International Journal of Medical Microbiology 303 (2013) 574–582

Contents lists available at ScienceDirect

International Journal of Medical Microbiology

journal homepage: www.elsevier.com/locate/ijmm

Isolation and characterization of gallium resistant *Pseudomonas aeruginosa* mutants

Rodolfo García-Contreras[a,*], Elizabeth Lira-Silva[a], Ricardo Jasso-Chávez[a], Ismael L. Hernández-González[b], Toshinari Maeda[c], Takahiro Hashimoto[c], Fred C. Boogerd[d], Lili Sheng[e,f], Thomas K. Wood[e], Rafael Moreno-Sánchez[a]

[a] *Department of Biochemistry, National Institute of Cardiology, Mexico, D.F., Mexico*
[b] *Department of Genetic Engineering, CINVESTAV, Irapuato, Guanajuato, Mexico*
[c] *Department of Biological Functions and Engineering, Kyushu Institute of Technology, Kitakyushu, Japan*
[d] *Department of Molecular Cell Physiology, VU University, Amsterdam, The Netherlands*
[e] *Department of Chemical Engineering, Pennsylvania State University, University Park, PA, USA*
[f] *State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai, China*

ARTICLE INFO

Article history:
Received 11 February 2013
Received in revised form 9 May 2013
Accepted 29 July 2013

Keywords:
Pseudomonas aeruginosa
Gallium
Resistance
HitAB permease
Pyocyanin

ABSTRACT

Pseudomonas aeruginosa PA14 cells resistant to the novel antimicrobial gallium nitrate (Ga) were developed using transposon mutagenesis and by selecting spontaneous mutants. The mutants showing the highest growth in the presence of Ga were selected for further characterization. These mutants showed 4- to 12-fold higher Ga minimal inhibitory growth concentrations and a greater than 8-fold increase in the minimum biofilm eliminating Ga concentration. Both types of mutants produced Ga resistant biofilms whereas the formation of wild-type biofilms was strongly inhibited by Ga. The gene interrupted in the transposon mutant was *hitA*, which encodes a periplasmic iron binding protein that delivers Fe^{3+} to the HitB iron permease; complementation of the mutant with the *hitA* gene restored the Ga sensitivity. This *hitA* mutant showed a 14-fold decrease in Ga internalization *versus* the wild-type strain, indicating that the HitAB system is also involved in the Ga uptake. Ga uptake in the spontaneous mutant was also lower, although no mutations were found in the *hitAB* genes. Instead, this mutant harbored 64 non-silent mutations in several genes including those of the phenazine pyocyanin biosynthesis. The spontaneous mutant produced 2-fold higher pyocyanin basal levels than the wild-type; the addition of this phenazine to wild-type cultures protected them from the Ga bacteriostatic effect. The present data indicate that mutations affecting Ga transport and probably pyocyanin biosynthesis enable cells to develop resistance to Ga.

Introduction

Pseudomonas aeruginosa, an opportunistic pathogen that causes infections with high mortality rate in individuals with severe burns, cancer, AIDS, immunosuppression or cystic fibrosis (Mutlu and Wunderink, 2006; Kerr and Snelling, 2009; Mahar et al., 2010; Lambert et al., 2011), is responsible for a plethora of infections, like those associated with ventilator-associated pneumonia, urinary and peritoneal dialysis, catheter infections, bacterial keratitis, otitis, burns, and wound and lung infections (Macé et al., 2008). It is therefore one of the main agents responsible for nosocomial infections (Hidron et al., 2008; Jones et al., 2009; Zhanel et al., 2010). In addition, it exhibits intrinsically high resistance to antimicrobials (Lambert, 2002; Poole, 2002) and develops fast antibiotic resistance (Poole, 2011). Recently, we demonstrated that *P. aeruginosa* can also acquire resistance to virulence inhibitors (Maeda et al., 2011), which were supposed to be impervious to resistance (Bjarnsholt et al., 2010; Rasko and Sperandio, 2010). In addition, there has been a notorious increase in multidrug resistant clinical strains (Paterson, 2006; Kerr and Snelling, 2009), and there are several pan-resistant strains treatable only with colistin. However, colistin is highly nephrotoxic and resistance against it has also been found in clinical and laboratory *P. aeruginosa* strains (Poole, 2011). Hence, it is expected that clinical, pan-resistant strains sensitive to colistin will eventually become resistant to colistin (Poole, 2011).

Therefore, it is relevant to develop novel anti-pseudomonal therapies. As an alternative to the utilization of current antibiotics, new approaches have been proposed such as (i) phage therapy (Wright et al., 2009); (ii) virulence inhibitors, including those

* Corresponding author at: Department of Biochemistry, National Institute of Cardiology, Juan Badiano #1 Col. Sección XVI, Del. Tlalpan, C.P 14080 México, D.F., Mexico. Tel.: +52 5556848625.
E-mail addresses: garrod13420@cardiologia.org.mx, rgarc@bq.unam.mx (R. García-Contreras).

1438-4221/$ – see front matter © 2013 Elsevier GmbH. All rights reserved.
http://dx.doi.org/10.1016/j.ijmm.2013.07.009

that inhibit either quorum sensing (Lesic et al., 2007; Rasko and Sperandio, 2010; Bjarnsholt et al., 2011), or the toxin secretion systems (Page and Heim, 2009); (iii) inhibitors of the antibiotic resistance pathways such as blockers of the drug efflux systems (Veesenmeyer et al., 2009); and (iv) gallium (Ga^{3+}), a "Trojan horse" which is a non reducible Fe^{3+} analog, that exerts both bacteriostatic and bactericidal effects against laboratory strains and clinical isolates (Kaneko et al., 2007).

Ga^{3+} promotes healing of pulmonary infections in mice (Kaneko et al., 2007), and bacterial keratitis in rabbits, when coupled to desferrioxamine and gentamicin (Banin et al., 2008). It inhibits biofilm formation at concentrations innocuous for bacterial growth, and gallium nitrate is already used in the clinical practice at high doses to treat malignant hypercalcemia (Kaneko et al., 2007). Resistance against Ga^{3+} was not found in 115 clinical isolates from cystic fibrosis patients (except for 1 outlier that exhibited a MIC90 which is approximately 24-fold higher than the PAO1 wild-type strain), and 23 of them were multi-resistant to antibiotics (Kaneko et al., 2007), suggesting that the mechanisms that confer antibiotic multiresistance do not confer cross resistance against Ga^{3+}.

Ga^{3+} enters the cell by unknown transporters and disrupts the iron uptake systems leading to cellular arrest or death (Kaneko et al., 2007). However, as it has detrimental effects on the survival of bacteria, it is anticipated that exposure to Ga^{3+} will cause the cells to develop resistance. In order to determine whether *P. aeruginosa* can develop resistance to Ga^{3+}, transposon-induced and spontaneous mutants able to tolerate high Ga^{3+} concentrations were generated, and the mutants were characterized.

