XXXI DOCTORAL PROGRAMME IN
BIOMEDICAL SCIENCES AND TECHNOLOGIES

Hijacking bacterial iron metabolism using the post-transition metal gallium

PhD candidate: Sarah Hijazi

PhD supervisors: Prof. Paolo Visca and Dr. Emanuela Frangipani

PhD school coordinator: Prof. Paolo Visca

To raise new questions, new possibilities, to regard old problems from a new angle, requires creative imagination and marks real advance in science.

Albert Einstein

Se questa scienza che grandi vantaggi porterà all'uomo, non servirà all'uomo per comprendere se stesso, finirà per rigirarsi contro l'uomo.

Giordano Bruno

L'objet de la science est de connaître la vérité; son occupation, de la chercher; son caractère, de l'aimer; les moyens de l'acquérir sont de renoncer aux passions, de fuir la dissipation et l'oisiveté.

Jean-Jacques Rousseau

SUMMARY

The inexorable rise in the incidence of antibiotic resistance in bacterial pathogens, coupled with the disappointingly low discovery rate of new and clinical useful antibiotics, has revised the attention on searching for alternatives to conventional antibacterial drugs. Considering the essential role of iron in bacterial physiology and pathogenicity, iron metabolism constitutes a promising but still poorly-exploited therapeutic target for the development of new treatments (Ballouche *et al.,* 2009; Foley and Simeonov, 2012). In the last years, the repurposing of existing Food and Drug Administration (FDA)-approved drugs for new clinical applications has become a major research area in drug discovery. In this context, the post-transition metal gallium [Ga(III)], that has a long history as a diagnostic and chemotherapeutic agent, was recently repurposed as an antibacterial (Rangel-Vega *et al.,* 2015; Soo *et al.,* 2017). Ga(III) has no proven function in biological systems, but acts as an iron-mimetic by replacing iron in many enzymes, impairing their function and ultimately hampering cell growth (Bonchi *et al.,* 2014; Minandri *et al.,* 2014; Goss *et al.,* 2018). Recently, there has been an expansion in the number of Ga(III)-based drugs displaying therapeutic potential, categorized in first-, second-, and third- generations of Ga(III) formulations, and ranging from simple salts such as Ga(III)-chloride ($GaCl_3$) and Ga(III)-nitrate (GaN), through metal-organic complexes such as Ga(III)-maltolate (GaM) (Bernstein *et al.,* 2000), and Ga(III)-protoporphyrin IX (GaPPIX) (Chitambar, 2017).

To investigate the effect of Ga(III) on Gram-negative bacteria, *Pseudomonas aerugionsa* was used as model of human pathogen. This bacterium represents an important nosocomial pathogen implicated in a wide range of hospital infections, especially in critically ill and immunocompromised patients, and the leading cause of chronic lung infections in patients suffering from cystic fibrosis (CF). Moreover, *P. aeruginosa* is intrinsically resistant to many classes of antibiotics, making the treatment of infected patients very problematic.

In the first part of this PhD thesis, the possibility of improving the anti-*P. aeruginosa* potency of Ga(III) by complex formation with PPIX (GaPPIX), that is actively up-taken by bacterial cells, was investigated. We demostrated that GaPPIX enters bacterial cells exclusively through the heme-uptake systems, primarily through *phu* system. Moreover, we provided evidences that GaPPIX inhibits the aerobic growth of *P. aeruginosa* by targeting cytochromes, thus interfering with cellular respiration.

Although *P. aeruginosa* is one of the most frequent cause of multidrug-resistance nosocomial infections, other pathogens belonging to the ESKAPE group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Enterobacter*

species) have reached an alarmingly high level of resistance, and infections caused by these pathogens are no longer treatable with conventional antibiotic therapies (Deplano *et al.,* 2005; Elemam *et al.,* 2009; Nowak *et al.,* 2017).

At present, there are no comparative data on the activity of Ga(III) against ESKAPE species under standard test conditions, representing a major pitfall to the repurposing of Ga(III) as last-resort antibacterial agent. This consideration prompted us to investigate the inhibitory activity of Ga(III) on all pathogens belonging to the ESKAPE group, using not only GaPPIX but other two Ga(III)-based compounds, the most widely studied GaN, and the orally-active compound GaM, which is endowed with high bioavailability and low toxicity (Bernstein *et al.,* 2000). Therefore, in the second part of this PhD thesis, the antibacterial activity of GaN, GaM and GaPPIX was investigated on a representative panel of ESKAPE pathogens, in three media characterized by different nutrients and iron content, namely: *i)* Mueller-Hinton broth (MHB), the standard medium used to determine the minimum inhibitory concentration (MIC) of antimicrobial agents; *ii)* iron-depleted MHB (DMHB) (Hackel *et al.,* 2018); and *iii)* RPMI-1640 tissue culture medium supplemented with 10% complement-free human serum (RPMI-HS), to better mimic the *in vivo* environment encountered by bacteria during infections (Antunes *et al.,* 2012; Thompson *et al.,* 2012; Bonchi *et al.,* 2015). We have defined suitable test conditions to assess the antibacterial activity of Ga(III) compounds *in vitro* (*i.e.,* RPMI-HS medium), and we found that ESKAPE pathogens are more susceptibile to Ga(III) compounds (MIC < 32 μM) in RPMI-HS compared with MHB and DMHB. In contrast, we demonstrated that GaPPIX lost its antibacterial activity in RPMI-HS, likely due to the presence of albumin, which binds GaPPIX, due to its similarity with heme, and counteracts GaPPIX inhibition. Moreover, we provided evidence that the presence of multiple heme-uptake systems strongly influences GaPPIX susceptibility. Interestingly, we showed that GaN and GaM exhibit a bacteriostatic effect, whereas GaPPIX exerts a bactericidal activity on some strains.

As anticipated, *P. aeruginosa* is also the leading cause of CF lung infection and the resulting of lung function deterioration. However, CF lung infections are sequential and sometimes composed of diverse bacterial pathogens, such as *Achromobacter xylosoxidans*, *Burkholderia cepacia* complex (*Bcc*), *Haemophilus influenzae*, *S. aureus, Stenotrophomonas maltophilia*, and *Streptococcus pneumoniae,* that coexist and evolve in the airways of CF patients (Folkesson *et al.,* 2012; Filkins and O'Toole, 2015; Parkins and Floto, 2015). The eradication of such infections is nearly impossible, due to the frequent and prolonged antibiotic administration, that exerts selective pressure for antibiotic resistance (López-Causapé *et al.,* 2013, 2015). Furthermore, if not treated, lung infections often become chronic, since generally associed with bacterial biofilm formation, becoming nearly impossible to eradicate with conventional antibiotics. The broad spectrum activity

of Ga(III) on the ESKAPE pathogens, together with the anti-biofilm activity previously demonstrated for Ga(III) (Bonchi *et al.,* 2014; Minandri *et al.,* 2014; Runci *et al.,* 2016), have prompted us to investigate the inhibitory activity of Ga(III) on CF-associated pathogens.

In the last part of this PhD thesis, we have investigated the antibacterial activity of GaN, GaM and GaPPIX on a representative collection of CF-associated pathogens, using standard media and biological fluids-mimetic growth media, such as RPMI-HS (Hijazi *et al.,* 2018), and the Artificial Sputum Medium (ASM; Kirchner et *al.,* 2012). Combining data obtained in all media tested, we found that Ga(III)-based compounds were effective against all CF-associated pathogens, except *S. pneumoniae*. Interestingly, some species (*i.e., S. aureus* and *H. influenzae*) were found to be extremely susceptible only to GaPPIX, while the other species were susceptible to more then one Ga(III)-based compounds.

In the worrisome scenario of expanding drug resistance among bacterial pathogens, our findings on the antibacterial activity of Ga(III) could open the way to valuable therapeutic options to cure otherwise untreatable bacterial infections.

RIASSUNTO

A causa dell'utilizzo indiscriminato degli antibiotici nella terapia convenzionale e la conseguente inarrestabile diffusione dell'antibiotico resistenza in numerose specie batteriche, negli ultimi anni, una maggior attenzione è stata dedicata alla ricerca di terapie antibatteriche alternative agli antibiotici. Dato il ruolo essenziale che il ferro esercita sulla crescita e sulla patogenicità batterica, il metabolismo del ferro rappresenta un promettente bersaglio per lo sviluppo di nuove molecole antibatteriche (Ballouche *et al.,* 2009; Foley and Simeonov, 2012). Negli ultimi anni, inoltre, il riutilizzo o la riproposizione di farmaci approvati dalla *Food and Drug Administration* (FDA) per nuovi usi clinici ha ampliato il campo di ricerca per la scoperta di nuovi farmaci. In questo contesto, il metallo di post-transizione gallio [Ga(III)], in passato utilizzato sia come antineoplastico che per il trattamento di patologie legate al metabolismo del tessuto osseo, è stato recentemente riproposto come molecola antibatterica (Rangel-Vega *et al.,* 2015; Soo *et al.,* 2017). È stato dimostrato come il Ga(III), grazie all'elevata somiglianza chimico-fisica con il ferro [Fe(III)], sia in grado di interferire con i processi ossido-riduttivi ferro-dipendenti delle cellule microbiche, alterandone il metabolismo e inibendone di conseguenza la crescita. La maggior parte dei sistemi biologici, infatti, non è capace di distinguere il Fe(III) dal Ga(III) a causa delle loro caratteristiche subatomiche molto simili (Bonchi *et al.,* 2014; Minandri *et al.,* 2014; Goss *et al.,* 2018). Recentemente inoltre, il numero di formulazioni del Ga(III) con un potenziale terapeutico è cresciuto, con farmaci definiti di prima, seconda e terza generazione, che spaziano dai semplici sali di questo metallo come il cloruro di Ga(III) ($GaCl_3$) e il nitrato di Ga(III) (GaN), ai complessi metallo-organici come Ga(III)-maltolato (GaM) (Bernstein *et al*., 2000), e Ga(III)-protoporfirina IX (GaPPIX) (Chitambar, 2017).

Per studiare l'effetto del Ga(III) su batteri Gram-negativi, è stato scelto come batterio modello *Pseudomonas aeruginosa*, che oggi rappresenta un preoccupante agente di infezioni nosocomiali, e la principale causa di infezioni polmonari croniche nei pazienti affetti da fibrosi cistica (FC). *P. aeruginosa* è naturalmente resistente alle principali classi di antibiotici, il che ne compromette in ultimo il trattamento delle infezioni.

In questa tesi, è stato studiato se l'effetto inibitorio del Ga(III) potesse essere aumentato quando complessato con PPIX (GaPPIX), molecola attivamente internalizzata da *P. aeruginosa.* È stato infatti osservato come il GaPPIX sia in grado di entrare nella cellula tramite i sistemi dell'acquisizione dell'eme, principalmente tramite il sistema *phu*. Inoltre, è stato evidenziato come il GaPPIX inibisca la crescita aerobica di *P. aeruginosa* interferendo con i citocromi, e in ultimo con la respirazione cellulare.

Sebbene *P. aeruginosa* rappresenti una delle cause più frequenti di infezioni nosocomiali multi-resistenti, altri patogeni appartenenti al gruppo ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Enterobacter* species) hanno raggiunto un livello di resistenza allarmante, rendendo intrattabili le infezioni causate da questi agenti patogeni con le convenzionali terapie antibiotiche (Deplano *et al.,* 2005; Elemam *et al.,* 2009; Nowak *et al.,* 2017). Inoltre, la mancanza di studi puntuali sull'attività del Ga(III) sulle specie ESKAPE in saggi standard, rappresenta un importante limite nell'utilizzo del Ga(III) come agente antibatterico. Per questo motivo, in questo lavoro di tesi, è stata saggiata l'attività inibitoria del Ga(III) sui patogeni appartenenti al gruppo ESKAPE. Oltre al precedentemente citato GaPPIX, altri due composti del Ga(III) sono stati scelti, il GaN, ampiamente studiato in letteratura, e il GaM, un composto attivo oralmente e dotato di un'elevata biodisponibilità oltre che di una bassa tossicità (Bernstein *et al.,* 2000).

Nella seconda parte di questa tesi, è stato dunque saggiato l'effetto inibitorio del GaN, GaM e GaPPIX su un pannello rappresentativo di patogeni ESKAPE, in tre terreni caratterizzati da diversi nutrienti e contenuto in ferro: *i)* il terreno standard Mueller-Hinton (MHB), comunemente utilizzato per determinare la minima concentrazione inibente dei agenti antimicrobici; *ii)* il MHB ferro-privo (DMHB) (Hackel *et al.,* 2018); e *iii)* il RPMI-1640, addizionato con il 10% di siero umano (RPMI-HS), che tende a simulare le condizioni incontrate *in vivo* dai batteri durante le infezioni (Antunes *et al.,* 2012; Thompson *et al.,* 2012; Bonchi *et al.,* 2015). Sono state definite le condizioni idonee per valutare l'attività antibatterica dei composti di Ga(III) *in vitro* (*i. e.*, in RPMI-HS), ed è stato dimostrato come in RPMI-HS i patogeni ESKAPE siano più suscettibili ai composti del Ga(III) (MIC < 32 μM) rispetto che in MHB o in DMHB. Tuttavia, è stato dimostrato un importante decremento dell'attività antibatterica del GaPPIX in RPMI-HS, probabilmente a causa della presenza dell'albumina umana, che potrebbe legare il GaPPIX a causa della sua somiglianza con l'eme, e neutralizzare quindi il suo effetto inibitorio. Inoltre, sono stati fornite evidenze di come la presenza di più sistemi per l'acquisizione dell'eme influenzi fortemente la suscettibilità al GaPPIX. Infine, è stato dimostrato l'effetto batteriostatico del GaN e GaM, e le proprietà battericide del GaPPIX.

Come precedentemente anticipato, *P. aeruginosa* rappresenta anche la principale causa d'infezione polmonare e di deterioramento della funzionalità polmonare in pazienti FC. Tuttavia, le infezioni polmonari in FC sono sequenziali e talvolta polimicrobiche, ossia causate contemporaneamente di batteri patogeni appartenenti a generi e specie diverse [come *Achromobacter xylosoxidans*, *Burkholderia cepacia* complex (*Bcc*), *Haemophilus influenzae*, *S. aureus, Stenotrophomonas maltophilia*, and *Streptococcus pneumoniae*], che coesistono e si evolvono nelle vie respiratorie dei

pazienti della FC (Folkesson *et al.,* 2012; Filkins and O'Toole, 2015; Parkins and Floto, 2015). Nel trattamento di queste infezioni, la frequente e prolungata somministrazione di antibiotici, che esercita sui patogeni una pressione selettiva determina lo sviluppo di resistenze agli antibiotici, rende l'eradicazione di queste infezioni virtualmente impossibile (López-Causapé *et al.,* 2013, 2015). Inoltre, le infezioni polmonari non trattate spesso cronicizzano e sono generalmente associate alla formazione di biofilm batterici impossibili da eradicare con gli antibiotici convenzionali. L'attività ad ampio spettro dei composti del Ga(III) sui patogeni ESKAPE, e l'attività anti-biofilm precedentemente dimostrata per il Ga(III) (Bonchi *et al.,* 2014, Minandri *et al.,* 2014; Runci *et al.,* 2016), ci hanno spinto a saggiare l'attività inibente del Ga(III) sui patogeni associati alla FC.

Nella terza e l'ultima parte di questo lavoro di tesi è stato saggiato l'effetto dei GaN, GaM e GaPPIX su un pannello rappresentativo di specie caratteristiche dell'infezione in FC, sia nei terreni standard che nei quelli che mimano i fluidi biologici, come RPMI-HS (Hijazi *et al.,* 2018) e l'Artificial Sputum Medium (ASM; Kirchner *et al.,* 2012). È stato dimostrato che i tre composti del Ga(III) sono in grado di inibire la crescita batterica di tutti i patogeni associati alla FC, ad eccezione di *S. pneumoniae*. In particolare, è stato dimostrato che alcune specie (*S. aureus* e *H. influenzae*) sono ipersensibili esclusivamente al GaPPIX, mentre gli altre specie sono suscettibili a più di un composto a base di Ga(III).

I risultati sull'attività antibatterica del Ga(III) ottenuti in questo lavoro di tesi fanno ben sperare per un possibile impiego del Ga(III) nel trattamento di infezioni batteriche altrimenti incurabili.

RÉSUMÉ

L'usage exagéré et inapproprié des antimicrobiens a accélèré l'apparition et la propagation de la résistance aux antibiotiques chez de nombreuses espèces bactériennes. Par conséquent, une attention particulière a été accordée récemment à la recherche des solutions alternatives. Étant donné le rôle essentiel du fer [Fe(III)] dans la croissance et la pathogénicité bactérienne, le métabolisme du Fe(III) a été évalué comme une cible possible pour le développement de nouvelles molécules antibactériennes (Ballouche *et al.,* 2009; Foley and Simeonov, 2012). Au cours des dernières années, la réutilisation des médicaments approuvés par la *Food and Drug Administration* (FDA) pour de nouvelles applications cliniques, est devenue un important domaine de recherche dans la découverte de nouveaux médicaments. Dans ce contexte, le métal de post-transition gallium [Ga(III)], connu come un agent de diagnostic et chimiothérapeutique, a été réutilisé récement comme antibactérien (Rangel-Vega *et al.,* 2015; Soo *et al.,* 2017). En fait, la plupart des systèmes biologiques ne sont pas capables de distinguer le Fe(III) de Ga(III) en raison de leurs caractéristiques subatomiques très similaires. De ce fait, en remplaçant le Fe(III) dans de nombreuses enzymes, le Ga(III) altère leur fonction et bloque par la suite la croissance cellulaire (Bonchi *et al.,* 2014; Minandri *et al.,* 2014; Goss *et al.,* 2018). Récemment, le nombre des composés à base de Ga(III), présentant un potentiel thérapeutique, a augmenté. Parmi ces composés, classifiés en première-, deuxième- et troisième- générations de formulations de Ga(III), se trouvent des sels simples tels que le chlorure de Ga(III) ($GaCl_3$) et le nitrate de Ga(III) (GaN), ou des complexes métallo-organiques, tels que le Ga(III)-maltolate (GaM) (Bernstein *et al.,* 2000) et le Ga(III)-protoporphyrine IX (GaPPIX) (Chitambar, 2017).

Pour étudier l'effet de Ga(III) sur les bactéries à Gram-négatif, *Pseudomonas aeruginosa* a été choisi comme modèle d'agent pathogène humain. Il s'agit de l'une des principales causes d'infections nososcomiales, ainsi que d'infections pulmonaires chroniques chez les personnes atteintes de mucoviscidose. En outre, *P. aeruginosa* est intrinsèquement résistant à de nombreuses classes d'antibiotiques, ce qui rend très problématique le traitement des patients infectés.

Dans ce travail de thèse, la possibilité d'améliorer la capacité anti-*P. aeruginosa* du Ga(III) a été étudiée, en utilisant un complexe de Ga(III), à savoir GaPPIX, qui est activement absorbé par cette bactérie. Nous avons démontré que le GaPPIX pénètre dans la cellule bactérienne, exclusivement en empruntant les voies specifiques de l'acquisition de l'hème, en particulier par le système *phu*. Nous avons également démontré que le GaPPIX entrave la croissance de *P. aeruginosa* en ciblant les cytochromes de la respiration cellulaire aerobie.

Bien que *P. aeruginosa* soit l'une des causes les plus fréquentes d'infections nosocomiales multirésistantes, d'autres agents pathogènes appartenant au groupe ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, et *Enterobacter* species) ont atteint un niveau alarmant de résistance, rendant difficile le traitment de ces infections par les antibiotiques conventionnels (Deplano *et al.,* 2005; Elemam *et al.,* 2009; Nowak *et al.,* 2017). Jusqu'à présent, la manque de données comparatives sur l'activité du Ga(III) sur les espèces du groupe ESKAPE dans des conditions standard de test, pose un problème pour la réutilisation de Ga(III) comme un agent antibactérien de dernier recours. Pour cette raison, en plus de GaPPIX, deux autres composés ont été selectionnés, à savoir, le GaN, plus largement étudié, et le GaM, oralement actif et doté d'une biodisponibilité élevée ainsi que d'une faible toxicité (Bernstein *et al.,* 2000).

Dans ce travail de thèse, l'activité antibactérienne des trois composés GaN, GaM et GaPPIX a été étudiée sur les bactéries pathogènes du groupe ESKAPE, dans trois milieux de croissance caractérisés par différents nutriments et teneur en fer, à savoir, *i)* le Mueller-Hinton (MHB), milieu de culture standard utilisé pour évaluer l'activité *in vitro* des agents antimicrobiens; *ii)* le MHB appauvri en fer (DMHB) (Hackel *et al.,* 2018); et *iii)* le milieu de culture RPMI-1640 contenant 10% de sérum humain (HS) sans complément (RPMI-HS), représentant des conditions plus proches de celles rencontrées *in vivo* par les bactéries durant l'infection (Antunes *et al.,* 2012; Thompson *et al.,* 2012; Bonchi *et al.,* 2015). Nous avons défini les conditions *in vitro* d'essai appropriées pour évaluer l'activité antibactérienne du Ga(III), représentées par le milieu RPMI-HS, dans lesquel nous avons constaté que les agents pathogènes ESKAPE sont plus sensibles aux composés de Ga(III) (MIC < 32 μM) par rapport au MHB et au DMHB. En revanche, nous avons démontré que le GaPPIX perd son activité antibactérienne dans le milieu RPMI-HS, probablement à cause de la présence d'albumine, qui pourrait se lier au GaPPIX, en raison de sa similitude avec l'hème, et freiner par la suite son effet inhibiteur. Nous avons également démontré que la présence de multiples systèmes d'acquisition de l'hème influence fortement la sensibilité au GaPPIX. Un fait intéressant, nous avons montré que les composés GaN et GaM provoquent un effet bactériostatique, alors que GaPPIX exerce une activité bactéricide.

P. aeruginosa est également la principale cause des infections pulmonaires ainsi que de la détérioration des fonctions pulmonaires chez les patients atteints de la mucoviscidose. Cependant, les infections pulmonaires de la mucoviscidose sont polymicrobiennes, soit causées par divers agents pathogènes, tels que *Achromobacter xylosoxidans*, *Burkholderia cepacia* complex (*Bcc*), *Haemophilus influenzae*, *S. aureus, Stenotrophomonas maltophilia*, et *Streptococcus pneumoniae*, qui coexistent et évoluent dans les voies respiratoires des ces patients (Folkesson *et al.,* 2012;

Filkins and O'Toole, 2015; Parkins and Floto, 2015). L'éradication de telles infections est presque impossible, en raison de l'administration fréquente et prolongée d'antibiotiques, qui par la suite exerce une pression sélective sur les bactéries et engendre ainsi la résistance aux antibiotiques (López-Causapé *et al.,* 2013, 2015). De plus, si ces infections échappent au traitment, elles deviennent souvent chroniques, et sont généralement associées à la formation de biofilms bactériens, impossibles à éradiquer avec les antibiotiques conventionnels. L'activité à large spectre du Ga(III) sur les agents pathogènes ESKAPE, ainsi que l'activité anti-biofilm précédemment démontrée pour le Ga(III) (Bonchi *et al.,* 2014; Minandri *et al.,* 2014; Runci *et al.,* 2016), nous ont incité à étudier son activité inhibitrice sur les agents pathogènes associés à la mucoviscidose.

Dans la dernière partie de cette thèse, nous avons étudié l'activité antibactérienne des composés GaN, GaM et GaPPIX, sur une liste representative des agents pathogènes associés à la mucoviscidose, en utilisant des milieux de culture standards ainsi que ceux reproduisant les conditions des fluides biologiques, tels que RPMI-HS (Hijazi *et al.,* 2018) et l'*artificial sputum medium* (ASM; Kirchner *et al.,* 2012). Nous avons démontré que les trois composés de Ga(III) sont capables d'inhiber la croissance bactérienne de tous les agents pathogènes associés à la mucoviscidose, à l'exception de *S. pneumoniae*. En particulier, nous avons démontré que certaines espèces (ex. *S. aureus* et *H. influenzae*) sont hypersensibles exclusivement au GaPPIX, tandis que les autres espèces ont démontré une sensibilité à plus d'un seul composé de Ga(III).

Dans le scénario inquiétant de l'augmentation de la résistance aux antibiotiques chez les bactéries pathogènes, nos résultats sur l'activité antibactérienne du Ga(III) pourraient ouvrir la voie à de nouvelles options thérapeutiques pour guérir des infections bactériennes autrement intraitables.

Table of contents

Chapter 1

Introduction and aims

Chapter 1

Introduction and aims

Antimicrobial resistance has become one of the most challenging problems of the healthcare system. The spread of antimicrobial-resistant pathogens has dramatic repercussions on mortality and morbidity rates, hence on global medical costs (Friedman *et al.,* 2016). In 2017, the World Health Organization (WHO) published its first ever list of antibiotic-resistant pathogens, divided in critical, high and medium priority, accordingly to the urgency of need for effective drugs to treat infections. The list of bacteria that are considered to be of critical priority includes *Pseudomonas aeruginosa*, which represents one of the most frequent cause of hospital-acquired infections (Blanc *et al.,* 1998), and the leading cause of chronic lung infections and mortality in patients suffering from cystic fibrosis (CF) disease (Moore and Mastoridis, 2017).

P. aeruginosa isolates most often show a multidrug resistant (MDR) phenotype, leaving few viable therapeutic options to clinicians, and calling for the development of novel anti-*Pseudomona*s therapies. In this scenario, the development of new non-antibiotic approaches to counteract bacterial infections represents an urgent need.

The aim of the research presented in this PhD thesis was to investigate the possibility to hijack bacterial iron metabolism, as an alternative approach to combact bacterial infections, using the post-transition metal Ga(III).

In Chapter 1, the state of the art on opportunistic bacterial pathogens, including clinical features, antibiotic resistance, pathogenicity traits and already available therapeutic strategies, is summarized.

1.1 The emergence of antibiotic resistance among opportunistic bacterial pathogens

Antimicrobial resistance represents one of the most important health concerns. In the last three decades, the emergence and spread of antibiotic resistance, coupled with the steep decline in the identification and development of new antibiotics from the pharmaceutical industry, have led to an urgent need to discover novel antibacterial drugs. Hospitals, and particularly intensive care units, represent an important breeding ground for the development and spread of antibiotic resistant bacteria. This is the consequence of inappropriate infection-control practices, including the

exorbitant and inappropriate antibiotic utilization (Gaynes and Edwards, 2005; Bassetti *et al.,* 2013).

Hospital-acquired infections (HAIs), like bloodstream infections, catheter-associated urinary tract infections, surgical site infections and ventilator-associated pneumonia (VAP), are often associated with multidrug-resistant (MDR, *i.e.,* non susceptible to at least one agent in three or more antimicrobial classes), extensively drug-resistant (XDR, *i.e.,* non susceptible to ≥ 1 agent in all but ≤ 2 categories), or even pandrug-resistant (PDR, *i.e.,* non susceptible to all antimicrobial agents) bacterial strains (Magiorakos *et al.,* 2012). Antimicrobial resistance genes may be carried on the bacterial chromosome, on plasmids, or transposons, and encode for several mechanisms, including drug inactivation/alteration, modification of drug binding sites/targets, changes in cell permeability, reduction of intracellular drug accumulation (*i.e.,* porins loss and expression of efflux pumps) (Sherrard *et al.,* 2014; Santajit and Indrawattana, 2016) (Figure 1).

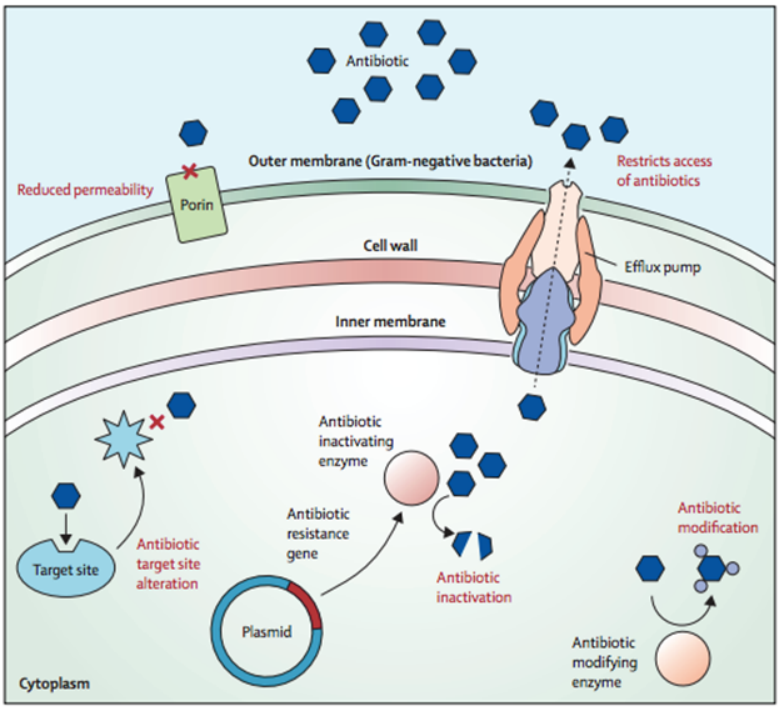


Figure 1. Examples of bacterial mechanisms of antiobiotic resistance. To escape the activity of antibiotics, bacteria have evolved many mechanisms, including the production of enzymes that irreversibly modify and inactivate the antibiotics, modification of the target sites to avoid access by antibiotics, and reduction of the intracellular drug accumulation *via* two possible ways. The first is through the loss of porins, which are proteins that form channels to allow the passage of many hydrophilic substances, including antibiotics, and consequently reduce the outer membrane permeability. The second way is through the expression of efflux pumps that increase the removal of antibiotics from the intracellular compartment (Sherrard *et al.,* 2014).

One paradigmatic examples of antibiotic resistance among bacterial pathogens is that to β-lactams. Several mechanisms have been contributed to the rapid bacterial evolution towards β-lactam antibiotic resistance. In fact, the production of β-lactam degrading enzymes (β-lactamases) has

played a major role in acquired β-lactam resistance in Enterobacteriacea, covering by that all major classes of potentially useful β-lactams. In addition to the pivotal role of acquired β-lactamases, cell impermeability and the active drug efflux have remarkably contributed to increase the extent of β-lactam resistance in *Acinetobacter baumannii* and *P. aeruginosa*. On the other hand, modification of target has evolved as the major mechanism of resistance in *Staphylococcus aureus* (Rossolini and Docquier, 2006).

All the above-mentioned pathogens are members of a small group of bacteria known as ESKAPE, which represent the main cause of nosocomial infection throughout the world. ESKAPE is an acronym for this group of bacteria, encompassing both Gram-negative and Gram-positive species, made up of *Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter* species, capable of "escaping" the biocidal effect of common antibacterial therapies (Rice, 2008; Boucher *et al.,* 2009). In addition to being the leading cause of nosocomial infections, these bacteria are extremely important, because they represent a paradigmatic example of pathogenesis, transmission and resistance, which developed in the hospital setting and is now expanding in the community (Rice, 2008).

The inexorable rise of antibiotic resistance reached by ESKAPE pathogens (Pendleton *et al.,* 2013), together with the drastic reduction in approval of new antibiotics during the past 25 years, have recently prompted the World Health Organization (WHO) to list these pathogens among the greatest threats to human health, and to boost research on new effective drugs to eradicate infections caused by these bacteria (World Health Organization, 2017) (Figure 2).

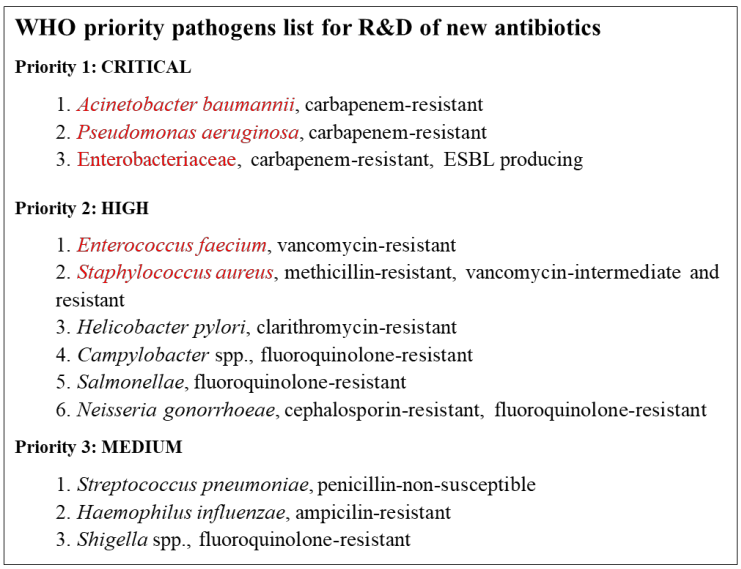


Figure 2. List of priority bacterial pathogens for research and development of new antibiotics. In 2017, the World Health Organization (WHO) published its first ever list of antibiotic-resistant priority pathogens, divided in critical, high and medium priority, accordingly to the urgency of need for effective drugs to treat infections. Bacterial pathogens belonging to the ESKAPE group are highlighted in red. *K. pneumoniae* and *Enterobacter* species are members of the Enterobacteriaceae family (World Health Organization, 2017).

Among ESKAPE pathogens, increasing biological and medical attention has been directed to *P. aeruginosa*, not only because it is one of the most frequent cause of MDR nosocomial infections, but also the leading cause of chronic lung infections responsible for morbidity and mortality of individuals suffering from CF (Moore and Mastoridis, 2017). CF, also known as mucoviscidosis, is the most common lethal genetic disease predominantly affecting the Caucasian population. The disease is caused by a mutation in the gene for Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), a protein found on the surface of epithelial cells. CF, a multisystem disease, is characterized by an overproduction of sticky and thick mucus in many organs, including the digestive system, sweat glands, the reproductive tract, but respiratory system is the most severely affected (Cohen and Prince, 2012) (Figure 3).

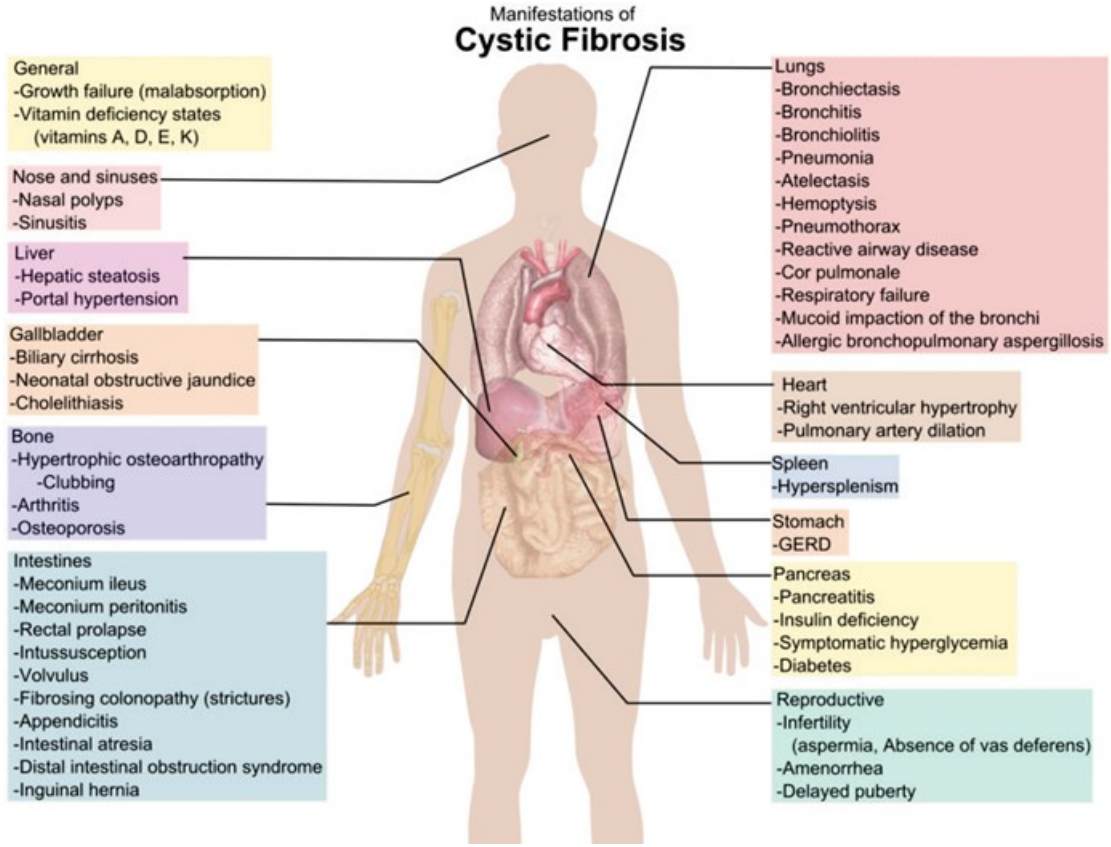


Figure 3. Manifestations of cystic fibrosis: CF is a multisystem disease, affecting the digestive system, sweat glands, the reproductive tract, and respiratory system, which is the most severally affected.

Although CFTR is expressed in many cell types, and CF manifests in various organs, the main cause of mortality and morbidity in patients with CF is due to respiratory problems, having the greatest impact on their quality of life and survival. The problem arises from bacterial respiratory infections, as the airways of patients with CF are colonized with complex polymicrobial communities (Filkins and O'Toole, 2015; Parkins and Floto, 2015). In the early age, the main colonizing organisms are *S. aureus* and *Haemophilus influenzae*, while during adulthood *P.*

aeruginosa and *Burkholderia cepacia* complex (*Bcc*) are predominantly present (Rogers et *al.,* 2003; Folkesson *et al.,* 2012), with *P. aeruginosa* becoming the most commun pathogen colonizing approximately 70% of patients by the age of 25 (Cystic Fibrosis Foundation Patient Registry, 2016) (Figure 4). Other emerging pathogens in CF include *Achromobacter xylosoxidans*, *Stenotrophomonas maltophilia*, and *Streptococcus pneumoniae*, which have also been found in the airways of CF patients (Rogers et *al.,* 2003; van der Gast *et al.,* 2011; Folkesson *et al.,* 2012).

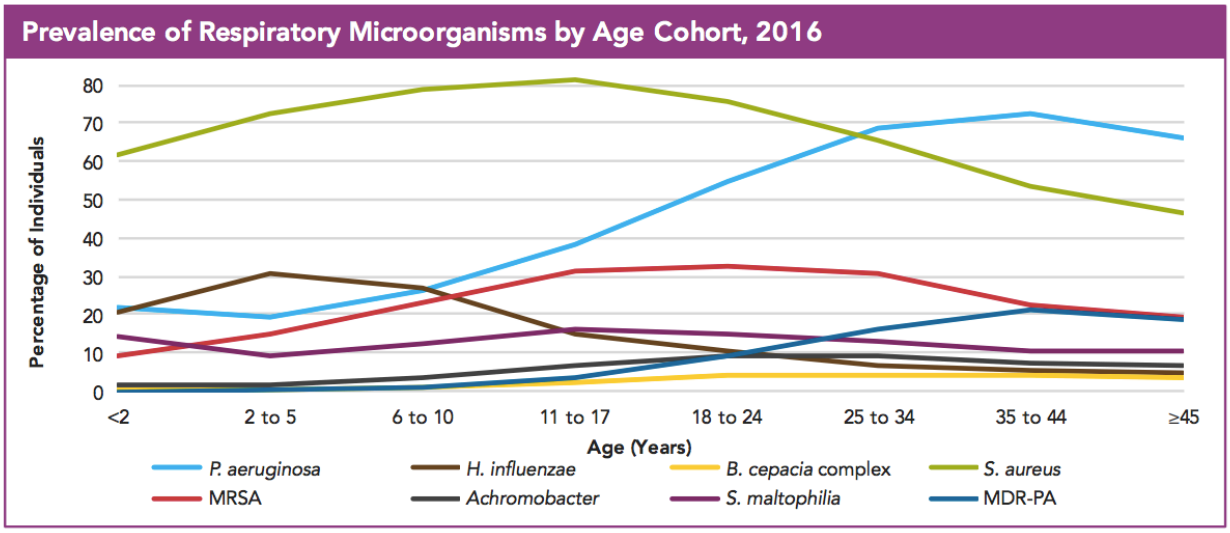


Figure 4. Prevalence of several common human respiratory pathogens in CF patients as a function of age. The natural history of CF is characterized by early colonization of the lung by *S. aureus* and occasionally by *H. influenzae*. As CF patients get older, multidrug-resistant *P. aeruginosa* (MDR-PA) and methicillin-resistant *S. aureus* (MRSA) can emerge. While most CF patients experience *P. aeruginosa* infection during their life, only a minority become infected with *B. cepacia* complex (*Bcc*) and other pathogens, such as *S. maltophilia* and *Achromobacter*, contributing to the complexity of the microbial flora in CF lungs and to pulmonary damage (Cystic Fibrosis Foundation Patient Registry, 2016).

Microbial communities in the CF lung are exposed to frequent and prolonged antiobitic stress, that exerts a selective pressure for antibiotic resistance, leading to the emergence of MDR bacterial isolates, which represent the most important issue for physicians who take care of patients with CF (López-Causapé *et al.,* 2013, 2015). Moreover, one of the most worrisome aspects of bacterial pathogenicity in CF is the development of chronic communities, which are associated with bacterial biofilm mode of growth, that is highly adapted and resilient to antibiotic treatment (Stewart and Costerton, 2001).

The loss of efficacy of antibiotics to treat polymicrobial infections, including biofilm-associated infections, urgently calls for the development of novel antibacterial therapies.

1.2 Bacterial iron metabolism as a target for new antibacterials

Since resistance to existing antibiotic drugs is constantly increasing and the antibiotic discovery pipeline is running dry, several efforts have been devoted to the search of new antibacterial strategies. Recently, attention has been directed to exploiting stresses already imposed on bacterial pathogens by the *in vivo* environment or host defenses, as novel antimicrobial approach to combact bacterial infections. Withholding of nutrients to prevent bacterial growth represents one of the first lines of defense against bacterial infection, a phenomen known as "nutritional immunity" (Skaar, 2010). Depriving bacteria of essential nutrients, such as iron, represents a promising strategy, since iron is an important micronutrient for virtually all cells, including bacteria. The requirement of iron for pathogens is based on its essential role in numerous biological processes, like DNA synthesis, respiration, and protection against oxidative stress (Andrews *et al.,* 2003). Iron can exist in either the reduced ferrous Fe(II) or the oxidized ferric Fe(III) states, which make iron a major redox mediator in biology (Wandersman and Delepelaire, 2004). In anaerobic and/or reducing environments, the prevalent iron species is the reduced and soluble form Fe(II), which is easily accessible by bacteria. Conversely, under physiological conditions (*i.e.,* in the presence of oxygen at neutral pH) iron is mainly present in the oxidized form Fe(III), which aggregates in insoluble oxy-hydroxyl polymers becoming not readily usable by bacteria. In mammals, pathogenic bacteria are faced with an additional challenge imposed by the presence of many high-affinity iron-binding proteins (*e.g.*, transferrin and lactoferrin) and/or heme-containing proteins (*e.g.,* hemoglobin and myoglobin), which sequester iron in human tissues and body fluids, preventing oxidative damage, and making iron inaccessible for bacteria (Ratledge and Dover, 2000).

Bacterial pathogens overcome nutritional immunity by using efficient strategies to acquire this essential metal, such as: *i)* the production of small molecules, termed siderophores, that bind and transport Fe(III) (Miethke and Marahiel, 2007); *ii)* the ability to utilize heme iron (Wandersman and Delepelaire, 2004); *iii)* the ability to utilize Fe(II) under anaerobic conditions (Cartron *et al.,* 2006); *iv)* the ability to acquire iron through siderophores produced by other bacteria or fungi (xenosiderophores) (Winkelmann, 2007; Traxler *et al.,* 2012); and *v)* the ability to acquire iron from transferrin, lactoferrin or ferritin (Döring *et al.,* 1988; Britigan *et al.,* 1993; Cornelissen, 2003).

The most common strategies used by Gram-negative and Gram-positive bacteria are the expression of siderophores and heme-acquisition systems. Siderophores are low-molecular-weight microbial chelators (≈ 1 kDa or less) synthesized through dedicated metabolic pathways, and secreted in the extracellular environment to form tight and stable complexes with Fe(III), that are recognized by specific receptors on the bacterial cell surface (Schalk *et al.,* 2008). The heme-uptake systems can

be divided into two general categories, those that transport heme by direct contact between the bacterium and the heme sources, and those that in addition to transporting heme, also sense and signal the presence of extracellular heme *via* secreted low-molecular-weight proteins (approximatively 20 kDa), termed hemophores, able to sequester heme from natural sources and/or host proteins, and deliver it to cognate surface receptors (Huang and Wilks, 2017). The hemophores-dependent heme-uptake sytems are only found in Gram-negative bacteria, and the best biochemically and structurally characterized is the *has* system of *Serratia marcescens* (Biville *et al.,* 2004) and *P. aeruginosa* (Ochsner *et al.,* 2000).

