. aeruginosa*. Tc^R	This study

605

606

607 **Table 2 | Primers used in this work**

Primer	Sequence (5'-3')	Description
EcoRI_PmexAB_Fw	GAATTCTGGTTTGGCCGAGTAAACCT	To amplify promoter region of *mexAB-oprM*
BamHI_PmexAB_Rv	GGATCCAGCGTTGTCCTCATGAGCGA	
EcoRI_PmexCD_Fw	GAATTCCGATGGGTCCCGGTTGGTTT	To amplify promoter region of *mexCD-oprJ*
BamHI_PmexCD_Rv	GGATCCGACACACCCGACCGTTGATT	
rpsL_Fw	CGCAGTGATTGTTACCGGTG	To check DNA contamination from RNA samples
rpsL_Rv	AGGCCTGAATGCCGGTGATC	
mexC_RTPCR_Fw	GTGGCGGTATCGAAGTCCT	To amplify *mexC* by real time-PCR
mexC_RTPCR_Rv	GACCTGCTGTTCCAGATCG	
rplU_RTPCR_Fw	GCAAGCGCATGGTCGACAAGA	To amplify the housekeeping gene *rplU* by real time-PCR
rplU_RTPCR_Rv	CGCTGTGCTCTTGCAGGTTGTG	
nfxB_Fw	CGGCCTCCTGTCGCTCTTCC	To amplify *nfxB* and the intergenic region between this gene and *mexC*
nfxB_Rv	AGCAACAGCAGGCCAGCCTT	

608

609

610 **Table 3. Potential inducer compounds of *mexCD-oprJ* and *mexAB-oprM* expression detected**
611 **by the Biolog screening.**

Efflux pump induced	Compound	Normalized luminescence value*			
		A	B	C	D
mexCD-oprJ	Benthezonium chloride**	1.71	**1.83**	**1.95**	**2.29**
	Dequalinium chloride**	**2.09**	**2.21**	**2.28**	**2.2**
	2, 2' - dipyridyl	1.54	1.62	**1.79**	IG
	Acriflavine	1.57	1.65	1.66	**1.91**
	Sanguinarine	1.72	**1.77**	**1.86**	**1.85**
	9 - aminoacridine	1.60	1.69	**1.92**	**2.2**
	Sodium cyanate	1.64	1.71	**1.83**	IG
	Procaine	1.57	1.64	**1.93**	IG
	Domiphen bromide**	1.65	**1.8**	**1.84**	**2.42**
	Alexidine**	1.32	1.53	1.67	**1.93**
	Cetylpyridinium chloride**	1.67	**1.81**	**1.81**	**1.76**
	Metyltrioctylammonium chloride	1.72	**1.77**	**1.83**	**1.96**
	Harmane	1.59	1.71	**1.93**	**1.87**
	Iodonitro tetrazolium violet	1.46	1.57	**1.79**	**2.19**
	Amitriptyline	1.50	1.60	**1.8**	**1.85**
	Orphenadrine	1.61	**1.82**	**2.11**	**1.9**
	Propanolol	1.70	**1.87**	**2.02**	**2.12**

	Atropine	**1.78**	**1.83**	**2.09**	IG
	Proflavine	1.49	1.53	**1.76**	**2.02**
	Dodine	1.67	1.72	**1.85**	**1.82**
mexAB-oprM	Amikacin	1.42	1.50	**1.72**	IG
	Azlocillin	1.10	1.10	1.13	**1.55**
	Acriflavine****	1.31	1.42	1.45	**1.65**
	Sanguinarine	1.34	1.40	1.52	**1.58**
	9 – aminoacridine****	1.31	1.47	1.47	**1.64**
	Sodium cyanate	1.30	1.48	**1.61**	IG
	Ethionamide	1.12	1.23	**1.75**	**1.55**
	Iodoacetate****	1.26	1.34	**1.75**	IG
	Penthachlorophenol***	1.14	1.16	1.39	**1.9**
	Proflavine****	1.18	1.27	1.50	**1.88**

* A, B, C and D represent the normalized luminescence values obtained in the four different concentrations of each compound present in the Biolog Phenotype Microarrays plates, being A the resulting normalized value for the lowest compound concentration and D the resulting normalized value for the highest compound concentration. All the normalized luminescence values for each compound that did not impair bacterial growth appear in the table and those above the threshold are highlighted in bold.