Material and methods

Strains and growth conditions

The strains used are listed in Table S1. Wild-type *P. aeruginosa* PA14 and isogenic mutants were kindly provided by Dr. Frederick Ausubel (Liberati et al., 2006). Cells were cultured for all experiments in minimal succinate medium (Ren et al., 2005) at 37 °C with orbital shaking at 200 rpm. All cultures were inoculated with overnight (15 h) pre-cultures in the same medium. Pre-cultures were started from single colonies grown on LB plates; the initial turbidity of each culture was adjusted to ~0.05. Cell turbidity was measured at 600 nm with a spectrophotometer (UV-1800, Shimadzu). Gentamicin (15 μg/mL) was added for culturing the isogenic mutants, and tetracycline (75 μg/mL) was added for culturing the transposon Ga^{3+} resistant mutants. *Escherichia coli* S17-1 (λ*pir*)/pUT-miniTn5 *luxAB*-Tc^R used for the generation of the transposon mutants was provided by Dr. de Lorenzo (1990) and grown in LB with tetracycline (10 μg/mL). Cell growth was evaluated by measuring turbidity at 600 nm. Gallium nitrate was from Sigma (St. Louis, MO, USA). All other chemicals were at least of reagent grade.

Isolation of spontaneous gallium resistant mutants

Cultures in succinate medium with 100 μM $Ga(NO_3)_3$ were inoculated with the PA14 wild-type strain at an initial O.D. 600 nm of 0.05 and further incubated at 37 °C with orbital shaking at 200 rpm. Under these conditions, no appreciable growth of the wild-type strain was apparent for about one week; afterwards growth became evident. After five sequential sub-cultures in the presence of 100 μM $Ga(NO_3)_3$, which were carried out by transferring an aliquot of the previous culture that had reached an O.D. of ~2.0 to fresh succinate medium (~16 generations; ~1:60 dilution), a significant increase in growth rates and final densities with turbidities higher than 2.0 was apparent for the Ga-resistant cultures (last 2 sub-cultures). This last step allowed the putative spontaneous Ga^{3+} resistant mutants to be purified by growing colonies on LB agar plates.

Isolation of transposon gallium resistant mutants

A PA14 mutant library was generated using conjugation with *E. coli* S17-1 (λpir)/pUT-miniTn5 *luxAB*-Tc^R, which delivered the Tn5-*luxAB* transposon (Ueda and Wood, 2009). After conjugation, cells (0.05 turbidity) were added to succinate medium with 100 μM $Ga(NO_3)_3$ and 75 μg tetracycline/mL to select for the transposon-carrying cells. After four sequential sub-cultures as described above (~16 generations), the transposon mutants were purified using LB tetracycline 75 μg/mL plates. The donor of the transposon *E. coli* S17-1 (λ*pir*)/pUT-miniTn5 *luxAB*-Tc^R was eliminated from the selective medium *via* the high tetracycline concentration.

Minimal concentration of $Ga(NO_3)_3$ to attain 50% inhibition of growth (MIC50)

Succinate medium (Ren et al., 2005) supplemented with 2.5 μM $FeCl_3$ was used in order to obtain higher cell densities, since this medium is iron limited. Cultures (12.5 mL in 125 mL flasks) were inoculated with pre-cultures of the wild-type, TC5 transposon or E12 spontaneous mutants at an initial turbidity of 0.05, and different concentrations of gallium nitrate were added. The solubility of $Ga(NO_3)_3$ in H_2O and succinate medium was verified by recording a decrease of less than 1% in the transmittance at 500 nm for solutions with up to 1 mM of gallium nitrate relative to water or medium.

Biofilm formation

Biofilms were generated on 1 g of glass wool (Corning, NY, USA) soaked in 50 mL of succinate medium supplemented with 2.5 μM $FeCl_3$ in 250 mL flasks. Prior to its utilization, the glass wool was washed three times with 50 mL of deionized water to remove contaminants. The cells were inoculated at a turbidity of ~0.05 and cultured at 37 °C and 200 rpm shaking for 15 h. To quantify the cells in the biofilms, the glass wool was removed and washed with 50 mL of deionized water and then another 50 mL of water was added and the glass wool was sonicated in a bath cleaner at 40 kHz (Branson 2510 Ultrasonic Cleaner, Branson) for 5 min. The turbidity at 600 nm was recorded to estimate the amount of cells in biofilms. The total cell numbers (planktonic + biofilm) were calculated from the turbidities of all the fractions (planktonic + wash + biofilm). For the cell density calculations, it was assumed that 1 mL of liquid with turbidity at 600 nm = 1 contains 1×10^9 cells (Ramos-González and Molin, 1998).

To determine the Ga minimum biofilm eliminating concentration (MBEC) (Sepandj et al., 2004), biofilm formation in 96 well plates was assessed with crystal violet staining (O'Toole et al., 1999). Biofilms were developed by adding planktonic cells from overnight pre-cultures to minimal succinate medium (without the addition of extra iron) at 37 °C for 24 h under static conditions. Thereafter, each well was washed with sterile 0.9% NaCl to remove planktonic cells and then $Ga(NO_3)_3$ dissolved in 0.9% NaCl was added at different concentrations (including a control with no Ga). The remaining well-wall attached cells (*i.e.*, biofilms) were further incubated for 24 h under the same conditions, washed with sterile 0.9% NaCl and re-incubated with fresh minimal succinate medium (Ga-free) for 24 h more. The final growth (O.D. 600 nm) was recorded. Ga-MBEC was determined as the lowest Ga concentration required to avoid cell re-growth in the last incubation (by killing all biofilm cells).

Pyoverdine

The production of pyoverdine was assayed in cell cultures without and with 10 or 25 μM gallium nitrate. Pyoverdine in the cell culture supernatants (in Tris 20 mM buffer, pH 8.0 at 37 °C) was determined spectrophotometrically by absorbance at 407 nm (Ren et al., 2005) and fluorometrically at 407 nm excitation and 460 nm emission. For the fluorescence measurements, all samples were assayed without and with gallium nitrate (up to 40 μM), to correct for the increase in the pyoverdine fluorescence due to the presence of Ga^{3+} (Greenwald et al., 2008).

Pyocyanin

This phenazine was determined spectrophotometrically after its extraction with chloroform and 0.2 N HCl (Essar et al., 1990). For the estimation of the pyocyanin concentration, the absorbance at 520 nm was used with a ε of 2.46 mM^{-1} cm^{-1} (O'Malley et al., 2004).

Gallium and iron determinations

To determine the extracellular and intracellular gallium and iron concentrations, cell cultures (0.05 initial turbidity at 600 nm) of 100 mL in succinate medium with 2.5 μM $FeCl_3$, and with or without 10 or 25 μM gallium nitrate, were grown to a turbidity of 2.5, approximately for 15 h, for all strains/Ga^{3+} concentrations, except for wild-type with 25 μM Ga^{3+} that needed ∼40 h to reach that turbidity, due the growth inhibitory Ga^{3+} effect. Thereafter, cells were centrifuged at 6000 rpm for 10 min at 4 °C. The cell-free supernatants were pooled, and the cell pellets were washed with 20 mM Tris pH 8.0 and 2 mM EGTA to remove the metals loosely bound to cells. The cells were again centrifuged, the washing buffer collected, and the cells re-suspended in Milli-Q H_2O. Aliquots of medium without cells, cell-free supernatants, washing buffer and cell pellets were digested with a mixture of nitric acid–sulfuric acid for iron determination or only with nitric acid for approximately seven days for gallium (the presence of H_2SO_4 interfered with this measurement). The volumes of the digested samples were adjusted with Milli-Q H_2O and the iron and gallium contents determined by atomic absorption spectrophotometry (AA-99 Varian) using calibration curves with AAS standard solutions of $Ga(NO_3)_3$ and $Fe(NO_3)_3$ from SIGMA (St. Louis Missouri, USA). The metal content–absorbance relationship was linear up to 0.036 μM iron and 0.29 μM gallium.