In Gram-negative bacteria, the transport of Fe(III)-siderophores or heme-containing molecules from the surface receptors into the cell requires the aid of the TonB-ExbB-ExbD protein complex, that transduces the proton motive force (PMF) of the cytoplasmic membrane into energy required for the transport of the iron compounds across the OM into the periplasmic space. Subsequent transport through the inner membrane involves specific periplasmic binding proteins (PBP) and inner membrane ABC transporters (Hood and Skaar, 2012). Unlike Gram-negative bacteria, Gram-positive bacteria do not express a TonB-ExbB-ExbD complex to mediate iron transport, because they have no OM. Consequently, the uptake of Fe(III)-siderophores or heme containing molecules involves a membrane-anchored binding protein, which is similar to the PBP in Gram-negative bacteria, as well as an ABC transporter (Hood and Skaar, 2012). Once inside the bacterial cell, heme can either be incorporated into proteins that require heme as a cofactor, or cleaved by cytoplasmic heme oxygenases to release free iron (Tiburzi *et al.,* 2009). Conversely, Fe(III) must always be dissociated from siderophores either by hydrolysis, or modification of the siderophore scaffold, or reduction of Fe(III) to Fe(II), which has lower affinity for siderophores. While the majority of Fe(III)-siderophore complexes are internalized into the cytoplasm prior to iron release, for some siderophores Fe(III) is dissociated in the periplasm and then transported into the cytosol by dedicated inner membrane transporters (Schalk and Guillon, 2013).

Iron acquisition must be finely controlled, since both iron deficiency and iron overload can damage the cell. In fact, while too low intracellular iron cannot support bacterial growth, too high intracellular Fe(II) is toxic, due to its propensity to react with oxygen, *via* the Fenton-type reactions, generating reactive oxygen species (ROS), which can damage biological macromolecules, such as lipids, proteins and DNA (Visca, 2004). Therefore, bacteria must sense intracellular iron levels and induce or repress iron-uptake systems in response to cytoplasmic iron deficiency or proficiency, respectively. This sensing typically involves the transcriptional control mediated by the iron-dependent repressor, known as Fur (ferric uptake regulator), which is widespread among both Gram-positive and Gram-negative bacteria, and directly or indirectly controls of the expression of

iron-uptake genes (Ochsner and Vasil, 1996; Achenbach *et al.,* 1997; Carpenter and Payne, 2014; Haley and Skaar, 2012; Mortensen and Skaar 2013; Latorre *et al.,* 2018). Under iron-replete conditions, Fur forms a homodimeric complex with Fe(II) and binds to the Fur boxes situated in the promoter region of iron-transport genes, thereby repressing their transcription. Upon iron limitation, the Fur-Fe(II) complex is not formed, so that the Fur protein cannot associate with promoters and iron-transport genes are expressed (Bagg and Neilands, 1987).

In addition, small regulatory RNAs (sRNAs), controlling the expression of iron-containing proteins, when intracellular iron levels are sufficiently high, have been identified in many bacterial pathogens. These sRNAs are directly repressed by Fur at the transcriptional level, and in turn repress the expression of target genes at the post-transcriptional level, thereby ensuring that iron-utilizing proteins are efficiently expressed only under conditions of intracellular iron availability (Salvail and Massé, 2012; Oglesby-Sherrouse and Murphy, 2013).

1.2.1 Novel strategies to interfere with bacterial iron metabolism

Given the crucial role of iron in bacterial physiology, and the very low amount of free iron in mammals, it is not surprising that mutations in specific iron-uptake systems have been found to dramatically reduce the ability of several bacteria to cause infection in a variety of animal models (Takase *et al.,* 2000; Velayudhan *et al.,* 2000; Brickman *et al.,* 2007; Fetherston *et al.,* 2010; Miranda-Casoluengo *et al.,* 2012; Thomas-Charles *et al.,* 2013; Wells *et al.,* 2013; Minandri *et al.,* 2016). These observations greatly support the therapeutic exploitation of bacterial iron homeostasis for the development of new antibacterials (Ballouche *et al.,* 2009; Foley and Simeonov, 2012). Novel strategies to interfere with bacterial iron metabolism have been proposed in search of new antibacterial therapies, and include: *i*) the use of iron chelators to out-compete binding of iron to siderophores; *ii*) the use of inhibitor compounds targeting enzymatic steps of iron metabolism; and *iii*) the "Trojan Horse" strategy to take advantage of the iron-uptake systems to deliver antimicrobial compounds inside the cell (Figure 5) (Ballouche *et al.,* 2009; Foley and Simeonov, 2012).

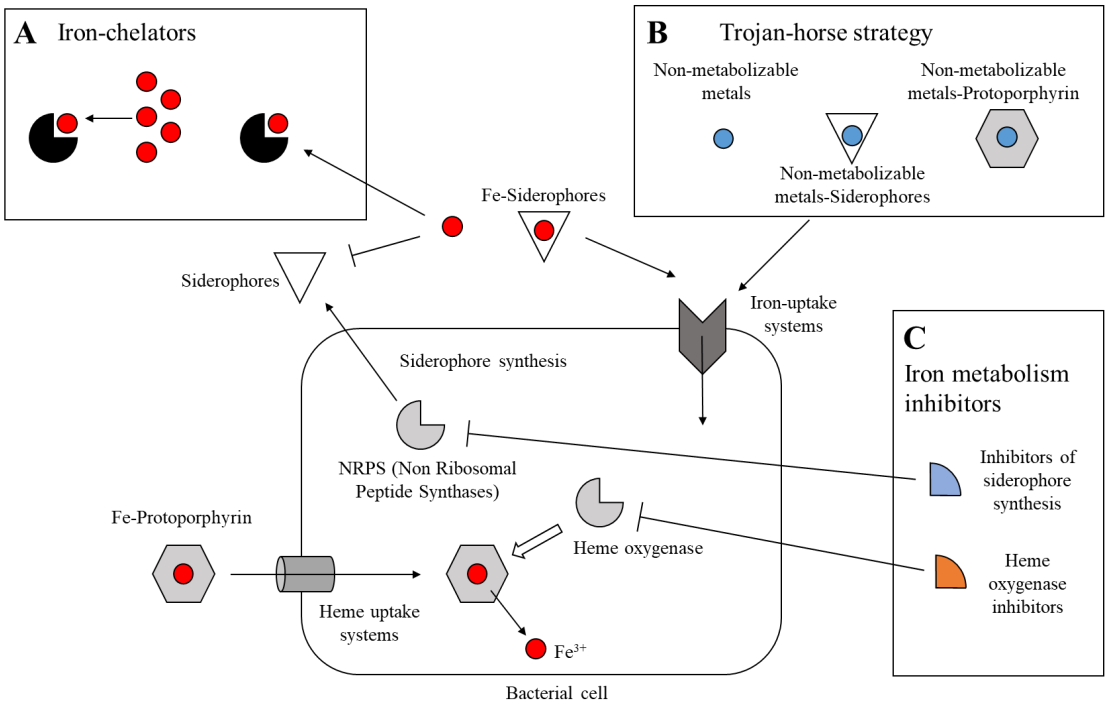


Figure 5. Antibacterial strategies interfering with iron metabolism. (A), Chelators deprive bacterial cells of iron. (B), Through the so-called "Trojan-horse" strategy, some antibacterial compounds take advantage of the natural iron-uptake routes to enter bacterial cells. Among these compounds, non-metabolizable metals which may enter bacterial cells either alone or complexed with siderophores or protoporphyrin. (C), Inhibitors of iron metabolism are compounds that interfere with different iron metabolism processes such as siderophore synthesis or heme degradation by heme oxygenase (modified from Ballouche *et al.,* 2009).

During the last years, several new iron chelators have been introduced in the clinical practice for the treatment of iron overload in humans, which could also withhold iron from invading bacterial pathogens, by competing with iron, and suppress their growth. Although at the infection site this role is usually carried out by Tf or Lf, iron chelators with low toxicity and good clinical tolerance (*e.g.,* deferoxamine, deferiprone and deferasirox) have been tested as antibacterial agents, to increase bacterial iron starvation (Ballouche *et al.,* 2009; Foley and Simeonov, 2012). Unfortunately, these compounds did not show strong inhibitory activity and, paradoxically, in some bacterial species they may even act as growth promoters rather than inhibitors. This is because some bacterial species (*e.g., P. aeruginosa* and *A. baumannii*) express surface receptors able to recognize and transport also synthetic chelators or xenosiderophores, and therefore they gain advantage from using such chelators as growth promoters (Kontoghiorghes *et al.,* 2010; Visca *et al.,* 2013). These aspects should be taken into account for the future development of iron chelators-based antimicrobial therapies.

Iron starvation can also be achieved using compounds that suppress the bacterial capacity to acquire or to utilize iron. Iron-acquisition mechanisms which are considered as "targetable" include siderophores biosynthesis. Although encouraging results have been obtained from studies focused on the identification of siderophore-biosynthetic inhibitors in different bacteria species (*e.g.,*

Escheriachia coli, *Yersinia pestis*, *A. baumannii* and *P. aeruginosa*) (Ballouche *et al.,* 2009; Foley and Simeonov, 2012), the redundancy and multiplicity of siderophore biosynthesis gene clusters (*e.g.,* two in *P. aeruginosa*, and at least four annotated in *A. baumannii*) make this strategy ineffective, since it should imply to find inhibitors for a great number of specific enzymes.

One of the most successful therapeutic approach to hijack bacterial iron metabolism, is the so-called "Trojan-horse strategy", *i.e.,* the delivery of antibacterial compounds, either alone or complexed with suitable carriers, that are actively up-taken by the bacterial cell (Ballouche *et al.,* 2009; Foley and Simeonov, 2012). Among these compounds, the most promising are non-metabolizable bactericidal metals, and in particular the post-transition metal gallium Ga(III), delivered alone or complexed with siderophores, chelators or PPIX (Frangipani *et al.,* 2014; Minandri *et al.,* 2014; Arivett *et al.,* 2015) to facilitate the internalization of antibacterial compounds by specific bacterial receptors, and consequently promote its inhibitory activity. Ga(III) was discovered in 1875 by the French chemist Lecoq de Boisbaudran, and took its name from the Gaul region (the latin "Gallia"). Although Ga(III) has no known physiologic function in living cells, the peculiar chemical characteristics make it to interact with cellular processes and biologically important proteins or enzymes, especially those involved in iron metabolism (Chitambar, 2010). The chemical properties of Ga(III) are nearly similar to those of Fe(III) and this allows Ga(III) to substitute Fe(III) in the prosthetic group of several enzymes (Bernstein, 1998). Ga(III) can perturb iron metabolism being incorporated into essential enzymes and proteins in place of Fe(III). Since Ga(III) can not be reduced under physiological condition, it cannot take part in redox reactions, inhibiting a number of essential functions. This has led to the development of Ga(III) compounds as diagnostic and therapeutic agents in medicine, especially in the area of metabolic bone disease, cancer, and infectious disease. In 2003, a pharmaceutical formulation of citrate-buffered gallium nitrate $Ga(NO_3)_3$ [Ganite® (Genta)] was approved by the FDA for the treatment of malignancy-associated hypercalcemia, preventing the release of calcium from bone in patients with metastatic lesions (Chitambar, 2010). Recently, Ga(III)-based drug, including simple salts such as Ga(III)-nitrate (GaN) and Ga(III)-chloride ($GaCl_3$), and metal-organic complexes such as Ga(III)-maltolate (GaM) (Bernstein *et al.,* 2000), and Ga(III)-protoporphyrin IX (GaPPIX), have then been developed, showing additional therapeutic properties such as immunomodulating and antimicrobial activity (Chitambar, 2017) (Figure 6). The fascinating possibility of developing Ga(III)-based therapies to treat bacterial infections is proved by ongoing clinical trials testing the pharmacokinetics, safety and tolerability of Ganite® administration to CF patients chronically infected by *P. aeruginosa* (ClinicalTrials.gov identifier: NCT01093521 and NCT02354859; Goss *et al*., 2012, 2018).

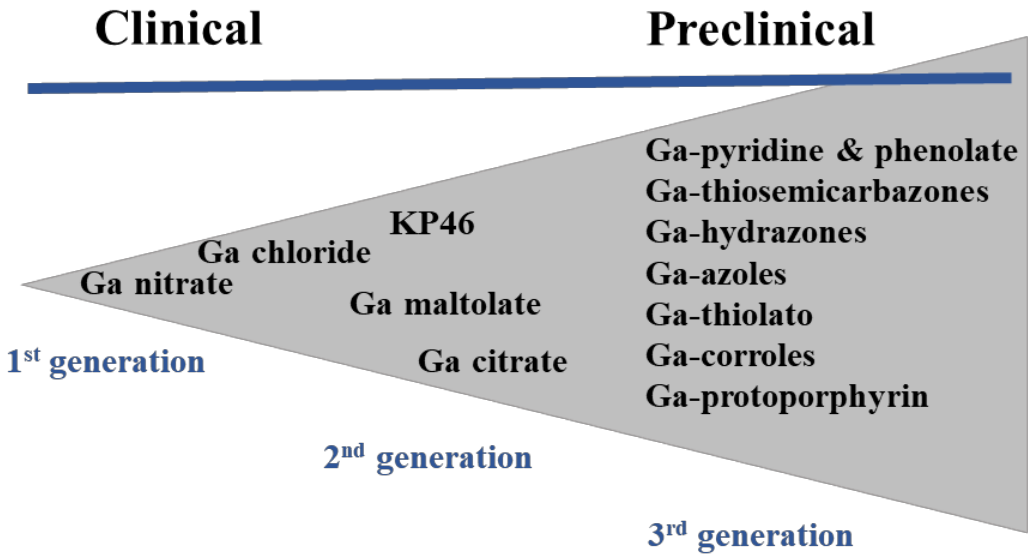


Figure 6. Development of Ga(III)-based compounds with therapeutic potential. Recently, there has been an expansion in the number of Ga(III)-based drugs showing therapeutic potential, sometimes categorized in first-, second-, and third- generations of Ga(III) formulations, and ranging from simple salts such as Ga(III) nitrate and Ga(III) chloride, through metal-organic complexes such as Ga(III)-maltolate and Ga(III)-protoporphyrin IX, that have demonstrated preclinical and clinical activity (Chitambar, 2017).

The successfully Trojan-horse strategy, using Ga(III) either alone or complexed with suitable carriers, was investigated on a wide range of pathogenic bacteria (Frangipani *et al.,* 2014; Minandri *et al.,* 2014; Arivett, *et al.,* 2015; Runci *et al.,* 2016).

Conjugation of Ga(III) with either siderophores or iron chelators is an attractive strategy to increase antibacterial potency, but it is not endowed with broad-spectrum activity, due to the species specificity of many iron-uptake systems (Hider and Kong, 2010). Therefore, these drug conjugates could represent alternative therapeutic options, but only for specific bacterial pathogens. On the other hand, the use of compounds that mimic normal iron sources, has been demonstrated a viable strategy to interfere with bacterial iron metabolism, since iron/heme mimetics are not species-specific compounds (Minandri *et al.,* 2014; Arivett, *et al.,* 2015; Runci *et al.,* 2016). For these reasons, three Ga(III) compounds, likely endowed with a broad-spectrum activity, namely GaN, GaM and GaPPIX, have been used in this PhD thesis to evaluate the possibility to interfere with iron metabolism of ESKAPE pathogens, and those associated with CF disease (Hijazi *et al.,* 2018 – Chapter 3, Chapter 4).

Unlike most antibiotics, which have a specific molecular target within the bacterial cell, Ga(III) is a typical multitarget drug that is predicted to impair several Fe(III)-dependent functions, as recently demonstrated (Goss *et al.,* 2018). Notably, selection of Ga(III)-resistant cells, seems less likely to occur compared to conventional antibiotics (Ross-Gillespie *et al.,* 2014; Goss *et al.,* 2018).

1.3 *Pseudomonas aeruginosa* as paradigmatic model of human pathogen: general characteristics and pathogenicity mechanisms

P. aeruginosa is a non-spore forming, Gram-negative, rod-shaped γ-proteobacterium measuring 0.5 to 0.8 μm by 1.5 to 3.0 μm (Figure 7).

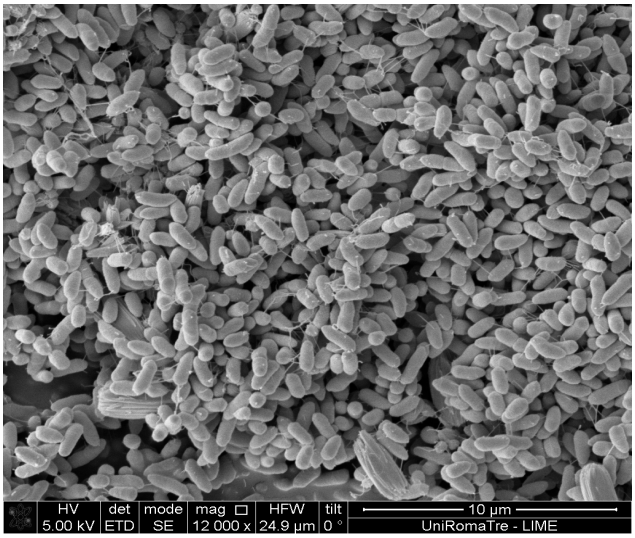


Figure 7. Scanning electron micrograph of *P. aeruginosa* strain PAO1.

It is ubiquitous and can occupy a variety of environmental niches, such as water, soil, animals, plants, sewage, hospitals, and humans (Zago and Chugani, 2016). The ubiquity could be attributed to its versatile metabolism. *P. aeruginosa* grows aerobically using multiple terminal oxidases, but it is also able to grow anaerobically using nitrate and nitrite as terminal electron acceptors (reviewed in Arai, 2011). Moreover, *P. aeruginosa* is also able to ferment arginine and pyruvate to produce energy (Vander Wauven *et al.,* 1984; Eshbach *et al.,* 2004).

P. aeruginosa is considered among the leading causes of HAIs. It is a typical opportunistic pathogen responsible for a wide range of infections, including lung, eyes, ears, urinary tract, burns, and bloodstream infections, especially in critically ill and immunocompromised patients (Lyczak *et al.,* 2000). The most important clinical manifestation of nosocomial *P. aeruginosa* infections is ventilator-associated-pneumonia (VAP), with approximatively 20% prevalence and dramatically high mortality and huge costs (Fujitani *et al.,* 2011; Kollef *et al.,* 2014). *P. aeruginosa* is considered the main bacterial pathogen responsible for severe lung deterioration in patients suffering from CF. Moreover, *P. aeruginosa* infection is also frequent among patients with chronic obstructive pulmonary disease (COPD), a progressive disorder of the lung parenchyma affecting old people and causing roughly 2.7 million deaths yearly (Döring *et al.,* 2011).

The remarkable large genome (typically between 6 and 7 Mb), containing a particularly large

proportion of regulatory genes, as well as a high number of genes involved in the catabolism, transport, and efflux of organic compounds (Stover *et al.,* 2000; Silby *et al.,* 2011), contributes to the ability of *P. aeruginosa* to cause wide range of infections (Sadikot *et al.,* 2005). During acute infections, the bacterium reversibly regulates gene expression to optimize its phenotypic adaptation in response to the environmental signals. Indeed, *P. aeruginosa* produces an array of virulence factors, including secreted factors, such as pyocyanin, elastase, proteases, phospholipase C, hydrogen cyanide, exotoxin A, exoenzyme S, and rhamnolipid, as well as cell-associated factors, such as, flagella, lipopolysaccharide and pili (Wagner and Iglewski, 2008). The production of many of these virulence factors is coordinatly regulated by small diffusable molecules, *via* a mechanism termed quorum sensing (Cornelis and Dingemans, 2013).

Once infection has been established, *P. aeruginosa* adapts chronic mode of survival (Smith *et al.,* 2006; Marvig *et al.,* 2015), in which reversibile gene regulation is often lost, leading to the development of mutants that vary genotypically and phenotypically from the originally infecting strain (Bragonzi *et al.,* 2009). Consequently, diverse phenotypic traits, including the loss of motility, acquisition of mucoidy phenotype caused by overproduction of the polysaccaharide alginate, and antibiotic resistance caused by efflux pump regulation, biofilm formation and persister cells, have been described in *P. aeruginosa* strains isolated at different stages during chronic lung infections in CF individuals (Bragonzi *et al.,* 2009; Winstanley *et al.,* 2016). *P. aeruginosa* switches its lifestyle from planktonic to sessile during chronic infections, forming a high-organized biofilm, an assemblage of microbial cells that irreversibly attach to one another and to a substratum, and that are encased in a self-produced polymer matrix, called Extracellular Polymeric Substance (EPS), which consists of polysaccharides, nucleic acids, lipids and proteins. Biofilm formation initiates when bacterial cells reach a surface and anchor to the site. Then, the biofilm begins to grow producing the exoplysaccharide that establishes the matrix, and then mature from microcolonies to multilayered cell bunches, and finally cells disperse by active or passive detachment (Figure 8). Biofilm can be mono- or poly-microbial, depending on the number (*i.e.,* one or many) of bacterial species that form it, and offers barrier and protection from external harms, such as toxic chemicals, host defence molecules and antibiotics, which makes biofilm-associated infections difficult to eradicate (Hall-Stoodley and Stoodley, 2009; Lieleg *et al.,* 2011). The clinical relevance of *P. aeruginosa* biofilms has augmented with the increasing prevalence of biofilm-associated infections (Costerton *et al.,* 1999; Costerton and Stewart, 2001; McDougald *et al.,* 2011).

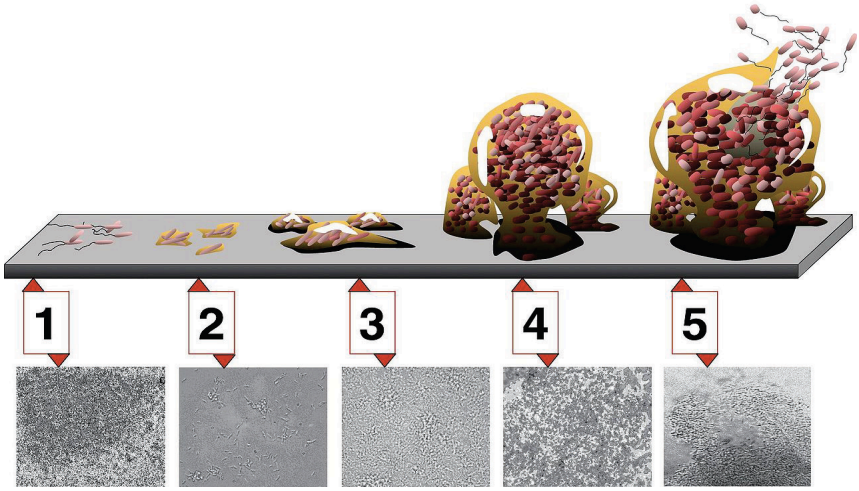


Figure 8. Five stages of biofilm development coupled with a photomicrograph of a developing *P. aeruginosa* biofilm. *P. aeruginosa* initiates the biofilm growth mode when free-floating cells adhere to a surface (1), then anchor themselves using cell adhesion structures, such as pili (2), and begin to grow by producing the exoplysaccharide that establishes matrix (3). Later, the biofilm become mature with a well-defined architecture of the matrix, and finally cells disperse by active or passive detachment (5) (Monroe, 2007).

Therapeutic options to treat acute and chronic *P. aeruginosa* infections are restricted, due to the ability of this bacterium to resist to a broad range of antimicrobial agents. Both intrinsic and acquired resistance to several antibiotics make *P. aeruginosa* a challenging bacterial pathogen, and lead to the spread of multidrug resistance (MDR) clinical isolates (Moore and Flaws, 2011). *P. aeruginosa* is intrinsically resistant to different antibiotics, as a consequence of the following mechanisms: *i)* the low permeability of its OM which reduces the entrance of the antimicrobial agents into the cell; *ii)* the constitutive expression of various efflux pump systems, which are transport proteins able to expel a wide range of substances out of the cell, including antibiotics (Benz and Hancock, 1981; Schweizer, 2003); and *iii)* the natural production of antibiotic-inactivating enzymes, such as the β-lactamase AmpC, which is localized in the periplasm, and whose expression is inducible by subinhibitory concentrations of certain β-lactams (Juan *et al.,* 2005).

P. aeruginosa can further acquire resistance mechanisms by horizontal genetic transfer from other bacteria, subsequently results in the generation of PDR strains, for which the commercially available antibiotics are no longer effective (Tseng *et al.,* 2009), warranting the inclusion of *P. aeruginosa* among the ESKAPE pathogens (Rice, 2008).

At present, few new anti-*Pseudomonas* drugs are available, and this has caused a return to old drugs. In particular, colistin (polymixin E), which acts on the OM of the bacteria by increasing its permeability and causing cell-death, was originally abandoned for long time by clinicians due to its toxic side effects (*i.e.,* nephrotoxicity and neurotoxicity), is now routinely administered *via* inhalation in CF patients suffering recurrent infections with MDR strains of *P. aeruginosa* (Falagas

and Kasiakou, 2006), and has recently been reformulated as injectable drug (Coly-Mycin M Parenteral). Unfortunately, the increase in use of colistin for treating MDR Gram-negative bacterial infections has resulted in the evolution of colistin-resistant strains, also including *P. aeruginosa* (Lee *et al.,* 2012, 2016). Therefore, new antibiotic formulations such as amikacin incorporated into liposomes, fosfomycin in combination with tobramycin, and inhaled solution of levofloxacin, are being explored to treat *P. aeruginosa* infections in CF patients (Smith *et al.,* 2017).

All the above reasons led to the definition of *P. aeruginosa* as one of the "red alert" pathogens that deeply menaces the utility of our antibacterial armamentarium (Maltezou, 2009; Poole, 2011).

The high morbidity and mortality associated with *P. aeruginosa* infections toghether with the scarcity of effective drugs to treat infections caused by MDR and PDR strains prompted the World Health Organization in 2017 to list *P. aeruginosa* on the high priority list for research and development of new drugs (World Health Organization, 2017).

1.3.1 The contribution of iron metabolism to *P. aeruginosa* pathogenicity

Like almost all pathogenic bacteria, *P. aeruginosa* has an absolute need for iron to cause infections and to persist within the host (Ratledge and Dover, 2000). This metal is involved in many cellular reactions and acts as a cofactor for a variety of enzymes (Andrews *et al.,* 2003). However, in the human host, the incorporation of iron into heme-containing molecules (*e.g.,* hemoglobin and myoglobin) and iron carrier proteins (*e.g.,* transferrin and lactoferrin) render this metal unavailabale for invading bacteria (Weinberg, 2009). As a consequence, *P. aeruginosa* has evolved multiple pathways to actively acquire this important nutrient, such as *i)* the production of two siderophores, namely pyoverdine (PVD, Meyer and Abdallah, 1978; Cox and Adams, 1985) and pyochelin (PCH, Cox *et al.,* 1981; Heinrichs *et al.,* 1991); *ii)* the ability to utilize a wide range of xenosiderophores (Cornelis and Matthijs, 2002; Cornelis *et al.,* 2009; Llamas *et al.,* 2014); *iii)* the ability to utilize heme-iron using two heme-uptake systems (Ochsner, 2000); and *iv)* the ability to acquire Fe(II) through the Feo system (Cartron *et al.,* 2006) (Figure 9).

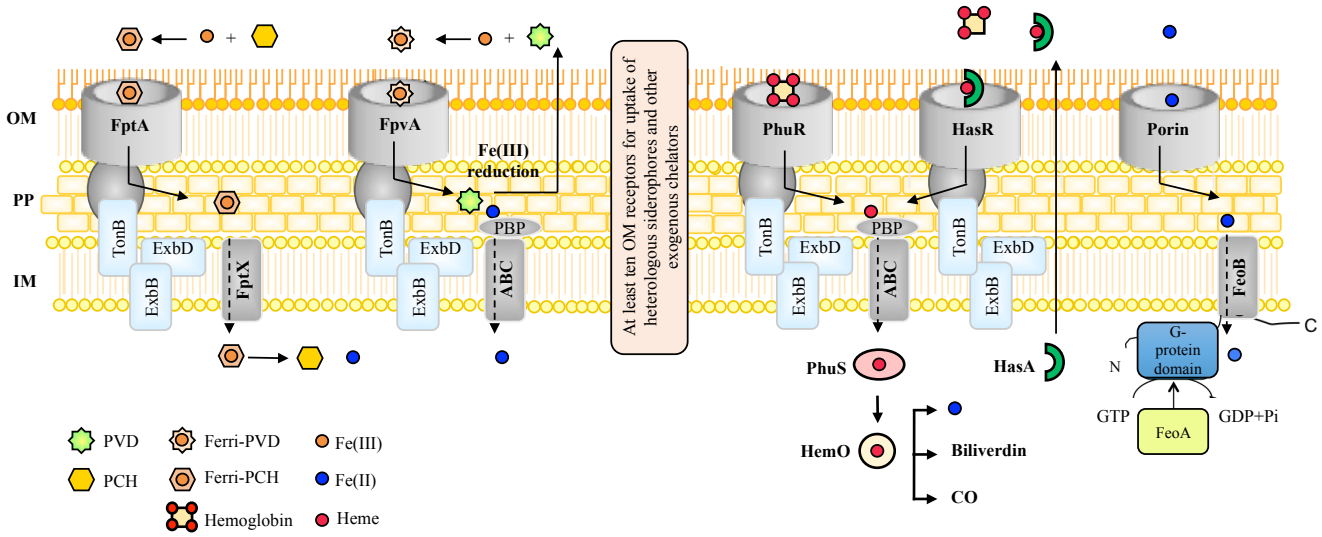


Figure 9. Schematic representation of iron-uptake systems of *P. aeruginosa*. To deal with iron starvation, *P. aeruginosa* has developed a plethora of mechanisms to actively acquire iron. It produces two siderophores PVD and PCH that bind Fe(III). The resulting ferri-siderophore complexes are transported across the outer membrane (OM) of the bacteria into the periplasm by specific TonB-dependent receptor proteins FpvA (PVD receptor) and FptA (PCH receptor). PVD remains in the periplasmic space, where Fe(II) is released after reduction of Fe(III), and then Fe(II) is sequestered by a specific periplasmic binding proteins (PBP) that transported it into the cytoplasm by an ABC transporter. Finally, a specific efflux pump (not shown) exports PVD molecules outside the bacterial cell (PVD recycling). Conversely, PCH is transported into the cytoplasm by the FptX permease, located on the inner membrane (IM), prior to Fe(II) release. In addition to the endogenous siderophores, *P. aeruginosa* has the ability to hijack heterologous siderophores and exogenous chelators to acquire iron (not shown). The bacterium has two heme-uptake systems (*phu* and *has*) to acquire heme iron. In the *phu* system, the tonB-dependent PhuR receptor binds directly hemoproteins removing heme, whereas the tonB-dependent HasR receptor in *has* sytem binds heme complexed to the secreted hemophore protein HasA. Both PhuR and HasR receptors transport heme into the periplasm, then heme binds a PBP that transports it to the cytoplasm, through an ABC transporter. Once in the cytoplasm, heme is bound to a heme chaperone PhuS that delivers it to the heme oxygenase HemO for releasing of Fe(II), biliverdin, and CO. Moreover, *P. aeruginosa* has also the ability to acquire Fe(II) through the Feo system. Different from all iron-acquisition sytems, which require the aid of the TonB-ExbB-ExbD protein complex, the soluble Fe(II) can presumably diffuse into the periplasm through undefined porins. Fe(II) is then transported across the IM into the cytoplasm by FeoB, thanks to a GTP-driven active transport process. The FeoB protein possesses a G-protein domain at its N-terminus and C-terminal integral-membrane domain is predicted to consist of eight transmembrane α-helices. FeoA is thought to be required for maximal FeoB activity and it can act by activating the GTPase function of FeoB. IM: bacterial inner; PP: periplasm; OM: bacterial outer membrane.

Receptor-mediated iron uptake in case of Fe(III) carriers is an energy-demanding process, which depends on proton consumption by the conserved TonB-ExbB-ExbD energy-transducing complex (see section 1.2). Depending on the iron availability, bioavalability of the iron pool, stage of infection and presence of competing pathogens, *P. aeruginosa* adapts and deploys specific iron-uptake systems.

The primary iron-uptake system used by *P. aeruginosa* is the production of the PVD siderophore, which binds Fe(III) with a very high affinity, and is even able to displace iron from Tf and deliver it into bacterial cell. Moreover, PVD is also a signal molecule since it triggers the production of virulence factors, such as the protease PrpL and the potent exotoxin A (Wilderman *et al.,* 2001; Lamont *et al.,* 2002; Tiburzi *et al.,* 2008), important for the colonization and invasion of human host. Given that PVD is involved in the regulation of virulence factors contributing to colonization

and invasion of the human host, it seems to be essential to cause acute infection (Cornelis and Dingemans, 2013). Interestingly, the prominent role of PVD in pathogenicity has been demonstrated in different mouse model of infections (Meyer *et al.,* 1996; Takase *et al.,* 2000; Xiong *et al.,* 2000; Imperi *et al.,* 2013; Minandri *et al,* 2016). PVD production is also important for biofilm formation, which is a typical trait of chronic infection. In fact, it has been demonstrated that mutants that do not produce PVD are unable to generate mature biofilms under iron-poor conditions, but the addition of exogenous pyoverdine to the growth media restores the biofilm formation ability of PVD defective mutants (Banin *et al.,* 2005; Patriquin *et al.,* 2008). Although PVD is involved in different biological processes, ranging from host invasion to biofilm formation, its production is energy consuming for *P. aeruginosa*, given that its biogenesis occurs through non ribosomal peptide synthesis and requires the activity of many different enzymes (Visca *et al.,* 2007).

The second siderophore produced by *P. aeruginosa* is PCH, and althought it has a lower affinity for Fe(III) compared with PVD, its biosynthesis is a more energy-efficient process (Ravel and Cornelis, 2003; Dumas *et al.,* 2013). It has been demonstrated that *P. aeruginosa* firstly produces pyochelin and then switches to pyoverdine when iron concentration becomes extremely low (Dumas *et al.,* 2013). In chronic infections, such as those encountered in CF lungs, the production of PCH could play a role in the sustained inflammatory response which is known to occur and cause damage to tissues (Lyczak *et al.,* 2002), and the involvement of PCH-mediated iron uptake in the pathogenicity of *P. aeruginosa* in mouse model of pulmonary infection has recently been reported (Minandri *et al.,* 2016).

In addition to the endogenous siderophores, *P. aeruginosa* has the ability to utilize a wide range of xenosiderophores, which could contribute to the pathogenicity of this bacterium in the CF lung infections, since the microbial flora of the CF respiratoy tract represents a complex and diverse ecosystem, in which multispecies communities coexist and evolve during infection.

Although the majority of heme in the host is bound to intracellular heme-proteins, primarily haemoglobin (Stojiljkovic and Perkins-Balding, 2002), free heme and haemoglobin are released by damaged cells during infection. Therefore, it is not surprisingly that successful pathogens, particularly *P. aeruginosa*, are adept to utilize heme iron. In fact, *P. aeruginosa* expresses two heme-uptake systems, namely *phu* (*Pseudomonas* heme uptake) and *has* (heme assimilation system) (Ochsner *et al.,* 2000).

The *phu* system allows the direct acquisition of heme from hemoproteins to the TonB-dependent OM receptor PhuR (Ochsner *et al.,* 2000), whereas the *has* system uses a secreted hemophore HasA to extract heme from hemoproteins and delivers it to the TonB-dependent OM receptor HasR (Létoffé *et al.,* 1998; Takase *et al.,* 2000). Once in the periplasm, heme binds to a periplasmic

binding protein (PBP) and enters the cytoplasm by the cognate ABC transporter. Once inside the cytoplams, heme is bound to heme chaperone PhuS before being delivered to the heme oxygenase HemO for releasing of Fe(II), CO and biliverdin (Bhakta and Wilks, 2006; Lansky *et al.,* 2006; Barker *et al.,* 2012; O'Neill *et al.,* 2012). Recently, it has been demonstrated that, during chronic lung infection in CF patients, heme uptake is a critical iron source for *P. aeruginosa*, with increasing utilization of heme and consistent expression of HemO during prolonged infection (Nguyen *et al.,* 2014). Moreover, another study by Marvig et al. (2015) showed an evolution of *P. aeruginosa* towards heme utilization within the host coupled with loss of siderophore PVD production.

Ferrous iron transport is generally essential for bacteria growing under low oxygen conditions and/or acidic environments, as those encountered in the lung of CF patients, conditions that promote ferrous iron stability. The highly soluble Fe(II) ions can freely diffuse through porins that constitute the OM channels then its uptake into the cytoplasm is mediated mainly *via* the Feo transport encoding by *feoABC* operon (Cartron *et al.,* 2006). It has been proposed that the Feo system could play a role in iron uptake during chronic lung infection in CF patients, due to the ability of phenazines, which are secondary metabolites produced by *P. aeruginosa*, to reduce Fe(III) to Fe(II). In fact, it has been shown that phenazines and Fe(II) accumulate in the lung of CF patients during chronic *P. aeruginosa* infection (Hunter *et al.,* 2012, 2013). In addition, deletion of the *feoB* gene results in the loss of *P. aeruginosa* ability to form biofilms and attenuates its virulence (Wang *et al.,* 2011).

Of note, *P. aeruginosa* has also developed mechanisms to release iron from iron-binding proteins such as Tf, during infection, through the expression of elastase, the alkaline protease AprA (Kim *et al.,* 2006), and the endoprotease PrpL (Wilderman *et al.,* 2001; Döring *et al.,* 1998). Moreover, *P. aeruginosa* proteases are alos effective in removing iron from ferritin, which is found to be abundant in the CF individuals (Dehner *et al.,* 2013).

The expression of all iron-acquisition systems is shut off in the presence of sufficient intracellular iron, due to the negative regulation exerted by the Fur repressor (Ochsner and Vasil, 1996; Poole and Mckay, 2003; Cartron *et al.,* 2006) (section 1.2). Fur is a 15 kDa protein, which has been proposed to be essential in *P. aeruginosa*, since several attempts to obtain knock-out mutants in the *fur* gene failed (Barton *et al.,* 1996; Hassett *et al.,* 1996; Llamas *et al.,* 2014). However, a *fur* conditional mutant endowed with very low levels of Fur has been recently generated (Pasqua *et al.,* 2017).

The redundancy and the complexity that characterize the iron-uptake mechanisms of *P. aeruginosa* underline the importance of this metal for its basic metabolism and for the outcome of infections caused by this pathogen.

1.3.2. The post-transition metal gallium as an anti-*Pseudomonas* drug

Serveral strategies have been used to interfere with iron metabolism of pathogenic bacteria, as outlined in section 1.2.1. One such promising alternative approach is the "Trojan horse" strategy using the post-transition metal Ga(III). Ga(III) was proven to be potent anti-bacterial and anti-biofilm activity against several pathogenic bacteria, including *P. aeruginosa* (Frangipani *et al.,* 2014; Minandri *et al.,* 2014; Arivett *et al.,* 2015; Runci *et al.,* 2016). The fascinating possibility of developing Ga(III)-based therapies to treat *P. aeruginosa* lung infection is proved by the ongoing of clinical trials testing the pharmacokinetics, safety and tolerability of Ganite® administration in CF patients chronically infected by *P. aeruginosa* (ClinicalTrials.gov identifier: NCT01093521 and NCT02354859; Goss *et al.,* 2012, 2018). Indeed, Phase 1 clinical trial on CF patients based on intravenous GaN administration (ClinicalTrial.gov, NCT01093521) has recently shown that Ga(III) improves lung function in people with CF and *P. aeruginosa* chronic lung infection (Goss *et al.,* 2018). Moreover, phase 2 clinical trial is in progress, to provide further evidence about safety and clinical efficacy in adults CF patients chronically infected with *P. aeruginosa* (www.clinicaltrial.gov; NCT02354859).

Although complete pathway by which Ga(III) is internalized by bacteria is not fully elucidated, many Ga(III) entrance routes have been investigated in *P. aeruginosa*. These include siderophore uptake pathways, and the periplasmic HitAB iron-binding proteins, that can transport Ga(III) ions accross the cell membrane (Kaneko *et al.,* 2007; García-Contreras *et al.,* 2013; Frangipani *et al.,* 2014). In this context, many Ga(III) compounds alone, such as, GaN, $GaCl_3$ and GaM, or complexed with organic compounds, including siderophores, iron chelators, and PPIX, have been used to interfere with bacterial iron metabolism (Banin *et al.,* 2008; Frangipani *et al.,* 2014). GaN at low concentrations was found to effectively inhibit the growth of *P. aeruginosa*, prevent biofilm formation, and kill both planktonic bacteria and established biofilms *in vitro*. Moreover, GaN showed effective protection in two different experimental animal infection models simulating acute lethal pneumonia and chronic lung biofilm infection (Kaneko *et al.,* 2007). Similarly to what observed with GaN, $GaCl_3$ showed a potent antibacterial activity against both *P. aeruginosa* in planktonic and biofilm mode of growth (Banin *et al.,* 2008). Interestingly, the orally active GaM, which is endowed with high bioavalability and low toxicity (Bernstein *et al.,* 2000), was shown to

be effective, even more than GaN, in preventing *P. aeruginosa* cell proliferation in a mouse burn wound model infection (DeLeon *et al.,* 2009). The solubility of GaM in both water and lipids, allowing for the penetration of cell walls and membranes, as opposed to the lack of lipophylicity of GaN, probably accounts for much of the difference in biologic effects between the two compounds (Bernstein *et al.,* 2000; DeLeon *et al.,* 2009). In addition to simple organic and inorganic Ga(III) compounds, several studies have investigated the possibility of using Ga(III) complexed with organic compounds, including synthetic chelators, siderophores, or PPIX (as Trojan horses), with the aim to enhance Ga(III) delivery in *P. aeruginosa*, and consequently the antibacterial potency. Although, Ga(III) coupled with the iron chelator desferrioxamine DFO showed antimicrobial activity against *P. aeruginosa* by killing free-living bacteria and by blocking biofilm formation, its anti-*Pseudomonas* activity was slightly more effective than $GaCl_3$ alone (Banin *et al.,* 2008). Ga(III)-coupled with the iron chelator citrate [Ga(III)-citrate complex] showed higher bacteriostatic and bactericidal activities than Ga(III)-DFO, plausibly due to more efficient uptake of Ga(III)-citrate by *P. aeruginosa* cells (Rzhepishevska *et al.,* 2011). Interestingly, Ga(III)-PCH was found to be more efficient than GaN alone in inhibiting *P. aeruginosa* growth, and this was dependent on increased Ga(III) delivery through the PCH siderophore uptake pathway (Kaneko *et al.,* 2007; Frangipani *et al.,* 2014). Surprisingly, no effect of Ga(III) coupled with PPIX had been reported on *P. aeruginosa*, despite the presence of two heme-uptake systems in this bacterial species (Stojiljkovic *et al*., 1999). The fascinating hypothesis to use GaPPIX as an anti-*Pseudomonas* compound has been investigated in this PhD thesis (Hijazi *et al.,* 2017 – Chapter 2).

1.3. Aims of the thesis

The inexorable rise in the incidence of antibiotic resistance in bacterial pathogens, coupled with the disappointingly low discovery rate of new antibiotics, calls for the need to develop new antimicrobial therapies (Bonomo and Szabo, 2006; Poole, 2011; Roca *et al.,* 2012; López-Causapé *et al.,* 2013, 2015; Nowak *et al.,* 2017). All pathogenic bacteria, including *P. aeruginosa*, require iron as a cofactor for many vital enzymes. However, free iron is usually present at very low concentrations in body fluids of mammals, being sequestered by iron-containing proteins (Chapter 1). While iron withholding by the host is an important component of innate immunity, pathogens have fought back by developing high efficient iron-uptake systems to acquire this essential nutrient (Chapter 1). Recent studies have explored the possibility of using iron mimetics as novel therapeutics, which would interfere with iron metabolism and likely display pleiotropic effects, and therefore lowering the possibility to develop resistance. One such mimetics which holds promise as an antibacterial agent is Ga(III). The use of Ga(III) for anticancer therapy in humans has been approved long time ago by the FDA of USA, but only lately Ga(III) has been repurposed as an antimicrobial agent (Rangel-Vega *et al.,* 2015; Soo *et al.,* 2017), causing the inactivation of iron-dependent redox processes in bacterial cells (Bonchi *et al.,* 2014; Minandri *et al.,* 2014). Recently, there has been an expansion in the number of Ga(III)-based drugs showing therapeutic potential, categorized in first-, second-, and third- generations of Ga(III) formulations, and ranging from simple salts such as $GaCl_3$ and GaN, through metal-organic complexes such as GaM (Bernstein *et al.,* 2000) and GaPPIX (Chitambar, 2017) (Chapter 1).

The main aim of this PhD thesis was to explore the possibility to interfere with bacterial iron metabolism with the post-transition metal Ga(III), as an alternative approach to combact bacterial infections.

Using the paradigmatic model of human pathogen *P. aeruginosa*, the antibacterial effect of GaN in both planktonic- and biofilm-living bacteria has been well documented (Kaneko *et al.,* 2007; Banin *et al.,* 2008; DeLeon *et al.,* 2009). Moreover, Ga(III) complexed with PCH siderophore has been found to potentiate the inhibitory activity of Ga(III), thereby increasing its entrance into bacterial cells (Frangipani *et al.,* 2014). In the first part of this PhD work, the effect of Ga(III) coupled PPIX, which is a heme mimetic, was evaluated on *P. aeruginosa*, in order the improve the antibacterial activity of Ga(III).