** These compounds are disinfectants, a category of compounds known to be inducers of *mexCD-oprJ*,.(37)

*** Known inducer of *mexAB-oprM* (34).

622 **** These compounds generate oxidative stress, which is a known inducer condition of *mexAB-*

623 *oprM* (33).

624

625

626 **Table 4. Potential inhibitors of *mexAB-oprM* and *mexCD-oprJ* expression detected with the**
627 **Biolog screening.**

Efflux pump repressed	Compound	Normalized luminescence value*			
		A	B	C	D
mexCD-oprJ	Chloramphenicol	1.34	1.23	1.26	**1.09**
	Spectinomycin	1.29	1.29	1.11	**1.05**
	Spiramycin	1.14	**0.98**	**1.08**	**1.09**
	Nordihydroguaiaretic acid	1.38	1.38	1.21	**1.06**
	Gallic acid	1.49	1.42	1.38	**0.92**
mexAB-oprM	Protamine sulfate	1.01	0.98	0.89	**0.65**
	Chromium chloride	0.79	0.77	0.73	0.71
	Glycine hydroxamate	**0.73**	**0.70**	**0.69**	**0.67**
	Hygromycin	1.42	1.13	0.80	0.72
	Josamycin	1.01	0.98	0.86	0.71

628 * A, B, C and D represent the normalized luminescence values obtained in the four different
629 concentrations of each compound present in the Biolog Phenotype Microarrays plates, being A the
630 resulting normalized value for the lowest compound concentration and D the resulting normalized
631 value for the highest compound concentration. The normalized luminescence values for each
632 compound that were below the threshold and did not impair bacterial growth appear in the table..
633

634 **Table 5 | MIC (µg/ml) values of *P. aeruginosa* to ciprofloxacin, in presence or absence of**

635 **inducer compounds**

	No inducers	Dequalinium chloride (10 µg/ml)	Procaine (2 mg/ml)	Atropine (2 mg/ml)
PAO1	0.047	0.38	0.125	0.094
nfxB*	0.5	0.5	0.5	0.5
nfxB*ΔmexD	0.047	0.047	0.047	0.047

636

637

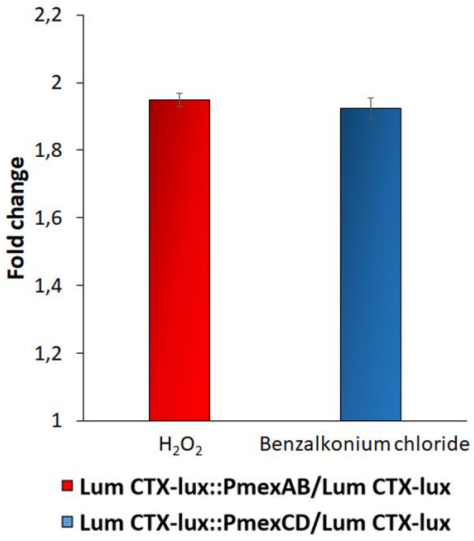


Figure 1. Effect of known inducers of the expression of *mexAB* and *mexCD* in the luminescence emitted by the *mexAB* and *mexCD* bioreporter strains . Luminescence and $OD600_{nm}$ of PAO1 CTX-lux::P*mexAB* and PAO1 CTX-lux::P*mexCD* was measured in presence of 0.136 µg/ml of H_2O_2 and 10 µg/ml benzalkonium chloride, respectively. Fold changes of the luminescence emitted by strains grown in the presence of the inducers *vs* the strains grown without any inducer are shown. Error bars represent standard deviation of three independent replicates. As shown, the known inducer compounds increase the production of luminescence of the bioreporter strains.