In addition, high density cultures of exponentially growing wild-type and TC5 cells were exposed to 50 μM Ga^{3+} for 1 h. For this experiment, cells were cultured as described above until a turbidity 600 nm ∼ 1.0. Then, cells from four 100 mL cultures were centrifuged at 6000 rpm for 10 min at 4 °C and resuspended in a total volume of 100 mL (concentrated 4-fold) of minimal succinate medium (without extra $FeCl_3$). Initial O.D. at 600 nm was determined and half of the culture was removed, centrifuged and the supernatant and cell pellet used for the determination of Ga (as a control to determine any unspecific Ga signal). The other half of the cultures was supplemented with 50 μM $Ga(NO_3)_3$ and further cultured for 1 h. Growth was determined after 1 h of incubation and then the culture was centrifuged, and Ga in the supernatant and cell pellets was determined. For these last experiments, Ga was determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES) using an ICPS-8000 (SHIMADZU) with argon as a carrier gas and emission at 294.3 nm specific for Ga. Calibration curves with AAS standard solutions of $Ga(NO_3)_3$ were used to estimate the concentration, where the metal content-absorbance relationship was linear up to 0.1 μM of gallium.

Pyocyanin protection assay

To determine whether pyocyanin was able to protect cells against the bacteriostatic effect of Ga^{3+}, cultures of the PA14 wild-type or mutant *feoB* (unable to transport Fe^{2+}) strains were supplemented with 200 μM $Ga(NO_3)_3$ at the mid-exponential phase (turbidity at 600 nm ∼ 0.5). The addition of Ga^{3+} arrested growth within the next cell division (approximately after 3.5 h). Where indicated, pyocyanin (Sigma) was added at 130 μM, and cell growth was monitored.

hitA cloning and complementation of hitA and TC5 mutants with pUCP20-hitA

The gene *hitA* was amplified by PCR using the oligonucleotides F-hitA (5′-GCG CGC GAA TTCAAC GGC ATC TAT ATC GAC GAA-3′), R-hitA (5′-GCG CGC AAG CTT GGC AGG AAC AGC CAG AAT G-3′) and genomic DNA from strain PA14 as the template. The PCR product (1306 bp) was cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA) and subcloned into EcoRI/HindIII digested pUCP20 plasmid (West et al., 1994), generating the plasmid pUCP20-*hitA*. The cloned *hitA* gene was sequenced and one synonymous mutation was found (A687G). The pUCP20-*hitA* plasmid was used to transform the strains *hitA* (Liberati et al., 2006), and TC5. The controls *hitA*-pUCP20 and TC5-pUCP20 strains were constructed by transforming the strains with the pUCP20 plasmid. Transformations were done as previously reported (Chakrabarty et al., 1975). All transformants were selected with 200 μg carbenicillin/mL.

Statistical analysis

The Student's *t* test (1-tailed) for non-paired samples was used.

Results

Isolation of Ga^{3+} resistant mutants

As $Ga(NO_3)_3$ is used at high doses to treat malignant hypercalcemia (Kaneko et al., 2007) and since it was the first Ga^{3+} formulation used as an antipseudomonal (Kaneko et al., 2007), this compound was selected for the generation of resistant mutants. We hypothesized that under the strong selective pressure imposed by this bacteriostatic/bactericidal metal, resistance would emerge by single or multiple mutations. To assess validity of this hypothesis, two different approaches were used. The first was to continuously expose wild-type cells to high, growth-inhibiting Ga^{3+} concentrations to induce, and select, spontaneous mutants. The second was to generate a PA14 transposon mutant library which was exposed to a high Ga^{3+} concentration to detect, and select, resistant transconjugants. Minimal succinate medium was used for the isolation of resistant mutants, because its low iron content limits growth (Fig. S1) and allows for strong growth inhibition by Ga^{3+}. PA14 wild-type cells were exposed to 100 μM $Ga(NO_3)_3$, a concentration at which there is no appreciable growth (no increase of O.D. 600 nm) for this strain after several days. However, after a lag phase of a week, growth was attained in the wild type strain and after five further sequential sub-cultures of growing cells in the same medium (∼16 generations), spontaneous mutants emerged. In turn, PA14 Tn mutants were identified by their faster growth rates in sequential liquid sub-cultures with 100 μM $Ga(NO_3)_3$ and 75 μg tetracycline/mL; hence, Ga^{3+} resistant Tn mutants were isolated after four sequential sub-cultures.

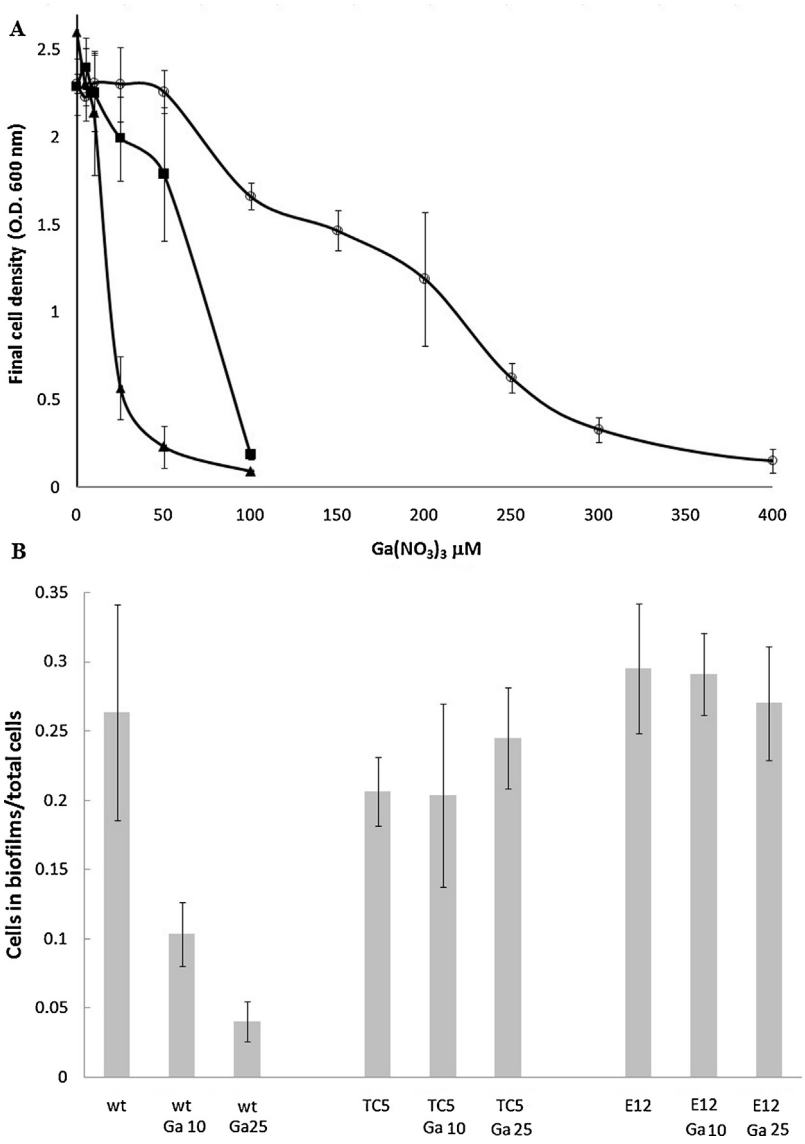

Fig. 1. Effect of Ga^{3+} on cell growth and biofilm formation. (A) PA14 wild-type strain (solid triangles), the Ga^{3+} resistant mutant generated by transposition "TC5" (solid squares), and the Ga^{3+} resistant mutant generated spontaneously "E12" (empty circles) were cultured with $Ga(NO_3)_3$ for 24 h. (B) Biofilms were allowed to develop in glass wool for 15 h with or without 10 μM or 25 μM of $Ga(NO_3)_3$. Total cells indicate the total number of planktonic cells + cells in the biofilm. Data shown represent the mean ± SD of three independent experiments.