The opportunistic human pathogen *P. aeruginosa* is a member of the ESKAPE group, responsible for high frequencies of MDR bacteria associated with nosocomial infections, for which the lack of comparative data on the activity of Ga(III) in standard conditions represents a major pitfall to the

repurposing of Ga(III) as last-resort antibacterial agent. In the second part of this thesis, the antibacterial activity of three Ga(III)-based compounds, namely GaN, GaM, and GaPPIX, was evalulated on all species belonging to the ESKAPE group.

Furthermore, *P. aeruginosa* is also a member of the polymicrobial community in the CF lungs, in which many bacterial pathogens have been found to coexist and evolve during infection (Filkins and O'Toole, 2015; Parkins and Floto, 2015). If not treated, they establish chronic lung infections, which are generally associated with biofilm formation that is nearly impossible to eradicate. In the last part of this PhD thesis, the inhibitory activity of GaN, GaM, and GaPPIX was assessed on a representative panel of CF-associated pathogens.

Chapter 1 references

Achenbach LA, Yang W. (1997). The fur gene from *Klebsiella pneumoniae*: characterization, genomic organization and phylogenetic analysis. Gene. **185**:201-207.

Andrews SC, Robinson AK, Rodríguez-Quiñones F. (2003). Bacterial iron homeostasis. FEMS Microbiol Rev. **27**:215-237.

Antunes LC, Imperi F, Minandri F, Visca P. (2012). *In vitro* and in *vivo* antimicrobial activities of gallium nitrate against multidrug-resistant *Acinetobacter baumannii*. Antimicrob Agents Chemother. **56**:5961-5970.

Arai H. (2011). Regulation and Function of Versatile Aerobic and Anaerobic Respiratory Metabolism in *Pseudomonas aeruginosa*. Front Microbiol. **2**:103.

Arivett BA, Fiester SE, Ohneck EJ, Penwell WF, Kaufman CM, Relich RF, Actis LA. (2015). Antimicrobial Activity of Gallium Protoporphyrin IX against *Acinetobacter baumannii* Strains Displaying Different Antibiotic Resistance Phenotypes. Antimicrob Agents Chemother. **59**:7657-7665.

Bagg A, Neilands JB. (1987). Ferric uptake regulation protein acts as a repressor, employing iron (II) as a cofactor to bind the operator of an iron transport operon in *Escherichia coli*. Biochemistry. **26**:5471-5477.

Ballouche M, Cornelis P, Baysse C. (2009). Iron metabolism: a promising target for antibacterial strategies. Recent Pat Antiinfect Drug Discov. **4**:190-205.

Banin E, Lozinski A, Brady KM, Berenshtein E, Butterfield PW, Moshe M, Chevion M, Greenberg EP, Banin E. (2008). The potential of desferrioxamine-gallium as an anti-Pseudomonas therapeutic agent. Proc Natl Acad Sci U S A. **105**:16761-16766.

Banin E, Vasil ML, Greenberg EP. (2005). Iron and *Pseudomonas aeruginosa* biofilm formation. Proc Natl Acad Sci USA. **102**:11076-11081.

Barker KD, Barkovits K, Wilks A. (2012). Metabolic flux of extracellular heme uptake in *Pseudomonas aeruginosa* is driven by the iron regulated heme oxygenase (HemO). J Biol Chem. **287**:18342-18350.

Barton HA, Johnson Z, Cox CD, Vasil AI, Vasil ML. (1996). Ferric uptake regulator mutants of *Pseudomonas aeruginosa* with distinct alterations in the iron-dependent repression of exotoxin A and siderophores in aerobic and microaerobic environments. Mol Microbiol. **21**:1001-1017.

Bassetti M, Merelli M, Temperoni C, Astilean A. (2013). New antibiotics for bad bugs: where are we? Ann Clin Microbiol Antimicrob. 12: 22.

Benz R, Hancock RE. (1981). Properties of the large ion-permeable pores formed from protein F of *Pseudomonas aeruginosa* in lipid bilayer membranes. Biochim Biophys Acta. **646**:298-308.

Bernstein LR, Tanner T, Godfrey C, Noll B. (2000). Chemistry and pharmacokinetics of gallium maltolate, a compound with high oral gallium bioavailability. Met Based Drugs. **7**:33-47.

Bernstein LR. (1998). Mechanisms of therapeutic activity for gallium. Pharmacol Rev. **50**:665-682.

Bhakta MN, Wilks A. (2006). The mechanism of heme transfer from the cytoplasmic heme binding protein PhuS to the delta-regioselective heme of *Pseudomonas aeruginosa*. Biochemistry. **45**:11642-11649.

Biville F, Cwerman H, Létoffé S, Rossi MS, Drouet V, Ghigo JM, Wandersman C. (2004). Haemophore-mediated signalling in *Serratia marcescens*: a new mode of regulation for an extra cytoplasmic function (ECF) sigma factor involved in haem acquisition. Mol Microbiol. **53**:1267-1277.

Blanc DS, Petignat C, Janin B, Bille J, Francioli P. (1998). Frequency and molecular diversity of *Pseudomonas aeruginosa* upon admission and during hospitalization: a prospective epidemiologic study. Clin Microbiol Infect. **4**:242-247.

Bonchi C, Frangipani E, Imperi F, Visca, P. (2015). Pyoverdine and proteases affect the response of *Pseudomonas aeruginosa* to gallium in human serum. Antimicrob. Agents Chemother. **59**:5641-5646.

Bonchi C, Imperi F, Minandri F, Visca P, Frangipani E. (2014). Repurposing of gallium-based drugs for antibacterial therapy. Biofactors. **40**:303-312.

Bonomo RA, Szabo D. (2006). Mechanisms of multidrug resistance in *Acinetobacter* species and *Pseudomonas aeruginosa*. Clin Infect Dis. **1**:43.

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.

Bragonzi A, Paroni M, Nonis A, Cramer N, Montanari S, Rejman J, Di Serio C, Döring G, Tümmler B. (2009). *Pseudomonas aeruginosa* microevolution during cystic fibrosis lung infection establishes clones with adapted virulence. Am. J Respir Crit Care Med. **180**:138-145.

Brickman TJ, Armstrong SK. (2007). Impact of alcaligin siderophore utilization on *in vivo* growth of Bordetella pertussis. Infect Immun. **75**:5305-5312.

Britigan BE, Hayek MB, Doebbeling BN, Fick RB Jr. (1993). Transferrin and lactoferrin undergo proteolytic cleavage in the *Pseudomonas aeruginosa*-infected lungs of patients with cystic fibrosis. Infect Immun. **61**:5049-5055.

Carpenter C, Payne SM. (2014). Regulation of iron transport systems in *Enterobacteriaceae* in response to oxygen and iron availability. J Inorg Biochem. **133**:110-117.

Cartron ML, Maddocks S, Gillingham P, Craven CJ, Andrews SC. (2006). Feo-transport of ferrous iron into bacteria. Biometals **19**:143-157.

Chitambar CR. (2010). Medical applications and toxicities of gallium compounds. Int J Environ Res Public Health. **7**:2337-2361.

Chitambar CR. (2017). The therapeutic potential of iron-targeting gallium compounds in human disease: From basic research to clinical application. Pharmacol Res. **115**:56-64.

Cohen TS, Prince A. (2012). Cystic fibrosis: a mucosal immunodeficiency syndrome. Nat Med. **18**:509-519.

Cornelis P, Dingemans J. (2013). *Pseudomonas aeruginosa* adapts its iron uptake strategies in function of the type of infections. Front Cell Infect Microbiol. **3**:75.

Cornelis P, Matthijs S, Van Oeffelen L. (2009). Iron uptake regulation in *Pseudomonas aeruginosa*. Biometals. **22**:15-22.

Cornelis P, Matthijs S. (2002). Diversity of siderophore-mediated iron uptake systems in fluorescent pseudomonads: not only pyoverdines. Environ Microbiol. **4**:787-7898.

Cornelissen CN. (2003). Transferrin-iron uptake by Gram-negative bacteria. Front Biosci. **8**:836-847.

Costerton JW, Stewart PS, Greenberg E. (1999). Bacterial biofilms: a common cause of persistent infections. Science. **284**:1318-1322.

Costerton JW, Stewart PS. (2001). Battling biofilms. Sci Am. **285**: 74-81.

Cox CD, Adams P. (1985). Siderophore activity of pyoverdin for *Pseudomonas aeruginosa*. Infect Immun. **48**: 130-138.

Cox CD, Rinehart KLJr, Moore ML, Cook JCJr. (1981). Pyochelin: novel structure of an iron-chelating growth promoter for *Pseudomonas aeruginosa*. Proc Natl Acad Sci. USA. **78**:4256-4260.

Cystic Fibrosis Foundation. (2016). Patient registry 2016 annual data report. Cystic Fibrosis Foundation, Bethesda, MD. https://www.cff.org/Research/Researcher-Resources/Patient-Registry/2016-Patient-Registry-Annual-Data-Report.pdf

Dehner C, Morales-Soto N, Behera RK, Shrout J, Theil EC, Maurice PA, Dubois JL. (2013). Ferritin and ferrihydrite nanoparticles as iron sources for *Pseudomonas aeruginosa*. J Biol Inorg Chem. **18**:371–381.

DeLeon K, Balldin F, Watters C, Hamood A, Griswold J, Sreedharan S, Rumbaugh KP. (2009). Gallium maltolate treatment eradicates *Pseudomonas aeruginosa* infection in thermally injured mice. Antimicrob Agents Chemother. **53**:1331-1337.

Deplano A, Denis O, Poirel L, Hocquet D, Nonhoff C, Byl B. Nordmann P, Vincent JL, Struelens MJ. 2005. Molecular characterization of an epidemic clone of panantibiotic-resistant *Pseudomonas aeruginosa*. J Clin Microbiol. **43**:1198-1204.

Döring G, Parameswaran IG, Murphy TF. (2011). Differential adaptation of microbial pathogens to airways of patients with cystic fibrosis and chronic obstructive pulmonary disease. FEMS Microbiol Rev. **35**:124-146.

Döring G, Pfestorf M, Botzenhart K, Abdallah MA. (1988). Impact of proteases on iron uptake of *Pseudomonas aeruginosa* pyoverdin from transferrin and lactoferrin. Infect Immun. **56**:291-293.

Dumas Z, Ross-Gillespie A, Kümmerli R. (2013). Switching between apparently redundant iron-uptake mechanisms benefits bacteria in changeable environments. Proc Biol Sci. **280**:20131055.

Elemam A, Rahimian J, Mandell W. 2009. Infection with panresistant *Klebsiella pneumoniae*: a report of 2 cases and a brief review of the literature. Clin Infect Dis. **49**:271-274.

Eschbach M, Schreiber K, Trunk K, Buer J, Jahn D, Schobert M. (2004). Long-term anaerobic survival of the opportunistic pathogen *Pseudomonas aeruginosa* via pyruvate fermentation. J Bacteriol. **186**:4596-4604.

Falagas ME, Kasiakou SK. (2006). Toxicity of polymyxins: a systematic review of the evidence from old and recent studies. Crit Care. **10**: R27.

Fetherston JD, Kirillina O, Bobrov AG, Paulley JT, Perry RD. (2010). The yersiniabactin transport system is critical for the pathogenesis of bubonic and pneumonic plague. Infect Immun. **78**:2045-2052.

Filkins LM, O'Toole GA. (2015). Cystic Fibrosis Lung Infections: Polymicrobial, Complex, and Hard to Treat. PLoS Pathog. **11**:e1005258.

Foley TL, Simeonov A. (2012). Targeting iron assimilation to develop new antibacterials. Expert Opin Drug Discov. **7**:831-847.

Folkesson A, Jelsbak L, Yang L, Johansen HK, Ciofu O, Høiby N, Molin S. (2012). Adaptation of *Pseudomonas aeruginosa* to the cystic fibrosis airway: an evolutionary perspective. Nat Rev Microbiol. **10**:841-851.

Frangipani E, Bonchi C, Minandri F, Imperi F, Visca P. (2014). Pyochelin potentiates the inhibitory activity of gallium on *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **58**:5572-5575.

Friedman ND, Temkin E, Carmeli Y. (2016). The negative impact of antibiotic resistance. Clin Microbiol Infect. **22**:416-422.

Fujitani S, Sun HY, Yu VL, Weingarten JA. (2011). Pneumonia due to *Pseudomonas aeruginosa*. Part I. Epidemiology, clinical diagnosis, and source. Chest. **139**:909 -919.

García-Contreras R, Lira-Silva E, Jasso-Chávez R, Hernández-González IL, Maeda T, Hashimoto T, Boogerd FC, Sheng L, Wood TK, Moreno-Sánchez R. (2013). Isolation and characterization of gallium resistant *Pseudomonas aeruginosa* mutants. Int J Med Microbiol. **303**:574-582.

Gaynes R, Edwards JR. (2005). National Nosocomial Infections Surveillance System. Overview of nosocomial infections caused by gram-negative bacilli. Clin Infect Dis. **41**:848-854.

Goss CH, Hornick DB, Aitken ML, Anderson G, Caldwell E. (2012). Phase 1 pharmacokinetic and safety study of intravenous GaniteTM (Gallium nitrate) in CF. Pediatric Pulmunol. **47**:303.

Goss CH, Kaneko Y, Khuu L, Anderson GD, Ravishankar S, Aitken ML, Lechtzin N, Zhou G, Czyz DM, McLean K, Olakanmi O, Shuman HA, Teresi M, Wilhelm E, Caldwell E, Salipante SJ, Hornick DB, Siehnel RJ, Becker L, Britigan BE, Singh PK. (2018). Gallium disrupts bacterial iron metabolism and has therapeutic effects in mice and humans with lung infections. Sci Transl Med. **10**. pii: eaat7520.

Hackel MA, Tsuji M, Yamano Y, Echols R, Karlowsky JA, Sahm DF. (2018). *In Vitro* activity of the siderophore cephalosporin, cefiderocol, against carbapenem-non-susceptible and multidrug-resistant isolates of gram-negative Bacilli collected worldwide in 2014 to 2016. Antimicrob Agents Chemother. **62**. pii:e01968-17.

Haley KP, Skaar EP. (2012). A battle for iron: host sequestration and *Staphylococcus aureus* acquisition. Microbes Infect. **14**:217-227.

Hall-Stoodley L, Stoodley P. (2009). Evolving concepts in biofilm infections. Cell Microbiol. **11**:1034-1043.

Hassett DJ, Sokol PA, Howell ML, Ma JF, Schweizer HT, Ochsner U, Vasil ML. (1996). Ferric uptake regulator (Fur) mutants of *Pseudomonas aeruginosa* demonstrate defective siderophore-mediated iron uptake, altered aerobic growth, and decreased superoxide dismutase and catalase activities. J Bacteriol. **178**:3996-4003.

Heinrichs DE, Young L, Poole K. (1991). Pyochelin-mediated iron transport in *Pseudomonas aeruginosa*: involvement of a high-molecular-mass outer membrane protein. Infect Immun. **59**:3680-3684.

Hider RC, Kong X. (2010). Chemistry and biology of siderophores. Nat Prod Rep. **27**:637–657.

Hijazi S, Visaggio D, Pirolo M, Frangipani E, Bernstein L, Visca P. (2018). Antimicrobial activity of gallium compounds on ESKAPE pathogens. Front Cell Infect Microbiol. **8**:316.

Hijazi S, Visca P, Frangipani E. (2017). Gallium-Protoporphyrin IX Inhibits *Pseudomonas aeruginosa* Growth by Targeting Cytochromes. Front Cell Infect Microbiol. **7**:12.

Hood MI, Skaar EP. (2012). Nutritional immunity: transition metals at the pathogen-host interface. Nat Rev Microbiol. 1**0**:525-537.

Huang W, Wilks A. (2017). Extracellular Heme Uptake and the Challenge of Bacterial Cell Membranes. Annu Rev Biochem. **86**:799-823.

Hunter RC, Asfour F, Dingemans J, Osuna BL, Samad T, Malfroot A, Cornelis P, Newman DK. (2013). Ferrous iron is a significant component of bioavailable iron in cystic fibrosis airways. MBio **4**, e00557-13.

Hunter RC, Klepac-Ceraj V, Lorenzi MM, Grotzinger H, Martin TR, Newman DK. (2012). Phenazine content in the cystic fibrosis respiratory tract negatively correlates with lung function and microbial complexity. Am J Respir Cell Mol Biol. 47738-47745.

Imperi F, Massai F, Facchini M, Frangipani E, Visaggio D, Leoni L, Bragonzi A, Visca P. (2013). Repurposing the antimycotic drug flucytosine for suppression of *Pseudomonas aeruginosa* pathogenicity. Proc Natl Acad Sci USA. **110**:7458-7463.

Juan C, Maciá MD, Gutiérrez O, Vidal C, Pérez JL, Oliver A. (2005). Molecular mechanisms of beta-lactam resistance mediated by AmpC hyperproduction in *Pseudomonas aeruginosa* clinical strains. Antimicrob Agents Chemother. **49**:4733-4738.

Kaneko Y, Thoendel M, Olakanmi O, Britigan BE, Singh PK. (2007). The transition metal gallium disrupts *P. aeruginosa* iron metabolism and has antimicrobial and antibiofilm activity. J Clin Invest. **117**:877-888.

Kim SJ, Park RY, Kang SM, Choi MH, Kim CM, Shin SH. (2006). *Pseudomonas aeruginosa* alkaline protease can facilitate siderophore-mediated iron-uptake via the proteolytic cleavage of transferrins. Biol Pharm Bull. **29**:2295-2300.

Kirchner S, Fothergill JL, Wright EA, James CE, Mowat E, Winstanley C. (2012). Use of artificial sputum medium to test antibiotic efficacy against *Pseudomonas aeruginosa* in conditions more relevant to the cystic fibrosis lung. J Vis Exp. **5**:e3857.

Kollef MH, Chastre J, Fagon JY, François B, Niederman MS, Rello J, Torres A, Vincent JL, Wunderink RG, Go KW, Rehm C. (2014). Global prospective epidemiologic and surveillance study of ventilator-associated pneumonia due to *Pseudomonas aeruginosa*. Crit Care Med. **42**:2178-2187.

Kontoghiorghes GJ, Kolnagou A, Skiada A, Petrikkos G. (2010). The role of iron and chelators on infections in iron overload and non iron loaded conditions: prospects for the design of new antimicrobial therapies. Hemoglobin. **34**:227-239.

Lamont IL, Beare PA, Ochsner U, Vasil AI, Vasil ML. (2002). Siderophore-mediated signaling regulates virulence factor production in *Pseudomonas aeruginosa*. Proc Natl Acad Sci USA. **99**:7072-7077.

Lansky IB, Lukat-Rodgers GS, Block D, Rodgers KR, Ratliff M, Wilks A. (2006). The cytoplasmic heme-binding protein (PhuS) from the heme uptake system of *Pseudomonas aeruginosa* is an intracellular heme-trafficking protein to the delta-regioselective heme oxygenase. J Biol Chem. **281**:13652-13662.

Latorre M, Quenti D, Travisany D, Singh KV, Murray BE, Maass A, Cambiazo V. (2018). The role of Fur in the transcriptional and iron homeostatic response of *Enterococcus faecalis*. Front Microbiol. **9**:1580.

Lee JY, Lim MH, Heo ST, Ko KS. (2012). Repeated isolation of *Pseudomonas aeruginosa* isolates resistant to both polymyxins and carbapenems from 1 patient. Diagn Microbiol Infect Dis. **72**:267-271.

Lee JY, Park YK, Chung ES, Na IY, Ko KS. (2016). Evolved resistance to colistin and its loss due to genetic reversion in *Pseudomonas aeruginosa*. Sci Rep. **6**:25543.

Létoffé S, Redeker V, Wandersman C. (1998). Isolation and characterization of an extracellular haem-binding protein from *Pseudomonas aeruginosa* that shares function and sequence similarities with the *Serratia marcescens* HasA haemophore. Mol Microbiol. **28**:1223-1234.

Lieleg O, Caldara M, Baumgärtel R, Ribbecka K. (2011). Mechanical robustness of *Pseudomonas aeruginosa* biofilms. **7**:3307-3314.

Llamas MA, Imperi F, Visca P, Lamont IL. (2014). Cell-surface signaling in *Pseudomonas*: stress responses, iron transport, and pathogenicity. FEMS Microbiol Rev. **38**:569-597.

López-Causapé C, Rojo-Molinero E, Macià MD, Oliver A. (2015). The problems of antibiotic resistance in cystic fibrosis and solutions. Expert Rev Respir Med. **9**:73-88.

López-Causapé C, Rojo-Molinero E, Mulet X, Cabot G, Moyà B, Figuerola J, Togores B, Pérez JL, Oliver A. (2013). Clonal dissemination, emergence of mutator lineages and antibiotic resistance evolution in *Pseudomonas aeruginosa* cystic fibrosis chronic lung infection. PLoS One. **8**;e71001.

Lyczak JB, Cannon CL, Pier GB. (2000). Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. Microbes Infect. **2**:1051-1060.

Lyczak JB, Cannon CL, Pier GB. (2002). Lung infections associated with cystic fibrosis. Clin Microbiol Rev. **15**:194-222.

Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, Harbarth S, Hindler JF, Kahlmeter G, Olsson-Liljequist B, Paterson DL, Rice LB, Stelling J, Struelens MJ, Vatopoulos A, Weber JT, Monnet DL. (2012). Multidrug-resistant, extensively drug resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect. **18**:268-281.

Maltezou HC. (2009). Metallo-beta-lactamases in Gram-negative bacteria: introducing the era of pan-resistance? Int J Antimicrob Agents. **33**:405.

Marvig RL, Sommer LM, Molin S, Johansen HK. (2015). Convergent evolution and adaptation of *Pseudomonas aeruginosa* within patients with cystic fibrosis. Nat Genet. **47**:57-64.

McDougald D, Rice SA, Barraud N, Steinberg PD, Kjelleberg S. (2011). Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal. Nat Rev Microbiol. **10**:39-50.

Meyer JM, Abdallah MA. (1978). The fluorescent pigment of *Pseudomonas fluorescens*: biosynthesis, purification and physicochemical properties. J Gen Microbiol. **107**:319-328.

Meyer JM, Neely A, Stintzi A, Georges C, Holder IA. (1996). Pyoverdin is essential for virulence of *Pseudomonas aeruginosa*. Infect Immun. **64**:518-523.

Miethke M, Marahiel MA. (2007). Siderophore-based iron acquisition and pathogen control. Microbiol Mol Biol Rev. **71**:413-451.

Minandri F, Bonchi C, Frangipani E, Imperi F, Visca P. (2014). Promises and failures of gallium as an antibacterial agent. Future Microbiol. **9**:379-397.

Minandri F, Imperi F, Frangipani E, Bonchi C, Visaggio D, Facchini M, Pasquali P, Bragonzi A, Visca P. (2016). Role of Iron Uptake Systems in *Pseudomonas aeruginosa* Virulence and Airway Infection. Infect Immun. **84**:2324-2335.

Miranda-Casoluengo R, Coulson GB, Miranda-Casoluengo A, Vázquez-Boland JA, Hondalus MK, Meijer WG. (2012). The hydroxamate siderophore rhequichelin is required for virulence of the pathogenic actinomycete *Rhodococcus* equi. Infect Immun. **80**:4106-4114.

Monroe D. (2007). Looking for chinks in the armor of bacterial biofilms. PLoS Biol. **5**:e307.

Moore JE, Mastoridis P. (2017). Clinical implications of *Pseudomonas aeruginosa* location in the lungs of patients with cystic fibrosis. J Clin Pharm Ther. **42**:259-267.

Moore NM, Flaws ML. (2011). Antimicrobial resistance mechanisms in *Pseudomonas aeruginosa*. Clin Lab Sci. Winter. **24**:47-51.

M**ortensen BL, Skaar EP.** (2013). The contribution of nutrient metal acquisition and metabolism to *Acinetobacter baumannii* survival within the host. Front Cell Infect Microbiol. **3**:95.

Nguyen AT, O'Neill MJ, Watts AM, Robson CL, Lamont IL, Wilks A, Oglesby-Sherrouse AG. (2014). Adaptation of iron homeostasis pathways by a *Pseudomonas aeruginosa* pyoverdine mutant in the cystic fibrosis lung. J Bacteriol. **196**:2265-2276.

Nowak J, Zander E, Stefanik D, Higgins PG, Roca I, Vila J, McConnell MJ, Cisneros JM, Seifert H, MagicBullet Working Group WP4. (2017). High incidence of pandrug-resistant *Acinetobacter baumannii* isolates collected from patients with ventilator-associated pneumonia in Greece, Italy and Spain as part of the MagicBullet clinical trial. J Antimicrob Chemother. **72**:3277-3282.

O'Neill MJ, Bhakta MN, Fleming KG, Wilks A. (2012). Induced fit on heme binding to the *Pseudomonas aeruginosa* cytoplasmic protein (PhuS) drives interaction with heme oxygenase (HemO). Proc Natl Acad Sci USA. **109**:5639-5644.

Ochsner UA, Johnson Z, Vasil ML. (2000). Genetics and regulation of two distinct haem-uptake systems, phu and has, in *Pseudomonas aeruginosa*. Microbiology. **146**:185-198.

Ochsner UA, Vasil ML. (1996). Gene repression by the ferric uptake regulator in *Pseudomonas aeruginosa*: cycle selection of iron-regulated genes. Proc Natl Acad Sci. USA. **93**:4409-4414.

Oglesby-Sherrouse AG, Murphy ER. (2013). Iron-responsive bacterial small RNAs: variations on a theme. Metallomics. **5**:276-286.

Parkins MD, Floto RA. (2015). Emerging bacterial pathogens and changing concepts of bacterial pathogenesis in cystic fibrosis. J Cyst Fibros. **14**:293-304.

Pasqua M, Visaggio D, Lo Sciuto A, Genah S, Banin E, Visca P, Imperi F. (2017). Ferric Uptake Regulator Fur Is Conditionally Essential in *Pseudomonas aeruginosa*. J Bacteriol. **199**. pii: e00472-17.

Patriquin GM, Banin E, Gilmour C, Tuchman R, Greenberg EP, Poole K. (2008). Influence of quorum sensing and iron on twitching motility and biofilm formation in *Pseudomonas aeruginosa*. J Bacteriol. **190**:662-671.

Pendleton JN, Gorman SP, Gilmore BF. (2013). Clinical relevance of the ESKAPE pathogens. Expert Rev Anti Infect Ther. **3**:297-308.

Poole K, McKay GA. (2003). Iron acquisition and its control in *Pseudomonas aeruginosa*: many roads lead to Rome. Front. Biosci. **8**:661-686.

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

Rangel-Vega A, Bernstein LR, Mandujano-Tinoco EA, García-Contreras SJ, García-Contreras R. (2015). Drug repurposing as an alternative for the treatment of recalcitrant bacterial infections. Front Microbiol. **6**:282.

Ratledge C, Dover LG. (2000). Iron metabolism in pathogenic bacteria. Annu Rev Microbiol. **54**:881-941.

Ravel J, Cornelis P. (2003). Genomics of pyoverdine-mediated iron uptake in pseudomonads. Trends Microbiol. **11**:195-200.

Rice LB. (2008). Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE. J Infect Dis. **197**:1079-1081.

Roca I, Espinal P, Vila-Farrés X, Vila J. (2012). The *Acinetobacter baumannii* Oxymoron: Commensal Hospital Dweller Turned Pan-Drug-Resistant Menace. Front Microbiol. **3**:148.

Rogers GB, Hart CA, Mason JR, Hughes M, Walshaw MJ, Bruce KD. (2003). Bacterial diversity in cases of lung infection in cystic fibrosis patients: 16S ribosomal DNA (rDNA) length heterogeneity PCR and 16S rDNA terminal restriction fragment length polymorphism profiling. J Clin Microbiol. **41**:3548-58.

Ross-Gillespie A, Weigert M, Brown SP, Kümmerli R. (2014). Gallium-mediate siderophore quenching as an evolutionarily robust antibacterial treatment. Evol. Med. Public Health. 18-29.

Rossolini GM, Docquier JD. (2006). New beta-lactamases: a paradigm for the rapid response of bacterial evolution in the clinical setting. Future Microbiol. **1**:295-308.

Runci F, Bonchi C, Frangipani E, Visaggio D, Visca P. (2016). *Acinetobacter baumannii* Biofilm Formation in Human Serum and Disruption by Gallium. Antimicrob Agents Chemother. **61**. pii: e01563-16.

Rzhepishevska O, Ekstrand-Hammarström B, Popp M, Björn E, Bucht A, Sjöstedt A, Antti H, Ramstedt M. (2011). The antibacterial activity of Ga3+ is influenced by ligand complexation as well as the bacterial carbon source. Antimicrob Agents Chemother. **55**:5568-5580.

Sadikot RT, Blackwell TS, Christman JW, Prince AS. (2005). Pathogen-host interactions in *Pseudomonas aeruginosa* pneumonia. Am J Respir Crit Care Med. **171**:1209-1223.

Salvail H, Massé E. (2012). Regulating iron storage and metabolism with RNA: an overview of posttranscriptional controls of intracellular iron homeostasis. Wiley Interdiscip Rev RNA. **3**:26-36.

Santajit S, Indrawattana N. (2016). Mechanisms of Antimicrobial Resistance in ESKAPE Pathogens. Biomed Res Int. **2016**:2475067.

Schalk IJ, Guillon L. (2013). Fate of ferrisiderophores after import across bacterial outer membranes: different iron release strategies are observed in the cytoplasm or periplasm depending on the siderophore pathways. Amino Acids. **44**:1267-1277.

Schalk IJ. (2008). Metal trafficking via siderophores in Gram-negative bacteria: specificities and characteristics of the pyoverdine pathway. J Inorg Biochem. **102**:1159-1169.

Schweizer HP. (2003). Efflux as a mechanism of resistance to antimicrobials in *Pseudomonas aeruginosa* and related bacteria: unanswered questions. Genet Mol Res. **2**:48-62.

Sherrard LJ, Tunney MM, Elborn JS. (2014). Antimicrobial resistance in the respiratory microbiota of people with cystic fibrosis. Lancet. **384**:703-713.

Silby MW, Winstanley C, Godfrey SA, Levy SB, Jackson RW. (2011). *Pseudomonas* genomes: diverse and adaptable. FEMS Microbiol Rev. **35**:652-680.

Skaar EP. (2010). The battle for iron between bacterial pathogens and their vertebrate hosts. PLoS Pathog. **6**:e1000949.

Smith EE, Buckley DG, Wu Z, Saenphimmachak C, Hoffman LR, D'Argenio DA, Miller SI, Ramsey BW, Speert DP, Moskowitz SM. (2006). Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. Proc Natl Acad Sci USA. **103**:8487-8492.

Smith WD, Bardin E, Cameron L, Edmondson CL, Farrant KV, Martin I, Murphy RA, Soren O, Turnbull AR, Wierre-Gore N, Alton EW, Bundy JG, Bush A, Connett GJ, Faust SN, Filloux A, Freemont PS, Jones AL, Takats Z, Webb JS, Williams HD, Davies JC. (2017). Current and future therapies for *Pseudomonas aeruginosa* infection in patients with cystic fibrosis. FEMS Microbiol Lett. 364(14).

Soo VW, Kwan BW, Quezada H, Castillo-Juárez I, Pérez-Eretza B, García-Contreras SJ, Martínez-Vázquez M, Wood TK, García-Contreras R. (2017). Repurposing of anticancer drugs for the treatment of bacterial infections. Curr Top Med Chem. **17**:1157-1176.

Stewart PS, Costerton JW. (2001). Antibiotic resistance of bacteria in biofilms. Lancet. **358**:135-138.

Stojiljkovic I, Perkins-Balding D. (2002). Processing of heme and heme-containing proteins by bacteria. DNA Cell Biol. **21**:281-295.

Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, Brinkman FS, Hufnagle WO, Kowalik DJ, Lagrou M, Garber RL, Goltry L, Tolentino E, Westbrock- Wadman S, Yuan Y, Brody LL, Coulter SN, Folger KR, Kas A, Larbig K, Lim R, Smith K, Spencer D, Wong GK, Wu Z, Paulsen IT, Reizer J, Saier MH, Hancock RE, Lory S, Olson MV (2000). Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature. **406**:959-964.

Takase H, Nitanai H, Hoshino K, Otani T. (2000). Impact of siderophore production on *Pseudomonas aeruginosa* infections in immunosuppressed mice. Infect Immun **68**:1834-1839.

Thomas-Charles CA, Zheng H, Palmer LE, Mena P, Thanassi DG, Furie MB. (2013). FeoB-mediated uptake of iron by Francisella tularensis. Infect Immun. **81**:2828-2837.

Thompson MG, Corey BW, Si Y, Craft DW, Zurawski DV. (2012). Antibacterial activities of iron chelators against common nosocomial pathogens. Antimicrob Agents Chemother. **56**:5419-5421.

Tiburzi F, Imperi F, Visca P. (2008). Intracellular levels and activity of PvdS, the major iron starvation sigma factor of *Pseudomonas aeruginosa*. Mol Microbiol. **67**:213-227.

Tiburzi F, Imperi F, Visca P. (2009). Is the host heme incorporated in microbial heme-proteins? IUBMB Life. **61**:80-83.

Traxler MF, Seyedsayamdost MR, Clardy J, Kolter R. (2012). Interspecies modulation of bacterial development through iron competition and siderophore piracy. Mol Microbiol. **86**:628-644.

Tseng SP, Tsai JC, Teng LJ, Hsueh PR. (2009). Dissemination of transposon Tn6001 in carbapenem-non-susceptible and extensively drug-resistant *Pseudomonas aeruginosa* in Taiwan. J Antimicrob Chemother. **64**:1170-1174.

van der Gast CJ, Walker AW, Stressmann FA, Rogers GB, Scott P, Daniels TW, Carroll MP, Parkhill J, Bruce KD. (2011). Partitioning core and satellite taxa from within cystic fibrosis lung bacterial communities. ISME J. **5**:780-91.

Vander Wauven C, Piérard A, Kley-Raymann M, Haas D. (1984). *Pseudomonas aeruginosa* mutants affected in anaerobic growth on arginine: evidence for a four-gene cluster encoding the arginine deiminase pathway. J Bacteriol. **160**:928-934.

Velayudhan J, Hughes NJ, McColm AA, Bagshaw J, Clayton CL, Andrews SC, Kelly DJ. (2000). Iron acquisition and virulence in *Helicobacter pylori*: a major role for FeoB, a high-affinity ferrous iron transporter. Mol Microbiol. **37**:274-286.

Visca P, Bonchi C, Minandri F, Frangipani E, Imperi F. (2013). The Dual Personality of Iron Chelators: Growth Inhibitors or Promoters? Antimicrob Agents Chemother. **57**:227-239.

Visca P, Imperi F, Lamont IL. (2007). Pyoverdine siderophores: from biogenesis to biosignificance. Trends Microbiol. **15**:22-30.

Visca P. (2004). Iron regulation and siderophore signalling in virulence by *Pseudomonas aeruginosa*. In: *Pseudomonas*: virulence and gene regulation (Volume 2). Ramos JL (Ed.), Kluwer Academic Publishers, Dordrecht, NL, 69-123.

Wagner VE, Iglewski BH. (2008). *P. aeruginosa* Biofilms in CF Infection. Clin Rev Allergy Immunol. **35**:124-134.

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

Wang Y, Wilks JC, Danhorn T, Ramos I, Croal L, Newman DK. (2011). Phenazine-1-carboxylic acid promotes bacterial biofilm development via ferrous iron acquisition. J Bacteriol.**193**:3606-3617.

Weinberg ED. (2009). Iron availability and infection. Biochem. Biophys. Acta **1790**:600-605.

Wells RM, Jones CM, Xi Z, Speer A, Danilchanka O, Doornbos KS, Sun P, Wu F, Tian C, Niederweis M. (2013). Discovery of a siderophore export system essential for virulence of *Mycobacterium tuberculosis*. PLoS Pathog. 9:e1003120.

Wilderman PJ, Vasil AI, Johnson Z, Wilson MJ, Cunliffe HE, Lamont IL, Vasil ML. (2001). Characterization of an endoprotease (PrpL) encoded by a PvdS-regulated gene in *Pseudomonas aeruginosa*. Infect Immun. **69**:5385-5394.

Winkelmann G. (2007). Ecology of siderophores with special reference to the fungi. Biometals. **20**:379-392.

Winstanley C, O'Brien S, Brockhurst MA. (2016). *Pseudomonas aeruginosa* Evolutionary Adaptation and Diversification in Cystic Fibrosis Chronic Lung Infections. Trends Microbiol. **24**:327-337.

World Health Organization. (2017). List of antibiotic resistant "priority pathogens". Available online at: http://www.who.int/mediacentre/news/releases/2017/bacteria-antibiotics-needed/en/

Xiong YQ, Vasil ML, Johnson Z, Ochsner UA, Bayer AS. (2000). The oxygen- and iron-dependent sigma factor pvdS of *Pseudomonas aeruginosa* is an important virulence factor in experimental infective endocarditis. J Infect Dis. **181**:1020-1026.

Zago A, Chugani S. (2016). *Pseudomonas*. Encyclopedia of Microbiology (Third Edition). 2009 ed. 245-260.

Chapter 2

Gallium-protoporphyrin IX inhibits *Pseudomonas aeruginosa* growth by targeting cytochromes

Sarah Hijazi, Paolo Visca, and Emanuela Frangipani

Department of Science, University of Roma Tre, Rome, Italy

Front Cell Infect Microbiol, 2017; 7:12, doi: 10.3389/fcimb.2017.00012.

Chapter 2

Gallium-protoporphyrin IX inhibits *Pseudomonas aeruginosa* growth by targeting cytochromes

Complexation of antibacterial drugs with organic compounds, such as siderophores and PPIX (drug conjugates), which are actively transported into the bacterial cell (Trojan horse strategy), represents an attractive strategy to enhance drugs delivery to bacteria, and therefore antibacterial potency.

Pseudomonas aeruginosa is a challenging bacterial pathogen due to both its innate and acquired resistance to numerous antibiotics (Moore and Flaws, 2011). *P. aeruginosa* is capable of causing a variety of infections, most notably chronic lung infection, which represents the main cause of morbidity and mortality in individuals suffering from cystic fibrosis (CF) (Murphy 2006; Davies *et al.,* 2007). As anticipated in Chapter 1, iron is an essential nutrient for bacterial growth *in vivo*, and the multiplicity of iron uptake systems in *P. aeruginosa* suggests that iron acquisition contributes to the success of *P. aeruginosa* as a human pathogen. In addition to the endogenous siderophores pyoverdine (PVD) and pyochelin (PCH), *P. aeruginosa* is able to acquire iron through other systems, including heme-uptake systems, which are expressed during infection (Konings *et al.,* 2013) (paragraph 1.3.1).

In the last years, the FDA-approved citrated-buffered $Ga(NO_3)_3$ (brand name Ganite®) has been successfully repurposed as an antimicrobial agent. Due to the chemical similarity between Ga(III) and Fe(III), Ga(III) substitutes for Fe(III) in several enzymes, thereby perturbing bacterial metabolism. GaN has already been shown to exert a potent antibacterial activity against *P. aeruginosa* (Kaneko *et al.,* 2007). Moreover, the antimicrobial properties of Ga(III) was enhanced by supplying this metal complexed with the PCH siderophore (Frangipani *et al.,* 2014). In the following article, Ga(III) coupled with the heme precursor protoporphyrin IX (GaPPIX), that is actively uptaken by *P. aeruginosa* through the heme-uptake systems, was shown to be capable of inhibiting the growth of *P. aeruginosa* by targeting cytochromes, thus interfering with cellular respiration.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 26 January 2017
doi: 10.3389/fcimb.2017.00012

Gallium-Protoporphyrin IX Inhibits *Pseudomonas aeruginosa* Growth by Targeting Cytochromes

*Sarah Hijazi, Paolo Visca and Emanuela Frangipani **

Department of Science, Roma Tre University, Rome, Italy

Pseudomonas aeruginosa is a challenging pathogen due to both innate and acquired resistance to antibiotics. It is capable of causing a variety of infections, including chronic lung infection in cystic fibrosis (CF) patients. Given the importance of iron in bacterial physiology and pathogenicity, iron-uptake and metabolism have become attractive targets for the development of new antibacterial compounds. *P. aeruginosa* can acquire iron from a variety of sources to fulfill its nutritional requirements both in the environment and in the infected host. The adaptation of *P. aeruginosa* to heme iron acquisition in the CF lung makes heme utilization pathways a promising target for the development of new anti-*Pseudomonas* drugs. Gallium [Ga(III)] is an iron mimetic metal which inhibits *P. aeruginosa* growth by interfering with iron-dependent metabolism. The Ga(III) complex of the heme precursor protoporphyrin IX (GaPPIX) showed enhanced antibacterial activity against several bacterial species, although no inhibitory effect has been reported on *P. aeruginosa.* Here, we demonstrate that GaPPIX is indeed capable of inhibiting the growth of clinical *P. aeruginosa* strains under iron-deplete conditions, as those encountered by bacteria during infection, and that GaPPIX inhibition is reversed by iron. Using *P. aeruginosa* PAO1 as model organism, we show that GaPPIX enters cells through both the heme-uptake systems *has* and *phu*, primarily *via* the PhuR receptor which plays a crucial role in *P. aeruginosa* adaptation to the CF lung. We also demonstrate that intracellular GaPPIX inhibits the aerobic growth of *P. aeruginosa* by targeting cytochromes, thus interfering with cellular respiration.

Keywords: aerobic respiration, antibacterial, cystic fibrosis, gallium, heme, infection, iron-uptake, terminal oxidases

OPEN ACCESS

Edited by:
Pierre Cornelis,
Vrije Universiteit Brussel, Belgium

Reviewed by:
Isabelle Schalk,
Ecole Superieure de Biotechnologie de Starsbourg, France
Christine Baysse,
University of Rennes 1, France

****Correspondence:***
Emanuela Frangipani
emanuela.frangipani@uniroma3.it

Received: *15 November 2016*
Accepted: *10 January 2017*
Published: *26 January 2017*

Citation:
Hijazi S, Visca P and Frangipani E (2017) Gallium-Protoporphyrin IX Inhibits Pseudomonas aeruginosa Growth by Targeting Cytochromes. Front. Cell. Infect. Microbiol. 7:12. doi: 10.3389/fcimb.2017.00012

INTRODUCTION

Pseudomonas aeruginosa is a challenging bacterial pathogen due to both innate and acquired resistance to several antibiotics (Moore and Flaws, 2011). This bacterium is capable of causing a variety of infections, including chronic lung infection, which represents the main cause of morbidity and mortality in patients suffering from cystic fibrosis (CF) (Murphy, 2006; Davies et al., 2007). The success of *P. aeruginosa* as an opportunistic pathogen relies, at least in part, on its metabolic versatility, including the ability to obtain energy from different sources under a variety of environmental conditions (Williams et al., 2007; Arai, 2011). *P. aeruginosa* possesses a branched respiratory chain terminated by oxygen or nitrogen oxides, to allow growth by aerobic respiration or by denitrification under anaerobic conditions, respectively (reviewed in Arai, 2011). Moreover,

P. aeruginosa is able to ferment arginine and pyruvate anaerobically (Vander et al., 1984; Eschbach et al., 2004). Aerobic respiration in *P. aeruginosa* relies on five terminal oxidases (Matsushita et al., 1982, 1983; Fujiwara et al., 1992; Cunningham and Williams, 1995; Cunningham et al., 1997; Stover et al., 2000; Comolli and Donohue, 2002, 2004). Three of these enzymes, the aa_3 terminal oxidase (Cox), the cbb_3-1 (Cco-1), and the cbb_3-2 (Cco-2) are cytochrome *c*-type oxidases, while the other two, i.e., the cyanide-insensitive oxidase (Cio) and the bo_3 oxidase (Cyo), are quinol oxidases (**Figure 1**). All these terminal oxidases contain heme, and are differentially expressed depending on the growth conditions, likely as a consequence to their different affinity for oxygen (Alvarez-Ortega and Harwood, 2007; Kawakami et al., 2010). Denitrification is ensured by a set of enzymes which sequentially convert nitrate (NO_3^-) to molecular nitrogen (N_2). Among the denitrification enzymes, only nitrite reductase (Nir) and nitric oxide reductase (Nor) contain heme as a cofactor (**Figure 1**).