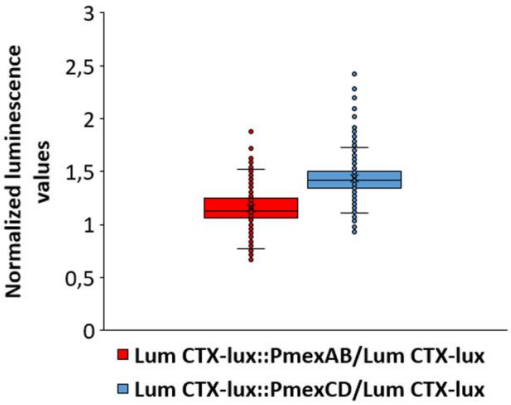


Figure 2. Effect of different compounds in the expression of either *mexAB-oprM* or *mexCD-oprJ*. The figure shows the normalized luminescence values produced by PAO1 CTX-*lux*::P*mexAB* and PAO1 CTX-*lux*::P*mexCD* in presence of 4 different concentrations of 240 compounds from Biolog plates. The outliers of the boxplot represent the conditions in which there was a potential overexpression or repression of the genes encoding the studied efflux pumps: those values above 1.53 for PAO1 CTX-*lux*::P*mexAB* and 1.73 for PAO1 CTX-*lux*::P*mexCD* indicate overexpression, while the values below 0.7 for PAO1 CTX-*lux*::P*mexAB* and 1.1 for PAO1 CTX-*lux*::P*mexCD* indicate repression

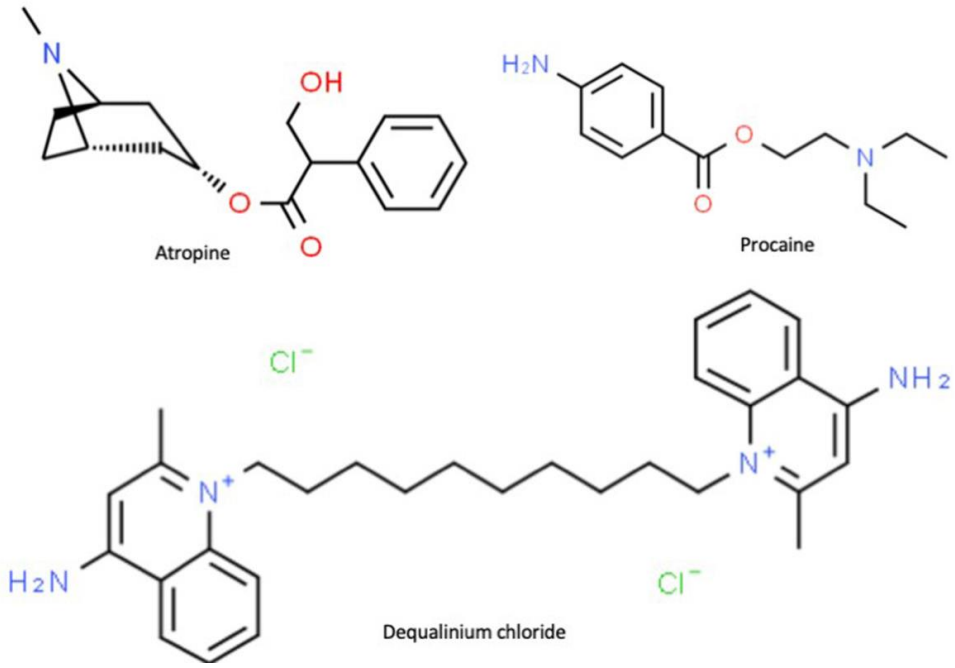


Figure 3. Structures of inducers of the expression of *mexCD-oprJ*. The structures of atropine, procaine and dequalinium chloride were obtained from the Chemspider database (http://www.chemspider.com).