The isolated mutants and their biofilms are gallium resistant

The Tn mutant TC5 and spontaneous mutant E12 were selected for assessing the degrees of resistance to Ga^{3+}, because these showed the highest growth rates and maximal cell densities in the presence of 50 μM Ga^{3+} (data not shown), which were similar to those of the wild type without Ga^{3+}. Indeed, high levels of resistance to Ga^{3+} were achieved by the mutants analyzed (Fig. 1A); the MIC50 after 24 h were 16 ± 2 μM ($n = 3$) for the wild-type, 64 ± 1.4 μM ($n = 3$) for TC5 (4-fold higher than wild-type; $P < 0.001$) and 194 ± 16 μM ($n = 3$) for E12 (12-fold higher than wild-type; $P < 0.001$).

In addition to its antibiotic properties, Ga^{3+} also shows inhibition on the formation of bacterial biofilms (Kaneko et al., 2007). Therefore, the effect of Ga^{3+} on the biofilm formation of the wild-type and the E12 and TC5 mutants was tested by two different assays: biofilms generated on glass wool (Fig. 1B) and the standard 96-well plate biofilm method (Fig. S2). As expected, at 10 μM and 25 μM, the biofilm cell number of the wild-type decreased by 40–60% and 50–85%, respectively ($P < 0.005$), whereas the biofilms of the TC5 and E12 mutants remained unaltered (Fig. 1B), with no significant changes in total cell numbers (Fig. S3). Moreover, the MBEC for Ga^{3+} in the wild-type strain was 200 μM, and for both the TC5 and E12 mutants the MBEC was greater than 1600 μM. However, the relationship of an OD of 1 being equivalent to 1×10^9 cells (Ramos-González and Molin, 1998) might not hold for cells under Ga^{3+} stress; hence, the Ga^{3+} resistance values determined might slightly change.

Molecular characterization of the transposon mutant

To gain insights into the molecular mechanisms involved in the resistance to Ga^{3+}, the identification of the interrupted gene in the transposon mutant was determined (see supplementary materials). The gene interrupted in TC5 was *hitA* (1008 bp) at position 800 bp (Arg 266) from the start of the coding region. This gene codifies a periplasmic protein that in *Haemophilus influenza*e is part of the Fe^{3+} transporter HitABC (Adhikari et al., 1995), suggesting

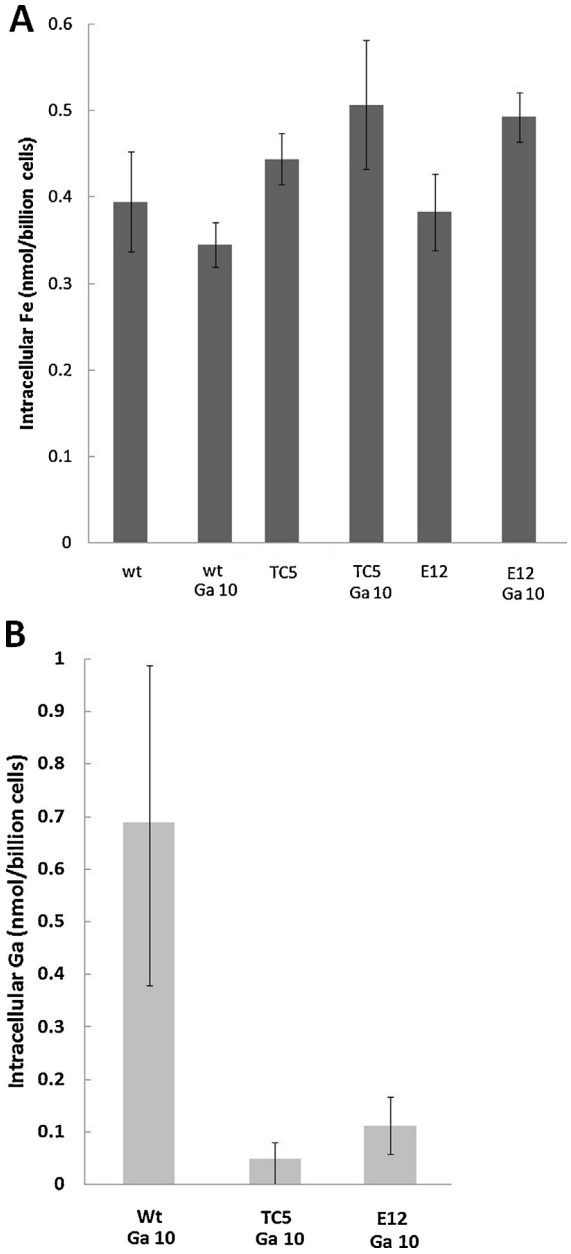

Fig. 2. Intracellular iron and gallium concentrations. Cells were grown in minimal succinate medium for 15 h. Iron (A) and gallium (B) levels are indicated in nmol/billion cells. A billion = 1×10^9 cells. Data shown represent the mean ± SEM of three and five independent experiments for Fe and Ga, respectively.

that this transporter may internalizes Ga^{3+} and that, once disrupted, Ga^{3+} uptake is prevented. This is in agreement with the lower intracellular levels of Ga^{3+} found in the TC5 mutant (see below). To further assess the role of the *hitA* disruption in Ga^{3+} resistance, the effect of Ga^{3+} on independent *hitA* and *hitB* mutants, both acquired from the transposon mutant library (Liberati et al., 2006), was determined. In agreement with the TC5 results, both *hit* mutants were resistant to 25 μM Ga^{3+} ($P < 0.005$) and showed MIC50 values 2.8-fold higher than the wild-type (Fig. 3).

Complementation of the TC5 mutant

To further establish that the interruption of *hitA* in the TC5 mutant was responsible for the Ga^{3+} resistance, the Ga^{3+} resistance phenotype of the TC5 mutant was reversed by the addition of the wild-type *hitA* gene. In addition, the isogenic PA14 *hitA* mutant from the transposon mutant library (Liberati et al., 2006) was also complemented using the same construction. The presence of 50 μM Ga^{3+} severely arrested wild-type strain growth while the *hitA* and TC5 mutants showed 6.5- and 9-fold higher cell density after 24 h (Fig. 4; $P < 0.005$ for both mutants). Complementation of the mutants with the *hitA* gene decreasedGa^{3+} resistance as expected ($P < 0.005$).