Like almost all pathogenic bacteria, *P. aeruginosa* has an absolute need for iron to cause infections and to persist within the host (Ratledge and Dover, 2000). Iron is required as a cofactor of many key enzymes involved in respiration, DNA synthesis and defense against reactive oxygen species (Andrews et al., 2003). However, in the human host, iron is poorly available to bacteria due to its incorporation into heme-containing molecules (e.g., hemoglobin and myoglobin) and iron carrier proteins (e.g., transferrin and lactoferrin) (Weinberg, 2009). This iron-withholding capacity represents the first line of the host defense against invading pathogens, a phenomenon known as "nutritional immunity" (Skaar, 2010). To circumvent iron-limitation, *P. aeruginosa* possesses several systems that actively acquire this essential metal, such as (i) the production of the siderophores pyoverdine (Pvd, Meyer and Abdallah, 1978; Cox and Adams, 1985) and pyochelin (Pch, Cox et al., 1981; Heinrichs et al., 1991); (ii) the ability to utilize a wide range of siderophores synthesized by other organisms (Cornelis and Matthijs, 2002; Cornelis et al., 2009); (iii) the ability to acquire Fe(II) through the Feo system (Cartron et al., 2006). In addition, *P. aeruginosa* can utilize heme-iron, by expressing two distinct heme-uptake systems, namely *phu* and *has* (Ochsner et al., 2000). The *phu* system allows the direct acquisition of heme from hemoproteins, which bind to the outer membrane receptor PhuR (Ochsner et al., 2000). In the *has* system a secreted hemophore HasA withdraws heme from hemoproteins and delivers it to the outer membrane receptor HasR (Létoffé et al., 1998). Given the similarity with the well-known *has* system of *Serratia marcescens* (Rossi et al., 2003; Létoffé et al., 2004), it is likely that the *has* system of *P. aeruginosa* positively regulates its own expression, *via* the sigma factor HasI and anti-sigma HasS, upon interaction of heme-loaded HasA with the HasR receptor (Llamas et al., 2014). The expression of both *has* and *phu* heme-uptake systems is shut down in the presence of sufficient intracellular iron, due to the negative regulation exerted by the ferric-uptake regulator (Fur) protein (Ochsner et al., 2000).

It has been shown that *P. aeruginosa* aerobic respiration and iron-uptake capabilities play pivotal roles during chronic lung

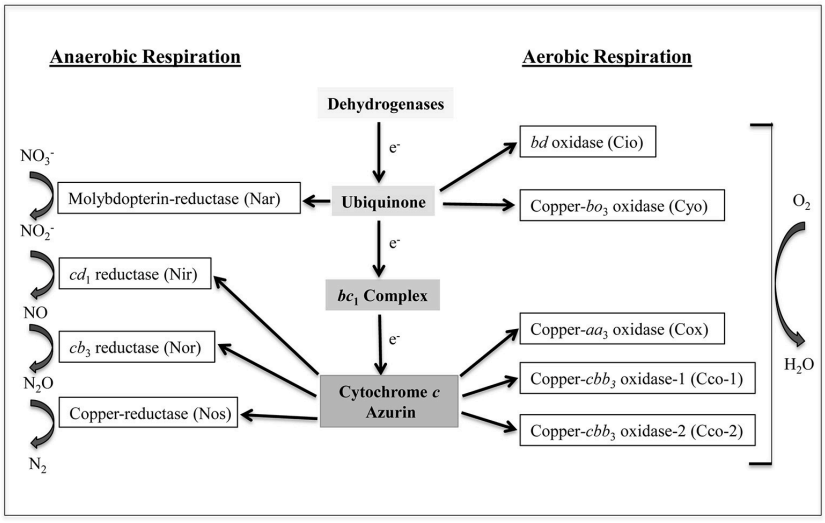


FIGURE 1 | Branched respiratory chain of *P. aeruginosa*. Cio, Cyo, Cox, Cco-1, and Cco-2 represent the five terminal oxidases that reduce oxygen to water under aerobic conditions. Cio and Cyo are quinol oxidases while Cox, Cco-1, and Cco-2 are cytochrome *c* oxidases. Nar, Nir, Nor, and Nos are nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase, respectively. These enzymes transfer electron to nitrogen oxides under anaerobic conditions. Nar receives electrons directly from the quinone pool while the other three receive electrons *via* the cytochrome *c* or from the small blue-copper protein azurin. *a*, *b*, *c,* and *d* represent different types of low-spin heme while a_3, b_3, d_1, and o_3 indicate the high-spin ones (modified from Arai, 2011).

infection in CF patients. In particular, three terminal oxidases (Cco-1, Cco-2, and Cio) sustain bacterial growth in the CF lung, a particular environment where *P. aeruginosa* iron-uptake abilities are sought to evolve toward heme utilization (Alvarez-Ortega and Harwood, 2007; Marvig et al., 2014; Nguyen et al., 2014).

The paucity of effective antibiotics to treat *P. aeruginosa* infections have made bacterial respiration and/or iron metabolism promising targets for the development of new anti-*Pseudomonas* drugs (Ballouche et al., 2009; Foley and Simeonov, 2012; Imperi et al., 2013). The possibility of using iron mimetics as novel therapeutics to interfere with iron metabolism has been exploited (Kaneko et al., 2007; Banin et al., 2008; Minandri et al., 2014). $Ga(NO_3)_3$, the active component of the FDA-approved formulation Ganite®, has successfully been repurposed as an antimicrobial drug (Bonchi et al., 2014; Rangel-Vega et al., 2015). Interestingly, $Ga(NO_3)_3$ has been shown to be very active against *P. aeruginosa*, by interfering with iron-dependent metabolic pathways (Kaneko et al., 2007; Bonchi et al., 2015). The antibacterial proprieties of Ga(III) reside in the fact that, different from Fe(III), Ga(III) cannot be reduced under physiological conditions. However, redox cycling is critical for many of iron-dependent biological functions, including respiration (Breidenstein et al., 2011). Moreover, the heme-mimetic GaPPIX [i.e., Ga(III) coupled with the heme precursor protoporphyrin IX] has been shown to possess a good antibacterial activity against several bacterial species, including *Staphylococcus aureus* and *Acinetobacter baumannii* (Stojiljkovic et al., 1999; Arivett et al., 2015; Chang et al., 2016). GaPPIX is likely to exploit heme-uptake routes to enter bacterial cells, where it could substitute for heme in heme-containing enzymes, including cytochromes, catalases, and peroxidases, resulting in the perturbation of vital cellular functions (Stojiljkovic et al., 1999). Due to the similarity between GaPPIX and heme, GaPPIX is predicted to interfere with heme-dependent *b*-type cytochromes, thus impairing their function and ultimately inhibiting bacterial respiration.

In this work, the *in vitro* effect of GaPPIX on *P. aeruginosa* was tested under iron-depleted conditions, as those encountered during infection. The entrance routes of GaPPIX into *P. aeruginosa* cells and possible targets of GaPPIX were investigated. We demonstrate that the sensitivity of *P. aeruginosa* to GaPPIX depends on both intracellular iron levels and the expression of heme-uptake systems. Furthermore, we show that GaPPIX enters *P. aeruginosa* cells mainly through the heme-uptake receptor PhuR. Evidence is also provided that intracellular GaPPIX inhibits the aerobic growth of *P. aeruginosa* by targeting heme-dependent *b*-type cytochromes.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Strains and plasmids used in this work are listed in **Table 1**. *P. aeruginosa* clinical isolates are listed in Table S1. *P. aeruginosa* strains from frozen cultures were maintained on Luria Bertani (LB) agar before being transferred to liquid culture media. Bacteria were cultured in iron-free Casamino Acids medium (DCAA, Visca et al., 1993) supplemented or not with 100 μM of $FeCl_3$ at 37°C, with vigorous shaking. When required, antibiotics were added to the media at the following concentrations for *Escherichia coli*, with the concentrations used for *P. aeruginosa* shown in parentheses: Ampicillin 100 μg/ml; carbenicillin (300 μg/ml in LB and 200 μg/ml in DCAA); and tetracycline 12.5 μg/ml (100 μg/ml). DCAA agar plates were prepared by the addition of 15 g/l bacteriological agar (Acumedia, Neogen corporation). When GaPPIX was required, a 50 mM of stock solution of GaPPIX (Frontier Scientific) was prepared in dimethyl sulfoxide (DMSO) and stored at 4°C in the dark. When $Ga(NO_3)_3$ was required, a 100 mM of stock solution of $Ga(NO_3)_3$ (Sigma-Aldrich), was prepared in double-distilled water and stored at −20°C.

Susceptibility Testing

The activity of GaPPIX, $Ga(NO_3)_3$ and Hemin (Hm) (Sigma-Aldrich) on *P. aeruginosa* was tested in 96-well microtiter plates (Falcon). Briefly, bacterial cells were grown over-night in DCAA supplemented with 100 μM $FeCl_3$ in order to obtain high cell densities, then washed in saline and diluted to an OD_{600} of 0.01 in 200 μl of DCAA containing increasing concentrations (0–100 μM) of GaPPIX, $Ga(NO_3)_3$ or Hm. Microtiter plates were incubated for 24 h at 37°C with gentle shaking (120 rpm). Growth (OD_{600}) was measured in a Wallac 1420 Victor3 V multilabel plate reader (PerkinElmer). The minimum inhibitory concentration (MIC) of gallium compounds was visually determined as the lowest concentration that completely inhibited *P. aeruginosa* growth. As a control experiment the same procedure was performed, except that 100 μM $FeCl_3$ was added in the medium containing the highest concentration of gallium compounds tested (100 μM).

The antibacterial activity of gallium compounds was also assessed by disk diffusion assays. Briefly, cells from an over-night culture in DCAA supplemented with 100 μM $FeCl_3$ were washed and diluted in saline to $OD_{600} = 0.1$, then seeded on the surface of DCAA agar plates supplemented or not with $FeCl_3$. Sterile 6-mm blank disks (ThermoFisher-Oxoid) soaked with 10 μl of a 15 mM solution of either GaPPIX or $Ga(NO_3)_3$ were deposited on the agar surface and the Zone Of growth Inhibition (ZOI) was measured (in mm) after 16 h of incubation at 37°C.

To observe the rescue effect of Hm and Hemoglobin (Hb), disks were soaked with 10 μl of a 7.5 mg/ml solution of bovine hemin chloride (Sigma-Aldrich) in 10 mM NaOH or bovine hemoglobin (Sigma-Aldrich) in phosphate buffered saline (PBS) and deposited on the plate surface nearby the disk soaked with GaPPIX. The appearance of a half-moon-shaped growth area around the disk soaked with Hm or Hb was detected after 16 h of incubation at 37°C.

Construction of Plasmids for the Expression of Heme Receptors

Plasmid preparations and DNA cloning were performed according to standards methods (Sambrook et al., 1989). Restriction and DNA modifying enzymes were used following the instructions of the manufacturers. Oligonucleotide primers are listed in **Table 1**. To express *hasR* in the Δ*hasR*Δ*phuR*

TABLE 1 | Bacterial strains and plasmids used in this study.

Strain or plasmid	Genotype and/or relevant characteristics	Reference or source
STRAINS		
P. aeruginosa		
PAO1	ATCC15692 (wild type, prototroph)	American type culture collection
Δ*hasR*	PAO1Δ*hasR*	This work
Δ*phuR*	PAO1Δ*phuR*	This work
Δ*hasR*Δ*phuR*	PAO1Δ*hasR*Δ*phuR*	Minandri et al., 2016
Δ*pvdA*	PAO1Δ*pvdA*	Imperi et al., 2008
Δ*pchD*	PAO1Δ*pchD*	Frangipani et al., 2014
Δ*pvdA*Δ*pchD*	PAO1Δ*pvdA*Δ*pchD*	Visca et al., 2013
Δ*cio*	PAO1 containing a 2400-bp deletion in the *cioAB* locus	This work
Δ*cox*	PAO1 containing a 4109-bp deletion in the *coxBA*-PA0107-*coIII* locus	This work
Δ*cyo*	PAO1 containing a 4830-bp deletion in the *cyoABCDE* operon	This work
Δ*cco*	PAO1 containing a 6445-bp deletion in the two adjacent *ccoNOQP1* and *ccoNOQP2* operons	This work
Δ*cyo*Δ*cio*	PAO1 mutated in both *cyo* and *cio*	This work
Δ*cyo*Δ*cco*	PAO1 mutated in both *cyo* and *cco*-1,2	This work
Δ*cyo*Δ*cio*Δ*cox*	PAO1 mutated in *cyo*, *cio* and *cox*	This work
Δ*cyo*Δ*cco*Δ*cox*	PAO1 mutated in *cyo*, *cco* and *cox*	This work
E. coli		
DH5αF′	*recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1* Δ(*lacZYA-argF*) U169 [Φ*80dlacZ*Δ*M15*] Nal^R	Sambrook et al., 1989
S17-1 λpir	*recA*, *thi*, *pro*, *hsdR*-M+RP4: 2-Tc:Mu: Km Tn7 λpir, Tp^R Sm^R	Simon et al., 1983
PLASMIDS		
pDM4	Suicide vector; *sacBR*, *oriR6K*, Cm^R	Milton et al., 1996
pME7541	Suicide construct used for deletion of the *cioAB* operon; Tc^R	Frangipani et al., 2008
pME9302	Suicide construct used for deletion of the *coxB-coIII* cluster; Tc^R	Frangipani et al., 2008
pME9303	Suicide construct used for deletion of the *cyoABCDE* operon; Tc^R	Frangipani et al., 2008
pME9308	Suicide construct used for deletion of the two adjacent *ccoNOQP* operons; Tc^R	Frangipani et al., 2008
pUCP18	*E. coli-Pseudomonas* shuttle vector derived from pUC18; ColE1, pRO1600, Ap^R, Cb^R	Schweizer, 1991
pUCP*hasR*	pUCP18 derivative carrying the coding sequence of *hasR* with its own promoter	This study
pUCP*phuR*	pUCP18 derivative carrying the coding sequence of *phuR* with its own promoter	This study
pUCP*phuRhasR*	pUCP18 derivative carrying the coding sequence of *phuR* and *hasR* with their own promoters	Minandri et al., 2016
pDM4Δ*hasR*	pDM4 derivative carrying the flanking regions of the *hasR* coding sequence	Minandri et al., 2016
pDM4Δ*phuR*	pDM4 derivative carrying the flanking regions of the *phuR* coding sequence	Minandri et al., 2016

Oligonucleotides	Sequence 5′-3′	Restriction site
hasR compl FW	CGGGGTACCGGCGGGAGTGACGCTGC	KpnI
hasR compl RV	GAAGATCTCCTTCACTGGGCAAAACGG	BglII
phuR compl FW	CCGGAATTCGAAAGGCTGGGAGTGCTG	EcoRI
phuR compl RV	CGGGGTACCACCTGTGGCATGGAAAGC	KpnI

Tc^R, tetracycline resistant; Ap^R, ampicillin resistant; Cm^R, chloramphenicol resistant; Cb^R, carbenicillin resistant; restriction sites in the oligonucleotides are underlined.

mutant, a 2932 bp fragment containing the *hasR* gene with its own promoter region was amplified by PCR from the PAO1 genome using primers *hasR* compl FW and *hasR* compl RV (**Table 1**). The product was then digested with KpnI and BglII and directionally cloned into the corresponding sites of the shuttle vector pUCP18, giving plasmid pUCP*hasR*. To express *phuR* in Δ*hasR*Δ*phuR* mutant, a 2575 bp fragment containing the *phuR* gene with its own promoter region was amplified by PCR from the PAO1 genome using primers *phuR* compl FW and *phuR* compl RV (**Table 1**). The product was then digested with EcoRI and KpnI and directionally cloned into the corresponding sites of the shuttle vector pUCP18, giving plasmid pUCP*phuR*. To express *hasR* and *phuR* in the Δ*hasR*Δ*phuR* mutant strain, the pUCP*hasRphuR* plasmid previously described (Minandri et al., 2016) was used.

Generation of *P. aeruginosa* Mutants

For mutant construction, *E. coli* and *P. aeruginosa* strains were grown in LB, with or without antibiotics, at 37 and 42°C, respectively, with vigorous aeration. Previously described suicide plasmids (**Table 1**) were used according to procedures detailed elsewhere (Milton et al., 1996; Frangipani et al., 2008).

Measurement of Cytochrome *c* Oxidase Activity in *P. aeruginosa* Intact Cells

Cytochrome *c* oxidase activity was assayed by using the artificial electron donor *N,N,N',N'*tetramethyl-*p*-phenylene diamine (TMPD) (Fluka). Briefly, bacteria were grown over-night in DCAA supplemented with 100 μM $FeCl_3$, then washed in saline and inoculated in DCAA to a final $OD_{600} = 0.05$. When the mid-exponential growth phase was reached (≈6 h post inoculum), cells were washed once in saline and adjusted to an $OD_{600} = 1$ (corresponding to $\approx 10^9$ CFU/ml).

Then, 10^8 bacterial cells (100 μl) were suspended in 1.4 ml of 33 mM potassium phosphate buffer (KPi, pH 7.0). The reaction was started by the addition of 5 μl of a 0.54 M TMPD solution to the sample cuvette. The rate of TMPD oxidation was recorded spectrophotometrically at 520 nm for 8 min at 25°C. Results were expressed as μmol TMPD oxidized/$min^{-1}/10^8$cells using 6.1 as the millimolar extinction coefficient of TMPD (Matsushita et al., 1982).

Isolation of Outer Membrane Proteins (OMPs) and SDS-PAGE Analysis

OMPs were isolated following the sarcosyl solubilization method (Filip et al., 1973), with some modifications. Briefly, bacteria from over-night cultures in DCAA supplemented with 100 μM $FeCl_3$ and 200 μg/ml Cb were washed in saline, then diluted to $OD_{600} = 0.05$ in 60 ml DCAA supplemented with 200 μg/ml Cb, and incubated over-night at 37°C. Cells were collected by centrifugation (2500 × *g*, 20 min), washed with 5 ml of 30 mM Tris HCl (pH 8, Sigma-Aldrich) and suspended in 1 ml of the same buffer. Bacteria were lysed by sonication in an ice bath (8 × 20 s cycles in a Sonics Vibra-Cell™ VCX 130 sonicator), punctuated by 20 s intervals (50% power). Phenyl methyl sulfonyl fluoride (PMSF, Sigma-Aldrich) was added to cell lysate at 1 mM final concentration. Unbroken cells were removed by centrifugation at 2400 × *g* for 20 min, and supernatants were transferred to fresh tubes. Sarcosyl (N-laurylsarcosinate sodium salt, Sigma) was added to the supernatant to a final concentration of 2%. After 1 h incubation at room temperature with gentle shaking, the mixture was centrifuged for 2 h at 55,000 × *g* at 4°C. OMP pellets were suspended in 40 μl 2 x SDS-PAGE loading dye (Sambrook et al., 1989), boiled for 10 min, then separated by 8% SDS-PAGE and visualized by Coomassie brilliant blue staining.

Statistical Analysis

Statistical analysis was performed with the software GraphPad Instat (GraphPad Software, Inc., La Jolla, CA), using One-Way Analysis of Variance (ANOVA), followed by Tukey-Kramer Multiple Comparisons Test.

RESULTS

P. aeruginosa is Inhibited by GaPPIX under Iron-Deplete Conditions

It has been previously reported that GaPPIX has no effect on *P. aeruginosa* (Stojiljkovic et al., 1999). This results is quite surprising given that *P. aeruginosa* is able to utilize heme as an iron source, by expressing two heme-uptake systems, i.e., *has* and *phu* (Ochsner et al., 2000). However, since the effect of GaPPIX has previously been investigated in iron-rich media (Stojiljkovic et al., 1999), we sought that under these conditions iron availability would have impaired Ga(III) activity. To verify this hypothesis, we preliminary tested the effect of GaPPIX on *P. aeruginosa* PAO1 growth using the iron-poor medium DCAA (Visca et al., 1993), supplemented with increasing concentrations of GaPPIX, the iron-binding porphyrin Hemin, or $Ga(NO_3)_3$, the latter resulting very active on *P. aeruginosa* in this medium (Bonchi et al., 2015). $Ga(NO_3)_3$ completely inhibited *P. aeruginosa* growth at 12.5 μM, and its activity was abrogated by the addition of $FeCl_3$ (**Figure 2A**) consistent with previous findings (Kaneko et al., 2007; Frangipani et al., 2014). Although the minimal inhibitory concentration (MIC) could not be determined for up to 100 μM GaPPIX (**Figure 2A**), exposure of PAO1 to GaPPIX reduced bacterial growth by 50% (IC_{50}) at 12.5 μM (**Figure 2A**). Also in the case of GaPPIX, growth inhibition was completely reversed by the addition of $FeCl_3$ (**Figure 2A**). As expected, exposure *P. aeruginosa* PAO1 to Hemin promoted bacterial growth at concentrations ranging between 1.55 and 25 μM, in line with the ability of *P. aeruginosa* to use Hemin as an iron source (Ochsner et al., 2000).

The GaPPIX susceptibility of *P. aeruginosa* PAO1 was also tested using the disk diffusion assays in DCAA agar plates supplemented or not with an excess of $FeCl_3$ (600 μM) (**Figure 2B**). In $FeCl_3$-supplemented DCAA, both GaPPIX and $Ga(NO_3)_3$ caused no inhibition of PAO1 growth. Conversely, in DCAA a clear ZOI was observed around the GaPPIX and $Ga(NO_3)_3$ disks (**Figure 2B**). Different from the ZOI formed by $Ga(NO_3)_3$, the ZOI formed by GaPPIX was less transparent (**Figure 2B**), consistent with the evidence that no MIC (full inhibition) could be determined for GaPPIX in liquid DCAA (**Figure 2A**). Although more transparent, the ZOI caused by $Ga(NO_3)_3$ was smaller than that of GaPPIX (**Figure 2B**). These preliminary data indicate that iron-deplete conditions render *P. aeruginosa* PAO1 susceptible to GaPPIX-mediated growth inhibition.

The Response of *P. aeruginosa* Cells to GaPPIX Depends on Intracellular Iron Carryover

The above results prompted us to investigate the effect of the intracellular iron content on GaPPIX-dependent growth inhibition. To this aim, the effect of GaPPIX was compared between *P. aeruginosa* PAO1 cells that had been pre-cultured in either DCAA containing 100 μM $FeCl_3$ (to increase the intracellular iron content) or DCAA without $FeCl_3$ (to lower the intracellular iron content). Iron-starved bacterial cells were significantly more susceptible to GaPPIX ($P < 0.001$) compared with those pre-cultured with $FeCl_3$ (**Figure 3A**). In particular, upon the addition of 0.38 μM GaPPIX, the growth of iron-starved PAO1 cells was reduced by 40% compared with cells pre-cultured in the presence of 100 μM $FeCl_3$ (**Figure 3A**).

To further investigate the correlation between the intracellular iron content and GaPPIX-dependent growth inhibition, GaPPIX

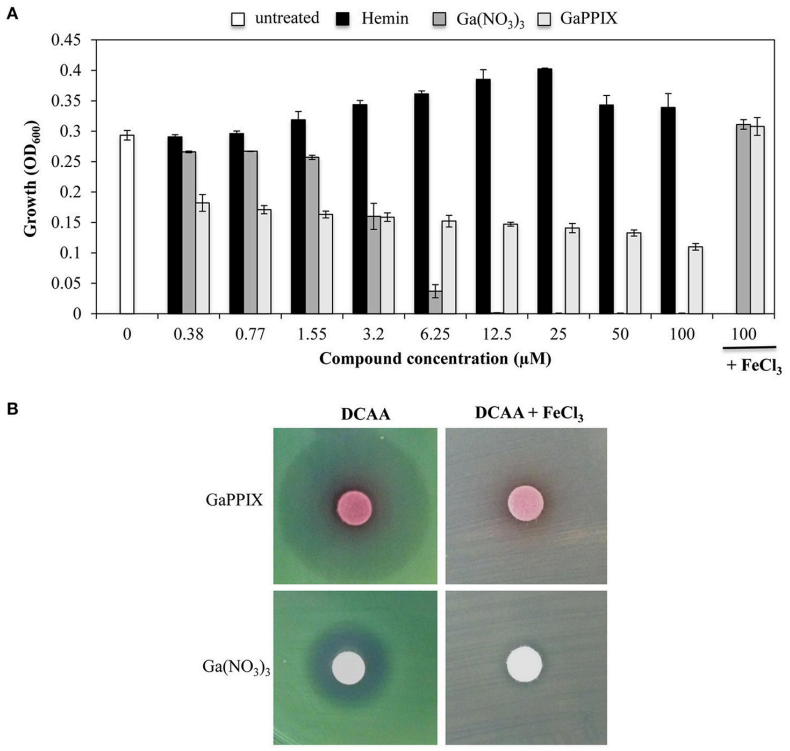


FIGURE 2 | GaPPIX inhibits *P. aeruginosa* PAO1 growth under iron-deplete conditions. (A) *P. aeruginosa* PAO1 was grown for 24 h at 37°C in DCAA in the presence of different concentrations of Hemin (black bars), $Ga(NO_3)_3$ (dark gray bars), GaPPIX (light gray bars), or nothing (white bar). Control cultures were supplemented with 100 μM $FeCl_3$ and 100 μM of either $Ga(NO_3)_3$ or GaPPIX. Values are the mean of 3 independent experiments, each one performed in duplicate ± the standard deviation. **(B)** Approximately 5×10^6 *P. aeruginosa* PAO1 cells were seeded on the surface of DCAA agar plates, supplemented or not with 600 μM $FeCl_3$, as indicated on top. Then, disks soaked with 10 μl of a 15 mM solution of either GaPPIX or $Ga(NO_3)_3$ were deposited on the agar surface, as indicated on the left. Plates were incubated for 16 h at 37°C. Images are representative of three independent experiments yielding similar results.

susceptibility was evaluated on *P. aeruginosa* mutants impaired in Fe(III)-siderophore uptake systems, i.e., mutants unable to synthesize pyoverdine (Δ*pvdA*), pyochelin (Δ*pchD*), or both siderophores (Δ*pvdA*Δ*pchD*) (**Figure 3B**). While GaPPIX-dependent growth inhibition was similar in the wild type and the Δ*pchD* mutant, both Δ*pvdA* and Δ*pvdA*Δ*pchD* mutants were extremely sensitive to GaPPIX (**Figure 3B**). In particular, 0.38 μM GaPPIX inhibited the growth of the Δ*pvdA* and Δ*pvdA*Δ*pchD* mutant strains by 75 and 78%, respectively, compared with the untreated cultures, while it reduced the growth of the wild-type strain and of the Δ*pchD* mutant by only 40 and 30%, respectively (**Figure 3B**). Altogether, these data indicate that the response of *P. aeruginosa* PAO1 to GaPPIX also depends on the carryover of intracellular iron.

GaPPIX is Preferentially Uptaken *via* the *P. aeruginosa* PhuR Receptor

To investigate the hypothesis that GaPPIX may enter *P. aeruginosa* cells by exploiting the same routes as heme, *P. aeruginosa* mutants carrying a deletion of either of the known heme receptors (Δ*hasR* and Δ*phuR* mutants; **Table 1**) were generated. The effect of GaPPIX on these mutants, as well as on a Δ*hasR*Δ*phuR* double mutant lacking both heme receptors (Minandri et al., 2016), was investigated in DCAA in the presence of 12.5 μM GaPPIX (IC_{50}; **Figure 4A**). While all strains showed the same growth profiles in the untreated medium, both Δ*phuR* and Δ*hasR*Δ*phuR* mutants grew better than the wild type or the Δ*hasR* mutant in the presence of 12.5 μM GaPPIX, displaying ≈50% higher growth levels relative to the wild type or the Δ*hasR* mutant (**Figure 4A**). These data suggest that, among the *P. aeruginosa* heme-uptake systems, *phu* has a more prominent role than *has* in the uptake of GaPPIX. Then, the effect of GaPPIX on heme-receptor mutants was evaluated in DCAA agar plates, by performing the disk diffusion assays (**Figure 4B**). Results showed a similar ZOI (27.6 ± 2.0 mm) for both the wild-type strain and the Δ*hasR* mutant, while a smaller ZOI (24.5 ± 0.7 mm) was observed for the Δ*phuR* mutant, indicating a less susceptible phenotype (**Figure 4B,** Table S2).

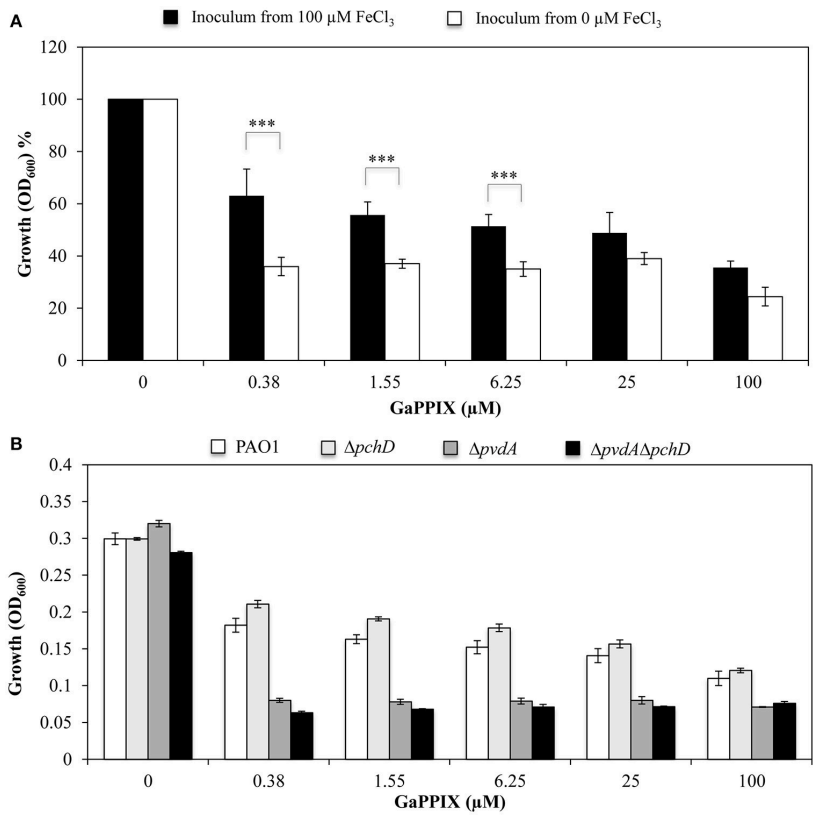


FIGURE 3 | The response of *P. aeruginosa* to GaPPIX depends on intracellular iron carryover. (A) *P. aeruginosa* PAO1 cells from an inoculum with 100 μM $FeCl_3$ (black bars) and without $FeCl_3$ (white bars) were grown in DCAA supplemented with increasing concentrations of GaPPIX for 24 h at 37°C. The values are expressed as the percentage relative to the untreated cultures, and represent the mean of four independent experiments, each one performed at least in duplicate ± the standard deviation. Asterisks indicate statistically significant differences relative to a culture derived from cells grown in the presence of 100 μM $FeCl_3$ (***$P <$ 0.001). **(B)** *P. aeruginosa* PAO1 (white bars), Δ*pchD* (light gray bars), Δ*pvdA* (dark gray bars), and Δ*pvdA*Δ*pchD* (black bars) were grown in DCAA supplemented with increasing concentrations of GaPPIX for 24 h at 37°C. Values are the mean of two independent experiments, each one performed in duplicate ± the standard deviation.

In addition, no ZOI was observed for the Δ*hasR*Δ*phuR* double mutant, indicating a fully resistant phenotype (**Figure 4B**). These observations indicate that both *has* and *phu* systems are implicated in GaPPIX transport, although the *phu* system appears to be the preferential route for the entrance of GaPPIX in *P. aeruginosa* cells (**Figure 4B**).

The Sensitivity of *P. aeruginosa* to GaPPIX Depends on the Expression of the Heme-Uptake Receptors

To further investigate the contribution of the HasR and PhuR receptors to GaPPIX-uptake, we individually expressed multicopy *hasR*, *phuR*, or both *hasR* and *phuR* in the Δ*hasR*Δ*phuR* mutant strain (using plasmids pUCP*hasR*, pUCP*phuR*, or pUCP*hasRphuR*, respectively) (**Figure 5A**). The effect of GaPPIX on these strains was initially tested by the disk diffusion assays (**Figure 5A**). While, the empty pUCP18 vector did not alter the susceptibility of Δ*hasR*Δ*phuR* to GaPPIX (**cfr Figures 5A**, **4B**), the expression of *hasR* from the multicopy plasmid pUCP*hasR* made the Δ*hasR*Δ*phuR* mutant more susceptible to GaPPIX (ZOI = 27.6 ± 2.0 mm) (**Figure 5A,** Table S2). The effect of GaPPIX was even more pronounced in the Δ*has*Δ*phuR* mutant overexpressing either *phuR* (Δ*has*Δ*phuR* carrying the multicopy plasmid pUCP*phuR*; ZOI = 34.0 ± 1.0 mm) or both *hasR* and *phuR* (Δ*has*Δ*phuR* carrying the multicopy plasmid pUCP*hasRphuR*; ZOI = 33.3 ± 0.5 mm) (**Figure 5A,** Table S2). GaPPIX sensitivity of the Δ*has*Δ*phuR* strain expressing *hasR*, *phuR*, or both genes, was also evaluated in DCAA liquid medium, in the presence of different concentrations of GaPPIX (**Figure 5B**). All strains grew equally in the untreated medium, and GaPPIX did not

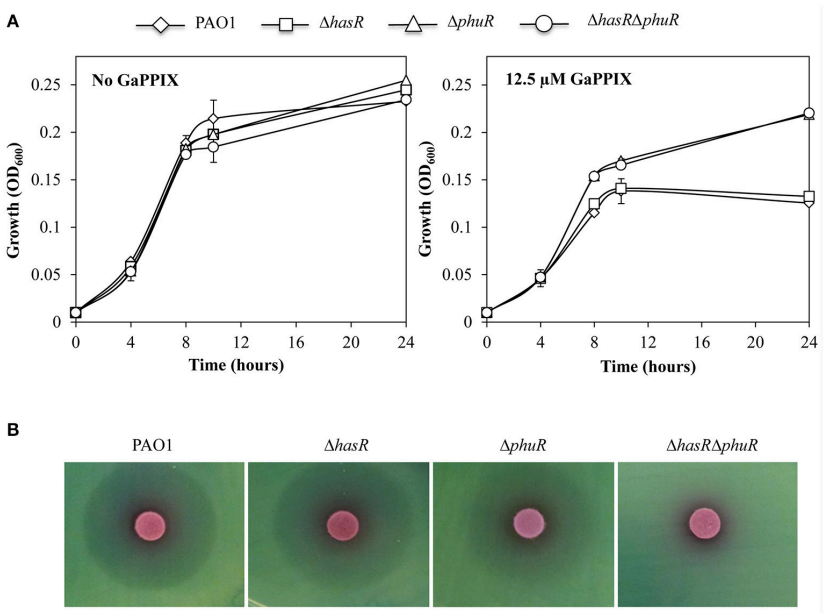


FIGURE 4 | GaPPIX enters *P. aeruginosa* cells through the heme-uptake systems. (A) Growth of *P. aeruginosa* wild type (diamond) and Δ*hasR* (square), Δ*phuR* (triangle), and Δ*hasR*Δ*phuR* (circle) mutant strains in DCAA supplemented or not with 12.5 μM GaPPIX at 37°C. Values are the mean of two independent experiments, each one performed in duplicate ± the standard deviation. **(B)** Approximately 5 × 10^6 bacterial cells were seeded on DCAA agar plates, then disks soaked with 10 μl of a 15 mM solution of GaPPIX were deposited on the agar surface. Plates were incubated for 16 h at 37°C. Images are representative of three independent experiments yielding similar results.

affect the growth of Δ*hasR*Δ*phuR*/pUCP18 up to 25 μM (**Figure 5B**). Conversely, strains Δ*hasR*Δ*phuR*/pUCP*hasR*, Δ*hasR*Δ*phuR*/pUCP*phuR*, and Δ*hasR*Δ*phuR*/pUCP*hasRphuR* were very sensitive to GaPPIX. In particular, 0.38 μM GaPPIX reduced the growth of the Δ*hasR*Δ*phuR*/pUCP*hasR* strain by 56%, and by >80% in both Δ*hasR*Δ*phuR*/pUCP*phuR* and Δ*hasR*Δ*phuR*/pUCP*hasRphuR* strains (**Figure 5B**). This effect was much more pronounced than that observed for the parental strain PAO1 (**Figure 2A**). Of note, no further growth reduction was observed for both the Δ*hasR*Δ*phuR*/pUCP*phuR* and Δ*hasR*Δ*phuR*/pUCP*hasRphuR* mutant strains at > 0.38 μM GaPPIX. The increased sensitivity of the Δ*hasR*Δ*phuR* strain expressing either *hasR* or *phuR*, relative to the wild type, can be explained by the overexpression of heme receptors from the multicopy plasmid pUCP18 (**Figure 5A**). To confirm this hypothesis, HasR and PhuR protein levels were visualized by SDS-PAGE analysis of OMPs purified from the different *P. aeruginosa* strains cultured in DCAA (**Figure 5C**). By comparing *P. aeruginosa* outer-membrane-proteins profiles of the wild type, the Δ*phuR* or the Δ*hasR*Δ*phuR* mutant strains, the lack of a *ca.* 75 kDa protein in the Δ*phuR* or the Δ*hasR*Δ*phuR* mutants, was observed. This was in good agreement with a predicted molecular mass of 82 kDa for the mature PhuR receptor. Moreover, a protein band at that position was evident in SDS-PAGE electropherograms of the Δ*hasR*Δ*phuR*/pUCP*phuR* and the Δ*hasR*Δ*phuR*/pUCP*hasRphuR* complemented mutants (**Figure 5C**). Similarly, a protein band corresponding to *ca.* 94 kDa, consistent with the HasR receptor mass, was absent in the Δ*hasR* and Δ*hasR*Δ*phuR* mutants, while it was clearly detectable in the Δ*hasR*Δ*phuR*/pUCP*hasR* and Δ*hasR*Δ*phuR*/pUCP*hasRphuR* complemented mutants (**Figure 5C**). In line with previous results (Ochsner et al., 2000), protein levels greatly differed between PhuR and HasR, the latter being poorly expressed in wild-type PAO1. These results confirm that both HasR and PhuR direct GaPPIX entrance in *P. aeruginosa* cells, and argue for a prominent role of PhuR as a consequence of its higher expression levels, compared with HasR.

To confirm the specificity of GaPPIX for both heme-uptake systems, we investigated whether the growth inhibitory effect of GaPPIX could be rescued by the presence of Hemin (Hm) or Hemoglobin (Hb), which are known to deliver iron *via* heme-uptake receptors (Ochsner et al., 2000). To this aim, the heme-uptake mutant Δ*hasR*Δ*phuR* overexpressing either PhuR or HasR was tested in the GaPPIX disk diffusion assays in the presence of Hm and Hb (**Figure 5D**). Both Hm and Hb partly rescued the growth of the Δ*hasR*Δ*phuR* mutant overexpressing either PhuR (from pUCP*phuR*) or HasR (from pUCP*hasR*) thus

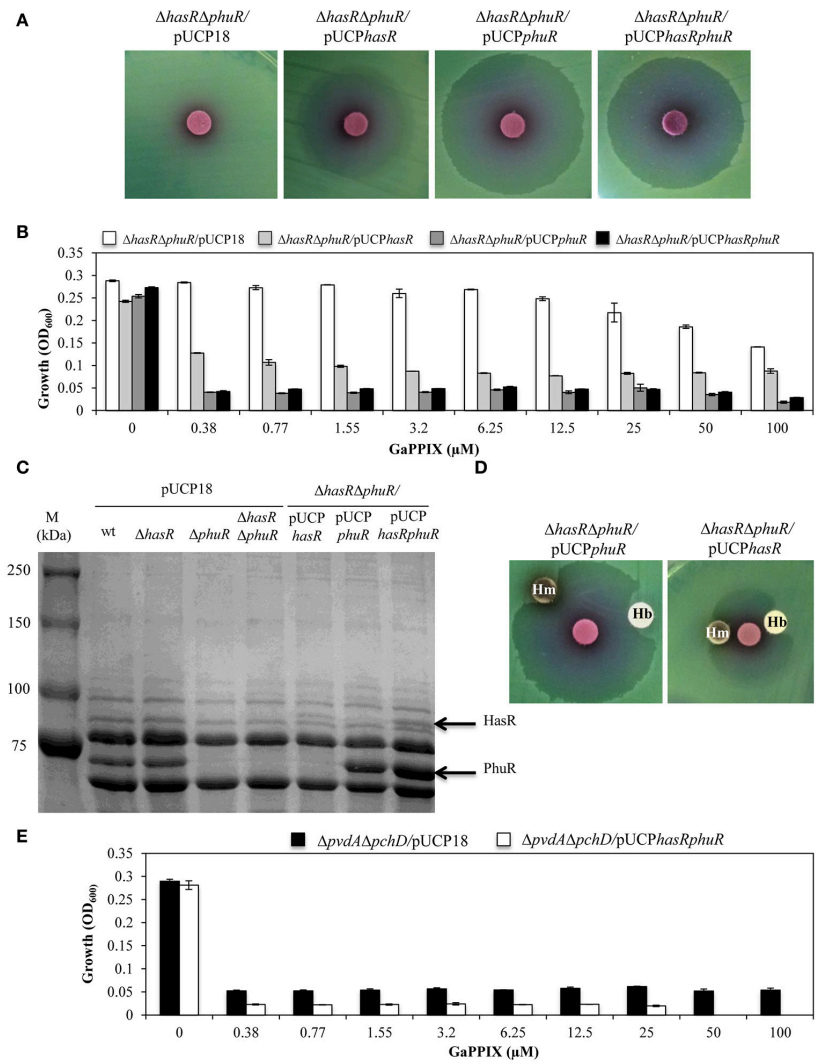


FIGURE 5 | The sensitivity of *P. aeruginosa* to GaPPIX depends on the expression of heme-uptake systems. (A) Approximately 5 × 10^6 bacterial cells were seeded on DCAA agar plates containing 200 μg/ml Cb, then disks soaked with 10 μl of a 15 mM solution of GaPPIX were deposited on the agar surface. Plates were incubated for 16 h at 37°C. **(B)** Growth of the *P. aeruginosa* Δ*hasR*Δ*phuR* mutant strain carrying the empty vector pUCP18 (white bars), or overexpressing *hasR* from plasmid pUCP*hasR* (light gray bars) or *phuR* from plasmid pUCP*phuR* (dark gray bars), or both *hasR* and *phuR* from plasmid pUCP*hasRphuR* (black bars), in DCAA supplemented with increasing concentrations of GaPPIX for 24 h at 37°C. **(C)** SDS-PAGE analysis of the outer membrane proteins of different *P. aeruginosa* PAO1 strains. Strains and plasmids are indicated on the top of each lane. M is the molecular mass marker (kDa), with band sizes on the left. The position of the putative 82 kDa PhuR and 94 kDa HasR outer membrane receptors is indicated by arrows on the right. **(D)** Rescue effect of hemin (Hm) or hemoglobin (Hb) from GaPPIX growth inhibition in the Δ*hasR*Δ*phuR* mutant strain overexpressing either *phu* from plasmid pUCP*phuR* or *hasR* from plasmid pUCP*hasR*. Approximately 5×10^6 bacterial cells were seeded on DCAA agar plates containing 200 μM Cb. Blank discs were soaked with 10 μl of a 15 mM solution of GaPPIX or with 75 μg of either Hm or Hb. Plates were incubated at 37°C for 16 h. **(E)** Growth of the *P. aeruginosa* Δ*pvdA*Δ*pchD* mutant strain carrying the empty vector pUCP18 (black bars), or overexpressing both *hasR* and *phuR* from plasmid pUCP*hasRphuR* (white bars), in DCAA supplemented with increasing concentrations of GaPPIX for 24 h at 37°C. Values are the mean of two independent experiments, each one performed in triplicate ± the standard deviation. Images in **B** and **D** are representative of two independent experiments yielding similar results.

confirming that (i) Hm, Hb and GaPPIX compete with heme receptors and (ii) GaPPIX enters *P. aeruginosa* cells through PhuR and HasR (**Figure 5D**).

It has been observed that *P. aeruginosa* isolates evolving during chronic lung infection in CF patients tend to accumulate mutations in siderophore loci, concomitant with preferential utilization of heme iron (Cornelis and Dingemans, 2013; Marvig et al., 2014; Andersen et al., 2015). To simulate this situation, we tested GaPPIX susceptibility of a siderphore-defective *P. aeruginosa* mutant overexpressing both PhuR and HasR receptors (Δ*pvdA*Δ*pchD*/pUCP*hasRphuR*). Whereas, exposure of the Δ*pvdA*Δ*pchD* mutant to GaPPIX reduced bacterial growth by 82% at 0.38 μM, expression of both *hasR* and *phuR* from multicopy plasmid pUCP*hasRphuR* made the Δ*pvdA*Δ*pchD* mutant extremely susceptible to GaPPIX, displaying 90% growth reduction (IC_{90}) at 0.38 μM (**Figure 5E**). Notably, full inhibition of the Δ*pvdA*Δ*pchD*/pUCP*hasRphuR* strain was observed upon challenge with 50 μM GaPPIX.