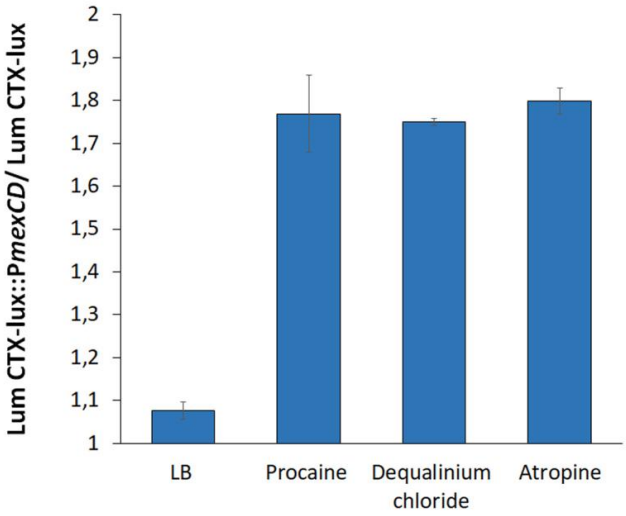


Figure 4. Effect of dequalinium chloride, procaine and atropine in the expression of *mexCD-oprJ*. The Figure shows the normalized luminescence values produced by the reporter strain PAO1 CTX-*lux*::P*mexCD* in presence of 10 µg/ml of dequalinium chloride, 2 mg/ml of procaine and 2 mg/ml of atropine. Luminescence was normalized to that produced by PAO1 CTX-*lux* in presence of inducer. As shown, expression of *mexCD-oprJ* is induced by the three tested compounds. Error bars represent standard deviation of three independent replicates.

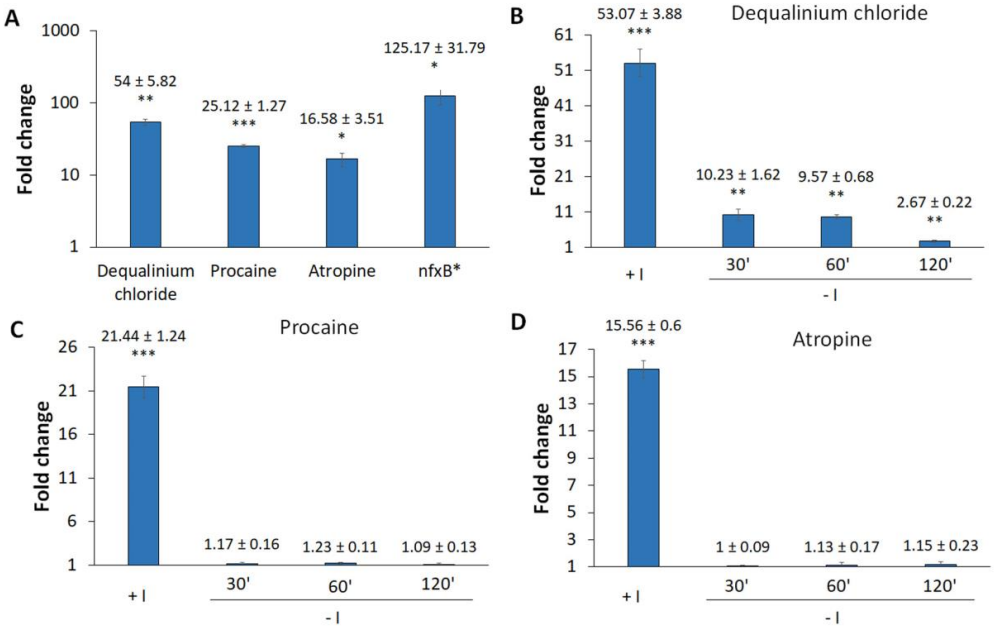


Figure 5. Analysis of *mexCD-oprJ* expression by real time-PCR in the presence and after removal of dequalinium chloride, procaine or atropine. (A) *mexC* expression after 90 minutes of incubation with 10 µg/ml of dequalinium chloride, 2 mg/ml of procaine, 2 mg/ml of atropine or without inducer. The *nfxB** strain grown in the absence of any inducer was used as a control of overexpression. As shown, expression of *mexCD-oprJ* is induced by the three tested compounds. (B, C, D) *mexC* expression after 30´, 90´and 120´of removal of the inducers (-I) and compared with the expression level after 90 minutes of induction (+I) with 10 µg/ml of dequalinium chloride (B), 2 mg/ml of procaine (C) and 2 mg/ml of atropine (D). As shown, overexpression of *mexCD-oprJ* is reduced after 30 minutes of removal of procaine and atropine to similar levels to the untreated bacteria levels; 30 and 60 minutes after removal of dequalinium chloride the expression level is reduced to 10 times the untreated bacteria levels and to 2 times after 120 minutes. Fold changes were calculated regarding the expression in *P. aeruginosa* PAO1 untreated. Each represented value is the average of three biological replicates. Statistically significant differences regarding PAO1 untreated were calculated with t-test for paired samples assuming equal variances: *p < 0.05; **p < 0.005; ***p < 0.0005.