Molecular characterization of the spontaneous mutant

To characterize the molecular changes that led to the high Ga^{3+} resistance in the spontaneous mutant E12, its complete genome was sequenced (see supplementary materials). The analysis showed 64 non-silent point mutations in the following functional categories: amino acid and lipid metabolism, transport, cell division, detoxification, DNA recombination/repair, signal transduction, energy production, synthesis of siderophores, and phenazines biosyntheses (Table S2). The mutations identified in the last two categories and the phenotypes associated with Ga^{3+} resistance for E12 (increase in the pyocyanin and pyoverdine contents) may be linked, since mutations were found in the genes that encode enzymes for the biosynthesis of pyoverdine and pyocyanin including pyoverdine synthetase D (PvdD; K2233E), phenazine biosynthesis protein D (PhzD2; K28E) and phenazine biosynthesis protein E (PhzE2; F231L and I266L).

Production of pyoverdine and pyocyanin and Ga intracellular contents

In order to assess the mechanisms involved in the high resistance to Ga^{3+}, phenotypes linked to this resistance were examined. Pyoverdine was determined since (i) it is the main siderophore of *P. aeruginosa*; (ii) it is required for capturing extracellular Fe^{3+}; and (iii) its over-production by the induction of the sigma factor *pvdS* or its addition to cultures confers protection against Ga (Kaneko et al., 2007). Pyoverdine basal levels were similar between the wild-type and both mutants, and remained unchanged in the presence of 10 μM Ga^{3+} (Fig. S4A). With 25 μM Ga^{3+}, the pyoverdine content decreased by 0.5-fold in the wild-type, as expected (Kaneko et al., 2007), but was unchanged in the Tn mutant, and increased by 1.4-fold in the spontaneous mutant. With 50 μM Ga^{3+}, pyoverdine levels further increased in the spontaneous mutant (2.3 ± 0.48-fold; $n = 3$; $P < 0.005$). These results indicated that pyoverdine overproduction is not involved in TC5 resistance but it may be a mechanism used by the spontaneous mutant at high Ga^{3+} concentrations.

As pyocyanin is involved in the delivery of iron to the cell by promoting the reduction of transferrin-bound Fe^{3+} to Fe^{2+}, and by directly reducing free Fe^{3+} (Cox, 1986), the levels of this phenazine were determined. The E12 mutant showed significantly higher levels of pyocyanin (2 ± 0.32-fold, $n = 3$, $P < 0.005$) compared to both the wild-type and Tn mutant in the absence of Ga^{3+}. The presence of 10 μM Ga^{3+} increased pyocyanin by ~50% in the wild-type and TC5 and 80% in the E12 mutant (Fig. S4B). Although the addition of 200 μM Ga^{3+} to wild-type during the mid-exponential phase arrested cell growth, the simultaneous addition of pyocyanin and Ga^{3+} rescued the growth (Fig. S5A). Furthermore, the isogenic *feoB* mutant, unable to transport Fe^{2+} (Wang et al., 2011) was not protected by pyocyanin (Fig. S5B). Two independent mutants unable to produce pyocyanin (*phzM* and *phzS*) showed lower resistance to Ga^{3+} than the wild-type (Fig. S6). These results revealed that pyocyanin overproduction can help cells to cope against Ga^{3+} growth inhibitory effects.

Rhamnolipids and the exopolysaccharide alginate secreted by PA14 are both able to bind metals; the levels of these two types of

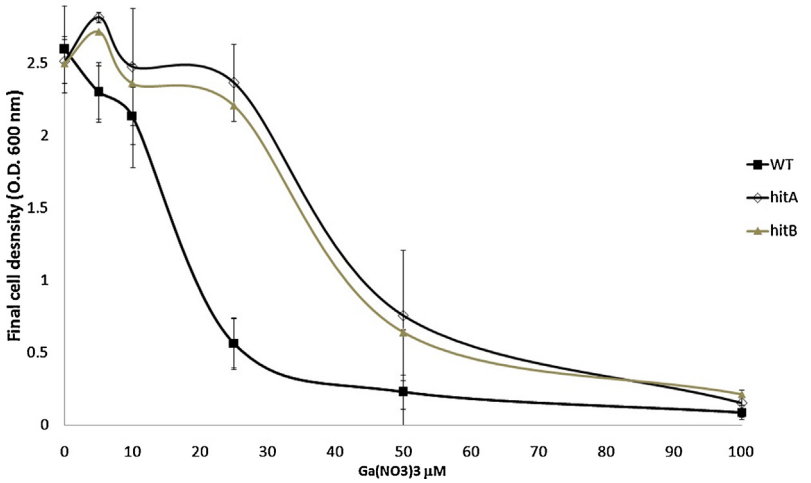

Fig. 3. Independent *hitA* and *hitB* transposon mutants from the Ausubel collection are Ga resistant. PA14 wild-type and the transposon mutant were cultured with $Ga(NO_3)_3$ for 24 h. Data shown represent the mean ± SD of three independent experiments.

compounds were similar in all strains in the absence of gallium, and their concentrations in TC5 and E12 remained unchanged upon the addition of 10 and 25 μM Ga (data not shown), indicating that the overproduction of these compounds is not linked to Ga^{3+} resistance in the mutants.

The intracellular iron levels in both mutants were similar to those of the wild-type (Fig. 2A), whereas the intracellular Ga^{3+} levels were 7- and 14-fold lower ($P < 0.05$) in the E12 and TC5 mutants, respectively, than in the wild-type in the presence of 10 μM Ga^{3+} (Fig. 2B). Upon addition of 25 μM Ga, the intracellular content of Ga in the E12 mutant was also 2-fold lower than in the wild type (data not shown). Accordingly, the external Ga^{3+} remaining after 1 h-incubations with 50 μM Ga^{3+} in the high cell density experiments was 2-fold higher (24 ± 10 μM; $n = 3$; $P < 0.05$) in the Tn strain than in the wild-type (12 ± 0.3 μM; $n = 3$). These results indicated that decreasing intracellular levels of Ga^{3+} contribute to the resistance observed in both mutants.

Discussion

The development of antimicrobials with novel action mechanisms against *P. aeruginosa* is urgent. In this regard Ga^{3+} is a novel antipseudomonal that decreases bacterial Fe^{3+} uptake and interferes with iron signaling through the alternative sigma factor PvdS (Kaneko et al., 2007). Indeed, Ga^{3+} and Fe^{3+} have nearly identical ionic radii, but oxidation/reduction cycling of iron occurs, which is critical for many of its biological functions (Chitambar and Narasimhan, 1991), whereas Ga^{3+} cannot be reduced. Thus, Ga^{3+} might interfere with several processes requiring the reduction–oxidation of iron (Kaneko et al., 2007) such as (a) DNA synthesis, since the ribonucleotide reductase is iron dependent (Fontecave et al., 1990), (b) protection against oxidative stress by catalase (Frederick et al., 2001) and superoxide dismutase, since both enzymes are iron-dependent (Hassett et al., 1993), and (c) electron transport by the respiratory chain since the cytochromes