GaPPIX Targets the Aerobic Respiration of *P. aeruginosa*

GaPPIX has been proven effective against a wide range of pathogenic bacteria by targeting metabolic pathways that require heme as an enzymatic cofactor, such as cellular respiration (Stojiljkovic et al., 1999). Thus, we investigated whether GaPPIX could interfere with the activity of terminal oxidases implicated in *P. aeruginosa* aerobic respiration. In particular, we focused on Cco-1, Cco-2, and Cio, which have been shown to sustain *P. aeruginosa* growth under low oxygen conditions, as those encountered in the lung of CF patients (Alvarez-Ortega and Harwood, 2007; Kawakami et al., 2010). To this aim, we initially tested the sensitivity of cytochrome *c* oxidases (i.e., Cox, Cco-1, and Cco-2) to GaPPIX. Strains deleted of the whole operon encoding the terminal oxidase Cox (Δ*cox*) or both the Cco-1 and Cco-2 terminal oxidases (Δ*cco*) were generated in the same parental strain used to generate the heme-receptor mutants (**Table 1**). The effect of GaPPIX was then assayed on these cytochrome-defective mutants using the TMPD redox indicator, which is an artificial electron donor to the cytochrome *c* (Matsushita et al., 1982). Oxidation of TMPD to a blue indophenol compound indicates electron flow to the cytochrome *c* terminal oxidases. Thus, cytochrome *c* oxidase activity was measured on *P. aeruginosa* PAO1 and in the Δ*cox* and Δ*cco* mutants grown in DCAA supplemented or not with a sub-inhibitory concentration of GaPPIX (4 μM). In whole cells cultured in the untreated medium, no cytochrome *c* oxidase activity could be measured in the Δ*cco* strain (**Figure 6A**), confirming that in our conditions the TMPD test mainly measures the activity of Cco. Indeed, the Δ*cox* mutation does not affect the TMPD oxidase activity (**Figure 6A**), as previously reported (Frangipani and Haas, 2009). This is because Cox is known to be poorly expressed during *P. aeruginosa* exponential growth (Kawakami et al., 2010). Interestingly, 4 μM GaPPIX reduced the respiratory activity by more than 50% in the wild-type strain PAO1 and the Δ*cox* mutant, compared with the untreated condition (**Figure 6A**). These observations suggest that Cco-1 and Cco-2 terminal oxidases are sensitive to GaPPIX. To confirm these preliminary results, the effect of GaPPIX was tested on a mutant expressing only Cco-1 and Cco-2. To this aim, a Δ*cyo*Δ*cio*Δ*cox* triple mutant strain was generated. Disk diffusion assays showed that the Δ*cyo*Δ*cio*Δ*cox* mutant was more sensitive to GaPPIX than the wild type (ZOI = 34.6 ± 1.24 vs 27.6 ± 2.0 mm, respectively) (**Figure 6B,** Table S2). Similar results were obtained in DCAA liquid cultures. PAO1 wild type and the Δ*cyo*Δ*cio*Δ*cox* mutant showed a similar growth profile in the untreated medium (**Figure 6C**), whereas exposure to 0.38 μM GaPPIX reduced bacterial growth by 40% and 68%, respectively, relative to the untreated cultures (**Figure 6C**). Interestingly, it was possible to determine an IC_{90} at 82 μM for the Δ*cyo*Δ*cio*Δ*cox* mutant strain (**Figure 6C**). These results confirm that Cco-1 and Cco-2 are targeted by GaPPIX.

Then, the effect of GaPPIX on the Cio terminal oxidase was assessed. To this purpose, sodium azide (NaN_3) was used as a specific inhibitor of copper-dependent oxidases, i.e., all terminal oxidases except Cio (Cunningham and Williams, 1995). Preliminarily, we determined the minimal NaN_3 concentration inhibiting all terminal oxidases except Cio in DCAA, by comparing the growth of wild-type PAO1 and the Δ*cio* mutant in the presence of increasing NaN_3 concentrations (250–1000 μM). We observed that 350 μM of NaN_3 completely inhibited the Δ*cio* mutant without affecting PAO1 growth (data not shown). Then, the sensitivity of Cio to GaPPIX was tested by performing a GaPPIX disk diffusion assays with wild-type PAO1 in DCAA supplemented or not with 350 μM NaN_3. It was observed that PAO1 remains sensitive to GaPPIX in the presence of 350 μM NaN_3, displaying a ZOI even greater than that obtained for PAO1 without NaN_3 (36.6 ± 3.0 vs 27.6 ± 2.0 mm, respectively) (**Figure 7A,** Table S2). This result provides evidence that Cio is a target for GaPPIX. To strengthen this evidence, a *P. aeruginosa* Δ*cyo*Δ*cco*Δ*cox* triple mutant, which expresses only Cio (**Table 1**) was constructed and assayed for GaPPIX susceptibility. Disk diffusion assay results showed that the Δ*cyo*Δ*cco*Δcox mutant was more sensitive to GaPPIX than the wild-type PAO1 (ZOI = 30.0 ± 0.7 vs 27.6 ± 2.0 mm, respectively) (**Figure 7A,** Table S2). Similar results were also obtained in DCAA liquid medium, showing that GaPPIX significantly reduced ($P < 0.001$) the growth of the Δ*cyo*Δ*cco*Δcox mutant relative to the wild type, at concentrations ranging between 0.38 and 6.25 μM (**Figure 7B**). Altogether, the above results indicate that *P. aeruginosa* Cco-1, Cco-2, and Cio terminal oxidases are targets for GaPPIX.

P. aeruginosa Clinical Isolates Are Sensitive to GaPPIX

The expression of *P. aeruginosa* genes encoding heme-uptake systems has recently been detected in sputum samples collected from CF patients (Konings et al., 2013), and an evolution toward preferential heme utilization has been documented in *P. aeruginosa* during the course of chronic lung infection in CF patients (Marvig et al., 2014; Nguyen et al., 2014). Given the importance of heme in sustaining *P. aeruginosa* growth during infection, we have comparatively assessed the response to $Ga(NO_3)_3$ and GaPPIX in a collection of *P. aeruginosa* clinical

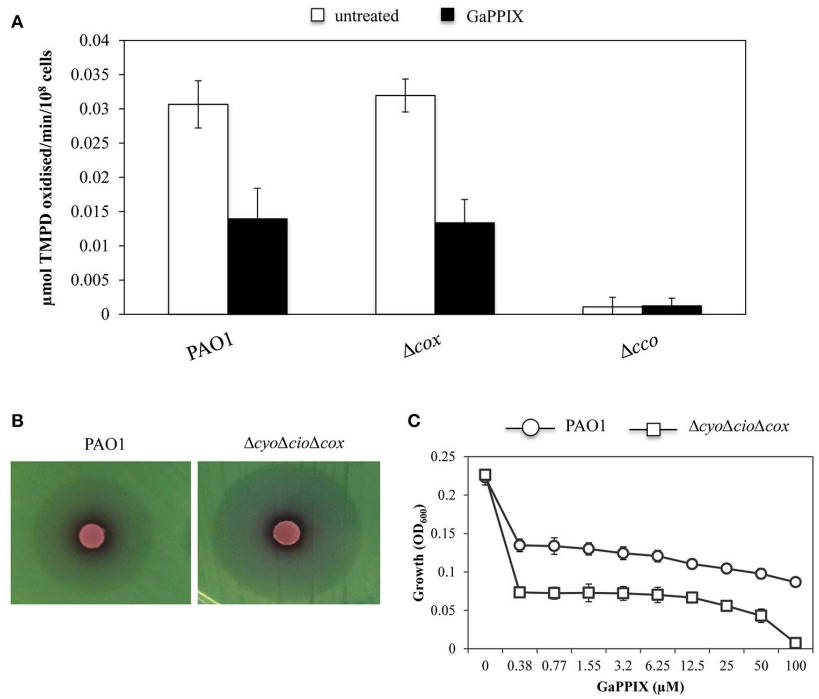


FIGURE 6 | GaPPIX inhibits *P. aeruginosa* PAO1 growth by targeting Cco. (A) TMPD oxidase activity on whole cells of wild-type PAO1, Δ*cox* and Δ*cco* mutants grown for 6 h in DCAA supplemented (black bars) or not (white bars) with 4 μM GaPPIX. Activity is expressed as μmol TMPD oxidized/min^{-1}/10^8 cells at pH 7.0 and 25°C. Each value is the average of three independent experiments, each one performed in triplicate ± the standard deviation. **(B)** Approximately 5 × 10^6 bacterial cells of wild-type PAO1 and the Δ*cyo*Δ*cio*Δ*cox* triple mutant were seeded on DCAA agar plates, then disks soaked with 10 μl of a 15 mM solution of GaPPIX were deposited on the agar surface. Plates were incubated for 16 h at 37°C. Images are representative of two independent experiments giving similar results. **(C)** Growth of wild type PAO1 (open circle) and the Δ*cyo*Δ*cio*Δ*cox* triple mutant (open square) in DCAA supplemented with increasing GaPPIX concentrations, for 24 h at 37°C. Values are representative of two independent experiments, each one performed in triplicate ± the standard deviation.

isolates from CF and non-CF patients (**Figure 8**, Table S1). Although GaPPIX (up to 100 μM) never abolished *P. aeruginosa* growth, the majority of clinical isolates (>70%) was sensitive to GaPPIX, displaying an IC_{50} values in the range 0.1–15.2 μM (Table S1). Moreover, all but one *P. aeruginosa* clinical isolates were significantly more susceptible than the reference PAO1 strain (**Figure 8**). In line with previous reports (Bonchi et al., 2015), all clinical isolates except one (FM1, Table S1) were very sensitive to $Ga(NO_3)_3$, showing IC_{50} values ranging from 0.2 to 9 μM (Table S1).

DISCUSSION

The ability of pathogenic bacteria to colonize the host and cause infections is dependent on their capability to acquire iron and generate energy to sustain *in vivo* growth (Ratledge and Dover, 2000; Alvarez-Ortega and Harwood, 2007; Hammer et al., 2013). The success of *P. aeruginosa* as a pathogen relies on the presence of several iron-uptake systems (reviewed in Llamas et al., 2014), as well as on a multiplicity of terminal oxidases which allow bacterial respiration *in vivo*. Both iron-uptake systems and respiratory cytochromes have been shown to contribute to *P. aeruginosa* fitness during chronic lung infection in CF patients (Alvarez-Ortega and Harwood, 2007; Konings et al., 2013). Recent observations have documented an adaptation of *P. aeruginosa* toward heme iron acquisition in the CF lung, where bacterial energy metabolism mainly relies on the three terminal oxidases Cco-1, Cco-2, and Cio, all of which have high affinity for oxygen (Alvarez-Ortega and Harwood, 2007). These data suggest that heme utilization pathways and respiratory cytochromes could represent candidate targets for the development of new anti-*Pseudomonas* drugs (Alvarez-Ortega and Harwood, 2007; Marvig et al., 2014; Nguyen et al., 2014). Indeed, targeting bacterial membrane functions such as cellular respiration, are considered promising therapeutic opportunities, especially in the case of persistent or chronic infections (Hurdle et al., 2011). Given that all terminal oxidases require heme as a cofactor, and that heme-uptake systems are expressed during chronic lung infection, in this work we have investigated the effect of the heme-mimetic GaPPIX against *P. aeruginosa*. We focused on

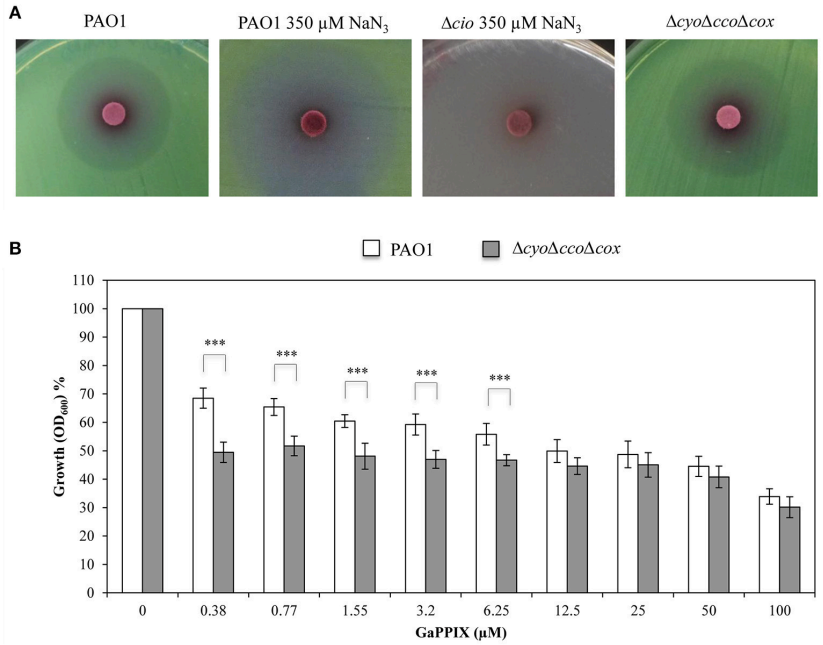


FIGURE 7 | GaPPIX inhibits *P. aeruginosa* PAO1 growth by targeting Cio. (A) Approximately 5×10^6 bacterial cells were seeded on DCAA agar plates, then disks soaked with 10 μl of a 15 mM solution of GaPPIX were deposited on the surface of DCAA agar plates supplemented or not with 350 μM NaN_3. Plates were incubated for 16 h at 37°C. Strains and conditions are indicated on top of each panel. Images are representative of two independent experiments giving similar results. **(B)** Growth inhibition (%) of wild type PAO1 (white bars) and the Δ*cyo*Δ*cco*Δ*cox* triple mutant (gray bars) in DCAA supplemented with increasing concentrations of GaPPIX relative to the untreated controls (no GaPPIX). Growth was measured after 24 h incubation at 37°C. Values are representative of four independent experiments, each one performed at least in duplicate ± the standard deviation. Asterisks indicate statistically significant differences between the wild type PAO1 and the Δ*cyo*Δ*cco*Δ*cox* triple mutant ($P < 0.001$).

Cco-1, Cco-2, and Cio since *P. aeruginosa* uses any of these three terminal oxidases to support the microaerobic growth necessary to thrive in the lung of CF patients. Cox and Cyo are not expressed or strongly repressed under these conditions (Alvarez-Ortega and Harwood, 2007).

We have initially demonstrated that GaPPIX is able to reduce the growth of *P. aeruginosa* only under iron-limiting growth conditions. However, different from $Ga(NO_3)_3$, bacterial growth was never completely inhibited at GaPPIX concentrations up to 100 μM (**Figure 2A**), in line with the fact that the ZOI for GaPPIX was less transparent compared with that generated by $Ga(NO_3)_3$ in the disk diffusion assays (**Figure 2B**). This diverse response of *P. aeruginosa* upon exposure to GaPPIX or $Ga(NO_3)_3$ (**Figures 2A,B**) could be explained by the fact that GaPPIX and $Ga(NO_3)_3$ enter bacterial cells through different pathways. $Ga(NO_3)_3$ may enter *P. aeruginosa* cells (i) by diffusion; (ii) through the HitAB iron transport proteins (García-Contreras et al., 2013); or (iii) *via* the siderophore Pch (Frangipani et al., 2014). On the other hand, we have demonstrated that GaPPIX can cross the *P. aeruginosa* outer membrane only through the heme-receptors HasR and PhuR, since a Δ*hasR*Δ*phuR* mutant is fully resistant to GaPPIX (**Figure 4**). Indeed, overexpression of heme receptors in the Δ*hasR*Δ*phuR* mutant makes this strain susceptible to GaPPIX, at even lower GaPPIX concentrations compared with wild-type PAO1 (**Figures 5A,B**). However, it should also be taken into consideration that GaPPIX and $Ga(NO_3)_3$ likely have different targets. In fact, while $Ga(NO_3)_3$ is known to target a variety of essential iron-containing enzymes (Bernstein, 1998; Soo et al., 2016), less is known about GaPPIX targets. Several studies have demonstrated that the antibacterial activity of GaPPIX relies on the molecule as a whole, since GaPPIX cannot be cleaved by bacterial enzymes (Stojiljkovic et al., 1999; Hammer et al., 2013). In fact, we demonstrated that the homolog of GaPPIX (Hemin) did not affect *P. aeruginosa* PAO1 growth. Indeed, Hemin promoted bacterial growth at concentrations ranging between 1.55 and 25 μM (**Figure 2A**), likely as a consequence of iron delivery to the cell, combined with positive regulation of the *has* system (Llamas et al., 2014). Hence, GaPPIX might be erroneously incorporated in heme-containing proteins such as cytochromes. However, due to the multiplicity of pathways involving cytochromes, exposure to GaPPIX never results in a complete growth inhibition. This

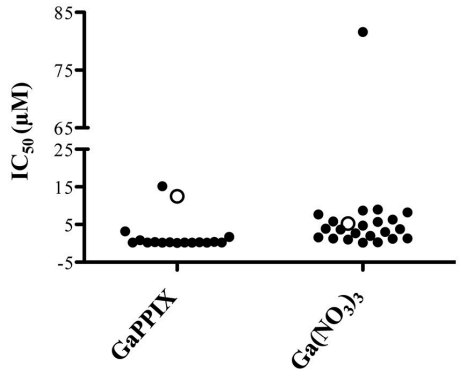


FIGURE 8 | Susceptibility of *P. aeruginosa* clinical isolates to GaPPIX. IC_{50} values of GaPPIX or $Ga(NO_3)_3$ in a collection of *P. aeruginosa* clinical isolates (black) and in the reference strains PAO1 (white). Bacteria were grown for 24 h at 37°C in DCAA with increasing concentrations of GaPPIX or $Ga(NO_3)_3$ to determine the IC_{50}. The IC_{50} of PAO1 was 12.5 μM for GaPPIX and 5.2 μM for $Ga(NO_3)_3$.

hypothesis is supported by the observation that GaPPIX is more active against *P. aeruginosa* mutants deleted in some of the cytochrome-dependent terminal oxidases (**Figures 6**, **7**). In fact, a *P. aeruginosa* mutant that only expresses the terminal oxidases Cco-1 and Cco-2 (Δ*cyo*Δ*cio*Δ*cox*) is much more sensitive to GaPPIX than the wild-type strain. In addition, the Δ*cyo*Δ*cio*Δ*cox* mutant showed a 68% growth reduction in liquid DCAA at 0.38 μM GaPPIX, compared to the untreated cultures, and an IC_{90} of 82 μM (**Figures 6B,C**). Along the same lines, a *P. aeruginosa* strain that only relies on the terminal oxidase Cio to respire oxygen, is more sensitive to GaPPIX than the wild-type strain (**Figure 7**). Taken together, our results demonstrate that GaPPIX targets *P. aeruginosa* respiratory cytochromes Cco-1, Cco-2, and Cio, which are exclusively found in bacteria (Cunningham and Williams, 1995; Pitcher and Watmough, 2004), although we cannot discriminate which of the Cco cytochromes is preferentially targeted by GaPPIX (the Δ*cco*-1,2 strain is mutated in both). Moreover, it is tempting to speculate that GaPPIX may also inhibit the other terminal oxidases Cyo and Cox (**Figure 1**), as well as some of the enzymes involved in denitrification, such as the heme-containing protein complexes Nir and Nor (**Figure 1**). Moreover, GaPPIX could also be incorporated into heme-containing enzymes involved in the protection from oxidative stress, increasing the susceptibility of *P. aeruginosa* to reactive oxygen species.

Although it was not possible to determine the MIC of GaPPIX for wild-type PAO1, it is worth to point out that GaPPIX was extremely active against a *P. aeruginosa* mutant impaired in siderophore production (Δ*pvdA*Δ*pchD*) and overexpressing both HasR and PhuR heme receptors from plasmid pUCP*hasRphuR* (**Figure 5**). Ninety percent growth reduction and full inhibition were observed upon exposure of this mutant to 0.38 and 50.0 μM GaPPIX, respectively. It is tempting to speculate that such strong inhibition could also occur in the CF lung, where siderophore-defective *P. aeruginosa* variants emerge during chronic infection, and heme represents the principal iron source (Marvig et al., 2014; Nguyen et al., 2014). Inhibition could further be enhanced under the microaerobic conditions encountered by *P. aeruginosa* in the CF airways (Hogardt and Heesemann, 2010), where the three high affinity terminal oxidases targeted by GaPPIX (Cco-1, Cco-2, and Cio) are essential for bacterial growth (Alvarez-Ortega and Harwood, 2007). Irrespective of the Ga(III) delivery system and of the energy metabolism adopted by *P. aeruginosa*, the balance between Fe(III) and Ga(III) availability *in vivo* will be the main determinant of Ga(III) efficacy. The inhibitory activity of GaPPIX was not limited to the prototypic strain PAO1, as it was also exerted on a representative collection of *P. aeruginosa* clinical isolates (Table S1). The great majority of clinical isolates (>70%) was sensitive to GaPPIX, irrespective of their origin, and all but one were significantly more susceptible than PAO1 ($IC_{50} \leq 3.2$ μM, Table S1).

Interestingly, studies on several human cell lines report that GaPPIX does not show cytotoxicity at concentrations ≤ 128 μM (Stojiljkovic et al., 1999; Chang et al., 2016), far above the concentrations that we found active on *P. aeruginosa* clinical isolates. Moreover, GaPPIX did not show to affect the health and behavior of mice, when administered by intraperitoneal injections (25–30 mg/kg) followed by four daily doses of (10–12 mg/kg) (Stojiljkovic et al., 1999), though it reduced the survival of *Galleria mellonella* larvae by 50% (LC_{50}) when injected at 25 mM (Arivett et al., 2015).

Although further studies are needed to assess the effect of GaPPIX against *P. aeruginosa* infection *in vivo*, our work should encourage future research directed to the development of heme-mimetic drugs targeting cellular respiration for the treatment of *P. aeruginosa* chronic lung infection.

AUTHOR CONTRIBUTIONS

PV and EF designed research; SH performed research; SH, EF, and PV analyzed data; SH, EF, and PV wrote the paper.

ACKNOWLEDGMENTS

This work was supported by grants from the Italian Ministry of University and Research PRIN-2012 (prot. 2012WJSX8K) and from the Italian Cystic Fibrosis Foundation (grant FFC#21/2015) to PV.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcimb.2017.00012/full#supplementary-material

REFERENCES

Alvarez-Ortega, C., and Harwood, C. S. (2007). Responses of *Pseudomonas aeruginosa* to low oxygen indicate that growth in the cystic fibrosis lung is by aerobic respiration. *Mol. Microbiol.* 65, 153–165. doi: 10.1111/j.1365-2958.2007.05772.x

Andersen, S. B., Marvig, R. L., Molin, S., Krogh Johansen, H., and Griffin, A. S. (2015). Long-term social dynamics drive loss of function in pathogenic bacteria. *Proc. Natl. Acad. Sci. U.S.A.* 112, 10756–10761. doi: 10.1073/pnas.1508324112

Andrews, S. C., Robinson, A. K., and Rodríguez-Quiñones, F. (2003). Bacterial iron homeostasis. *FEMS Microbiol. Rev.* 27, 215–237. doi: 10.1016/S0168-6445(03)00055-X

Arai, H. (2011). Regulation and function of versatile aerobic and anaerobic respiratory metabolism in *Pseudomonas aeruginosa*. *Front. Microbiol.* 2:103. doi: 10.3389/fmicb.2011.00103

Arivett, B. A., Fiester, S. E., Ohneck, E. J., Penwell, W. F., Kaufman, C. M., Relich, R. F., et al. (2015). Antimicrobial activity of gallium protoporphyrin IX against *Acinetobacter baumannii* strains displaying different antibiotic resistance phenotypes. *Antimicrob. Agents Chemother.* 59, 7657–7665. doi: 10.1128/AAC.01472-15

Ballouche, M., Cornelis, P., and Baysse, C. (2009). Iron metabolism: a promising target for antibacterial strategies. *Recent Pat. Antiinfect. Drug. Discov.* 4, 190–205. doi: 10.2174/157489109789318514

Banin, E., Lozinski, A., Brady, K. M., Berenshtein, E., Butterfield, P. W., Moshe, M., et al. (2008). The potential of desferrioxamine-gallium as an anti-*Pseudomonas* therapeutic agent. *Proc. Natl. Acad. Sci. U.S.A.* 105, 16761–16766. doi: 10.1073/pnas.0808608105

Bernstein, L. R. (1998). Mechanisms of therapeutic activity for gallium. *Pharmacol. Rev.* 50, 665–682.

Bonchi, C., Frangipani, E., Imperi, F., and Visca, P. (2015). Pyoverdine and proteases affect the response of *Pseudomonas aeruginosa* to gallium in human serum. *Antimicrob. Agents Chemother.* 59, 5641–5646. doi: 10.1128/AAC.01097-15

Bonchi, C., Imperi, F., Minandri, F., Visca, P., and Frangipani, E. (2014). Repurposing of gallium-based drugs for antibacterial therapy. *Biofactors* 40, 303–312. doi: 10.1002/biof.1159

Breidenstein, E. B., de la Fuente-Núñez, C., and Hancock, R. E. (2011). *Pseudomonas aeruginosa*: all roads lead to resistance. *Trends Microbiol.* 19, 419–426. doi: 10.1016/j.tim.2011.04.005

Cartron, M. L., Maddocks, S., Gillingham, P., Craven, C. J., and Andrews, S. C. (2006). Feo-transport of ferrous iron into bacteria. *Biometals* 19, 143–157. doi: 10.1007/s10534-006-0003-2

Chang, D., Garcia, R. A., Akers, K. S., Mende, K., Murray, C. K., Wenke, J. C., et al. (2016). Activity of gallium meso-and protoporphyrin IX against biofilms of multidrug-resistant *Acinetobacter baumannii* isolates. *Pharmaceuticals* 9:16. doi: 10.3390/ph9010016

Comolli, J. C., and Donohue, T. J. (2002). *Pseudomonas aeruginosa* RoxR, a response regulator related to *Rhodobacter sphaeroides* PrrA, activates expression of the cyanide-insensitive terminal oxidase. *Mol. Microbiol.* 45, 755–768. doi: 10.1046/j.1365-2958.2002.03046.x

Comolli, J. C., and Donohue, T. J. (2004). Differences in two *Pseudomonas aeruginosa* cbb_3 cytochrome oxidases. *Mol. Microbiol.* 51, 1193–1203. doi: 10.1046/j.1365-2958.2003.03904.x

Cornelis, P., and Dingemans, J. (2013). *Pseudomonas aeruginosa* adapts its iron uptake strategies in function of the type of infections. *Front. Cell. Infect. Microbiol.* 3:75. doi: 10.3389/fcimb.2013.00075

Cornelis, P., and Matthijs, S. (2002). Diversity of siderophore-mediated iron uptake systems in fluorescent pseudomonads: not only pyoverdines. *Environ. Microbiol.* 4, 787–7898. doi: 10.1046/j.1462-2920.2002.00369.x

Cornelis, P., Matthijs, S., and Van Oeffelen, L. (2009). Iron uptake regulation in *Pseudomonas aeruginosa*. *Biometals* 22, 15–22. doi: 10.1007/s10534-008-9193-0

Cox, C. D., and Adams, P. (1985). Siderophore activity of pyoverdin for *Pseudomonas aeruginosa*. *Infect. Immun.* 48, 130–138.

Cox, C. D., Rinehart, K. L. Jr., Moore, M. L., and Cook, J. C. Jr. (1981). Pyochelin: novel structure of an iron-chelating growth promoter for *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. U.S.A.* 78, 4256–4260.

Cunningham, L., Pitt, M., and Williams, H. D. (1997). The *cioAB* genes from *Pseudomonas aeruginosa* code for a novel cyanide-insensitive terminal oxidase related to the cytochrome *bd* quinol oxidases. *Mol. Microbiol.* 24, 579–591. doi: 10.1046/j.1365-2958.1997.3561728.x

Cunningham, L., and Williams, H. D. (1995). Isolation and characterization of mutants defective in the cyanide-insensitive respiratory pathway of *Pseudomonas aeruginosa*. *J. Microbiol.* 177, 432–438. doi: 10.1128/jb.177.2.432-438.1995

Davies, J. C., Alton, E. W., and Bush, A. (2007). Cystic fibrosis. *BMJ* 335, 1255–1259. doi: 10.1136/bmj.39391.713229.AD

Eschbach, M., Schreiber, K., Trunk, K., Buer, J., Jahn, D., Schobert, M., et al. (2004). Long-term anaerobic survival of the opportunistic pathogen *Pseudomonas aeruginosa* via pyruvate fermentation. *J. Bacteriol.* 186, 4596–4604. doi: 10.1128/JB.186.14.4596-4604.2004

Filip, C., Fletcher, G., Wulff, J. L., and Earhart, C. F. (1973). Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl sarcosinate. *J. Bacteriol.* 115, 717–722.

Foley, T. L., and Simeonov, A. (2012). Targeting iron assimilation to develop new antibacterials. *Expert Opin. Drug. Discov.* 7, 831–847. doi: 10.1517/17460441.2012.708335

Frangipani, E., Bonchi, C., Minandri, F., Imperi, F., and Visca, P. (2014). Pyochelin potentiates the inhibitory activity of gallium on *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 58, 5572–5575. doi: 10.1128/AAC.03154-14

Frangipani, E., and Haas, D. (2009). Copper acquisition by the SenC protein regulates aerobic respiration in *Pseudomonas aeruginosa* PAO1. *FEMS Microbiol.* 298, 234–240. doi: 10.1111/j.1574-6968.2009.01726.x

Frangipani, E., Slaveykova, V. I., Reimmann, C., and Haas, D. (2008). Adaptation of aerobically growing *Pseudomonas aeruginosa* to copper starvation. *J. Bacteriol.* 190, 6706–6717. doi: 10.1128/JB.00450-08

Fujiwara, T., Fukumori, Y., and Yamanaka, T. (1992). A novel terminal oxidase, cytochrome baa_3 purified from aerobically grown *Pseudomonas aeruginosa*: it shows a clear difference between resting state and pulsed state. *J. Biochem.* 112, 290–298.

García-Contreras, R., Lira-Silva, E., Jasso-Chávez, R., Hernández-González, I. L., Maeda, T., Hashimoto, T., et al. (2013). Isolation and characterization of gallium resistant *Pseudomonas aeruginosa* mutants. *Int. J. Med. Microbiol.* 303, 574–582. doi: 10.1016/j.ijmm.2013.07.009

Hammer, N. D., Reniere, M. L., Cassat, J. E., Zhang, Y., Hirsch, A. O., Hood, M. I., et al. (2013). Two heme-dependent terminal oxidases power *Staphylococcus aureus* organ-specific colonization of the vertebrate host. *MBio* 4, e00241–e00213. doi: 10.1128/mBio.00241-13

Heinrichs, D. E., Young, L., and Poole, K. (1991). Pyochelin-mediated iron transport in *Pseudomonas aeruginosa*: involvement of a high-molecular-mass outer membrane protein. *Infect. Immun.* 59, 3680–3684.

Hogardt, M., and Heesemann, J. (2010). Adaptation of *Pseudomonas aeruginosa* during persistence in the cystic fibrosis lung. *Int. J. Med. Microbiol.* 300, 557–562. doi: 10.1016/j.ijmm.2010.08.008

Hurdle, J. G., O'Neill, A. J., Chopra, I., and Lee, R. E. (2011). Targeting bacterial membrane function: an underexploited mechanism for treating persistent infections. *Nat. Rev. Microbiol.* 9, 62–75. doi: 10.1038/nrmicro2474

Imperi, F., Massai, F., Facchini, M., Frangipani, E., Visaggio, D., Leoni, L., et al. (2013). Repurposing the antimycotic drug flucytosine for suppression of *Pseudomonas aeruginosa* pathogenicity. *Proc. Nat1. Acad. Sci. U.S.A.* 110, 7458–7463. doi: 10.1073/pnas.1222706110

Imperi, F., Putignani, L., Tiburzi, F., Ambrosi, C., Cipollone, R., Ascenzi, P., et al. (2008). Membrane-association determinants of the ω-amino acid monooxygenase PvdA, a pyoverdine biosynthetic enzyme from *Pseudomonas aeruginosa*. *Microbiology* 154, 2804–2813. doi: 10.1099/mic.0.2008/018804-0

Kaneko, Y., Thoendel, M., Olakanmi, O., Britigan, B. E., and Singh, P. K. (2007). The transition metal gallium disrupts *P. aeruginosa* iron metabolism and has antimicrobial and antibiofilm activity. *J. Clin. Invest.* 117, 877–888. doi: 10.1172/JCI30783

Kawakami, T., Kuroki, M., Ishii, M., Igarashi, Y., and Arai, H. (2010). Differential expression of multiple terminal oxidases for aerobic respiration in *Pseudomonas aeruginosa*. *Environ. Microbiol.* 12, 1399–1412. doi: 10.1111/j.1462-2920.2009.02109.x

Konings, A. F., Martin, L. W., Sharples, K. J., Roddam, L. F., Latham, R., Reid, D. W., et al. (2013). *Pseudomonas aeruginosa* uses multiple pathways to acquire iron during chronic infection in cystic fibrosis lungs. *Infect. Immun.* 81, 2697–2704. doi: 10.1128/IAI.00418-13

Létoffé, S., Delepelaire, P., and Wandersman, C. (2004). Free and hemophore-bound heme acquisitions through the outer membrane receptor HasR have different requirements for the TonB-ExbB-ExbD complex. *J. Bacteriol.* 186, 4067–4074. doi: 10.1128/JB.186.13.4067-4074.2004

Létoffé, S., Redeker, V., and Wandersman, C. (1998). Isolation and characterization of an extracellular haem-binding protein from *Pseudomonas aeruginosa* that shares function and sequence similarities with the *Serratia marcescens* HasA haemophore. *Mol. Microbiol.* 28, 1223–1234. doi: 10.1046/j.1365-2958.1998.00885.x

Llamas, M. A., Imperi, F., Visca, P., and Lamont, I. L. (2014). Cell-surface signaling in *Pseudomonas*: stress responses, iron transport, and pathogenicity. *FEMS Microbiol. Rev.* 38, 569–597. doi: 10.1111/1574-6976.12078

Marvig, R. L., Damkiær, S., Khademi, S. H., Markussen, T. M., Molin, S., Jelsbak, L., et al. (2014). Within-host evolution of *Pseudomonas aeruginosa* reveals adaptation toward iron acquisition from hemoglobin. *MBio* 5, e00966–e00914. doi: 10.1128/mBio.00966-14

Matsushita, K., Shinagawa, E., Adachi, O., and Ameyama, M. (1982). *o*-Type cytochrome oxidase in the membrane of aerobically grown *Pseudomonas aeruginosa*. *FEBS Lett.* 139, 255–258. doi: 10.1016/0014-5793(82)80864-8

Matsushita, K., Yamada, M., Shinagawa, E., Adachi, O., and Ameyama, M. (1983). Membrane-bound respiratory chain of *Pseudomonas aeruginosa* grown aerobically. A KCN-insensitive alternate oxidase chain and its energetics. *J. Microbiol.* 93, 1137–1144.

Meyer, J. M., and Abdallah, M. A. (1978). The fluorescent pigment of *Pseudomonas fluorescens*: biosynthesis, purification and physicochemical properties. *J. Gen. Microbiol.* 107, 319–328. doi: 10.1099/00221287-107-2-319

Milton, D. L., O'Toole, R., Horstedt, P., and Wolf-Watz, H. (1996). Flagellin A is essential for the virulence of *Vibrio anguillarum*. *J. Bacteriol.* 178, 1310–1319. doi: 10.1128/jb.178.5.1310-1319.1996

Minandri, F., Bonchi, C., Frangipani, E., Imperi, F., and Visca, P. (2014). Promises and failures of gallium as an antibacterial agent. *Future Microbiol.* 9, 379–397. doi: 10.2217/fmb.14.3

Minandri, F., Imperi, F., Frangipani, E., Bonchi, C., Visaggio, D., Facchini, M., et al. (2016). Dissecting the role of iron uptake systems in *Pseudomonas aeruginosa* virulence and airways infection. *Infect. Immun.* 84, 2324–2335. doi: 10.1128/IAI.00098-16

Moore, N. M., and Flaws, M. L. (2011). Antimicrobial resistance mechanisms in *Pseudomonas aeruginosa*. *Clin. Lab. Sci.* 24, 47–51.

Murphy, T. F. (2006). The role of bacteria in airway inflammation in exacerbations of chronic obstructive pulmonary disease. *Curr. Opin. Infect. Dis.* 19, 225–230. doi: 10.1097/01.qco.0000224815.89363.15

Nguyen, A. T., O'Neill, M. J., Watts, A. M., Robson, C. L., Lamont, I. L., Wilks, A., et al. (2014). Adaptation of iron homeostasis pathways by a *Pseudomonas aeruginosa* pyoverdine mutant in the cystic fibrosis lung. *J. Bacteriol.* 196, 2265–2276. doi: 10.1128/JB.01491-14

Ochsner, U. A., Johnson, Z., and Vasil, M. L. (2000). Genetics and regulation of two distinct haem-uptake systems, *phu* and *has*, in *Pseudomonas aeruginosa*. *Microbiology* 146, 185–198. doi: 10.1099/00221287-146-1-185

Pitcher, R. S., and Watmough, N. J. (2004). The bacterial cytochrome cbb_3 oxidases. *Biochim. Biophys. Acta* 1655, 388–399. doi: 10.1016/j.bbabio.2003.09.017

Rangel-Vega, A., Bernstein, L. R., Mandujano Tinoco, E. A., García-Contreras, S. J., and García-Contreras, R. (2015). Drug repurposing as an alternative for the treatment of recalcitrant bacterial infections. *Front. Microbiol.* 6:282. doi: 10.3389/fmicb.2015.00282

Ratledge, C., and Dover, L. G. (2000). Iron metabolism in pathogenic bacteria. *Annu. Rev. Microbiol.* 54, 881–941. doi: 10.1146/annurev.micro.54.1.881

Rossi, M. S., Paquelin, A., Ghigo, J. M., and Wandersman, C. (2003). Haemophore-mediated signal transduction across the bacterial cell envelope in *Serratia marcescens*: the inducer and the transported substrate are different molecules. *Mol. Microbiol.* 48, 1467–1480. doi: 10.1046/j.1365-2958.2003.03516.x

Sambrook, J., Fitsch, E. F., and Maniatis, T. (1989). *Molecular Cloning*: *A Laboratori Manual*, *2nd Edn.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Schweizer, H. P. (1991). *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19. *Gene* 97, 109–112. doi: 10.1016/0378-1119(91)90016-5

Simon, R., Priefer, U., and Puhler, A. (1983). A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. *Nat. Biotech.* 1, 784–791. doi: 10.1038/nbt1183-784

Skaar, E. P. (2010). The battle for iron between bacterial pathogens and their vertebrate hosts. *PLoS Pathog.* 6:e1000949. doi: 10.1371/journal.ppat.1000949

Soo, V. W., Kwan, B. W., Quezada, H., Castillo-Juárez, I., Pérez-Eretza, B., García-Contreras, S. J., et al. (2016). Repurposing of anticancer drugs for the treatment of bacterial infections. *Curr. Top. Med. Chem.* doi: 10.2174/1568026616666160930131737. [Epub ahead of print].

Stojiljkovic, I., Kumar, V., and Srinivasan, N. (1999). Non-iron metalloporphyrins: potent antibacterial compounds that exploit haem/Hb uptake systems of pathogenic bacteria. *Mol. Microbiol.* 31, 429–442. doi: 10.1046/j.1365-2958.1999.01175.x

Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., et al. (2000). Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* 406, 959–964. doi: 10.1038/35023079

Vander, W. C., Piérard, A., Kley-Raymann, M., and Haas, D. (1984). *Pseudomonas aeruginosa* mutants affected in anaerobic growth on arginine: evidence for a four-gene cluster encoding the arginine deiminase pathway. *J. Bacteriol.* 160, 928–934.

Visca, P., Bonchi, C., Minandri, F., Frangipani, E., and Imperi, F. (2013). The dual personality of iron chelators: growth inhibitors or promoters? *Antimicrob. Agents Chemother.* 57, 2432–2433. doi: 10.1128/AAC.02529-12

Visca, P., Ciervo, A., Sanfilippo, V., and Orsi, N. (1993). Iron-regulated salicylate synthesis by *Pseudomonas* spp. *J. Gen. Microbiol.* 139, 1995–2001. doi: 10.1099/00221287-139-9-1995

Weinberg, E. D. (2009). Iron availability and infection. *Biochem. Biophys. Acta* 1790, 600–605. doi: 10.1016/j.bbagen.2008.07.002

Williams, H. D., Zlosnik, J. E., and Ryall, B. (2007). Oxygen, cyanide and energy generation in the cystic fibrosis pathogen *Pseudomonas aeruginosa*. *Adv. Microb. Physiol.* 52, 1–71. doi: 10.1016/S0065-2911(06)52001-6

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary material

Table S1. *P. aeruginosa* clinical isolates used in this study.

Strain	Origin	Source	GaPPIX IC_{50} (μM)	$Ga(NO_3)_3$ IC_{50} (μM)	Reference or source
TB73	CF	rs	-	9	Bragonzi *et al*., 2009
KK2	CF	rs	3.2	2.7	Bragonzi *et al*., 2009
KK27	CF	rs	-	3.9	Bragonzi *et al*., 2009
TR1	CF	rs	0.27	3	Bragonzi *et al*., 2009
AA11	CF	rs	-	5.7	Bragonzi *et al*., 2009
AA44	CF	rs	0.25	1.2	Massai *et al*., 2011
FM1	CF	rs	0.32	81.6	Massai *et al*., 2011
FM2	CF	rs	0.26	1.3	Massai *et al*., 2011
FM4	CF	rs	0.2	1.6	Massai *et al*., 2011
FM13	CF	rs	1.7	6.3	Massai *et al*., 2011
FM17	CF	rs	0.38	4.7	Massai *et al*., 2011
SP1	non-CF	ws	-	8.7	Bonchi *et al*., 2015
SP6	non-CF	blood	15.2	3.7	Bonchi *et al*., 2015
SP9	non-CF	cvc	-	5.8	Bonchi *et al*., 2015
SP10	non-CF	blood	0.2	0.3	Bonchi *et al*., 2015
SP11	non-CF	ascites	0.83	8.2	Bonchi *et al*., 2015
SP15	non-CF	pe	0.21	0.2	Bonchi *et al*., 2015
SP18	non-CF	bile	0.25	1.96	Bonchi *et al*., 2015
SP20	non-CF	blood	0.19	1	Bonchi *et al*., 2015
SP21	non-CF	pe	0.22	1.26	Bonchi *et al*., 2015
SP24	non-CF	bile	-	7.7	Bonchi *et al*., 2015
PA14	non-CF	burn	0.1	3.8	Rahme *et al*., 1995
PAO1	non-CF	ATCC15692	12.5	5.25	American type culture collection

Abbreviations: CF, cystic fibrosis; rs, respiratory secretions; ws, wound swab; cvc, central venous catheter; pe, pleural exudate; - resistant.

Table S2. Effect of GaPPIX on different *P. aeruginosa* strains determined by mean of disk diffusion assays.

Strain	**GaPPIX susceptibility (avg ZOI ± SD)**[a]
PAO1	27.6 ± 2.0
Δ*hasR*	27.6 ± 2.0
Δ*phuR*	24.5 ± 0.7
Δ*hasR*Δ*phuR*/pUCP*hasR*	27.6 ± 2.0
Δ*hasR*Δ*phuR*/pUCP*phuR*	34.0 ± 1.0
Δ*hasR*Δ*phuR*/pUCP*hasRphuR*	33.3 ± 0.5
Δ*cyo*Δ*cio*Δ*cox*	34.6 ± 1.24
PAO1 in the presence of 350 μM NaN_3	36.6 ± 3.0
Δ*cyo*Δ*cco*Δ*cox*	30.0 ± 0.7

[a] ZOIs were measured in mm; Average (avg) is the mean of at least three independent experiments.

Additional references not included in the main text:

Bragonzi, A., Paroni, M., Nonis, A., Cramer, N., Montanari, S., Rejman, J., Tümmler, B., et al. (2009). *Pseudomonas aeruginosa* microevolution during cystic fibrosis lung infection establishes clones with adapted virulence. *Am. J. Respir. Crit Care Med*. 180, 138-145. doi: 10.1164/rccm.200812-1943OC

Massai, F., Imperi, F., Quattrucci, S., Zennaro, E., Visca, P., Leoni, L., et al. (2011). A multitask biosensor for micro-volumetric detection of N-3-oxo-dodecanoyl-homoserine lactone quorum sensing signal. *Biosens. Bioelectron*. 26, 3444-3449. doi: dx.doi.org/10.1016/j.bios.2011.01.022

Rahme, L. G., Stevens, E. J., Wolfort, S. F., and Shao, J. (1995). Common virulence factors for bacterial pathogenicity in plants and animals. *Science* 268, 1899.

Chapter 3

Antimicrobial activity of gallium compounds on ESKAPE pathogens

Sarah Hijazi[a], Daniela Visaggio[a], Mattia Pirolo[a], Emanuela Frangipani[a], Lawrence Bernstein[b], and Paolo Visca[a]

[a]*Department of Science, University of Roma Tre, Rome, Italy*
[b]*Terrametrix, Menlo Park, CA 94025, USA*

Front Cell Infect Microbiol, 2018; 8:316. doi: 10.3389/fcimb.2018.00316.

Chapter 3

Antimicrobial activity of gallium compounds on ESKAPE pathogens

The paucity of antibiotics retaining activity on multidrug-resistant bacteria of the ESKAPE group (Chapter 1), poses the urgent need to discover novel antibacterial drugs. Considering the essential role of iron in bacterial physiology and pathogenicity, iron uptake and metabolism are regarded as druggable targets for new antibacterial strategies (Ballouche *et al.,* 2009; Foley and Simeonov, 2012) (Chapter 1). With the aim of targeting bacterial iron metabolism as a new therapeutic option, iron-acquisition routes have been hijacked using the post-transition metal Ga(III), which has a long history as a diagnostic and chemotherapeutic agent, and has recently been repurposed as an antibacterial agent. Lately, there has been an increase in the number of Ga(III) compounds displaying therapeutic potential, ranging from simple salts such as Ga(III)-nitrate $Ga(NO_3)_3$ (GaN), and Ga(III)-chloride ($GaCl_3$), through metal-organic complexes such as Ga(III)-maltolate (GaM) (Bernstein *et al.,* 2000) and Ga(III)-protoporphyrin IX (GaPPIX), belonging to the first-, second-, and third- generations of Ga(III) formulations, respectively (Chitambar, 2017).