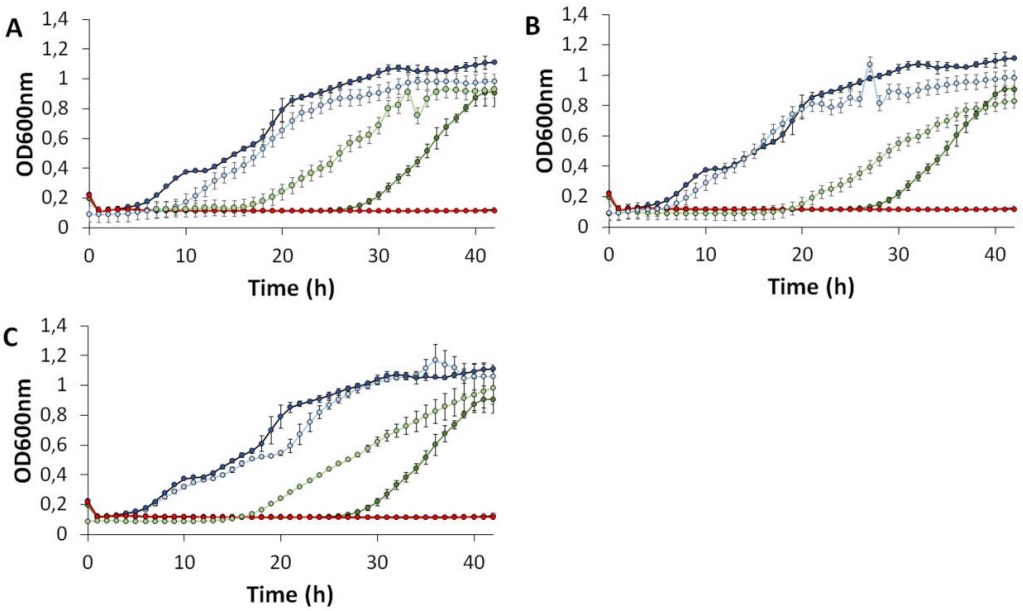


Figure 6. Effect of inducers in the growth of *P. aeruginosa* in presence of ciprofloxacin. Growth curves of *P. aeruginosa* PAO1 (green), *nfxB** (blue) and of *nfxB**Δ*mexD* (red) in LB medium containing 0.25 µg/ml ciprofloxacin, with (light) or without (dark) 10 µg/ml of dequalinium chloride (A), 2 mg/ml of procaine (B) or 2 mg/ml of atropine (C). As shown, the wild-type *P. aeruginosa* strain grows better in presence of ciprofloxacin when the inducers are added. Each OD_{600nm} represented value is the average of three biological replicates.