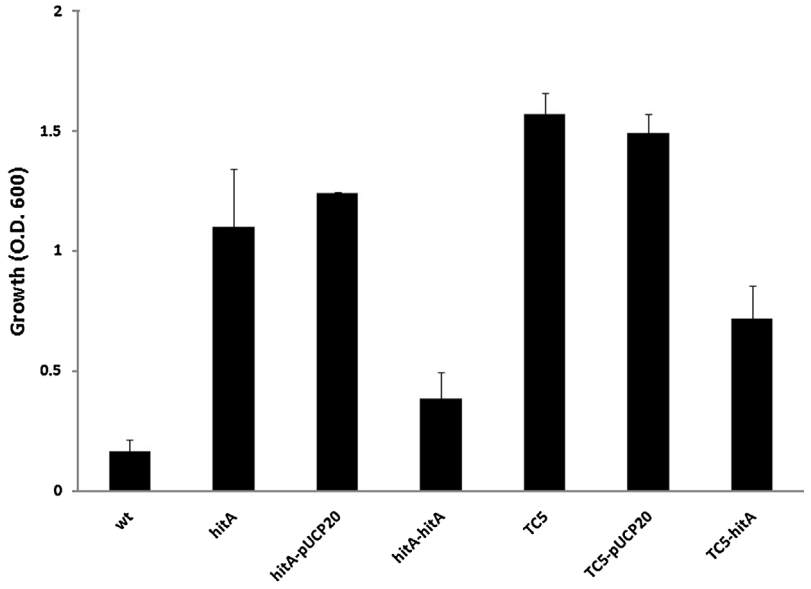

Fig. 4. Complementation of TC5 and *hitA* mutants. Final cell growth (O.D. 600 nm) of the strains in minimal succinate medium (supplemented with 2.5 μM of $FeCl_3$) in the presence of 50 μM $Ga(NO_3)_3$ after 24 h. pUCP20 indicates the presence of the empty vector and pUCP20-*hitA* is the plasmid with *hitA+* of *P. aeruginosa* PA14. Data shown represent the mean ± SEM of three independent experiments.

and Fe–S proteins in the respiratory complexes engage in redox reactions mediated by iron (Anraku, 1988).

In contrast, single inactivation of the pyoverdine, pyochelin or ferric citrate iron transport systems and double inactivation of the pyoverdine and pyochelin systems neither prevent the entrance of Ga^{3+} to the cell nor render PAO1 resistant. These observations agree with the hypothesis that mutations in single targets will not generate high resistance and also indicate that Ga^{3+} is not internalized by the major iron uptake systems (Kaneko et al., 2007).

However, we demonstrate here that resistance against the inhibition of both planktonic growth and biofilms exerted by Ga^{3+} can readily emerge, either by transposon inactivation of a single target (*hitA*) or by an undefined number of gene changes in the spontaneous mutant. For the transposon TC5 mutant, our results show that the main resistance mechanism is a decrease in the Ga^{3+} intake *via* the HitAB system. This finding is relevant since this is the first report in which a putative Ga^{3+} transporter is described.

Interestingly, the addition of pUPC20-*hitA* to the *hitA* mutant was able to increase the Ga^{3+} sensitivity to levels similar to those of the wild-type strain; these results firmly establish one molecular mechanism of Ga^{3+} resistance in *P. aeruginosa*. Complementation of the TC5 mutant with *hitA* also increased Ga^{3+} sensitivity although at significantly lower levels than that shown by the wild-type strain. This TC5 mutant behavior may be related to a polar effect of the transposon insertion on the expression of *hitB*, since TC5 DNA microarrays showed a 18-fold decrease in its expression relative to wt strain (data not shown).

In the spontaneous E12 mutant, high levels of pyocyanin were induced and mutations in two pyocyanin biosynthetic genes were found. This phenazine, which is a marker of *P. aeruginosa* virulence (McDermott et al., 2012), catalyzes the reduction of either free or protein bound Fe^{3+} to Fe^{2+} (Cox, 1986). However, addition of pyocyanin protected cells from Ga^{3+} only for the wild-type strain but not for the *feoB* mutant (*cf.* Fig. S5). The explanation is that *feoB* mutants are unable to take up Fe^{2+}, a transport reaction catalyzed by the Fe^{2+} FeoB transporter (Marlovits et al., 2002); the transporter is expected not to be inhibited by Ga^{3+} (Logan et al., 1981). Therefore, iron can only be internalized in these mutants as Fe^{3+} (Wang et al., 2011), and hence Ga^{3+} can competitively inhibit the Fe^{3+} transporter activity (Kaneko et al., 2007) and affect growth in *feoB* mutants. In contrast, in the spontaneous E12 mutant, with unaltered FeoB transporter, excess pyocyanin promotes Fe^{2+} generation and uptake, attenuating Ga^{3+} toxicity.

Thus, a strain with enhanced Fe^{2+} uptake capacity (for example by overproducing FeoB or other heterologous Fe^{2+} transporters) is expected to show increased Ga^{3+} resistance, as compared to the wild-type strain; and pyocyanin would exert a stronger protection against Ga^{3+} stress. On the other hand, inhibition of pyocyanin production, by deleting pyocyanin biosynthesis genes in wild type, TC5 or E12, is expected to decrease Ga^{3+} resistance.

In contrast to the significantly lower intracellular levels of Ga (7–14 fold), the iron content in both mutants was similar to that of wild type cells. This observation suggests that iron homeostasis is not involved while prevention of Ga^{3+} accumulation is indeed