At present, there are no comparative data on the activity of Ga(III) compounds against ESKAPE species under standard test conditions, representing a major pitfall to the repurposing of Ga(III) as last-resort antibacterial agent. Prompted by the promising results obtained with GaPPIX on *P. aeruginosa* (Chapter 2), we have widen our investigations on the inhibitory effect of Ga(III) on all pathogens belonging to the ESKAPE group, using not only GaPPIX but other two Ga(III)-based compounds, the most widely studied GaN, as well as GaM which is endowed with high bioavailability and low toxicity (Bernstein *et al.,* 2000). The antibacterial activity of Ga(III) compounds was investigated in different media characterized by different iron concentrations and nutrient compositions, namely Mueller- Hinton broth (MHB), iron-depleted MHB (DMHB) (Hackel *et al.,* 2018), and RPMI-1640 tissue culture medium supplemented with 10% complement-free human serum (RPMI-HS), to better mimic the *in vivo* environment (Antunes *et al.,* 2012; Thompson *et al.,* 2012; Bonchi *et al.,* 2015).

In this work, we have determined suitable test conditions, represented by RPMI-HS, to assess the antibacterial activity of Ga(III) compounds *in vitro*. The presence of HS in RPMI-HS reduces iron availability thanks to the presence of transferrin, thereby providing a more realistic milieu for testing the antibacterial activity of iron-mimetic compounds. We found that, in RPMI-HS, the antibacterial activity of Ga(III) compounds, in particular GaN and GaM, was more than in MHB and DMHB. However, the presence of serum albumin in RPMI-HS and the type and number of

bacterial heme-uptake systems strongly influenced GaPPIX susceptibility. Intriguingly, we found that GaPPIX exerted a bactericidal activity on some strains, whereas GaN and GaM invariably exhibited bacteriostatic effects.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 10 September 2018
doi: 10.3389/fcimb.2018.00316

Antimicrobial Activity of Gallium Compounds on ESKAPE Pathogens

Sarah Hijazi[1], *Daniela Visaggio*[1], *Mattia Pirolo*[1], *Emanuela Frangipani*[1], *Lawrence Bernstein*[2] *and Paolo Visca*[1]*

[1] Department of Science, Roma Tre University, Rome, Italy, [2] Terrametrix, Menlo Park, CA, United States

ESKAPE bacteria are a major cause of multidrug-resistant infections, and new drugs are urgently needed to combat these pathogens. Given the importance of iron in bacterial physiology and pathogenicity, iron uptake and metabolism have become attractive targets for the development of new antibacterial drugs. In this scenario, the FDA-approved iron mimetic metal Gallium [Ga(III)] has been successfully repurposed as an antimicrobial drug. Ga(III) disrupts ferric iron-dependent metabolic pathways, thereby inhibiting microbial growth. This work provides the first comparative assessment of the antibacterial activity of $Ga(NO_3)_3$ (GaN), Ga(III)-maltolate (GaM), and Ga(III)-protoporphyrin IX (GaPPIX), belonging to the first-, second- and third-generation of Ga(III) formulations, respectively, on ESKAPE species, including reference strains and multidrug-resistant (MDR) clinical isolates. In addition to the standard culture medium Mueller Hinton broth (MHB), iron-depleted MHB (DMHB) and RPMI-1640 supplemented with 10% human serum (HS) (RPMI-HS) were also included in Ga(III)-susceptibility tests, because of their different nutrient and iron contents. All ESKAPE species were resistant to all Ga(III) compounds in MHB and DMHB (MIC $> 32\,\mu M$), except *Staphylococcus aureus* and *Acinetobacter baumannii*, which were susceptible to GaPPIX. Conversely, the antibacterial activity of GaN and GaM was very evident in RPMI-HS, in which the low iron content and the presence of HS better mimic the *in vivo* environment. In RPMI-HS about 50% of the strains were sensitive (MIC < 32) to GaN and GaM, both compounds showing a similar spectrum of activity, although GaM was more effective than GaN. In contrast, GaPPIX lost its antibacterial activity in RPMI-HS likely due to the presence of albumin, which binds GaPPIX and counteracts its inhibitory effect. We also demonstrated that the presence of multiple heme-uptake systems strongly influences GaPPIX susceptibility in *A. baumannii*. Interestingly, GaN and GaM showed only a bacteriostatic effect, whereas GaPPIX exerted a bactericidal activity on susceptible strains. Altogether, our findings raise hope for the future development of Ga(III)-based compounds in the treatment of infections caused by multidrug-resistant ESKAPE pathogens.

Keywords: antibacterial, ESKAPE, gallium maltolate, gallium nitrate, gallium protoporphyrin IX, iron uptake

OPEN ACCESS

Edited by:
Rodolfo García-Contreras, Universidad Nacional Autónoma de México, Mexico

Reviewed by:
Rolf Kümmerli, Universität Zürich, Switzerland
Gian Maria Rossolini, Università degli Studi di Firenze, Italy

***Correspondence:**
Paolo Visca
paolo.visca@uniroma3.it

Received: *01 June 2018*
Accepted: *20 August 2018*
Published: *10 September 2018*

Citation:
Hijazi S, Visaggio D, Pirolo M, Frangipani E, Bernstein L and Visca P (2018) Antimicrobial Activity of Gallium Compounds on ESKAPE Pathogens. Front. Cell. Infect. Microbiol. 8:316. doi: 10.3389/fcimb.2018.00316

INTRODUCTION

ESKAPE species (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) are among the most common bacterial pathogens in nosocomial infections, causing extensive morbidity and mortality, especially in critically ill and immunocompromised patients (Rice, 2010). All these species are characterized by a high level of antibiotic resistance (Pendleton et al., 2013), which recently prompted the World Health Organization to list ESKAPE pathogens among the greatest threats to human health, and to boost research on new effective drugs for treatment of antibiotic-resistant infections (World Health Organization, 2017). Among Gram-negative ESKAPE species, *K. pneumoniae*, *A. baumannii* and *P. aeruginosa* have reached an alarmingly high level of resistance, causing infections which are no longer treatable with conventional antibiotic therapies (Deplano et al., 2005; Elemam et al., 2009; Nowak et al., 2017). Depriving bacteria of essential nutrients, such as iron, is a viable strategy for the development of new antibacterials. Iron is a key nutrient for nearly all forms of life, including bacteria, being a cofactor of many vital enzymes (e.g., those involved in cellular respiration, DNA synthesis, and defense against reactive oxygen species) (Andrews et al., 2003). During infection, bacteria are faced with iron scarcity in body fluids, and must gain access to iron bound to transferrins (e.g., transferrin and lactoferrin) and/or heme-containing proteins (e.g., hemoglobin and myoglobin) (Weinberg, 2009). To counteract iron-limitation, bacteria have developed high-affinity iron-uptake strategies, such as: (i) the production of low-molecular-weight compounds, called siderophores, which bind Fe(III) and actively transport the metal into the cell (Miethke and Marahiel, 2007); (ii) the ability to utilize heme iron, by producing hemophores and/or specific transport systems for heme and heme-binding proteins (Wandersman and Delepelaire, 2004); and (iii) the active transport of Fe(II) through the cytoplasmic membrane *via* the Feo system (Cartron et al., 2006). Given the essential role of iron in bacterial physiology and pathogenicity, iron uptake and metabolism have become attractive targets for the development of new antibacterials (Ballouche et al., 2009; Foley and Simeonov, 2012). In this regard, the ferric iron [Fe(III)] mimetic ion gallium [Ga(III)] has been shown to inhibit the growth of many bacterial and fungal species by interfering with iron-dependent metabolic pathways (Bastos et al., 2010; Minandri et al., 2014). Given the chemical similarity between Fe(III) and Ga(III), microorganisms cannot easily distinguish between these two ions, so that Ga(III) competes with Fe(III) for incorporation into essential proteins and enzymes. However, unlike Fe(III), Ga(III) cannot be reduced under physiological conditions, resulting in the inhibition of several iron-dependent redox pathways (Bernstein, 1998).

For more than three decades, Ga(III) compounds have been employed as diagnostic tools in medicine. Radioactive ^{67}Ga allows localization of malignant cells and inflammatory or infective foci (Edwards and Hayes, 1969). Citrated $Ga(NO_3)_3$ (GaN, brand name Ganite®, Genta, NJ, USA) was approved by the US FDA for the treatment of cancer-associated hypercalcemia, though it is no longer available. Recently, there has been an expansion in the number of Ga(III) compounds showing therapeutic potential, sometimes categorized in first, second, and third generations, and ranging from simple salts such as $GaCl_3$ and GaN, through metal-organic complexes such as Ga(III)-maltolate (GaM) (Bernstein et al., 2000) and Ga(III)-protoporphyrin IX (GaPPIX) (Chitambar, 2017) (**Figure 1**). It is noted that GaN has very low oral bioavailability and must be parenterally administered, whereas GaM has high oral bioavailability and has been safely administered orally to people (Bernstein et al., 2000).

At present, neither standard protocols nor reference media for Ga(III)-susceptibility testing have been defined, though several lines of evidence indicate that iron irreversibly suppresses the antibacterial properties of Ga(III) (Kaneko et al., 2007; Antunes et al., 2012). Moreover, there are no comparative data on the activity of different Ga(III) compounds against ESKAPE species under standard test conditions, representing a major pitfall to the repurposing of Ga(III) as last-resort antibacterial agent.

In this study, the antibacterial activity of three compounds belonging to the first-, second-, and third-generation Ga(III) formulations, i.e., GaN, GaM, and GaPPIX, was tested on ESKAPE pathogens in culture media characterized by different iron content, namely Mueller-Hinton broth (MHB), iron-deprived MHB (DMHB) (Hackel et al., 2018) and RPMI-1640 tissue culture medium supplemented with 10% complement-free human serum (RPMI-HS), to better mimic the *in vivo* environment (Antunes et al., 2012; Thompson et al., 2012; Bonchi et al., 2015). ESKAPE bacteria resulted more susceptible to Ga(III) compounds in RPMI-HS than in MHB and DMHB. However, the presence of serum albumin in RPMI-HS and the type and number of bacterial heme-uptake systems strongly influenced GaPPIX susceptibility. Intriguingly, GaPPIX exerted a bactericidal activity on some strains, whereas GaN and GaM invariably exhibited bacteriostatic effects.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Bacterial strains used in this work are listed in **Table S1**. Bacteria were routinely cultured for 18 h in Tryptic Soy Broth (TSB, Acumedia) with vigorous shaking. When required, tetracycline (Tc), gentamicin (Gm), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, Sigma) were added to the media. For *A. baumannii*, 50 μg/ml Tc, and 100 μg/ml Gm were used. For *Escherichia coli* 10 μg/ml Gm and 40 μg/ml X-Gal were used. When vitamins (Vit) were required, 19 μg /ml of nicotinic acid (Sigma-Aldrich), and 2 μg /ml of pyridoxal hydrochloride (MERCK) were added to the media. Bovine hemin chloride (Sigma-Aldrich) was freshly prepared in 10 mM NaOH. Bovine serum albumin (BSA, Sigma-Aldrich) was freshly prepared and added to the media at the final concentration of 5 mg/ml.

Media and Ga(III) Compounds

Three media have been used in this study: (i) BBL Mueller Hinton II (Cation-Adjusted) Broth (MHB, Becton Dickinson) was prepared according to the manufacturer's instructions; (ii)

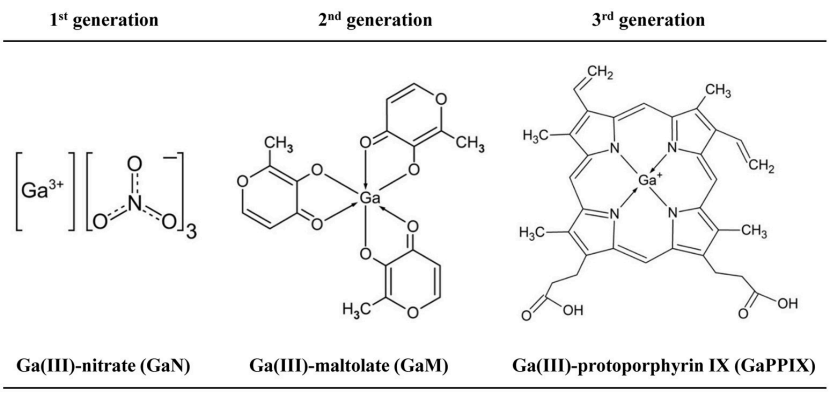


FIGURE 1 | Chemical structures of the three Ga(III) compounds used in this study.

DMHB was prepared following the approved CLSI protocol for antimicrobial susceptibility testing (Hackel et al., 2018). Briefly, MHB was treated for 16 h at 4°C with 100 g/l of the metal-chelating Chelex® 100 resin (Bio-Rad) under moderate stirring, then filtered through Whatman no. 1 filter paper and pH adjusted to 7.3. After autoclaving, $CaCl_2$ and $MgSO_4$ were added to DMHB at the final concentrations of 22.5 and 11.25 μg/ml, respectively; and (iii) RPMI-1640 (Sigma-Aldrich) supplemented with 10% complement-free human serum (RPMI-HS). Human serum was collected from 140 healthy donors, pooled, filtered, and inactivated (30 min, 56°C), as previously described (Antunes et al., 2012). Bulk serum chemistry was: total serum proteins 80 mg/ml; total iron 0.70 μg/ml; ferritin 0.243 μg/ml; transferrin 2.63 mg/ml; total iron binding capacity 4.27 mg/ml (20% transferrin saturation).

Three Ga(III) compounds were used in this study: (i) GaN ($Ga(NO_3)_3 \times 6H_2O$, Sigma-Aldrich; quality tested), freshly prepared as a 100 mM stock solution in water; (ii) GaM (NORAC Pharma, provided by Dr. Bernstein), freshly prepared as a 22 mM stock solution in water; and (iii) GaPPIX (Frontier Scientific), prepared as a 25 mM stock solution in dimethyl sulfoxide (DMSO), and stored at 4°C in the dark.

Iron Content Measurement, Siderophore Production, and β-Galactosidase (LacZ) Activity Assays

The iron concentration of MHB, DMHB, and RPMI-HS was measured by inductively coupled plasma optical emission spectrometry (ICP-OES) using an ICP-OES 710 Varian Spectrometer (Agilent Technologies). Briefly, the medium was mixed with 5% HNO_3, heated for 1 h at 90°C, and filtered through a Millipore membrane (pore size 0.45 μm) prior to ICP-OES analysis.

Siderophore production was determined by the chrome azurol S-Fe(III)-hexadecyltrimethylammonium bromide method (Schwyn and Neilands, 1987). Activity of the *basA*::*lacZ* reporter gene fusion carried by plasmid pMP220::P*basA* (Antunes et al., 2012) was tested in the reference strain *A. baumannii* ATCC 17978, and β-galactosidase levels were expressed as Miller units (Miller, 1972).

Susceptibility Testing of Ga(III) Compounds

The inhibitory activity of Ga(III) compounds on ESKAPE pathogens was assessed by the microdilution method (Clinical Laboratory Standards Institute, 2015), with minor modifications. Bacteria were grown for 18 h in TSB, then washed in saline and diluted to obtain *ca.* 5×10^5 CFU/ml in 200 μl of MHB, DMHB, or RPMI-HS, in the presence of increasing concentrations (0 to 128 μM) of each Ga(III) compound [GaN or GaM or GaPPIX], using 96-well microtiter plates. Plates were incubated for 24 h at 37°C with orbital shaking (110 rpm). The MIC of Ga(III) compounds was determined as the lowest concentration that completely inhibited bacterial growth as detected by the unaided eye (Clinical Laboratory Standards Institute, 2015). To test the effect of Fe(III) and hemin on GaPPIX antibacterial activity, freshly prepared $FeCl_3$ (Sigma-Aldrich) or bovine hemin chloride (Sigma-Aldrich) were added at the indicated final concentrations, into 200 μl of MHB inoculated with *ca.* 5×10^5 CFU/ml, in the presence of GaPPIX supplied at the MIC. Microtiter plates were incubated for up to 24 h at 37°C and bacterial growth {optical density at 600 nm [OD_{600}]} was periodically measured using SPARK 10M TECAN reader.

The antibacterial activity of GaPPIX on *A. baumannii* strains was also assessed by disk diffusion assays. Briefly, 18 h cultures in TSB were washed and diluted in saline to $OD_{600} = 0.1$, then seeded with a sterile swab on the surface of RPMI-HS supplemented with 15 g/l agar (Acumedia). Sterile 6-mm blank disks (ThermoFisher-Oxoid) soaked with 10 μl of a 15 mM solution of GaPPIX were deposited on the agar surface, and the growth inhibition halo was detected after 18 h incubation at 37°C.

Time-Kill Assays

Time-kill kinetic assays of Ga(III) compounds were performed on eleven ESKAPE pathogens according to a previously described procedure (Principe et al., 2009), with minor modifications.

Briefly, tubes containing 1 ml of RPMI-HS supplemented with 28 μM of GaN, GaM or GaPPIX were inoculated with bacteria to a density *ca.* 5×10^5 CFU/ml, and incubated at 37°C with gentle shaking (120 rpm). Aliquots were removed at time 0, 3, 6, and 24 h post-inoculation, and serially diluted in saline for determination of viable counts on Luria Bertani (LB) agar plates.

Identification and Cloning of the Heme-Utilization Gene Clusters in *A. baumannii*

Previously described oligonucleotides and PCR conditions were used to check the presence of genes belonging to the heme iron-uptake gene cluster 2 (hereafter termed *hemT* cluster), and the heme iron-uptake gene cluster 3 (Antunes et al., 2011), which includes the *hemO* gene, hence named *hemO* cluster (Ou et al., 2015).

The 9,833 bp DNA fragment encompassing eight genes of the *hemO* cluster of ACICU (from ACICU_00873 to ACICU_00880 locus) was obtained by PCR amplification using primers HemO_FW (5′-CATTTGGTTTCCGAGTCTCG-3′) and HemO_RV (5′-CCATGATGCGTACCATGCA-3′). The PCR product was purified by the PCR Clean-Up System (Promega) and blunt-end ligated to the SmaI site of pVRL1 (Lucidi et al., 2018), yielding plasmid pVRL1*hemO*. The pVRL1*hemO* plasmid was introduced in *A. baumannii* ATCC 17978 by electroporation according to published procedures (Yildirim et al., 2016). Transformants were selected on LB agar plates supplemented with 100 μg/ml Gm.

RESULTS

DMHB and RPMI-HS Are Iron-Poor Media That Support the Growth of ESKAPE Species

For a comparative assessment of the antibacterial effect of the three Ga(III) compounds, a representative collection of ESKAPE species was used, including reference strains and multidrug-resistant (MDR) clinical isolates (**Table S1**). Since Ga(III) is an Fe(III)-mimetic acting as a metabolic competitor of Fe(III), its antibacterial activity depends on the iron concentration in the test medium, being enhanced by conditions of relative iron scarcity (Minandri et al., 2014). Therefore, both chemical analyses and functional assays were performed to probe iron content and availability in MHB, DMHB, and RPMI-HS media, prior to Ga(III)-susceptibility testing. ICP-OES measurements (**Figure S1**) showed that the iron concentrations in DMHB (0.43 μM) and RPMI-HS (1.95 μM) were lower than in MHB (3.38 μM). The relatively high iron concentration of RPMI-HS can be ascribed to partially (*ca.* 20%) iron-saturated transferrin in human serum, since only iron traces (0.11 μM) are present in serum-free RPMI-1640 (data not shown). To evaluate whether DMHB and RPMI-HS are perceived by bacteria as iron-poor media, both siderophore production and iron-repressible gene expression were investigated by using *A. baumannii* ATCC 17978 as a biosensor organism. Notably, high siderophore levels were produced in both DMHB and RPMI-HS, as opposed to MHB (**Figure S1**). Moreover, a transcriptional fusion between the promoter of the iron-repressible *basA* gene and the reporter *lacZ* gene (Antunes et al., 2012) was expressed by *A. baumannii* ATCC 17978 at higher levels in DMHB and RPMI-HS than in MHB (**Figure S1**). These data indicate that DMHB and RPMI-HS are low-iron media that induce an iron-starvation response during bacterial growth. ESKAPE pathogens share similar iron-mediated regulatory mechanisms of gene expression, all possessing the Ferric uptake regulator protein Fur, which drives the expression of iron-repressible genes, including those for siderophore-biosynthesis (i.e., *basA*). Therefore, it can be assumed that the *basA*::*lacZ* transcriptional fusion provides an indirect estimate of the intracellular iron levels of ESKAPE bacteria grown in different media (Ochsner and Vasil, 1996; Achenbach and Yang, 1997; Haley and Skaar, 2012; Mortensen and Skaar, 2013; Carpenter and Payne, 2014; Latorre et al., 2018). The ability of ESKAPE pathogens to grow in DMHB and RPMI-HS was then tested for the reference strains of each species (**Figure S2**). For all strains tested, evident growth reduction (12–60%) was observed in DMHB compared with MHB. Addition of 3 μM $FeCl_3$ to DMHB (i.e., restoring the iron concentration of MHB before Chelex® 100 treatment) rescued the growth of all strains, except *E. faecium* ATCC 19434 and *S. aureus* ATCC 25923 (**Figure S2A**). For these two species, the residual growth reduction observed in iron-replete DMHB is likely due to the removal of other metabolically relevant metals, besides iron. Moreover, all but one strain grew in RPMI-HS, although at different rates (**Figure S2B**). The only exception was *E. faecium* ATCC 19434, whose growth was rescued by the addition of two vitamins, namely nicotinic acid and pyridoxal hydrochloride. These cofactors were added to RPMI-HS to allow Ga-susceptibility testing of *E. faecium* (**Figure S2C**). These preliminary experiments allowed us to establish iron-poor culture conditions in conventional media that support the growth of all ESKAPE strains tested, thus being suitable for comparative testing of the antibacterial activity of Ga(III) compounds.

Susceptibility of ESKAPE Pathogens to Ga(III) Compounds

The activity of the three Ga(III) compounds was tested on a total of 24 ESKAPE strains in three selected media (**Table 1**). Arbitrarily assuming the resistance breakpoint at MIC > 32 μM, which roughly corresponds to the peak serum concentration of Ga(III) achievable during human therapy (Bernstein, 1998; Collery et al., 2002), all strains were resistant to the three Ga(III) compounds tested in MHB and DMHB, except *S. aureus* and *A. baumannii*, which were susceptible to GaPPIX (MIC ≤ 32 μM). Notably, the MIC of GaPPIX for *S. aureus* was extremely low in both MHB and DMHB (0.06–0.12 μM). Moreover, no differences in MIC values for *S. aureus* and *A. baumannii* were observed between MHB and DMHB, in spite of their different iron content, and thus of the different iron starvation status of bacteria (**Figure S1**). This suggests a mechanism of action of GaPPIX that is not responsive to iron. In fact, addition to MHB of 100 μM $FeCl_3$, i.e., in excess over GaPPIX, did not abrogate the growth inhibitory effect of GaPPIX for all *S. aureus*

TABLE 1 | MIC (μM) of Ga(III) compounds for ESKAPE strains.

Bacterial strain	MHB			DMHB			RPMI-HS[b]		
	GaN	GaM	GaPPIX	GaN	GaM	GaPPIX	GaN	GaM	GaPPIX
E. faecalis ATCC 29212	>128	>128	>128	>128	>128	>128	>128	64	>128
E. faecalis ATCC 700802	>128	>128	>128	>128	>128	>128	>128	128	>128
E. faecium[T] ATCC 19434	>128	>128	>128	>128	>128	>128	>128	64	>128
E. faecium BM4147	>128	>128	>128	>128	>128	>128	ND	ND	ND
S. aureus ATCC 25923	>128	>128	**0.12**	>128	>128	**0.06**	>128	>128	>128
S. aureus ATCC 43300	>128	>128	**0.12**	>128	>128	**0.06**	>128	128	>128
S. aureus Sau117[a]	>128	>128	**0.06**	>128	>128	**0.06**	>128	128	>128
S. aureus UD95[a]	>128	>128	**0.12**	>128	>128	**0.12**	>128	128	>128
K. pnemoniae ATCC 27736	>128	>128	>128	>128	>128	>128	>128	>128	>128
K. pnemoniae Kp3[a]	>128	>128	>128	>128	>128	>128	>128	>128	>128
K. pnemoniae 17830[a]	>128	>128	>128	>128	>128	>128	**4**	**2**	>128
K. pnemoniae 16855[a]	>128	>128	>128	>128	>128	>128	**16**	**16**	>128
A. baumannii ATCC 17978	>128	>128	**16**	> 128	> 128	**16**	**1**	**1**	128
A. baumannii AYE[a]	>128	>128	**32**	>128	>128	**32**	**2**	**1**	128
A. baumannii ACICU[a]	>128	>128	**16**	>128	>128	**16**	**2**	**1**	**1**
A. baumannii C13-373[a]	>128	>128	**32**	>128	>128	**32**	**2**	**1**	**0.25**
P. aeruginosa ATCC 15692 (PAO1)	>128	>128	>128	64	>128	>128	**8**	**4**	128
P. aeruginosa PA14	>128	>128	>128	64	>128	>128	**16**	**8**	128
P. aeruginosa LesB58[a]	>128	>128	>128	>128	>128	>128	**0.5**	**0.5**	**8**
P. aeruginosa SP-13[a]	>128	>128	>128	64	>128	>128	**8**	**4**	128
E. aerogenes[T] ATCC 13048	>128	>128	>128	>128	>128	>128	>128	>128	>128
E. aerogenes 84-6792	>128	>128	>128	>128	>128	>128	>128	>128	>128
E.cloacae[T] ATCC 13047	>128	>128	>128	>128	>128	>128	>128	>128	>128
E. cloacae 78-6303	>128	>128	>128	>128	>128	>128	**8**	**8**	>128

[T], type strain; ND, not determined due to the poor growth. [a], MDR strain; [b], only in the case of Enterococcus species, RPMI-HS was supplemented with nicotinic acid and pyridoxine to allow bacterial growth. Arbitrarily assuming the resistance breakpoint at MIC > 32 μM, the MIC values for susceptible isolates are shown in bold type.

and *A. baumannii* strains tested (**Figure 2**), while 100 μM $FeCl_3$ alone, used as control in MHB, did not influence bacterial growth (data not shown). Since GaPPIX is likely to exert its antibacterial effect by acting as a heme analog (Stojiljkovic et al., 1999; Hijazi et al., 2017), we wondered whether hemin might counteract bacterial GaPPIX susceptibility. To verify this hypothesis, several concentrations of hemin (from 5 to 400 μM) were added to MHB together with GaPPIX, provided at the MIC (**Table 1**). The addition of 5 μM hemin was sufficient to completely abrogate the activity of GaPPIX against all *S. aureus* strains, except *S. aureus* UD95, for which a higher hemin concentration (50 μM) was required (**Figure 2A**). Conversely, not even the highest hemin concentration tested (400 μM) was able to fully reverse the growth-inhibitory effect of GaPPIX in *A. baumannii* (**Figure 2B**). Of note, also $FeCl_3$ provided at 400 μM (i.e., equimolar with the highest hemin concentration tested) did not rescue the growth of all *A. baumannii* strains tested.

Surprisingly, a dramatic loss of GaPPIX activity was observed in RPMI-HS for *S. aureus* and *A. baumannii* (MICs ≥ 128 μM for 4/4 and 2/4 strains, respectively), as opposed to the activity of GaN and GaM which was enhanced for almost all species tested in RPMI-HS, compared with MHB or DMHB (**Table 1**). Non-fermenting Gram-negative species, *P. aeruginosa* and *A. baumannii*, showed low MIC values for both GaN and GaM (0.5 μM < MIC < 16 μM) with some intra-species variability. Intriguingly, the hypervirulent *P. aeruginosa* LesB58 (Liverpool strain) showed the lowest MIC values for all Ga(III) compounds, and was the only GaPPIX-sensitive *P. aeruginosa* strain (**Table 1**). Interestingly, half of *K. pneumoniae* strains and one isolate of *E. cloacae* were also susceptible to GaN and GaM in RPMI-HS (MIC ≤ 16 μM). Moreover, no GaPPIX MIC could be determined in RPMI-HS for all Enterobacteriaceae tested (MIC > 128 μM) (**Table 1**). Of note, Ga(III) susceptibility was not limited to antibiotic-sensitive ESKAPE strains, but also exerted on MDR clinical isolates (**Table 1**).

Bactericidal Activity of Ga(III) Compounds

A bactericidal activity has previously been reported for GaN and GaPPIX on *P. aeruginosa* and *A. baumannii*, respectively. However, previous killing assays were conducted in rich laboratory media, which do not mimic the condition encountered *in vivo* by infecting pathogens (Kaneko et al., 2007; Arivett et al., 2015). For this reason, we devised to assess the bactericidal activity of the three Ga(III) compounds in RPMI-HS, i.e., under conditions that trend to mimic biological fluids. Only susceptible strains showing MIC values < 32 μM (**Table 1**) were selected for bactericidal activity

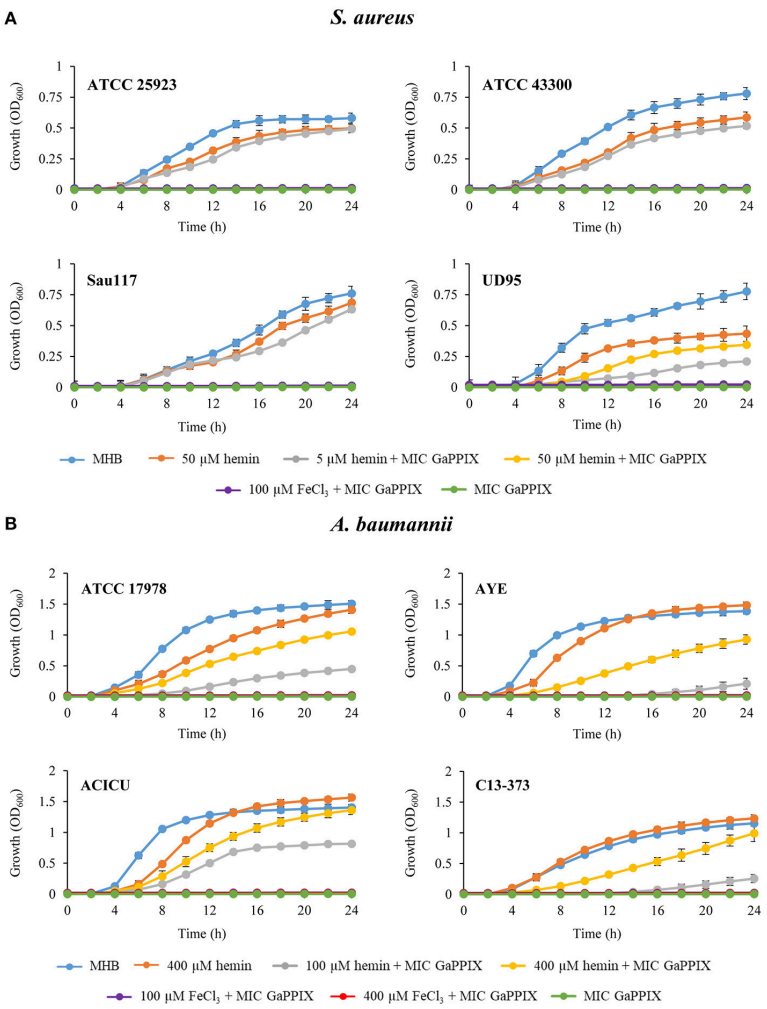


FIGURE 2 | Effect of iron and hemin on the inhibitory activity of GaPPIX. Strains were grown in MHB supplemented or not with GaPPIX at the MIC. Hemin and $FeCl_3$ were supplied at different concentrations, either alone or in combination with GaPPIX, as indicated. **(A)** *S. aureus* ATCC 25923, ATCC 43300, Sau117, UD95, and **(B)** *A. baumannii* strains ATCC17978, AYE, ACICU and C13–373. OD_{600} was monitored periodically for up to 24 h. Data are the mean ± standard deviation of triplicate experiments.

testing of GaN, GaM and GaPPIX, all provided at 28 µM, which corresponds to the peak serum concentration of Ga(III) achievable during human therapy (Bernstein, 1998; Collery et al., 2002). Interestingly, GaN and GaM showed only a bacteriostatic effect (**Figure S3**, **Figure 3**), whereas the response to GaPPIX varied among the susceptible strains (**Figure 3**). The presence of 28 µM GaPPIX caused 2–3 log reduction of viable cells (CFU counts) of *A. baumannii* ACICU, *A. baumannii* C13-373, and *P. aeruginosa* LesB58 after 6 h of incubation at 37°C (**Figure 3**), indicating a bactericidal effect of GaPPIX in RPMI-HS.

Serum Albumin Counteracts the Activity of GaPPIX

In many bacterial pathogens, severe iron limitation induces the expression of heme-uptake systems, therefore increasing the susceptibility to GaPPIX (Stojiljkovic et al., 1999; Hijazi et al., 2017). Intriguingly, *S. aureus* was very sensitive to GaPPIX in both MHB and DMHB, while no MIC was determined for GaPPIX in RPMI-HS (**Table 1**), even though RPMI-HS is iron-poor and induces an iron-starvation response in bacteria (**Figure S1**). Likewise, *A. baumannii* ATCC 17978 and AYE were much more susceptible to GaPPIX in MHB and DMHB than in

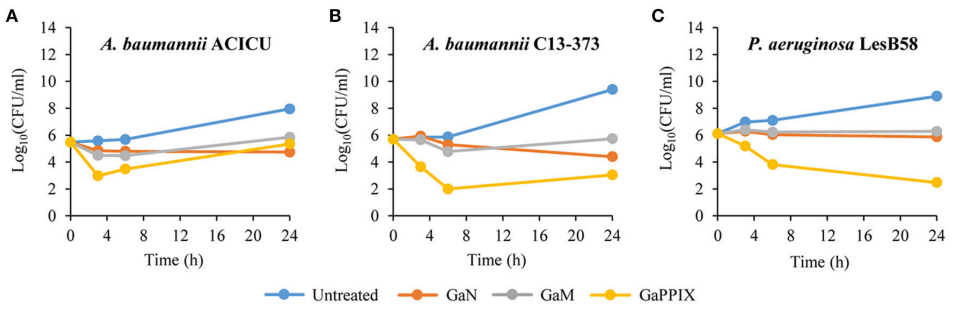


FIGURE 3 | Bactericidal activity of Ga(III) compounds on susceptible strains. GaN, GaM, and GaPPIX time-kill kinetics were determined by plate counts after 0-, 3-, 6-, and 24-h incubation in RPMI-HS supplemented with 28 μM of each Ga(III) compound. **(A)** *A. baumannii* ACICU, **(B)** *A. baumannii* C13-373 and **(C)** *P. aeruginosa* LesB58. Panels show one representative experiment of three independent replicates yielding similar results.

TABLE 2 | Effect of BSA on the MIC (μM) of GaPPIX for *S. aureus* and *A. baumannii*.

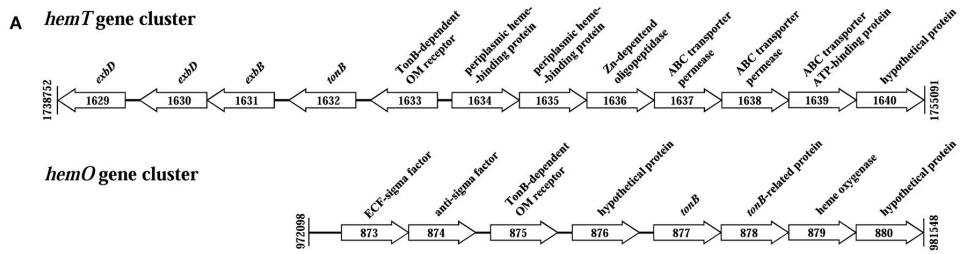
Bacterial strains	MHB		RPMI-1640	
	no BSA	5 mg/ml BSA	no BSA	5 mg/ml BSA
S. aureus ATCC 25923	0.12	32	0.12	8
S. aureus ATCC 43300	0.12	16	0.12	32
S. aureus Sau117	0.06	16	0.25	16
S. aureus UD95	0.12	64	0.12	64
A. baumannii ATCC 17978	16	> 128	8	128
A. baumannii AYE	32	> 128	8	128
A. baumannii ACICU	16	> 128	ND	ND
A. baumannii C13-373	32	> 128	16	128

ND, not determined due to the poor bacterial growth.

RPMI-HS (**Table 1**). However, while all *S. aureus* strains were resistant to GaPPIX in RPMI-HS (MIC > 128 μM, **Table 1**), they became extremely sensitive to GaPPIX in RPMI-1640 without HS (MIC ≤ 0.25 μM, **Table 2**). Likewise, *A. baumannii* ATCC 17978 and AYE became sensitive to GaPPIX in RPMI-1640 without HS (MIC = 8 μM, **Table 2**), although showing high GaPPIX MIC in RPMI-HS (128 μM, **Table 1**). These results argue for the presence of HS compound(s) capable of counteracting the antibacterial activity of GaPPIX. Since human serum albumin (HSA), the most abundant plasma protein, binds a variety of ligands including heme (Adams and Berman, 1980), we hypothesized that HSA could bind GaPPIX, due to its chemical similarity with heme, thus impairing its translocation across the cell membrane *via* heme-uptake systems. To test this hypothesis, the susceptibility of *S. aureus* and *A. baumannii* to GaPPIX was determined in MHB and RPMI-1640 supplemented or not with BSA, a protein sharing 76% sequence identity and the same heme-binding properties as HSA (Goncharova et al., 2013). The amount of added BSA was 5 mg/ml, equaling the final concentration of HSA in RPMI-HS. Consistent with our hypothesis, addition of 5 mg/ml BSA to RPMI-1640 dramatically increased (67 to 533 fold) the MICs of GaPPIX on both *S. aureus* and *A. baumannii* (**Table 2**). A similar effect was also observed upon addition of BSA to MHB (**Table 2**). Taken together, these results suggest that the poor susceptibility to GaPPIX observed for *S. aureus* and *A. baumannii* in RPMI-HS is due to the presence of HSA which binds GaPPIX and neutralizes its inhibitory effect.

Multiple Heme-Uptake Systems Influence *A. baumannii* Susceptibility to GaPPIX

The wide range of GaPPIX susceptibility observed among *A. baumannii* isolates led us to hypothesize that the presence of different GaPPIX-uptake capabilities could be the source of this variability. GaPPIX is known to exploit heme-uptake routes to enter bacterial cells (Stojiljkovic et al., 1999; Hijazi et al., 2017), and all *A. baumannii* strains sequenced so far, including ATCC 17978, AYE and ACICU, possess the *hemT* heme-uptake cluster (homolog of the iron-uptake gene cluster 2 in Antunes et al., 2011). Interestingly, *A. baumannii* ACICU possesses an additional heme-uptake cluster, named *hemO* (iron-uptake gene cluster 3 in Antunes et al., 2011) (**Figure 4A**). Since *A. baumannii* ACICU showed an extremely low GaPPIX MIC (1 μM), we hypothesized that the presence of *hemO* could be responsible for the increased GaPPIX susceptibility, likely providing a more efficient GaPPIX uptake route. Indeed, PCR analysis revealed the presence of both *hemO* and *hemT* clusters in *A. baumannii* C13-373 (data not shown), another strain showing very low GaPPIX MIC (0.25 μM), similar to *A. baumannii* ACICU (**Figure 4B**). To shed more light on the relationship between heme uptake and the antibacterial activity of GaPPIX in *A. baumannii*, the whole *hemO* cluster of strain ACICU (9,833 bp), was cloned and expressed *in trans* in *A. baumannii* ATCC 17978. GaPPIX susceptibility of *A. baumannii* ATCC 17978 expressing the whole *hemO* cluster from plasmid pVRL1*hemO* or harboring the empty vector (pVRL1) was then assessed in both solid and liquid RPMI-HS (**Figure 4B**). In RPMI-HS agar plates, a much larger growth inhibition halo was observed around the GaPPIX-soaked disk for *A. baumannii* ATCC 17978 (pVRL1*hemO*), compared with *A. baumannii* ATCC 17978 (pVRL1), indicating that expression of the *hemO* cluster from a multicopy plasmid *in trans* greatly



B

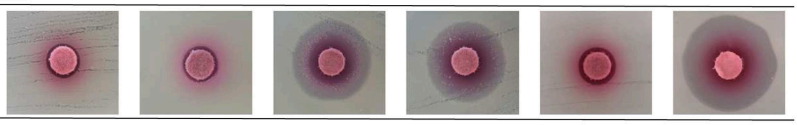
A. baumannii strain	ATCC 17978	AYE	ACICU	C13-373	ATCC 17978 pVRL1	ATCC 17978 pVRL1*hemO*
Heme utilization cluster						
hemT	+	+	+	+	+	+
hemO	-	-	+	+	-	++
GaPPIX disk diffusion assay						
GaPPIX MIC (μM)						
RPMI-HS	128	128	1	0.25	128	0.25

FIGURE 4 | Influence of heme-uptake systems on *A. baumannii* susceptibility to GaPPIX. **(A)** Schematic representation of the two gene clusters for heme uptake, *hemT* and *hemO*, of *A. baumannii* ACICU. Coding sequences are shown as arrows (not to scale) oriented according to the predicted direction of transcription. **(B)** Top; presence (+) or absence (-) of heme clusters in different *A. baumannii* strains (++, overexpression from the multicopy plasmid pVRL1*hemO*). Middle; GaPPIX disk diffusion assay in RPMI-HS agar plates inoculated with different *A. baumannii* strains. Bottom; MICs of GaPPIX for different *A. baumannii* strains in RPMI-HS.

increases *A. baumannii* ATCC 17978 susceptibility to GaPPIX (**Figure 4B**). As expected, the presence of the empty vector pVRL1 did not affect the susceptibility of *A. baumannii* ATCC 17978 to GaPPIX (**Figure 4B**). In line with the results of the disk diffusion assay, MIC data confirmed that *A. baumannii* ATCC 17978 (pVRL1*hemO*) is more susceptible to GaPPIX than *A. baumannii* ATCC 17978 (pVRL1), the former showing a MIC of 0.25 μM in RPMI-HS (**Figure 4B**). Altogether, these data indicate that the susceptibility of *A. baumannii* to GaPPIX is increased by the presence of the *hemO* gene cluster.

DISCUSSION

Antimicrobial resistance has become one of the most challenging problems of the healthcare system. The spread of antimicrobial-resistant pathogens has dramatic repercussions on mortality and morbidity rates, hence on global medical costs (Friedman et al., 2016). ESKAPE pathogens rank among the most prevalent causative agents of healthcare-associated infections, and pose a serious therapeutic challenge due to their resistance to available antibiotics (Boucher et al., 2009). However, while new antibiotics are urgently needed, the pipeline of antibiotic discovery is almost dry (Luepke and Mohr, 2017). Iron uptake and metabolism are regarded as druggable targets for new antibacterial strategies (Ballouche et al., 2009; Foley and Simeonov, 2012). In this context, the iron mimetic metal Ga(III) has been shown to inhibit bacterial growth, by interfering with iron-dependent metabolic pathways (Minandri et al., 2014).

In this work, we report the first comparative evaluation of the antibacterial properties of three Ga(III) compounds on ESKAPE species in conventional susceptibility testing media characterized by different iron concentrations and nutrient compositions.

Our data demonstrate that the bacterial susceptibility to Ga(III) compounds varies among species and among strains within the same species, and is influenced by the iron concentration and nutrient composition of the medium. Ga(III)-susceptibility tests conducted in MHB and DMHB showed elevated MIC values ($> 32\,\mu M$) for all species, except *S. aureus* and *A. baumannii*, which were sensitive to GaPPIX only. Conversely, Ga(III) testing in RPMI-HS gave a better response, with an overall higher susceptibility to GaN and GaM for *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *E. cloacae* (MIC $\leq 16\,\mu M$ for 50 to 100% of strains tested). Among the

ESKAPE bacteria tested, aerophilic species, which preferentially adopt a respiratory metabolism, namely *A. baumannii* and *P. aeruginosa*, were in general more susceptible to Ga(III) in RPMI-HS than fermenting species, such as Enterobacteriaceae, *S. aureus* and enterococci. This is probably due to Ga(III)-dependent impairment of iron-demanding processes, such as respiration and response to oxidative stress (Stojiljkovic et al., 1999; Hijazi et al., 2017). Moreover, GaN and GaM showed a similar spectrum of activity, although GaM was more potent than GaN in 9 out of 13 isolates for which a MIC could be determined. Previous experiments in a mouse burn wound model infection are in line with this observation, given that much lower GaM concentrations were needed to prevent *P. aeruginosa* and *A. baumannii* proliferation, compared with GaN (DeLeon et al., 2009). The solubility of GaM in both water and lipids, allowing for the penetration of cell walls and membranes, as opposed to the lack of lipophylicity of GaN, probably accounts for much of the difference in biologic effects between the two compounds (Bernstein et al., 2000; DeLeon et al., 2009). Although the presence of non-selective entrance routes for Ga(III) cannot be excluded, Ga(III) mainly exploits iron-uptake systems to enter bacterial cells (Minandri et al., 2014). Therefore, the variable susceptibility to GaN and GaM observed for *K. pneumoniae* and *E. cloacae* in RPMI-HS could be explained by the variable number and type of iron-acquisition systems in these two species (Podschun et al., 1992; Koczura and Kaznowski, 2003) and perhaps their varying growth rates. This holds true also in *P. aeruginosa*, where siderophores, either pyoverdine (PVD) or pyochelin (PCH), have opposite effects on Ga(III) activity; while PCH shuttles Ga(III) into *P. aeruginosa* cells, PVD sequesters it away in the periplasmic space, therefore protecting bacterial cells from Ga(III)-mediated toxicity (Kaneko et al., 2007; Frangipani et al., 2014).