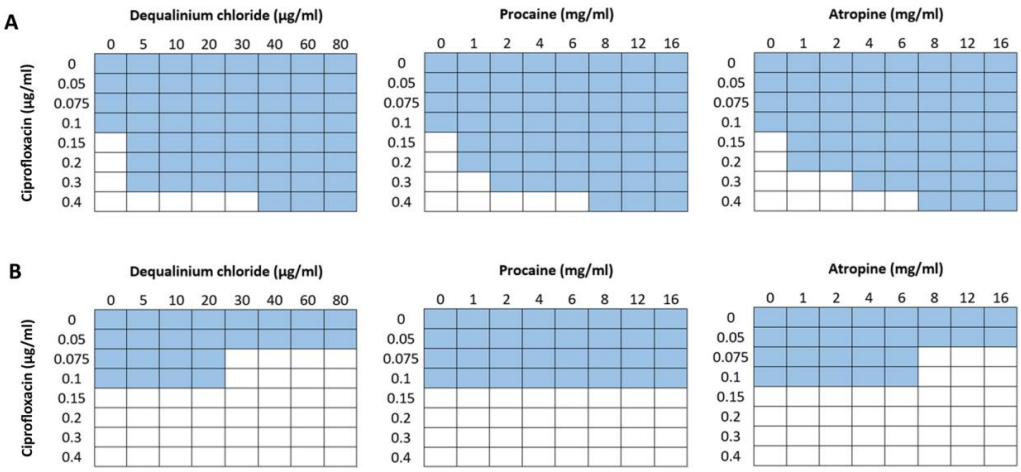
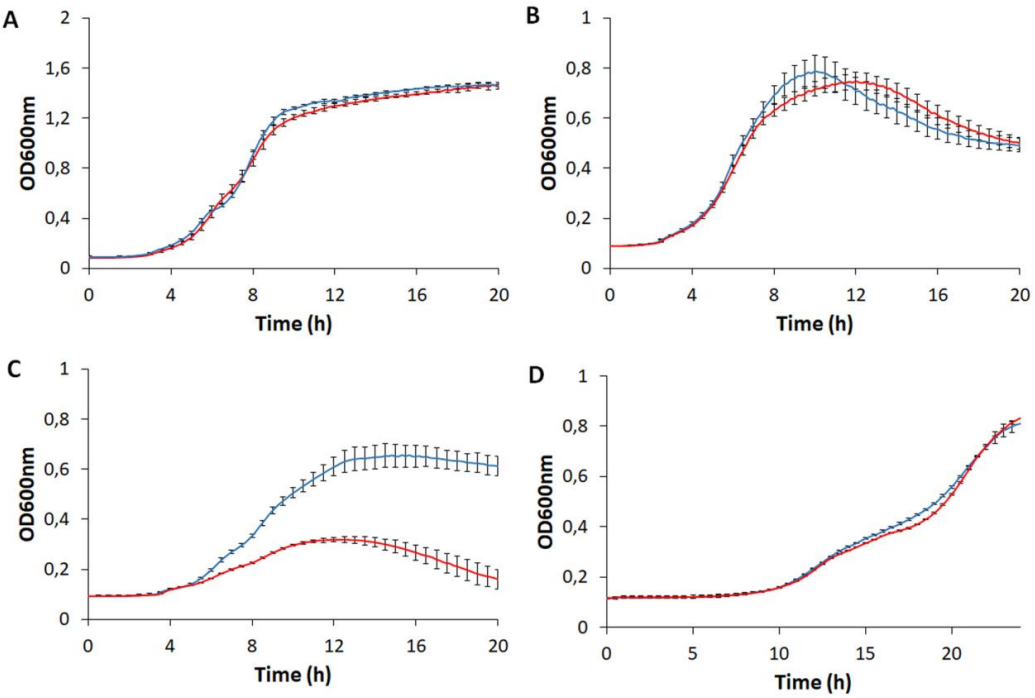


Figure 7. Checkerboard analysis for ciprofloxacin and *mexCD-oprJ* inducer compounds. Checkerboard analysis were performed with *P. aeruginosa* PAO1 wild type strain (A) and *nfxB**Δ*D* strain (B). Wells with bacterial growth are represented in blue and wells in which there was not growth are represented in white. Inducer compounds showed antagonistic effect with ciprofloxacin in the case of PAO1, while no antagonistic effect was observed for the *mexD* deficient strain.