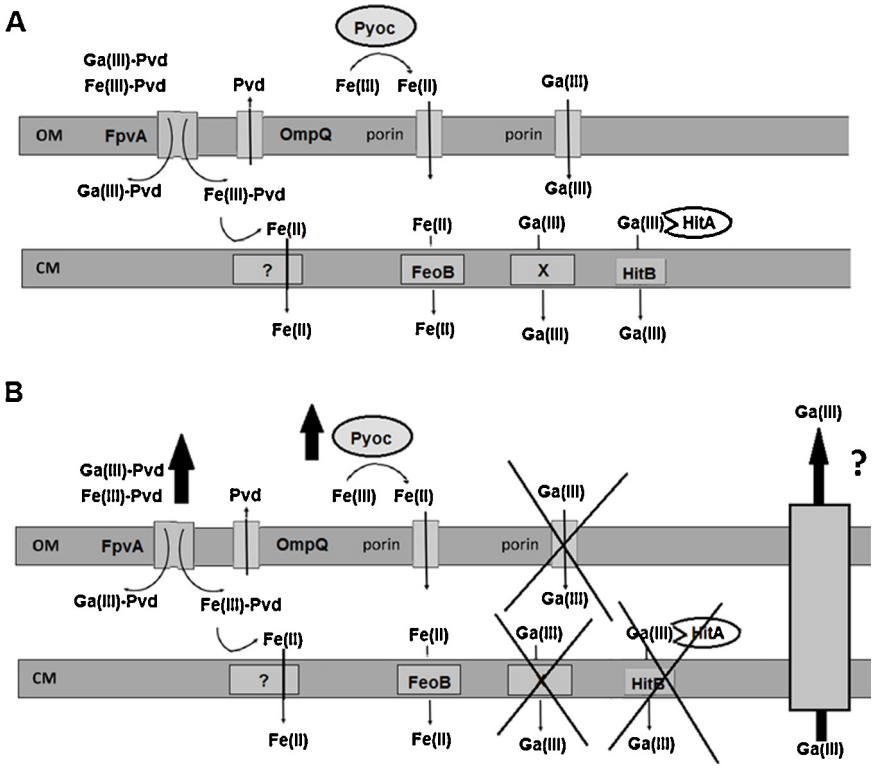

Fig. 5. Gallium and Iron transport model and the possible Ga^{3+} resistance mechanisms in *Pseudomonas aeruginosa*. (A) Gallium and Iron internalization: Outside the cell, Ga^{3+} and Fe^{3+} bind the siderophore pyoverdine (Pvd), and these complexes enter the periplasm *via* the FpvA transporter (Wandersman and Delepelaire, 2004). Fe^{3+} is reduced to Fe^{2+} intracellularly by a yet unknown protein, Fe^{2+} is released by pyoverdine, and free pyoverdine is pumped outside the cell by the OmpQ efflux system (Yeats et al., 2004). As Ga^{3+} cannot be reduced, it remains sequestered in the periplasmic space bound to pyoverdine. In addition, Fe^{3+} can be reduced by pyocyanin (Cox, 1986), and then enter the periplasm *via* a porin. Fe^{2+} can also enter the cell by the FeoB transporter (Marlovits et al., 2002). Ga^{3+} may also enter the periplasm *via* porins and then the cytosol *via* an unknown transporter or the HitB permease, if presented by HitA (Adhikari et al., 1995). (B) Ga^{3+} resistance mechanisms: (1) An increase in pyoverdine has the dual effect of increasing Fe^{3+} and decreasing Ga^{3+} internalization by its sequestration in the periplasm (Kaneko et al., 2007). (2) An increase in pyocyanin contributes to increase the reduction of Fe^{3+} to Fe^{2+}, a metal ion species that would be impervious to the Ga^{3+} competitive inhibition effects. (3) An inactivation of the Ga^{3+} uptake transporters (crossed), including the HitAB transporter mutated in TC5. (4) The activation of a putative Ga^{3+} efflux mechanism. *Abbreviations*: Pvd; pyoverdine; OM; outer membrane; CM; cytoplasmic membrane.

part of the resistance mechanisms toward Ga^{3+} in both mutants. Therefore, diminished Ga^{3+} uptake, active Ga^{3+} efflux, or both processes are very likely involved in the resistance to Ga^{3+} shown by the spontaneous mutant. In this regard, it is noteworthy that nine mutations in four transport proteins were found in the E12 spontaneous mutant, four in the multidrug efflux pump component ArmB (MexY) and two in a putative permease.

Pyoverdine was apparently not involved in the resistance developed by TC5 because its levels were not different from those of the wild-type in the presence of 10 μM Ga^{3+}. However, at 25 μM Ga^{3+}, pyoverdine production of the wild-type was inhibited but not that of TC5, suggesting involvement of this process in the development of resistance toward Ga^{3+}. On the other hand, the pyoverdine levels in the spontaneous E12 mutant were significantly higher at 50 μM Ga^{3+}. It is plausible that high pyoverdine levels confer Ga^{3+} resistance since the addition of exogenous pyoverdine as well as its overproduction lead to Ga^{3+} tolerance, likely mediated by sequestration of the extracellular Ga^{3+} (Kaneko et al., 2007).

Future work with the Ga^{3+} resistant mutants cultured with different carbon sources (Rzhepishevska et al., 2011) and, in particular with an artificial sputum medium which may mimic the conditions faced by the bacterium in the human host, may allow understanding of the emergence and nature of the Ga^{3+} resistance mechanisms under a more physiological context.

Fig. 5 indicates how Ga^{3+} may enter the cell and interfere with Fe^{3+} delivery and Fe^{2+} uptake as well as the potential Ga^{3+} resistant mechanisms described in this work: (i) decrease in Ga^{3+} uptake due inactivation of the HitAB transporter in the transposon mutant, (ii) decrease in Ga^{3+} uptake due the inactivation of other unknown transporters/activation of efflux in the spontaneous mutant, and (iii) increase in Fe^{2+} concentration mediated by pyocyanin Fe^{3+} reduction. Thus, the present work demonstrates for the first time that resistance against the novel antimicrobial gallium nitrate can readily develop in *P. aeruginosa.* In addition, this is the first report unveiling the molecular basis of the resistance mechanisms involved. This knowledge may have clinical relevance if Ga^{3+} is used as an antipseudomonal drug.

Acknowledgments

We thank Dr. Frederick Ausubel from Harvard Medical School for the PA14 strains, Dr. Victor de Lorenzo from Centro Nacional de Biotecnología, Madrid for *E. coli* S17-1 (λpir)/pUT-miniTn5 *luxAB*-Tc^R, Dr. Carlos Cervantes from Universidad Michoacana of Mexico for the pUCP20 vector, Dr. José Salud Rodriguez-Zavala from the National Institute of Cardiology of Mexico for helpful discussions, Dr. Héctor Quezada from the National Institute of Cardiology of Mexico for his help with *hitA* cloning, and Abril Ruiz and Wendy Rangel for help with some experiments. This work was partially supported by grants from SEP/CONACyT-México Nos. 123636 and 152794, and ICyTDF No. PICS08-5 and No. PICSA 11-78. TKW is the Biotechnology Endowed Chair at the Pennsylvania State University.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijmm.2013.07.009.

References

Adhikari, P., Kirby, S.D., et al., 1995. Biochemical characterization of a *Haemophilus influenzae* periplasmic iron transport operon. J. Biol. Chem. 270 (42), 25142–25149.

Anraku, Y., 1988. Bacterial electron transport chains. Annu. Rev. Biochem. 57, 101–132.

Banin, E., Lozinski, A., et al., 2008. The potential of desferrioxamine-gallium as an anti-*Pseudomonas* therapeutic agent. Proc. Natl. Acad. Sci. U.S.A. 105 (43), 16761–16766.

Bjarnsholt, T., Tolker-Nielsen, T., et al., 2010. Interference of *Pseudomonas aeruginosa* signalling and biofilm formation for infection control. Expert Rev. Mol. Med. 12, e11.

Bjarnsholt, T., Tolker-Nielsen, T., et al., 2011. Interference of *Pseudomonas aeruginosa* signalling and biofilm formation for infection control. Expert Rev. Mol. Med. 12, e11.

Cox, C.D., 1986. Role of pyocyanin in the acquisition of iron from transferrin. Infect Immun. 52 (1), 263–270.

Chakrabarty, A.M., Mylroie, J.R., et al., 1975. Transformation of *Pseudomonas putida* and *Escherichia coli* with plasmid-linked drug-resistance factor DNA. Proc. Natl. Acad. Sci. U.S.A. 72 (9), 3647–3651.

Chitambar, C.R., Narasimhan, J., 1991. Targeting iron-dependent DNA synthesis with gallium and transferrin-gallium. Pathobiology 59 (1), 3–10.

de Lorenzo, V., Herrero, M., et al., 1990. Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. J. Bacteriol. 172 (11), 6568–6572.

Essar, D.W., Eberly, L., et al., 1990. Identification and characterization of genes for a second anthranilate synthase in *Pseudomonas aeruginosa*: interchangeability of the two anthranilate synthases and evolutionary implications. J. Bacteriol. 172 (2), 884–900.

Fontecave, M., Gerez, C., et al., 1990. Reduction of the Fe(III)-tyrosyl radical center of *Escherichia coli* ribonucleotide reductase by dithiothreitol. J. Biol. Chem. 265 (19), 10919–10924.

Frederick, J.R., Elkins, J.G., et al., 2001. Factors affecting catalase expression in *Pseudomonas aeruginosa* biofilms and planktonic cells. Appl. Environ. Microbiol. 67 (3), 1375–1379.

Greenwald, J., Zeder-Lutz, G., et al., 2008. The metal dependence of pyoverdine interactions with its outer membrane receptor FpvA. J. Bacteriol. 190 (20), 6548–6558.