GaPPIX deserves a special comment. As documented for various bacterial species, GaPPIX exerts its activity when transported into the cell, implying that the presence and/or expression level of heme-uptake genes have a major impact on GaPPIX activity (Stojiljkovic et al., 1999; Hijazi et al., 2017). The *A. baumannii hemO* gene cluster encodes a very efficient heme-utilization system, responsible for an increased translocation of GaPPIX in the cell (de Léséleuc et al., 2014). Results presented here suggest a major role for the *hemO* system also in GaPPIX susceptibility, given that only strains possessing both *hemT* and *hemO* gene clusters were severely inhibited by GaPPIX in RPMI-HS (MIC 0.25–1.0 μM), whereas those possessing only the *hemT* cluster were not (MIC > 128 μM) (**Tables 1**, **2**). Notably, this phenomenon was only observed in RPMI-HS, where GaPPIX is bound by HSA, but not in MHB or DMHB where no albumin is present. This suggests that gene products of the *A. baumannii hemO* cluster efficiently withdraw HSA-bound GaPPIX and deliver it to its intracellular targets. On the other hand, the HemT and Isd heme-uptake systems, which are present in *A. baumannii* and *S. aureus*, respectively (Ascenzi et al., 2015), appear by themselves unable to confer GaPPIX susceptibility in RPMI-HS (but not in MHB and DMHB), probably because these two systems cannot efficiently extract HSA-bound GaPPIX for transport into the cell. These observations are in line with a previous report showing that the addition of 10% HS to MHB caused a 3-fold increase of the GaPPIX MIC for *A. baumannii* (Arivett et al., 2015). Intriguingly, the activity of GaPPIX against *S. aureus* and *A. baumannii* was independent of the iron content of the medium, given that: (i) it was similar in MHB and DMHB media, irrespective of their different iron content, and, (ii) amendment of MHB with an exceedingly high $FeCl_3$ concentration did not neutralize the antibacterial activity of GaPPIX (**Figure 2**), in agreement with previous reports (Stojiljkovic et al., 1999; Arivett et al., 2015). Interestingly, the addition of hemin partially reversed the antibacterial activity of GaPPIX in MHB, albeit in one *S. aureus* and in all *A. baumannii* strains tested not even a molar excess of hemin completely abrogated the effect of GaPPIX (**Figure 2**). This means that incorporation of GaPPIX in vital bacterial enzymes is only in part reversed by competition with hemin. This observation could have significant clinical implications, since the release of heme and/or iron from red blood cells and iron-binding proteins during inflammatory processes is unlikely to undermine the antibacterial activity of GaPPIX *in vivo*. These observations, together with the previously reported low toxicity of GaPPIX (Stojiljkovic et al., 1999; Arivett et al., 2015; Chang et al., 2016), support the potential use of GaPPIX as a therapeutic option to treat some bacterial infections. It should be noted that, different from GaN and GaM which exploit multiple routes to enter bacteria (Minandri et al., 2014), making the selection of Ga(III)-resistant cells less likely to occur compared with conventional antibiotic treatments (Ross-Gillespie et al., 2014), GaPPIX enters the cell through specialized heme-uptake systems (Stojiljkovic et al., 1999; Hijazi et al., 2017). While this could imply more frequent emergence of GaPPIX-resistant cells through loss of heme (hence GaPPIX) uptake capabilities, the preferential use of heme as iron source by bacterial pathogens *in vivo* argues against a dispensable role of these systems during infection.

In conclusion, we have determined suitable test conditions to assess the antibacterial activity of Ga(III) compounds *in vitro*. The presence of human serum (HS) in RPMI-HS reduces iron availability thanks to the presence of transferrin, thereby providing a more realistic milieu for testing the antibacterial activity of iron-mimetic compounds. Moreover, in RPMI-HS the presence of serum albumin, which interferes with GaPPIX but not of GaN or GaM, indicates that, among the three Ga(III)-compounds tested, the FDA-approved GaN and the orally active GaM were the most effective under conditions that mimic the *in vivo* environment, i.e., in RPMI-HS. With respect to the clinical repositioning of Ga(III) as an antibacterial agent, one should consider that the recommended dosing regimen of citrated GaN for the treatment of cancer patients (200 to 300 mg/m^2 body surface area, i.v. administration) ensures a peak serum concentration of Ga(III) of *ca.* 28 μM (Bernstein, 1998; Collery et al., 2002). Notably, we found that in RPMI-HS much lower GaN concentrations are needed to inhibit the growth of *A. baumannii*, *P. aeruginosa* and some Enterobacteriaceae. Ongoing clinical trials on patients with cystic fibrosis (IGNITE study, ClinicalTrials.gov Identifier: NCT02354859) will provide

important insights into *P. aeruginosa* inhibition during i.v. GaN treatment of chronic lung infection, hence on the actual potential of Ga(III) as an antibacterial agent. It is also noted that topical or other localized means of administration can readily provide millimolar levels of Ga(III) to sites of infection, including burn-associated infections (DeLeon et al., 2009). In fact, the topic use of Ga(III)-citrate has been shown to improve healing, reduce inflammation and favor reepithelization in a murine wound model of *K. pneumoniae* infection (Thompson et al., 2015).

Interestingly, we found that pre-existing resistance to multiple antibiotics in MDR strains did not compromise Ga(III) susceptibility, likely as a consequence of Ga(III) molecular targets (iron-binding proteins) being different from those of common antibiotics, toward which resistance has been selected. In conclusion, Ga(III) could represent a drug of last resort to combat infections sustained by otherwise untreatable pan-resistant bacteria.

AUTHOR CONTRIBUTIONS

DV and PV conceived and designed the experiments. SH and MP performed the experiments. SH, DV, EF, and PV analyzed the data. SH wrote the draft manuscript. SH, DV, MP, EF, LB, and PV revised the manuscript.

FUNDING

This work was supported by grants from the Italian Cystic Fibrosis Research Foundation (grants FFC#21/2015 and FFC#18/2017).

ACKNOWLEDGMENTS

We wish to thank Prof. Gian Maria Rossolini (Department of Experimental Medicine and Clinic, University of Florence, Italy) for providing *Enterobacter* strains, and Dr. Annalisa Pantosti (Department of Infectious, Parasitic and Immuno-mediated Diseases, Istituto Superiore di Sanità, Rome, Italy) for providing *S. aureus* and *K. pneumoniae* strains. We are also grateful to Dr. Federica Runci and the personnel of Policlinico Umberto I, Sapienza University of Rome, for collecting blood samples from healthy donors. The Grant of Excellence Departments, MIUR-Italy (ARTICOLO 1, COMMI 314–337 LEGGE 232/2016) is gratefully acknowledged.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2018.00316/full#supplementary-material

REFERENCES

Achenbach, L. A., and Yang, W. (1997). The fur gene from *Klebsiella pneumoniae*: characterization, genomic organization and phylogenetic analysis. *Gene* 185, 201–207. doi: 10.1016/S0378-1119(96)00642-7

Adams, P. A., and Berman, M. C. (1980). Kinetics and mechanism of the interaction between human serum albumin and monomeric haemin. *Biochem. J.* 191, 95–102. doi: 10.1042/bj1910095

Andrews, S. C., Robinson, A. K., and Rodríguez-Quiñones, F. (2003). Bacterial iron homeostasis. *FEMS Microbiol. Rev.* 27, 215–237. doi: 10.1016/S0168-6445(03)00055-X

Antunes, L. C., Imperi, F., Minandri, F., and Visca, P. (2012). *In vitro* and *in vivo* antimicrobial activities of gallium nitrate against multidrug-resistant *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 56, 5961–5970. doi: 10.1128/AAC.01519-12

Antunes, L. C., Imperi, F., Towner, K. J., and Visca, P. (2011). Genome-assisted identification of putative iron-utilization genes in *Acinetobacter baumannii* and their distribution among a genotypically diverse collection of clinical isolates. *Res. Microbiol.* 162, 279–284. doi: 10.1016/j.resmic.2010.10.010

Arivett, B. A., Fiester, S. E., Ohneck, E. J., Penwell, W. F., Kaufman, C. M., Relich, R. F., et al. (2015). Antimicrobial activity of gallium protoporphyrin IX against *Acinetobacter baumannii* strains displaying different antibiotic resistance phenotypes. *Antimicrob. Agents Chemother.* 59, 7657–7665. doi: 10.1128/AAC.01472-15

Ascenzi, P., di Masi, A., Leboffe, L., Frangipani, E., Nardini, M., Verde, C., et al. (2015). Structural biology of bacterial haemophores. *Adv. Microb. Physiol.* 67, 127–176. doi: 10.1016/bs.ampbs.2015.09.002

Ballouche, M., Cornelis, P., and Baysse, C. (2009). Iron metabolism: a promising target for antibacterial strategies. *Recent Pat. Anti Infect. Drug Discov.* 4, 190–205. doi: 10.2174/157489109789318514

Bastos, T.de O., Soares, B. M., Cisalpino, P. S., Mendes, I. C., dos Santos, R. G., and Beraldo, H. (2010). Coordination to gallium(III) strongly enhances the potency of 2-pyridineformamide thiosemicarbazones against *Cryptococcus* opportunistic fungi. *Microbiol. Res.* 165, 573–577. doi: 10.1016/j.micres.2009.10.005

Bernstein, L. R. (1998). Mechanisms of therapeutic activity for gallium. *Pharmacol. Rev.* 50, 665–682.

Bernstein, L. R., Tanner, T., Godfrey, C., and Noll, B. (2000). Chemistry and pharmacokinetics of gallium maltolate, a compound with high oral gallium bioavailability. *Met. Based Drugs* 7, 33–47. doi: 10.1155/MBD.2000.33

Bonchi, C., Frangipani, E., Imperi, F., and Visca, P. (2015). Pyoverdine and proteases affect the response of *Pseudomonas aeruginosa* to gallium in human serum. *Antimicrob. Agents Chemother.* 59, 5641–5646. doi: 10.1128/AAC.01097-15

Boucher, H. W., Talbot, G. H., Bradley, J. S., Edwards, J. E., Gilbert, D., Rice, L. B., et al. (2009). Bad bugs, no drugs: no ESKAPE! An update from the infectious diseases society of America. *Clin. Infect. Dis.* 48, 1–12. doi: 10.1086/595011

Carpenter, C., and Payne, S. M. (2014). Regulation of iron transport systems in Enterobacteriaceae in response to oxygen and iron availability. *J. Inorg. Biochem.* 133, 110–117. doi: 10.1016/j.jinorgbio.2014.01.007

Cartron, M. L., Maddocks, S., Gillingham, P., Craven, C. J., and Andrews, S. C. (2006). Feo-transport of ferrous iron into bacteria. *Biometals* 19, 143–157. doi: 10.1007/s10534-006-0003-2

Chang, D., Garcia, R. A., Akers, K. S., Mende, K., Murray, C. K., Wenke, J. C., et al. (2016). Activity of gallium meso- and protoporphyrin IX against biofilms of multidrug-resistant *Acinetobacter baumannii* isolates. *Pharmaceuticals* 9:E16. doi: 10.3390/ph9010016

Chitambar, C. R. (2017). The therapeutic potential of iron-targeting gallium compounds in human disease: from basic research to clinical application. *Pharmacol. Res.* 115, 56–64. doi: 10.1016/j.phrs.2016.11.009

Clinical and Laboratory Standards Institute (2015). *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*. 10th edn. Wayne, PA: CLSI (Approved standard M07–A10).

Collery, P., Keppler, B., Madoulet, C., and Desoize, B. (2002). Gallium in cancer treatment. *Crit. Rev. Oncol. Hematol.* 42, 283–296. doi: 10.1016/S1040-8428(01)00225-6

de Léséleuc, L., Harris, G., KuoLee, R., Xu, H. H., and Chen, W. (2014). Serum resistance, gallium nitrate tolerance and extrapulmonary dissemination are linked to heme consumption in a bacteremic strain of *Acinetobacter baumannii*. *Int. J. Med. Microbiol.* 304, 360–369. doi: 10.1016/j.ijmm.2013.12.002

DeLeon, K., Balldin, F., Watters, C., Hamood, A., Griswold, J., Sreedharan, S., et al. (2009). Gallium maltolate treatment eradicates *Pseudomonas aeruginosa* infection in thermally injured mice. *Antimicrob. Agents Chemother.* 53, 1331–1337. doi: 10.1128/AAC.01330-08

Deplano, A., Denis, O., Poirel, L., Hocquet, D., Nonhoff, C., Byl, B., et al. (2005). Molecular characterization of an epidemic clone of panantibiotic-resistant *Pseudomonas aeruginosa*. *J. Clin. Microbiol*. 43, 1198–1204. doi: 10.1128/JCM.43.3.1198-1204.2005

Edwards, C. L., and Hayes, R. L. (1969). Tumor scanning with ^{67}Ga citrate. *J. Nucl. Med*. 10, 103–105.

Elemam, A., Rahimian, J., and Mandell, W. (2009). Infection with panresistant *Klebsiella pneumoniae*: a report of 2 cases and a brief review of the literature. *Clin. Infect. Dis.* 49, 271–274. doi: 10.1086/600042

Foley, T. L., and Simeonov, A. (2012). Targeting iron assimilation to develop new antibacterials. *Expert. Opin. Drug Discovery* 7, 831–847. doi: 10.1517/17460441.2012.708335

Frangipani, E., Bonchi, C., Minandri, F., Imperi, F., and Visca, P. (2014). Pyochelin potentiates the inhibitory activity of gallium on *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 58, 5572–5575. doi: 10.1128/AAC.03154-14

Friedman, N. D., Temkin, E., and Carmeli, Y. (2016). The negative impact of antibiotic resistance. *Clin. Microbiol. Infect*. 22, 416–422. doi: 10.1016/j.cmi.2015.12.002

Goncharova, I., Orlov, S., and Urbanová, M. (2013). The location of the high- and low-affinity bilirubin-binding sites on serum albumin: ligand-competition analysis investigated by circular dichroism. *Biophys. Chem*. 180–181, 55–65. doi: 10.1016/j.bpc.2013.06.004

Hackel, M. A., Tsuji, M., Yamano, Y., Echols, R., Karlowsky, J. A., and Sahm, D. F. (2018). *In vitro* activity of the siderophore cephalosporin, cefiderocol, against carbapenem-non-susceptible and multidrug-resistant isolates of gram-negative *Bacilli* collected worldwide in 2014 to 2016. *Antimicrob. Agents Chemother*. 62:e01968-17. doi: 10.1128/AAC.01968-17

Haley, K. P., and Skaar, E. P. (2012). A battle for iron: host sequestration and *Staphylococcus aureus* acquisition. *Microbes Infect.*14, 217–227. doi: 10.1016/j.micinf.2011.11.001

Hijazi, S., Visca, P., and Frangipani, E. (2017). Gallium-protoporphyrin IX inhibits *Pseudomonas aeruginosa* growth by targeting cytochromes. *Front. Cell. Infect. Microbiol.* 7:12. doi: 10.3389/fcimb.2017.00012

Kaneko, Y., Thoendel, M., Olakanmi, O., Britigan, B. E., and Singh, P. K. (2007). The transition metal gallium disrupts *P.* aeruginosa iron metabolism and has antimicrobial and antibiofilm activity. *J. Clin. Invest*. 117, 877–888. doi: 10.1172/JCI30783

Koczura, R., and Kaznowski, A. (2003). Occurrence of the *Yersinia* high-pathogenicity island and iron uptake systems in clinical isolates of *Klebsiella pneumoniae*. *Microb. Pathog*. 35, 197–202. doi: 10.1016/S0882-4010(03)00125-6

Latorre, M., Quenti, D., Travisany, D., Singh, K. V., Murray, B. E., Maass, A., et al. (2018). The role of Fur in the transcriptional and iron homeostatic response of *Enterococcus faecalis*. *Front. Microbiol.* 9:1580. doi: 10.3389/fmicb.2018.01580

Lucidi, M., Runci, F., Rampioni, G., Frangipani, E., Leoni, L., and Visca, P. (2018). New shuttle vector for gene cloning and expression in multi-drug resistance *Acinetobacter* species. *Antimicrob. Agents Chemother*. 62:e02480-17. doi: 10.1128/AAC.02480-17

Luepke, K. H., and Mohr, J. F. (2017). The antibiotic pipeline: reviving research and development and speeding drugs to market. *Expert Rev. Anti Infect. Ther*. 15, 425–433. doi: 10.1080/14787210.2017.1308251

Miethke, M., and Marahiel, M. A. (2007). Siderophore-based iron acquisition and pathogen control. *Microbiol. Mol. Biol. Rev.* 71, 413–451. doi: 10.1128/MMBR.00012-07

Miller, J. H. (1972). *Experiments in Molecular Genetics*. New York, NY: Cold Spring Harbor Laboratory.

Minandri, F., Bonchi, C., Frangipani, E., Imperi, F., and Visca, P. (2014). Promises and failures of gallium as an antibacterial agent. *Future Microbiol.* 9, 379–397. doi: 10.2217/fmb.14.3

Mortensen, B. L., and Skaar, E. P. (2013). The contribution of nutrient metal acquisition and metabolism to *Acinetobacter baumannii* survival within the host. *Front. Cell. Infect. Microbiol.* 3:95. doi: 10.3389/fcimb.2013.00095

Nowak, J., Zander, E., Stefanik, D., Higgins, P. G., Roca, I., Vila, J., et al. (2017). High incidence of pandrug-resistant *Acinetobacter baumannii* isolates collected from patients with ventilator-associated pneumonia in Greece, Italy and Spain as part of the MagicBullet clinical trial. *J. Antimicrob. Chemother.* 72, 3277–3282. doi: 10.1093/jac/dkx322

Ochsner, U. A., and Vasil, M. L. (1996). Gene repression by the ferric uptake regulator in *Pseudomonas aeruginosa*: cycle selection of iron-regulated genes. *Proc. Natl. Acad. Sci. U.S.A.* 93, 4409–4414. doi: 10.1073/pnas.93.9.4409

Ou, H. Y., Kuang, S. N., He, X., Molgora, B. M., Ewing, P. J., Deng, Z., et al. (2015). Complete genome sequence of hypervirulent and outbreak-associated *Acinetobacter baumannii* strain LAC-4: epidemiology, resistance genetic determinants and potential virulence factors. *Sci. Rep*. 5:8643. doi: 10.1038/srep08643

Pendleton, J. N., Gorman, S. P., and Gilmore, B. F. (2013). Clinical relevance of the ESKAPE pathogens. *Expert Rev. Anti Infect. Ther*. 3, 297–308. doi: 10.1586/eri.13.12

Podschun, R., Fischer, A., and Ullmann, U. (1992). Siderophore production of *Klebsiella* species isolated from different sources. *Zentralbl. Bakteriol.* 276, 481–486. doi: 10.1016/S0934-8840(11)80673-0

Principe, L., D'Arezzo, S., Capone, A., Petrosillo, N., and Visca, P. (2009). *In vitro* activity of tigecycline in combination with various antimicrobials against multidrug resistant *Acinetobacter baumannii*. *Ann. Clin. Microbiol. Antimicrob.* 8:18. doi: 10.1186/1476-0711-8-18

Rice, L. B. (2010). Progress and challenges in implementing the research on ESKAPE pathogens. *Infect. Control Hosp. Epidemiol.* 1, S7–S10. doi: 10.1086/655995

Ross-Gillespie, A., Weigert, M., Brown, S. P., and Kümmerli, R. (2014). Gallium-mediate siderophore quenching as an evolutionarily robust antibacterial treatment. *Evol. Med. Public Health* 2014, 18–29. doi: 10.1093/emph/eou003

Schwyn, B., and Neilands, J. B. (1987). Universal chemical assay for the detection and determination of siderophores. *Anal. Biochem.* 160, 47–56. doi: 10.1016/0003-2697(87)90612-9

Stojiljkovic, I., Kumar, V., and Srinivasan, N. (1999). Non-iron metalloporphyrins: potent antibacterial compounds that exploit haem/Hb uptake systems of pathogenic bacteria. *Mol. Microbiol.* 31, 429–442. doi: 10.1046/j.1365-2958.1999.01175.x

Thompson, M. G., Corey, B. W., Si, Y., Craft, D. W., and Zurawski, D. V. (2012). Antibacterial activities of iron chelators against common nosocomial pathogens. *Antimicrob. Agents Chemother.* 56, 5419–5421. doi: 10.1128/AAC.01197-12

Thompson, M. G., Truong-Le, V., Alamneh, Y. A., Black, C. C., Anderl, J., Honnold, C. L., et al. (2015). Evaluation of gallium citrate formulations against a multidrug-resistant strain of *Klebsiella pneumoniae* in a murine wound model of infection. *Antimicrob. Agents Chemother*. 59, 6484–6493. doi: 10.1128/AAC.00882-15

Wandersman, C., and Delepelaire, P. (2004). Bacterial iron sources: from siderophores to hemophores. *Annu. Rev. Microbiol.* 58, 611–647. doi: 10.1146/annurev.micro.58.030603.123811

Weinberg, E. D. (2009). Iron availability and infection. *Biochem. Biophys. Acta* 1790, 600–605. doi: 10.1016/j.bbagen.2008.07.002

World Health Organization (2017). *List of Antibiotic Resistant Priority Pathogens*. Available online at: http://www.who.int/mediacentre/news/releases/2017/bacteria-antibiotics-needed/en/

Yildirim, S., Thompson, M. G., Jacobs, A. C., Zurawski, D. V., and Kirkup, B. C. (2016). Evaluation of parameters for high efficiency transformation of *Acinetobacter baumannii*. *Sci. Rep.* 6:22110. doi: 10.1038/srep22110

Conflict of Interest Statement: LB holds several patents for possible applications of GaM in human and veterinary medicine and is affiliated with a company (Gallixa LLC) that would like to obtain regulatory approval for topical gallium maltolate as a therapeutic agent. LB did not participate in data collection for this study.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary material

Table S1. List of bacterial strains used in this study.

Bacterial species	Strain	Country	Year	Source	Resistance	Reference
Enterococcus faecalis	ATCC 29212	ns	ns	urine	ns	American Type Culture Collection
E. faecalis	ATCC 700802	USA	1987	blood	VAN (*vanB*), GEN	Sahm et al., 1989
Enterococcus faecium^{T}	ATCC 19434	ns	ns	unknown	ns	Schleifer and Kilpper-Balz, 1984
E. faecium	BM4147	ns	ns	human clinical	VAN (*vanA*)	Bugg et al., 1991
Staphylococcus aureus	ATCC 25923	USA	1945	human clinical	susceptible	American Type Culture Collection
S. aureus	ATCC 43300	USA	ns	human clinical	MRSA	American Type Culture Collection
S. aureus	Sau117	Italy	2010	skin infection	MDR	Monaco et al., 2013
S. aureus	UD95	Italy	2011	human clinical	MDR	This work
Klebsiella pnemoniae	ATCC 27736	ns	ns	human clinical	ns	American Type Culture Collection
K. pnemoniae	Kp3	Italy	2011	blood	MDR	Villa et al., 2014
K. pnemoniae	17830	Italy	2008	sacral ulcer	MDR	Garcia-Fernandez et al., 2010
K. pnemoniae	16855	Italy	2008	wound	MDR	Garcia-Fernandez et al., 2010
Acinetobacter baumannii	ATCC 17978	France	1951	blood	SXT	Smith et al., 2007
A. baumannii	AYE	France	2001	urine	MDR	Poirel et al., 2003
A. baumannii	ACICU	Italy	2005	spinal fluid	MDR	Iacono et al., 2008
A. baumannii	C13-373	Italy	2007	blood	MDR	Migliavacca et al., 2013
Pseudomonas aeruginosa	ATCC 15692 (PAO1)	ns	ns	infected wound	ns	American Type Culture Collection
P. aeruginosa	PA14	USA	1995	burn	susceptible	Rahme et al., 1995
P. aeruginosa	LesB58	UK	1996	sputum	MDR	Cheng et al., 1996
P. aeruginosa	SP-13	Italy	ns	blood	MDR	Bonchi et al., 2015
Enterobacter aerogenes^{T}	ATCC 13048	USA	ns	sputum	ns	Bascomb et al., 1971
E. aerogenes	84-6792	Italy	2016	blood	susceptible	This work
Enterobacter cloacae^{T}	ATCC 13047	USA	ns	spinal fluid	susceptible	Hormaeche and Edwards, 1960
E. cloacae	78-6303	Italy	2016	blood	susceptible	This work

Abbreviations: GEN, gentamicin; MDR, multidrug resistant; MRSA, methicillin-resistant *Staphylococcus aureus*; SXT, trimethoprim-sulfamethoxazole; VAN, vancomycin; ns, not specified.

^{T} type strain.

References:

Bascomb, S., Lanpage, S. P., Willcox, W. R., and Curtis, M. A. (1971). Numerical Classification of the Tribe *Klebsielleae*. *J. Gen. Microbiol*. 66, 279-295. doi: 10.1099/00221287-66-3-279

Bonchi, C., Frangipani, E., Imperi, F. and Visca, P. (2015). Pyoverdine and proteases affect the response of *Pseudomonas aeruginosa* to gallium in human serum. *Antimicrob. Agents Chemother*. 59, 5641-5646. doi: 10.1128/AAC.01097-15

Bugg, T. D. H., Wright, G. D., Dutka-Malen, S., Arthur, M., Courvalin, P., and Walsh, C. T. (1991). Molecular basis for vancomycin resistance in *Enterococcus faecium* BM4147: biosynthesis of a depsipeptide peptidoglycan precursor by vancomycin resistance proteins VanH and VanA. *Biochem.* 30, 10408-10415. doi: 0.1021/bi00107a007

Cheng, K., Smyth, R. L., Govan, J. R., Doherty, C., Winstanley, C., Denning, N., et al. (1996). Spread of beta-lactam resistant *Pseudomonas aeruginosa* in a cystic fibrosis clinic. *Lancet* 348, 639-642 doi: 10.1016/S0140-6736(96)05169-0

García-Fernández, A., Miriagou, V., Papagiannitsis, C. C., Giordano, A., Venditti, M., Mancini, C., et al. (2010). An ertapenem-resistant extended-spectrum-β-lactamase-producing *Klebsiella*

pneumoniae clone carries a novel OmpK36 porin variant. *Antimicrob. Agents Chemother.* 54, 4178-4184. doi: 10.1128/AAC.01301-09

Hormaeche, E. and Edwards, P. R. (1960). Proposal for the rejection of the generic name *Cloaca* Castellani and Chalmers, and proposal of *Enterobacter* as a generic name with designation of the type species and its type culture. *Int. J. Syst. Evol. Microbiol*. 10, 75-76. doi: 10.1099/0096266X-10-2-75

Iacono, M., Villa, L., Fortini, D., Bordoni, R., Imperi, F., Bonnal, R. J., et al. (2008). Whole-genome pyrosequencing of an epidemic multidrug-resistant *Acinetobacter baumannii* strain belonging to the european clone II group. *Antimicrob. Agents Chemother.* 52, 2616-2625. doi: 10.1128/AAC.01643-07

Migliavacca, R., Espinal, P., Principe, L., Drago, M., Fugazza, G., Roca, I., et al. (2013). Characterization of resistance mechanisms and genetic relatedness of carbapenem-resistant *Acinetobacter baumannii* isolated from blood, Italy. *Diagn. Microbiol. Infect. Dis.* 75, 180-186. doi: 10.1016/j.diagmicrobio.2012.11.002

Monaco, M., Pedroni, P., Sanchini, A., Bonomini, A., Indelicato, A., and Pantosti, A. (2013). Livestock-associated methicillin-resistant *Staphylococcus aureus* responsible for human colonization and infection in an area of Italy with high density of pig farming. *BMC Infect. Dis.* 13:258. doi: 10.1186/1471-2334-13-258

Poirel, L., Menuteau, O., Agoli, N., Cattoen, C., and Nordmann, P. (2003). Outbreak of extended-spectrum β-lactamase VEB-1-producing isolates of *Acinetobacter baumannii* in a french hospital. *J. Clin. Microbiol.* 41, 3542-3547. doi: 10.1128/JCM.41.8.3542-3547.2003

Rahme, L. G., Stevens, E. J., Wolfort, S. F., Shao, J., Tompkins, R. G., and Ausubel, F. M. (1995). Common virulence factors for bacterial pathogenicity in plants and animals. *Science* 30, 1899-1902. doi: 10.1126/science.7604262

Sahm, D. F., Kissinger, J., Gilmore, M. S., Murray, P. R., Mulder, R., Solliday, J. et al. (1989). *In vitro* susceptibility studies of vancomycin-resistant *Enterococcus faecalis*. *Antimicrob. Agents Chemother.* 33,1588-1591.

Schleifer, K. H. and Kilpper-Balz, R. (1984). Transfer of *Streptococcus faecalis* and *Streptococcus faecium* to the genus *Enterococcus* norn. rev. as *Enterococcus faecalis* comb. nov. and *Enterococcus faecium* comb. *Nov. Int. J. Syst. Evol. Microbiol.* 34, 31-34.

Smith, M. G., Gianoulis, T. A., Pukatzki, S., Mekalanos, J. J., Ornston, L. N., Gerstein, M., et al. (2007). New insights into *Acinetobacter baumannii* pathogenesis revealed by high-density

pyrosequencing and transposon mutagenesis. *Genes Dev*. 21, 601-614. doi: 10.1101/gad.1510307

Villa, L., Feudi, C., Fortini, D., García-Fernández, A., and Carattoli, A. (2014). Genomics of KPC-producing *Klebsiella pneumoniae* sequence type 512 clone highlights the role of RamR and ribosomal S10 protein mutations in conferring tigecycline resistance. *Antimicrob. Agents Chemother*. 58, 1707-12. doi: 10.1128/AAC.01803-13

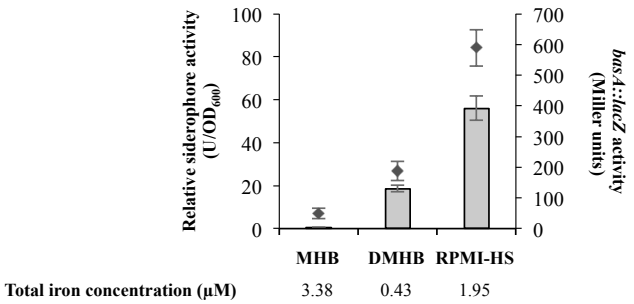


Figure S1. Probing the iron content of Ga(III)-susceptibility test media. Siderophore production (grey bars), and activity of the *basA*::*lacZ* iron-regulated reporter gene fusion (black diamonds) in *A. baumannii* ATCC 17978, grown in MHB, DMHB, or RPMI-HS for 24 h. Data are the mean ± standard deviation of triplicate experiments. The iron content of media, as determined by ICP-OES, is also reported.

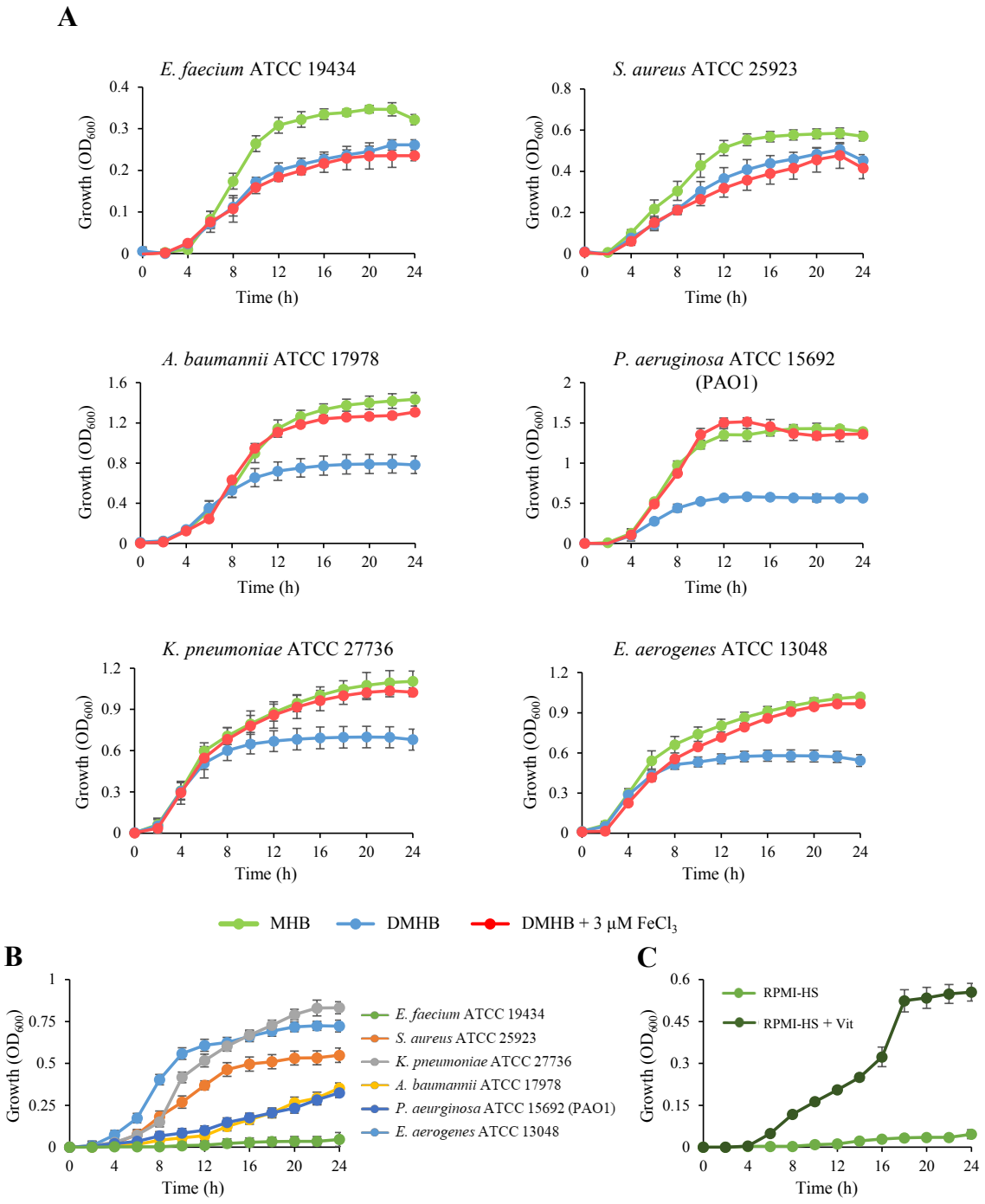


Figure S2. Growth profiles of ESKAPE reference strains in MHB, DMHB, and RPMI-HS. **(A)** Bacterial strains were grown for 18 h at 37 °C in TSB, diluted in saline and inoculated (5 x 10^5 CFU/ml) into 96-well microtiter plates containing MHB, or DMHB supplemented or not with 3.3 µM $FeCl_3$. **(B)** Same as **(A)**, but using RPMI-1640 supplemented with 10 % HS (RPMI-HS). **(C)** *E. faecium* growth rescue in RPMI-HS supplemented with 19 µg/ml of nicotinic acid and 2 µg/ml of pyridoxal hydrochloride (Vit). OD_{600} was monitored periodically for up to 24 h. Data are the means ± standard deviation of triplicate experiments.

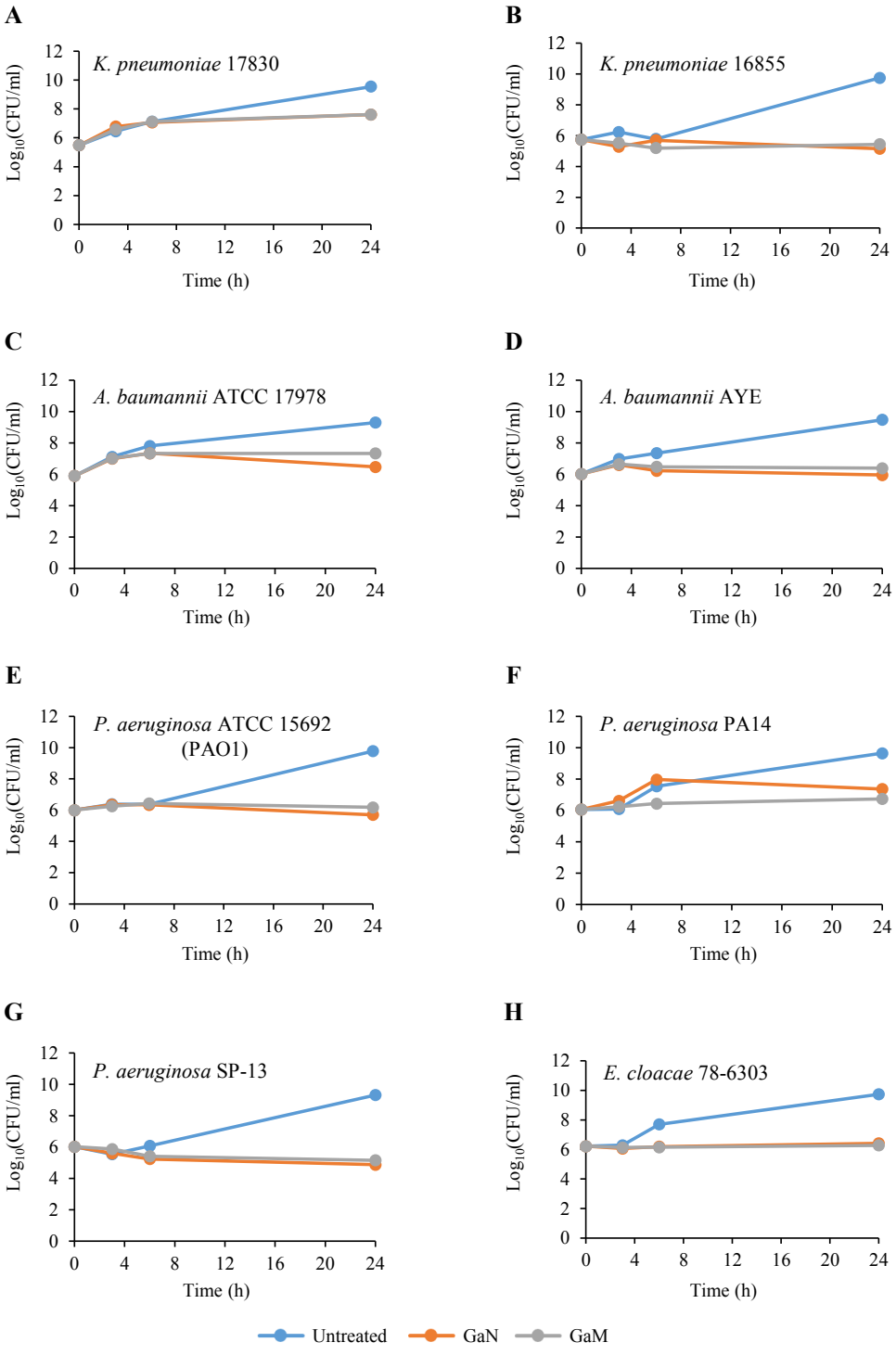


Figure S3. GaN and GaM time-kill assays. GaN and GaM time-kill kinetics were determined after 0-, 3-, 6- and 24-h incubation in RPMI-HS supplemented with 28 µM of GaN or GaM. **(A)** *K. pneumoniae* 17830, **(B)** *K. pneumoniae* 16855, **(C)** *A. baumannii* ATCC 17978, **(D)** *A. baumannii* AYE, **(E)** and *P. aeruginosa* PAO1, **(F)** *P. aeruginosa* PA14, **(G)** *P. aeruginosa* SP-13, and **(H)** *E. cloacae* 78-6303. Panels show one representative experiment of three independent replicates yielding similar results.

Chapter 4

Fighting cystic fibrosis infections with gallium-based compounds

Sarah Hijazi [a], Daniela Visaggio[a], Mattia Pirolo[a], Emanuela Frangipani[a], Lawrence Bernstein[b], and Paolo Visca[a]

[a]*Department of Science, University of Roma Tre, Rome, Italy*

[b]*Terrametrix, Menlo Park, CA 94025, USA*

Part of this work was presented at the 15th The XIV Congress of the Italian Federation of Life Sciences (FISV) – (September 18th-21th, 2018 - Rome, Italy).

Manuscript in preparation for submission to The Journal of Cystic Fibrosis

Chapter 4

Fighting cystic fibrosis infections with gallium-based compounds

The microbial flora of the respiratory tract of patients suffering from cystic fibrosis (CF) represents a complex and diverse ecosystem, in which multispecies communities can coexist and evolve during infection. Main pathogens in such communities are *Achromobacter xylosoxidans, Burkholderia cepacia* complex (*Bcc*) species, *Haemophilus influenzae*, *Pseudomonas aeruginosa, Staphylococcus aureus, Stenotrophomonas maltophilia,* and *Streptococcus pneumoniae* (Filkins and O'Toole, 2015; Parkins and Floto, 2015) (Chapter 1). The eradication of such infections in CF patients is very difficult, due to the frequent and prolonged antibiotic administration, that exerts selective pressure for antibiotic resistance (López-Causapé *et al.,* 2013, 2015), leading to the emergence of multidrug resistant (MDR) strains (Lechtzin *et al.,* 2006; Nguyen and Singh, 2006; Sass *et al.,* 2011). Moreover, one of the most worrisome aspects of bacterial pathogenicity in CF is the development of chronic infections, which is associated with bacterial communities in biofilm mode of growth, that are highly adapted and resilient to antibiotic treatment (Stewart and Costerton, 2001).

The lack of antibiotics to treat MDR bacterial infections and biofilm-associated infections, coupled with the disappointingly low discovery rate of new and clinical useful antibiotics, urgently call for the development of novel antibacterial therapies. In this scenario, interfering with iron, an essentiel nutrient required by all CF pathogens, may represens a good strategy to eradicate bacterial infections. Prompted by the promising results obtained in Chapter 3, in which Ga(III) has been shown to exhibit an antibacterial activity against a broad spectrum of MDR pathogens, coupled with previous studies showing that Ga(III) is also effective against biofilm-growing bacteria (Bonchi *et al.,* 2014; Minandri *et al.,* 2014; Runci *et al.,* 2016), we have widen our investigations on the effect of Ga(III) compounds on CF-associated pathogens.

In this work, the efficacy of Ga(III)-based compounds, namely $Ga(NO_3)_3$ (GaN), Ga(III)-maltolate (GaM) (Bernstein *et al.,* 2000) and Ga(III)-protoporphyrin IX (GaPPIX) belonging to the first-, second- and third-generation of Ga(III) formulations, respectively (Chitambar, 2017), was investigated on a representative collection of CF pathogens, using standard and biological fluids-mimetic growth media, such as RPMI-HS (Hijazi *et al.,* 2018), and Artificial Sputum Medium (ASM; Kirchner et *al.,* 2012). Combining data obtained in all media tested, we found that Ga(III)-based compounds were effective against all CF-associated pathogens, except *S. pneumoniae*.

Interestingly, some species (*i.e., S. aureus* and *H. influenzae*) were found to be extremely susceptible only to GaPPIX, while the other species were susceptible to more then one Ga(III) compound.

Abstract

Background: Polymicrobial chronic infection is a hallmark of cystic fibrosis (CF) patients, and multidrug-resistant pathogens are often associated with CF disease. Since the pipeline of antibiotic discovery is almost dry, new targets are urgently needed for the development of new therapeutic options. Given the essential role of iron in bacterial physiology and pathogenicity, iron uptake and metabolism are considered as druggable targets for the development of new treatments. On these premises, the post-transition metal gallium [Ga(III)] was recently repurposed as an antibacterial agent. Ga(III) has no proven function in biological systems, but acts as an iron-mimetic by replacing iron in many enzymes, impairing their function and ultimately hampering bacterial growth.

Methods: A representative panel of 30 CF pathogens as well as 10 clinical isolates of the main CF pathogen *Pseudomonas aeruginosa* were included in this study. The MICs of Ga(III) compounds on bacterial cells were determined using the broth microdilution method recommended by the Clinical and Laboratory Standards Institute guidelines, in standard media as well as in media mimicking biological fluids.

Results: After setting suitable susceptibility tests, the *in vitro* efficacy of Ga(III)-based compounds on CF-associated pathogens, except *Streptococcus pneumoniae* was demonstrated. Notably, *S. aureus* and *H. influenzae* were found to be extremely susceptible only to GaPPIX, while the other species were susceptible to more then one Ga(III)-based compounds.