Antimicrobial Agents and Chemotherapy



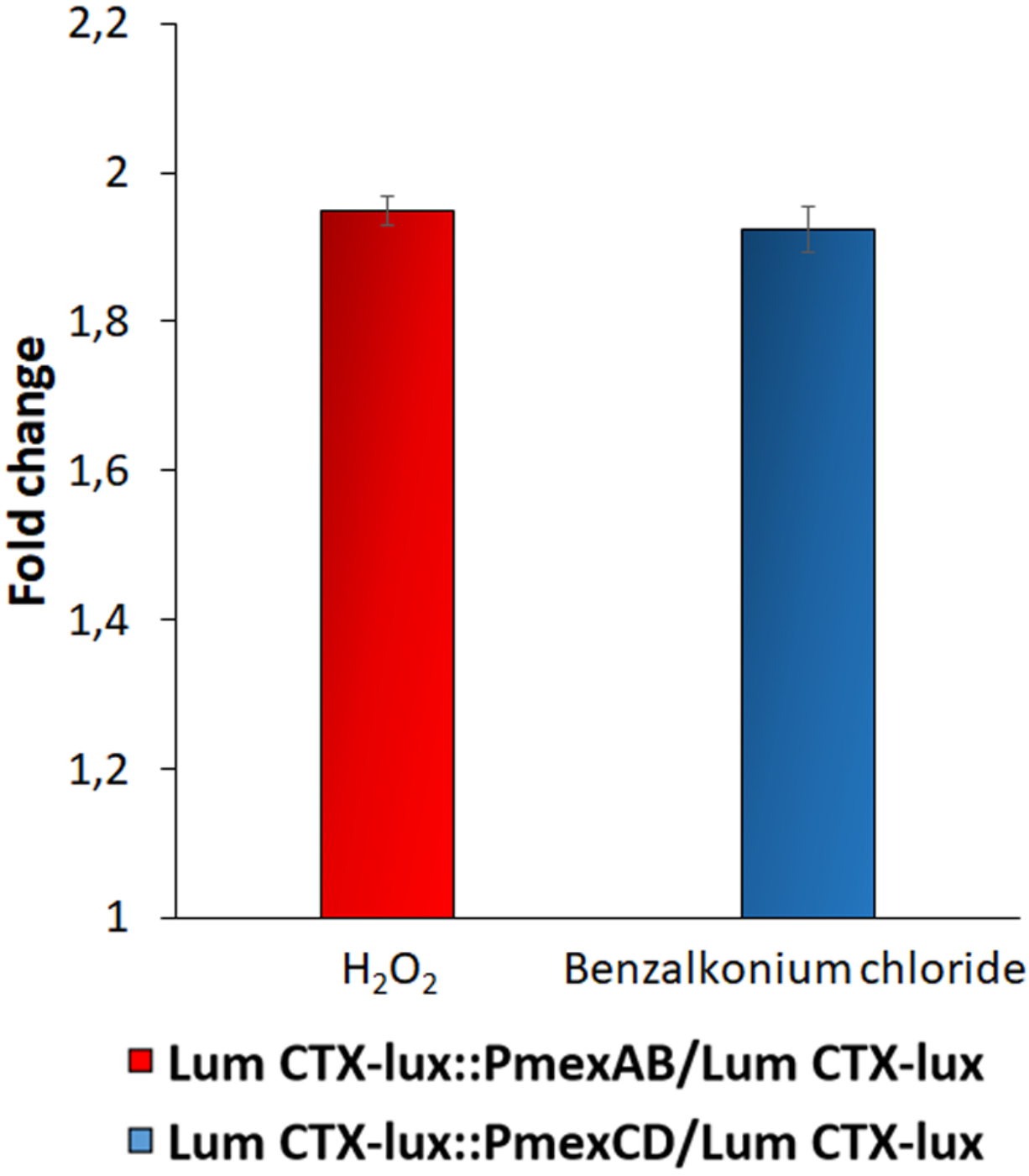
736

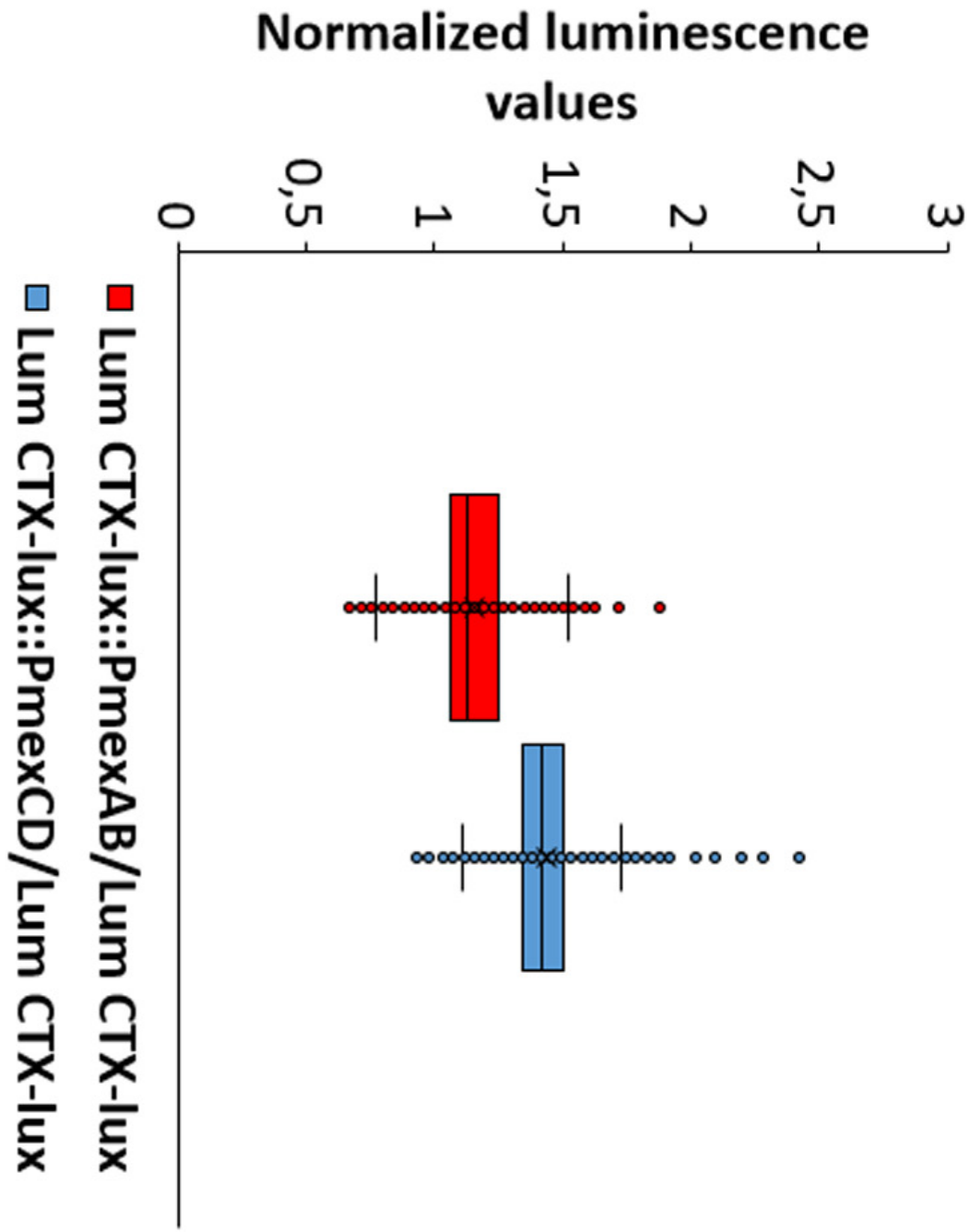
737

738 **Figure 8. Effect of MexCD-OprJ in the susceptibility of *P. aeruginosa* to *mexCD-oprJ***
739 **inducers.** PAO1 wild-type strain (blue) and *nfxB*ΔmexD* strain (red) were grown in LB as a
740 control (A) and in presence of 4 mg/ml of atropine (B), 4 mg/ml of procaine (C) or 20 µg/ml of
741 dequalinium chloride (D). As shown, the absence of MexCD-OprJ increases *P. aeruginosa*
742 susceptibility to procaine. Each OD_{600nm} represented value is the average of three biological
743 replicates.

744

745





Antimicrobial Agents and Chemotherapy