Hassett, D.J., Woodruff, W.A., et al., 1993. Cloning and characterization of the *Pseudomonas aeruginosa* sodA and sodB genes encoding manganese- and iron-cofactored superoxide dismutase: demonstration of increased manganese superoxide dismutase activity in alginate-producing bacteria. J. Bacteriol. 175 (23), 7658–7665.

Hidron, A.I., Edwards, J.R., et al., 2008. NHSN annual update: antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006–2007. Infect. Control Hosp. Epidemiol. 29 (11), 996–1011.

Jones, R.N., Stilwell, M.G., et al., 2009. Antipseudomonal activity of piperacillin/tazobactam: more than a decade of experience from the SENTRY Antimicrobial Surveillance Program (1997–2007). Diagn. Microbiol. Infect. Dis. 65 (3), 331–334.

Kaneko, Y., Thoendel, M., et al., 2007. The transition metal gallium disrupts *Pseudomonas aeruginosa* iron metabolism and has antimicrobial and antibiofilm activity. J. Clin. Invest. 117 (4), 877–888.

Kerr, K.G., Snelling, A.M., 2009. *Pseudomonas aeruginosa*: a formidable and ever-present adversary. J. Hosp. Infect. 73 (4), 338–344.

Lambert, M.L., Suetens, C., et al., 2011. Clinical outcomes of health-care-associated infections and antimicrobial resistance in patients admitted to European intensive-care units: a cohort study. Lancet Infect. Dis. 11 (1), 30–38.

Lambert, P.A., 2002. Mechanisms of antibiotic resistance in *Pseudomonas aeruginosa*. J. R. Soc. Med. 95 (Suppl. 41), 22L 6.

Lesic, B., Lepine, F., et al., 2007. Inhibitors of pathogen intercellular signals as selective anti-infective compounds. PLoS Pathog. 3 (9), 1229–1239.

Liberati, N.T., Urbach, J.M., et al., 2006. An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. Proc. Natl. Acad. Sci. U.S.A. 103 (8), 2833–2838.

Logan, K.J., Ng, P.K., et al., 1981. Comparative pharmacokinetics of ^{67}Ga and ^{59}Fe in humans. Int. J. Nucl. Med. Biol. 8 (4), 271–276.

Macé, C., Seyer, D., et al., 2008. Identification of biofilm-associated cluster (*bac*) in *Pseudomonas aeruginosa* involved in biofilm formation and virulence. PLoS ONE 3 (12), e3897.

Maeda, T., García-Contreras, R., et al., 2011. Quorum quenching quandary: resistance to antivirulence compounds? ISME J. 6 (3), 493–501.

Mahar, P., Padiglione, A.A., et al., 2010. *Pseudomonas aeruginosa* bacteraemia in burns patients: risk factors and outcomes. Burns 36 (8), 1228–1233.

Marlovits, T.C., Haase, W., et al., 2002. The membrane protein FeoB contains an intramolecular G protein essential for Fe(II) uptake in bacteria. Proc. Natl. Acad. Sci. U.S.A. 99 (25), 16243–16248.

McDermott, C., Chess-Williams, R., et al., 2012. Effects of *Pseudomonas aeruginosa* virulence factor pyocyanin on human urothelial cell function and viability. J. Urol. 187 (3), 1087–1093.

Mutlu, G.M., Wunderink, R.G., 2006. Severe pseudomonal infections. Curr. Opin. Crit. Care 12 (5), 458–463.

O'Malley, Y.Q., Reszka, K.J., et al., 2004. *Pseudomonas aeruginosa* pyocyanin directly oxidizes glutathione and decreases its levels in airway epithelial cells. Am. J. Physiol. Lung Cell Mol. Physiol. 287 (1), L94–L103.

O'Toole, G.A., Pratt, L.A., et al., 1999. Genetic approaches to study of biofilms. Methods Enzymol. 310, 91–109.

Page, M.G., Heim, J., 2009. Prospects for the next anti-*Pseudomonas* drug. Curr. Opin. Pharmacol. 9 (5), 558–565.

Paterson, D.L., 2006. The epidemiological profile of infections with multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter* species. Clin. Infect. Dis. 43 (Suppl 2), S43L 8.

Poole, K., 2002. Outer membranes and efflux: the path to multidrug resistance in Gram-negative bacteria. Curr. Pharm. Biotechnol. 3 (2), 77–98.

Poole, K., 2011. *Pseudomonas aeruginosa*: resistance to the max. Front. Microbiol. 2, 65.

Ramos-González, M.I., Molin, S., 1998. Cloning, sequencing, and phenotypic characterization of the rpoS gene from *Pseudomonas putida* KT2440. J. Bacteriol. 180 (13), 3421–3431.

Rasko, D.A., Sperandio, V., 2010. Anti-virulence strategies to combat bacteria-mediated disease. Nat. Rev. Drug Discov. 9 (2), 117–128.

Ren, D., Zuo, R., et al., 2005. Quorum-sensing antagonist (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone influences siderophore biosynthesis in *Pseudomonas putida* and *Pseudomonas aeruginosa*. Appl. Microbiol. Biotechnol. 66 (6), 689–695.

Rzhepishevska, O., Ekstrand-Hammarstrom, B., et al., 2011. The antibacterial activity of Ga^{3+} is influenced by ligand complexation as well as the bacterial carbon source. Antimicrob. Agents Chemother. 55 (12), 5568–5580.

Sepandj, F., Ceri, H., et al., 2004. Minimum inhibitory concentration (MIC) versus minimum biofilm eliminating concentration (MBEC) in evaluation of antibiotic sensitivity of gram-negative bacilli causing peritonitis. Perit. Dial. Int. 24 (1), 65–67.

Ueda, A., Wood, T.K., 2009. Connecting quorum sensing, c-di-GMP, pel polysaccharide, and biofilm formation in *Pseudomonas aeruginosa* through tyrosine phosphatase TpbA (PA3885). PLoS Pathog. 5 (6), e1000483.

Veesenmeyer, J.L., Hauser, A.R., et al., 2009. *Pseudomonas aeruginosa* virulence and therapy: evolving translational strategies. Crit. Care Med. 37 (5), 1777–1786.

Wandersman, C., Delepelaire, P., 2004. Bacterial iron sources: from siderophores to hemophores. Annu. Rev. Microbiol. 58, 611–647.

Wang, Y., Wilks, J.C., et al., 2011. Phenazine-1-carboxylic acid promotes bacterial biofilm development via ferrous iron acquisition. J. Bacteriol. 193 (14), 3606–3617.

West, S.E., Schweizer, H.P., et al., 1994. Construction of improved *Escherichia–Pseudomonas* shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in *Pseudomonas aeruginosa*. Gene 148 (1), 81–86.

Wright, A., Hawkins, C.H., et al., 2009. A controlled clinical trial of a therapeutic bacteriophage preparation in chronic otitis due to antibiotic-resistant *Pseudomonas aeruginosa*; a preliminary report of efficacy. Clin. Otolaryngol. 34 (4), 349–357.

Yeats, C., Rawlings, N.D., et al., 2004. The PepSY domain: a regulator of peptidase activity in the microbial environment? Trends Biochem. Sci. 29 (4), 169–172.

Zhanel, G.G., DeCorby, M., et al., 2010. Prevalence of antimicrobial-resistant pathogens in Canadian hospitals: results of the Canadian Ward Surveillance Study (CANWARD 2008). Antimicrob. Agents Chemother. 54 (11), 4684–4693.