Conclusions: The development of Ga(III)-based antibacterials could help to set patient-specific therapies based on the infection phase, depending on the prominent bacterial pathogens present in the CF lung. These therapies could be considered not only as last-resort drugs for the treatment of otherwise untreatable (pan-drug resistant) infections, but also as a first-line drug in order to delay the use of antibiotics and prevent the emergence of resistance.

Keywords: Cystic fibrosis; Polymicrobial, Chronic infection; Iron metabolism; Antibacterial; Gallium compounds

1. Introduction

Pulmonary manifestations in cystic fibrosis (CF) patients represent the main cause of morbidity and mortality associated with the disease (Stoltz *et al.,* 2015). Impairment of muco-ciliary transport causes the obstruction of distal airways with viscous secretions, which favours bacterial colonisation (Govan and Deretic, 1996). Trapped bacteria colonize the mucus and enable the development and persistence of bacterial pulmonary infection. The microbial flora of the respiratory tract of CF patients is an evolving ecosystem colonised by multispecies communities (Filkins and O'Toole, 2015; Parkins and Floto, 2015). During childhood, the main colonizing pathogens are *Staphylococcus aureus* and *Haemophilus influenzae*, while during adulthood *Pseudomonas aeruginosa* and occasionally *Burkholderia cepacia* complex (*Bcc*) are present (Rogers et *al.,* 2003; Folkesson *et al.,* 2012), with *P. aeruginosa* becoming the main pathogen affecting around 70% of adults with CF (Cystic Fibrosis Foundation Patient Registry, 2016). Other emerging pathogens in CF include *Achromobacter xylosoxidans*, *Stenotrophomonas maltophilia*, and *Streptococcus pneumoniae* (Rogers et *al.,* 2003; van der Gast *et al.,* 2011; Folkesson *et al.,* 2012). While frequent and long-lasting antibiotic treatments have considerably prolonged the longevity of CF patients (Cystic Fibrosis Foundation Patient Registry, 2016), they have exerted tremendous selective pressures for antibiotic resistance (Sherrad *et al.,* 2013; López-Causapé *et al.,* 2013, 2015). Therefore, multidrug-resistance (MDR) has been found in the respiratory tract of CF patients (Lechtzin *et al.,* 2006; Nguyen and Singh, 2006; Sass *et al.,* 2011), making the eradication of infecting bacteria extremely difficult. Furthermore, if not treated, CF pathogens, and in particular *P. aeruginosa*, lead to the development of chronic lung infections, which are characterized by the formation of microbial biofilm (Moore and Mastoridis, 2017). The biofilm matrix acts as a barrier protecting bacterial cells from external harms, especially antibiotics, making chronic lung infections nearly impossible to eradicate. The loss of efficacy of antibiotics to treat MDR CF-bacterial infections calls for the development of novel antibacterial therapies. A promising strategy to counteract bacterial infection in the CF lungs is the development of unconventional antibacterial

drugs impairing the acquisition of essential nutrients by CF pathogens. Iron is a key nutrient for all pathogenic bacteria, including those associated with CF, being a cofactor for many enzymes involved in critical cellular functions (Andrews *et al.,* 2003). However, the CF lung is typically an iron-poor environment where bacteria struggle with the host's iron withholding capacity (Reid *et al.,* 2009). Bacteria adopt various strategies to actively acquire this important nutrient, the most widespread being the production of siderophores and expression of heme-uptake systems. Given the essential role of iron in bacterial physiology and pathogenicity, disruption of bacterial iron metabolism represents a promising approach for the development of new antibacterials (Ballouche *et al.,* 2009; Foley and Simeonov, 2012). In this context, the post-transition iron mimetic metal gallium [Ga(III)] has been successfully repurposed as an antimicrobial agent (reviewed by Bonchi *et al.,* 2014; Minandri *et al.,* 2014; Rangel-Vega *et al.,* 2015). The chemical properties of Ga(III) are very similar to those of Fe(III), and this allows Ga(III) to replace Fe(III) in the prosthetic group of several enzymes. However, different from Fe(III), Ga(III) cannot be reduced under physiological conditions and, therefore, it cannot take part in redox reactions, ultimately impairing a number of essential functions (Bernstein, 1998, Chitambar, 2010). Previous work showed that Ga(III) is endowed with both anti-bacterial and anti-biofilm activities on a wide range of pathogenic bacteria (Minandri *et al.,* 2014; Runci *et al.,* 2016; Hijazi *et al.,* 2018; Goss *et al.,* 2018). The fascinating possibility of developing Ga(III)-based therapies to treat *P. aeruginosa* lung infection is corroborated by the conductance of clinical trials testing the pharmacokinetics, safety and tolerability of Ganite® [*i.e.,* a pharmaceutical formulation of citrate-buffered gallium nitrate approved by the FDA] in CF patients chronically infected by *P. aeruginosa* (ClinicalTrials.gov identifier: NCT01093521 and NCT02354859; Goss *et al.,* 2012, 2018).

However, while the antibacterial activity of different Ga(III) formulations has extensively investigated on *P. aeruginosa* (Kaneko *et al.,* 2008; Bonchi *et al.,* 2015; Hijazi *et al.,* 2017, 2018), there are no systematic studies, comparing the antibacterial activity of Ga(III) on the different bacterial species associated with CF lung infection.

The main aim of this work was to comparatively investigate the efficacy of Ga(III)-based compounds, namely Ga(III)-nitrate (GaN), Ga(III)-maltolate (GaM) (Bernstein *et al.,* 2000) and Ga(III)-protoporphyrin IX (GaPPIX) belonging to the first-, second- and third-generation of Ga(III) formulations, respectively (Chitambar, 2017), on a representative collection of CF pathogens, using standard test media as well as media which mimick biological fluids.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Bacterial strains used in this work are listed in supplementary Table S1 and Table S2. All CF bacterial strains, except fastidious pathogens (*H. influenzae* and *S. pneumoniae*), were routinely cultured for 18 h in Tryptic Soy Broth (TSB, Acumedia) with vigorous shaking at 37°C. For *H. influenzae* and *S. pneumoniae*, bacteria were cultured for 20 h in *Haemophilus* Test Medium (HTM) supplemented with 15 g/l agar (Acumedia) (HTMA), and Columbia agar supplemented with 5% sheep blood (Biomérieux), respectively. *S. pneumoniae* isolates were incubated in the presence of 5% of CO_2.

When needed, 10 mM protoporphyrin IX (PPIX, Sigma-Aldrich) stock solution was freshly prepared in 10 mM NaOH.

2.2. Test media and Ga(III) compounds

Several media were used for Ga(III)-susceptibility testing, namely: *i)* Cation-adjusted Mueller-Hinton broth II (MHB, Becton Dickinson); *ii)* the iron-depleted MHB (DMHB; Hijazi *et al.,* 2018); *iii)* HTM, depleted of heme; *iv)* the related iron-depleted HTM (DHTM), prepared by treating HTM with 100 g/l of the metal-chelating Chelex® 100 resin (Bio-rad); *v)* Todd-Hewitt Broth (THB, Oxoid) supplemented with 0.5% yeast extract (YE) (THYB) (Marshall *et al.,* 1993); *vi)* the iron-depleted THYB (DTHYB; Montañez, *et al.,* 2005); *vii)* RPMI-HS (Hijazi *et al.,* 2018), supplemented with 5% casaminoacids (CAA, Becton Dickinson) and/or supplements [*i.e.,* 3.3

µg/ml of hypoxanthine (Nutritional Biochemicals Corporation), 100 µg/ml of L-alanine (Merck), 55 µg/ml of L-cysteine hydrochloride (Merck), 6.6 µg/ml nicotinamide adenine dinucleotide (NAD, Boehringer Mannheim GmbH), and 8 µg/ml uracil (Sigma)], and *viii)* Artificial Sputum Medium (ASM; Kirchner *et al.,* 2012), but replacing the mix of 20 amino acids with 5 g/l CAA (Sriramulu *et al.,* 2005).

Three Ga(III) compounds were used in this study: *i)* GaN [$Ga(NO_3)_3 \times 6H_2O$, (Sigma-Aldrich)], freshly prepared as a 100 mM stock solution in water; *ii)* GaM (NORAC Pharma, provided by Dr. Bernstein), freshly prepared as a 22 mM stock solution in water; and *iii)* GaPPIX (Frontier Scientific), prepared as a 25 mM stock solution in dimethyl sulfoxide (DMSO), and stored at 4 °C in the dark.

2.3. Susceptibility testing of Ga(III) compounds

The antibacterial activity of Ga(III) compounds on planktonic CF pathogens was conducted using the microdilution method (CLSI, 2015), with minor modifications. Briefly, bacteria grown as detailed in par. 2.1, were inoculated to *ca.* 5 x 10^5 CFU/ml in 200 µl of MHB/DMHB, HTM/DHTM, THYB/DTHYB, RPMI-HS, and ASM, in the presence of increasing concentrations (0 to 128 µM) of each Ga(III) compound (GaN, GaM, and GaPPIX), using 96-well microtiter plates. All plates were incubated at 37 °C and, only in case of *S. pneumoniae*, in an atmosphere containing 5% CO_2. The minimum inhibitory concentration (MIC) of Ga(III) compounds, which corresponds to the lowest concentration that completely inhibited bacterial growth as detected by the unaided eye (CLSI, 2015), was determined after 24 h of incubation at 37 °C in all media tested. Only for *A. xylosoxidans* and *S. maltophilia*, MIC values were determined after 48 h of incubation at 37 °C in RPMI-HS + CAA, due to poor growth after 24 h.

2.4. Inhibition of biofilm formation and disruption of preformed biofilms

The ability of Ga(III) compounds to prevent *P. aeruginosa* biofilm formation was investigated. Bacteria were grown for 18 h in TSB, washed in saline and inoculated to *ca.* 5 x 10^5 CFU/ml in 200 µl of ASM in the presence of increasing concentrations (0 to 128 µM) of each Ga(III) compound (GaN, GaM, and GaPPIX), using 96-well microtiter plates. Microtiter plates were incubated for up to 24 h at 37°C, and bacterial growth (optical density at 600 nm [OD_{600}]) measured spectrophotometrically using a microtiter plate reader (SPARK 10M TECAN). Biofilm formation was quantified by a microtiter plate assay as described elsewhere, with some modifications (Runci *et al.,* 2016). Briefly, planktonic cells were removed, and the attached cells were gently washed with sterile saline solution, air dried, and stained for 30 min with 250 µl of 0.1% crystal violet (CV) at room temperature (RT). Wells were then gently washed twice with sterile water, and the surface-associated dye was dissolved in 200 µl of 30% acetic acid for 10 min at RT. The OD_{590} of the CV eluate was measured using a microtiter plate reader (SPARK 10M TECAN). For quantification of biofilm disruption in ASM, bacteria were inoculated to *ca.* 5 x 10^5 CFU/ml in 200 µl of ASM, incubated at 37 °C for 24 h to allow biofilm formation, and then challenged with ASM supplemented with 32 µM of each Ga(III) compound. Biofilm disruption was followed by CV microtiter plate assay for 72 h.

3. Results

3.1. Defining suitable test conditions for Ga(III)-based compounds

For a comparative assessment of the antibacterial effect of the three Ga(III) compounds, a collection of CF pathogens was used, including reference strains and clinical isolates (Table S1). Since Ga(III) is an Fe(III)-mimetic compound acting as a metabolic competitor of Fe(III), its antibacterial activity depends on the iron concentration of the test medium, being enhanced under conditions of iron scarcity (Minandri *et al.,* 2014). Therefore, in addition to the standard iron-rich medium MHB, recommended for antibacterial-susceptibility testing under strandard conditions (CLSI, 2015), the related iron-depleted medium DMHB (Hijazi *et al.,* 2018) was also used. In order to verify whether MHB and DMHB allow the growth of all CF pathogens included in this study, the reference strain of each species was cultured in both media. Fastidious microorganisms (*i.e., S. pneumoniae* and *H. influenzae*) were not included at this stage, as they have peculiar nutritional requirements, which prevent their growth in standard laboratory media, such as MHB (CLSI, 2015). All non-fastidious reference strains grew well in both MHB and DMHB, albeit an evident growth reduction was observed in DMHB compared with MHB, probably due to the limiting iron content (Fig. S1). According to the CLSI guidelines, susceptibility assays on fastidious microorganisms must be performed in HTM and MHB with 5% lysed horse blood for *Haemophilus* and *Streptococcus* species, respectively. Unfortunately, both HTM and MHB with 5% lysed horse blood media contain significant amount of iron, due to the presence of heme and blood, respectively, which could mask the antibacterial activity of Ga(III) compounds, especially GaPPIX (Stojiljkovic *et al.,* 1999; Hijazi *et al.,* 2017). However, the presence of heme is essential for *H. influenzae* growth, since this species is unable to synthesise the heme-precursor iron-free tetrapyrole PPIX (White and Granick, 1963). Therefore, to control iron supply, PPIX was used, in place of heme, so to ensure growth of *H. influenzae*. For these purposes, the growth of *H. influenzae* reference strain ATCC 49247 was tested in HTM (Fig. S2a), and DHTM (Fig. S2b), in the presence of increasing concentrations of PPIX, ranging from the CLSI-recommended heme concentration (*i.e.*, 23 μM) to 0.005 μM. Interestingly,

the presence of PPIX allows *H. influenzae* growth, both in HTM and in DHTM, in a dose-dependent manner (up to 0.032 μM and 0.063 μM in HTM and DHTM, respectively) (Fig. S2a, b). Then, *H. influenzae* growth yields reached a plateau at PPIX concentrations ≥ 0.063 μM and ≥ 0.125 μM in HTM and DHTM, respectively (Figure S2a, b), and no further growth yield increase was observed for concentrations of PPIX up to 23 μM (data not shown). Given these results, Ga(III)-susceptibility tests on *H. influenzae* were conducted in HTM and DHTM in the presence of 0.063 and 0.125 μM PPIX, respectively.

Similarly, the use of MHB with blood addition, as recommended by CLSI, was unsuitable to test the antibacterial activity of Ga(III) compounds on *S. pneumoniae*, due to the high iron content. Thus, THYB was used as an alternative medium to determine the MIC of antimicrobial compounds in *S. pneumoniae* (Marshall *et al.,* 1993), and its iron-depleted version (DTHYB; Montañez, *et al.,* 2005). *S. pneumoniae* ATCC 33400 was able to grow in both iron-rich and iron-poor media, although slightly less in DTHYB compared to THYB (Fig. S2c), likely due to iron scarcity. Though the media above are recommended to determine the MIC of antimicrobial compounds (CLSI, 2015), they do not mimic the *in vivo* conditions encountered by CF pathogens during lung infections. For this purpose, the ASM medium (Kirchner et *al.,* 2012) was also included in this study, as its composition better reflects the thick and stagnant airways mucus of CF patients. Almost all CF pathogens included in this study, except *S. pneumoniae* and *H. influenzae*, were able to grow in ASM, though with different growth rates (Fig. S3).

Although ASM ideally represents the best medium to mimic the growth of CF pathogens *in vivo*, the inability of *S. pneumoniae* and *H. influenzae* to grow in such medium prompted us to evaluate the possibility of using another cultural condition able to mimic the host biological fluids. We have recently demonstrated that RPMI-HS is a suitable medium to determine the MIC of Ga(III)-based compounds, due to the low iron content and the presence of HS, which simulates the *in vivo* environment (Hijazi *et al.,* 2018). Thus, the ability of CF pathogens to grow was initially evaluated in RPMI-HS (Hijazi *et al.,* 2018; Fig. S4a). Unfortunately, only *B. dolosa* LMG 18943, *B.*

multivorans LMG 31010, *P. aeruginosa* PAO1 and *S. aureus* ATCC 25923 (4/7 strains) grew well in RPMI-HS, while *A. xylosoxidans* ATCC 27061, *B. cenocepacia* LMG 16656 and *S. maltophilia* ATCC 13637, as well as fastidious microorganisms (*H. influenzae* ATCC 49247 and *S. pneumoniae* ATCC 33400) did not (Fig. S4). We then supplemented RPMI-HS with 0.5% CAA, as additional carbon source, due to its very low iron content (21 nM; Cunrath *et al.,* 2016), to promote bacterial growth. Indeed, the growth of all non-fastidious microorganisms was increased compared to RPMI-HS, except in *A. xylosoxidans* ATCC 27061 (Fig. S4a), while the addition of CAA to RPMI-HS did not support the growth of fastidious microorganisms (Fig. S4b). Finally, to allow the growth of fastidious microorganisms, RPMI-HS + CAA was supplemented with (3.3 μg/ml of hypoxanthine, 100 μg/ml of L-alanine, 55 μg/ml of L-cysteine hydrochloride, 6.6 μg/ml NAD, and 8 μg/ml uracil (Hasan *et al.,* 1997), which determined an evident growth increase of these strains (Fig. S4b). Therefore, RPMI-HS + CAA with or without supplements, were chosen for MIC determinations of Ga(III)-based compounds in fastidious and non-fastidious microorganisms, respectively.

3.2. Susceptibility of CF pathogens to Ga(III) compounds

Once established the suitable growth conditions, the MIC of Ga(III) compounds was determined for CF pathogens. Since the peak serum concentration of Ga(III) achievable during i.v. human therapy is *ca.* 30 μM (Bernstein, 1998; Collery *et al.,* 2002), we arbitrarily assumed the resistance breakpoint at MIC > 32 μM. In rich media (MHB, HTM and THYB), all CF pathogens were resistant to all Ga(III) compounds tested, except *S. aureus* and *H. influenzae* which were extremely susceptible to GaPPIX only (MIC = 0.06/0.012 μM for *S. aureus* and MIC ≤ 0.0075 μM for all *H. influenzae* strains*,* Table1). Moreover, no differences in MIC values for *S. aureus* and *H. influenzae* were observed between MHB/DMHB and HTM/DHTM, respectively, in spite of their different iron content, and thus of the different iron starvation status of bacteria. This suggests a mechanism of action of GaPPIX that is not responsive to iron in these two bacterial species. On the other hand, *A. xylosoxidans* was resistant to GaPPIX in MHB, whereas it was susceptible in DMHB (MIC = 16/32

μM), suggesting that the iron content of the growth medium strongly influences the susceptibility to GaPPIX in this species (Table 1). Moreover, no effect of Ga(III)-based compounds was observed in iron-depleted media (DMHB, DHTM, and DTHYB), with the exception of *A. xylosoxidans* strains and *P. aeruginosa* TR1 which were susceptible only to GaPPIX and GaN, respectively (Table 1). This preliminary screening allowed the identification of GaPPIX as the most promising Ga(III)-based compound, being extremely active against *A. xyloxodidans, H. influenzae*, and *S. aureus* (Table 1).

Table 1
MICs of Ga(III) compounds (μM) for CF pathogens in standard and their related iron-depleted media

Bacterial strain	MHB/HTM[a*]/THYB[b]			DMHB/DHTM[a*]/DTHYB[b]		
	GaN	**GaM**	**GaPPIX**	**GaN**	**GaM**	**GaPPIX**
Achromobacter xylosoxidans[T] ATCC 27061	> 128	> 128	> 128	> 128	> 128	**32**
A. xylosoxidans CF-2	> 128	> 128	> 128	> 128	> 128	**32**
A. xylosoxidans CF-3	> 128	> 128	> 128	> 128	> 128	**32**
A. xylosoxidans CF-4	> 128	> 128	> 128	> 128	> 128	**16**
Burkholderia cenocepacia[T] LMG 16656	> 128	> 128	> 128	> 128	> 128	> 128
B. cenocepacia FFC 0076	> 128	> 128	> 128	> 128	> 128	> 128
Burkholderia dolosa[T] LMG 18943	> 128	> 128	> 128	> 128	> 128	> 128
B. dolosa FFC 0305	> 128	> 128	> 128	> 128	> 128	> 128
Burkholderia multivorans[T] LMG 31010	> 128	> 128	> 128	> 128	> 128	> 128
B. multivorans 454	> 128	> 128	> 128	> 128	> 128	> 128
Haemophilus influenzae ATCC 49247[a]	> 128	> 128	**≤ 0.0075**	> 128	> 128	**≤ 0.0075**
H. influenzae ATCC 9833[a]	> 128	> 128	**≤ 0.0075**	64	> 128	**≤ 0.0075**
H. influenzae FC 89[a]	> 128	> 128	**≤ 0.0075**	> 128	> 128	**≤ 0.0075**
H. influenzae FC 104[a]	> 128	> 128	**≤ 0.0075**	> 128	> 128	**≤ 0.0075**
Pseudomonas aeruginosa PAO1 (ATCC 15692)	> 128	> 128	> 128	64	> 128	> 128
P. aeruginosa TR1	128	> 128	> 128	**32**	> 128	> 128
P. aeruginosa FM12	> 128	> 128	> 128	64	> 128	> 128
P. aeruginosa FM13	> 128	> 128	> 128	64	> 128	> 128
Staphylococcus aureus ATCC 25923	> 128	> 128	**0.12**	> 128	> 128	**0.06**
S. aureus ATCC 43300	> 128	> 128	**0.06**	> 128	> 128	**0.06**
S. aureus BG-1	> 128	> 128	**0.06**	> 128	> 128	**0.06**
S. aureus BG-6	> 128	> 128	**0.06**	> 128	> 128	**0.03**
Stenotrophomonas maltophilia[T] ATCC 13637	> 128	> 128	128	> 128	> 128	64
S. maltophilia K279a	> 128	> 128	> 128	> 128	> 128	128
S. maltophilia OBGTC23	> 128	> 128	> 128	> 128	> 128	64
S. maltophilia OBGTC26	> 128	> 128	> 128	> 128	> 128	64
Streptococcus pneumoniae[T] ATCC 33400 [b]	> 128	> 128	> 128	> 128	> 128	128
S. pneumoniae PFC-01[b]	> 128	> 128	128	> 128	> 128	64
S. pneumoniae PFC-02[b]	> 128	> 128	128	> 128	> 128	64
S. pneumoniae PFC-04[b]	> 128	> 128	64	> 128	> 128	64

Abbreviations: [T], type strain. Notes: [a], only in the case of *H. influenzae*, HTM/DHTM were used; [b], only in the case of *S. pneumoniae,* THYB/DHTYB were used; *, HTM and DHTM were supplemented with 0.063 μM and 0.125 μM PPIX, respectively, to allow *H. influenzae* growth. Arbitrarily assuming the resistance breakpoint at MIC > 32 μM, the MIC values for susceptible isolates are shown in bold type.

This result was confirmed by using the ASM medium, where GaPPIX displayed an even broader antimicrobial activity, also against 2/6 *Bcc*, *2*/4 *P. aeruginosa,* and 4/4 *S. maltophilia* strains. Conversely, no MIC was determined using GaN and GaM in case of *A. xylosoxidans*, *Bcc*, and *S. maltophilia* (Table 2). Interestingly, both GaN and GaM were very effective in inhibiting *P. aeruginosa* in ASM, and even more in RPMI-HS + CAA (Table 2), thus confirming the suitability of RPMI-HS + CAA to evaluate the antimicrobial activity of Ga(III)-based compounds. Indeed, using RPMI-HS + CAA a Ga(III)-sensitive response was observed also for *Bcc* and *S. maltophilia*, which became sensitive to both GaN and GaM (Table 2). Of note, differently from *A. xylosoxidans* ATCC 27061, 2/3 clinical isolates were able to grow in RPMI-HS + CAA and were sensitive to GaN and GaM (Table 2). However, the MIC of GaPPIX varied among susceptible strains grown in RPMI-HS + CAA compared with ASM, being higher in *A. xylosoxidans, Bcc*, *P. aeruginosa*, and lower in *S. maltophilia* (Table 2). *S. aureus* was completely resistant to GaPPIX in RPMI-HS + CAA, confirming the interference by compounds that bind GaPPIX and counteract its antibacterial activity (Hijazi *et al.,* 2018).

Unfortunately, none of the Ga(III) compounds included in this comparative study inhibited *S. pneumoniae* (Table 1 and Table 2).

Table 2
MICs of Ga(III) compounds (μM) for CF pathogens in biological fluids-mimetic media

Bacterial strain	**ASM**			**RPMI-HS + CAA***		
	GaN	**GaM**	**GaPPIX**	**GaN**	**GaM**	**GaPPIX**
Achromobacter xylosoxidans^{T} ATCC 27061	> 128	> 128	**≤ 0.0075**	ND	ND	ND
A. xylosoxidans CF-2	> 128	> 128	**0.12**	ND	ND	ND
A. xylosoxidans CF-3	> 128	> 128	**0.12**	**32**	**16**	64
A. xylosoxidans CF-4	> 128	> 128	**≤ 0.0075**	**8**	**4**	**2**
Burkholderia cenocepacia^{T} LMG 16656	> 128	> 128	**32**	**1**	**1**	**32**
B. cenocepacia FFC 0076	> 128	> 128	**4**	**2**	**2**	64
Burkholderia dolosa^{T} LMG 18943	> 128	> 128	> 128	**2**	**2**	128
B. dolosa FFC 0305	> 128	> 128	> 128	**4**	**4**	128
Burkholderia multivorans^{T} LMG 31010	> 128	> 128	> 128	**4**	**2**	128
B. multivorans 454	> 128	> 128	> 128	**4**	**2**	128
Haemophilus influenzae ATCC 49247	ND	ND	ND	> 128	> 128	**0.5**
H. influenzae ATCC 9833	ND	ND	ND	> 128	> 128	**0.5**
H. influenzae FC 89	ND	ND	ND	> 128	> 128	**2**
H. influenzae FC 104	ND	ND	ND	> 128	> 128	**2**
Pseudomonas aeruginosa PAO1 (ATCC 15692)	**16**	**8**	128	**8**	**8**	> 128
P. aeruginosa TR1	**16**	**16**	**0.015**	**2**	**2**	**4**
P. aeruginosa FM12	**16**	**16**	> 128	**16**	**8**	> 128
P. aeruginosa FM13	**16**	**16**	**16**	**8**	**4**	64
Staphylococcus aureus ATCC 25923	> 128	> 128	**2**	> 128	> 128	> 128
S. aureus ATCC 43300	> 128	> 128	**2**	> 128	128	> 128
S. aureus BG-1	> 128	> 128	**1**	> 128	128	64
S. aureus BG-6	> 128	> 128	**0.25**	> 128	64	128
Stenotrophomonas maltophilia^{T} ATCC 13637	> 128	> 128	**8**	**8**	**8**	**0.25**
S. maltophilia K279a	> 128	> 128	**4**	**4**	**4**	**0.06**
S. maltophilia OBGTC23	> 128	> 128	**0.03**	**0.25**	**0.25**	**0.03**
S. maltophilia OBGTC26	128	> 128	**0.03**	**4**	**4**	**0.12**
Streptococcus pneumoniae^{T} ATCC 33400	ND	ND	ND	> 128	128	> 128
S. pneumoniae PFC-01	ND	ND	ND	> 128	64	64
S. pneumoniae PFC-02	ND	ND	ND	> 128	64	> 128
S. pneumoniae PFC-04	ND	ND	ND	> 128	64	64

Abbreviations: ^{T}, type strain. ND, not determined due to the poor growth. *, only in the case of *H. influenzae and S. pneumoniae*, RPMI-HS + CAA was supplemented with 3.3 μg/ml of hypoxanthine, 100 μg/ml of L-alanine, 55 μg/ml of L-cysteine hydrochloride, 6.6 μg/ml of NAD, and 8 μg/ml uracil, to allow bacterial growth. Arbitrarily assuming the resistance breakpoint at MIC > 32 μM, the MIC values for susceptible isolates are shown in bold type.

3.3. Anti-bacterial activity of Ga(III) compounds on a collection of P. aeruginosa CF isolates

P. aeruginosa represents the main CF-associated pathogen, affecting most if not all adults with CF (Cystic Fibrosis Foundation Patient Registry, 2016), and causing persistent infections characterised with a rapid decline in lung function and survival (Moore and Mastoridis, 2017). These features prompted us to widen our investigations on the effect of Ga(III) compounds on a representative panel of *P. aeruginosa* clinical isolates from CF patients (Table S2). Thus, the antibacterial activity of GaN, GaM and GaPPIX was assessed in ASM and RPMI-HS + CAA growth media. All *P. aeruginosa* CF clinical isolates were susceptible to GaN and GaM in both media. The effect of GaN and GaM was even more pronounced in RPMI-HS + CAA (1 μM ≤ MIC ≤ 16 μM for GaN, and 1 μM ≤ MIC ≤ 8 μM for GaM) compared with ASM (Table 3). Conversely, GaPPIX was active against two *P. aeruginosa* isolates (KK71 and TR66) in ASM (MIC = 0.03 μM – 32 μM) and in RPMI-HS + CAA (MIC = 8 μM). In particular, the strain *P. aeruginosa* KK71 was extremely susceptible to GaPPIX in ASM (MIC = 0.03 μM) (Table 3). Intriguingly, the *P. aeruginosa* isolates AA43 was resistant in ASM and became susceptible to GaPPIX in RPMI-HS + CAA (MIC = 8 μM) (Table 3), indicating that some factors present in RPMI-HS + CAA may influence the susceptibility of clinical isolates to GaPPIX.

Table 3
MICs of Ga(III) compounds (μM) for CF *P. aeruginosa* clinical isolates

Bacterial strain	**ASM**			**RPMI-HS + CAA**		
	GaN	**GaM**	**GaPPIX**	**GaN**	**GaM**	**GaPPIX**
AA2	**16**	**32**	> 128	**8**	**8**	128
AA43	**16**	**32**	> 128	**2**	**2**	**8**
BT2	**16**	**32**	128	**8**	**8**	128
BT73	**16**	**32**	128	**8**	**4**	64
FM17	**16**	**32**	> 128	**16**	**8**	> 128
KK1	**16**	**16**	128	**8**	**4**	> 128
KK27	**16**	**8**	64	**8**	**4**	64
KK28	**16**	**8**	> 128	**8**	**4**	64
KK71	**16**	**8**	**0.03**	**2**	**1**	**8**
TR66	**16**	**8**	**32**	**1**	**1**	**8**

Arbitrarily assuming the resistance breakpoint at MIC > 32 μM, the MIC values for susceptible isolates are shown in bold type.

3.4. Activity of Ga(III) compounds on P. aeruginosa biofilm formation and disruption of preformed biofilm

Chronic lung infections in CF are associated with the biofilm mode of growth of *P. aeruginosa*, making the eradication of such infections extremely difficult. Therefore, we investigated whether Ga(III)-based compounds are able to impair *P. aeruginosa* biofilm formation. Firstly, the ability of selected *P. aeruginosa* strains (Table S1) to form biofilm was assessed in ASM for 24 h at 37 °C by the use of 96-well microtiter plate assay (O'Toole *et al.,* 2000). All *P. aeruginosa* strains were good biofilm producers, and biofilm formation by the clinical *P. aeruginosa* isolates TR1, FM12 and FM13 was higher than PAO1 (Fig. 1). Sub-inhibitory concentrations (1/2 MIC, Table 2) of each Ga(III)-based compound did not prevent biofilm growth by *P. aeruginosa* (Fig. 1).

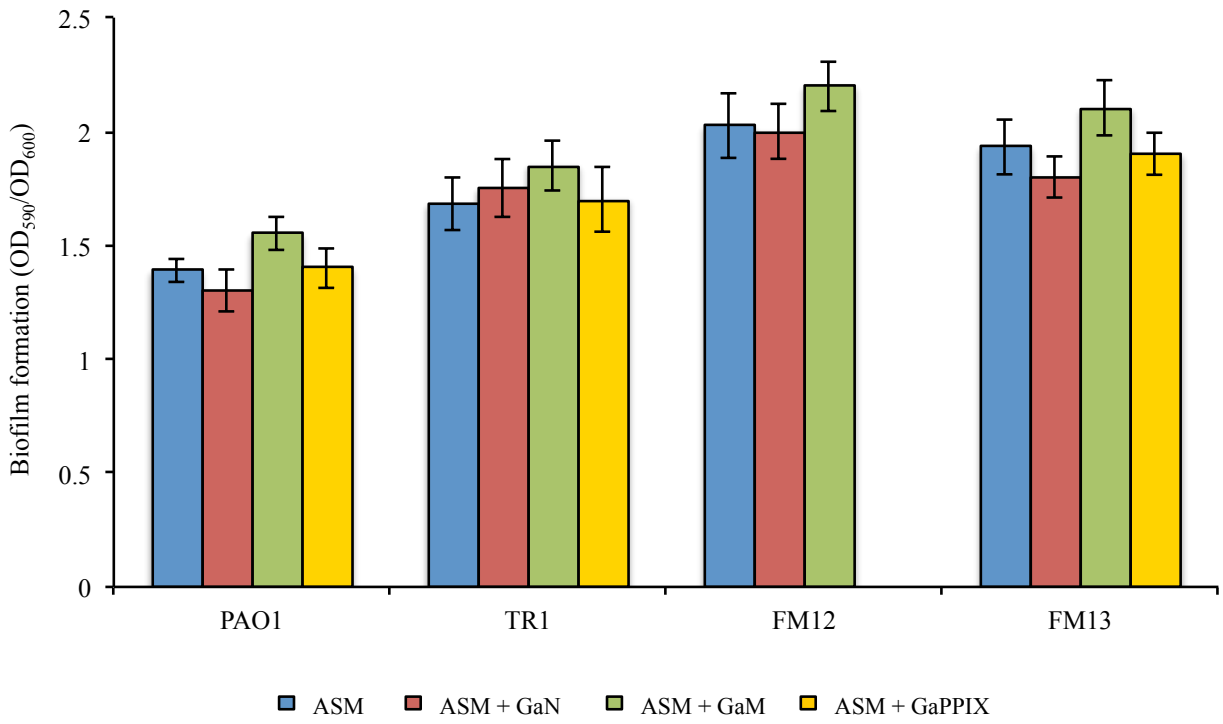


Fig. 1. Biofilm formation by different *P. aeruginosa* strains. Bacteria were grown in ASM for 24 h at 37 °C with or without GaN, GaM, or GaPPIX, supplemented at sub-inhibitory concentrations (1/2 MIC), when possible (*i.e.,* in susceptible strains). Biofilm formation (OD_{590}/OD_{600}) was quantified by crystal violet microtiter plate assay. Data are the means of two experiments in triplicate ± SD.

Whether Ga(III) compounds are able to disrupt preformed biofilm was then investigated. Preformed *P. aeruginosa* biofilms were exposed to GaN, GaM and GaPPIX at 32 μM, or fresh ASM medium as control, and biofilm was quantified at different times and expressed in percentage relative to the untreated ASM (Fig. 2). No difference in biofilm disruption was observed among treatments, at all time points considered (Fig. 2). Of note, GaN on *P. aeruginosa* PAO1 and GaPPIX on some clinical strains (TR1 and FM13) seemed to favour rather than disrupt preformed biofilms, although this phenomenon could be ascribed to experimental variation rather than Ga(III)-induced cellular stress (Fig. 2). Interestingly, biofilm challenge with either ASM alone or ASM supplemented with each Ga(III)-compound caused a reduction of preformed biofilm greater than 40% and 60% in all *P. aeruginosa* strains, after 48 h and 72 h, respectively (Fig. S5b, c), suggestive of a natural senescence-dependent biofilm dispersal mechanism, independent of Ga(III) treatment (Fig. S5).

Since Ga(III) has previously been shown to disrupt preformed *P. aeruginosa* biofilms in different media and experimental settings (Kaneko *et al.,* 2007; Banin *et al.,* 2008), further experiments are certainly required to clarify this issue.

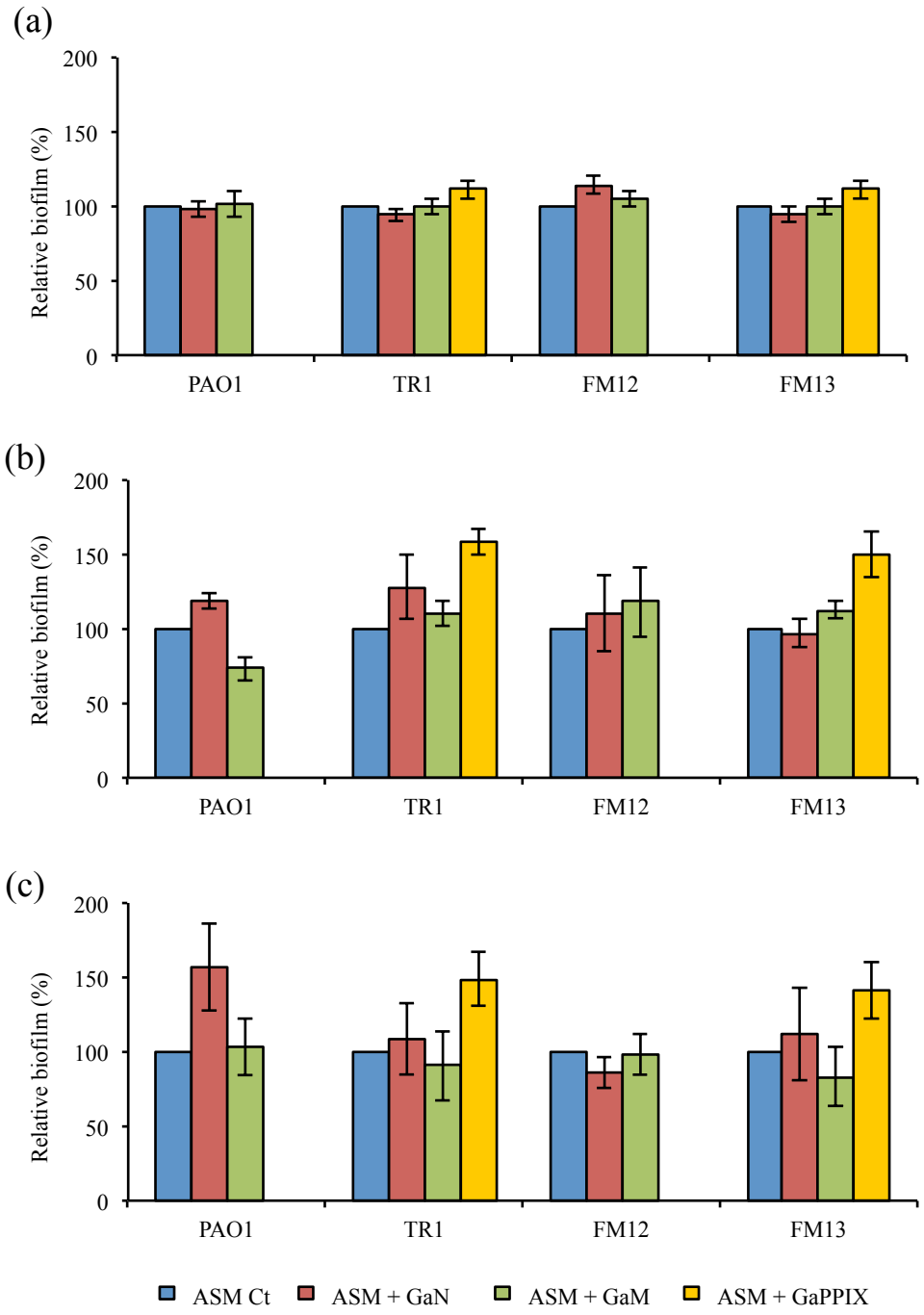


Fig. 2. Activity of Ga(III)-based compounds against *P. aeruginosa* preformed biofilm. Preformed 24 h *P. aeruginosa* biofilms were challenged with GaN, GaM and GaPPIX at 32 µM, or fresh ASM medium as control. Biofilm disruption was quantified by crystal violet microtiter plate assay after 24 h (a) 48 h (b) and 72 h (c) of incubation at 37 °C. The values are expressed as the percentage relative to the untreated ASM, and represent the means of two experiments in triplicate ± SD.

4. Discussion

Antibiotic resistance poses a significant challenge to the treatment of chronic lung infections in CF patients (Rutter et *al.,* 2017). Therefore, the discovery of novel strategies to combat infections caused by CF pathogens has become a matter of vital importance. Iron acquisition is crucial for almost all pathogenic bacteria, including those infecting CF airways, and it is implicated in pathogenicity (Tyrell and Callaghan, 2016). Recently, interest in the use of the post-transition metal Ga(III) as a novel approach to combat MDR bacterial pathogens has increased considerably, and different Ga(III)-based drugs showed promising results (Minandri *et al.,* 2014; Hijazi *et al.,* 2018). This because Ga(III) inhibits essential iron-containing bacterial enzymes, such as those involved in DNA synthesis, and oxidant defense, thereby taking the place of iron (Goss *et al.,* 2018).

Since *P. aeruginosa* represents the most dreaded pathogen in CF, several pre-clinical and clinical studies have been focused on the anti-*Pseudomonas* activity of different Ga(III) formulations (Goss *et al.,* 2012, 2018). However, *P. aeruginosa* is not the only bacterium colonizing the airways of CF patients. Indeed, colonisation by bacterial pathogens starts with *S. aureus* and *H. influenzae*, and then becomes more complex when non-fermenting Gram-negative bacteria such as *S. maltophilia*, *Bcc* species and *A. xylosoxidans* multiply in CF airways (Rajan *et al.,* 2002). Therefore, the lack of systematic study on the activity of different Ga(III) compounds against CF species represents a pitfall to the development of Ga(III)-based compounds as broad-spectrum antibacterial agents in CF. In the present work, we comparatively investigated the activity of three different Ga(III) formulations in different media, using a collection of CF-associated bacterial species, including but not limited to, *P. aeruginosa*. Combining data obtained in all media tested, GaPPIX had the broadest activity among the Ga(III)-based compounds tested, being able to inhibit the growth of bacteria belonging to all genera tested, except *S. pneumoniae* (Table 1 and Table 2). In particular, *H. influenzae* and *S. aureus*, resulted extremely susceptible to GaPPIX in standard media (*i.e.,* HTM/DHTM and MHB/DMHB, respectively) and in ASM, while *S. aureus* became completely resistant to GaPPIX in RPMI-HS + CAA, in line with previous results (Hijazi *et al.,* 2018). The

reduced antimicrobial effect of GaPPIX in RPMI-HS + CAA could be ascribed to the presence of human serum albumin (HSA), the most abundant plasma protein, which binds GaPPIX, thus neutralizing its inhibitory effect (Hijazi *et al.,* 2018). This phenomenon may also explain the loss or reduction of GaPPIX activity in RPMI-HS + CAA compared with ASM, on some strains belonging to *A. xylosoxidans* (CF-3 and CF-4), *B. cenocepacia* (FFC 0076), *P. aeruginosa* (TR1 and FM13) and *S. malthophilia* (OBGTC26). However, in the case of *S. malthophilia* (ATCC 13637, K279a), GaPPIX was more active in RPMI-HS + CAA than in ASM (Table 2), suggesting that others factors, besides the presence of HSA, may influence GaPPIX susceptibility, at least in this species. It is well known that GaPPIX exploits heme uptake systems to enter bacterial cells (Stojiljkovic *et al.,* 1999; Hijazi *et al.,* 2017), therefore the number, type and level of expression of heme-utilization systems could account for the variable susceptibility to GaPPIX (Hijazi *et al.,* 2017, 2018).

Contrary to what observed for GaPPIX, the acivity of GaN and GaM was comparable among strains belonging to the same species (Table 2). In particular, GaN and GaM were able to inhibit the growth of all *A. xylosoxidans*, *P. aeruginosa*, *S. maltophilia* and *Bcc* species in RPMI-HS + CAA. However, GaN and GaM lost their antibacterial activity against *A. xylosoxidans*, *S. maltophilia* and *Bcc* in ASM. Since Ga(III) mainly exploits iron-uptake systems to enter bacterial cells, the different susceptibility observed in RPMI-HS + CAA compared with ASM suggest a higher iron bioavailability in the latter medium, which induces a low expression of iron-uptake systems, hence limiting the entrance of GaN and GaM in bacterial cells (Wandersman and Delepelaire, 2004). Although ASM is considered a suitable medium to investigate the antibacterial proprieties of drugs against CF pathogens (Wright *et al.,* 2013; Schneider-Futschik *et al.,* 2018), the use of this medium to test inhibitors of bacterial iron metabolism is questionable.

P. aeruginosa deserves a special comment: planktonic growth of all clinical isolates was inhibited by GaN and GaM in both ASM and RPMI-HS + CAA, confirming the efficacy of Ga(III)-based compounds against *P. aeruginosa* clinical isolates (Kaneko *et al.,* 2007; Goss *et al.,* 2018), and supporting a future development as antimicrobials to treat *P. aeruginosa* infection in CF patients.

Indeed, Phase 1 clinical trial on CF patients based on intravenous GaN administration (ClinicalTrial.gov, NCT01093521) has recently shown that Ga(III) improves lung function in people with CF and *P. aeruginosa* chronic lung infection (Goss *et al.,* 2018). Moreover, phase 2 clinical trial is ongoing, to provide further information about safety and clinical efficacy in adults CF patients chronically infected with *P. aeruginosa* (www.clinicaltrial.gov; NCT02354859). However, since Ganite® is no longer available, more convenient Ga(III) formulations and routes of administration, could be envisaged.

This study adds to the body of literature supporting the future development of Ga(III)-based compounds to treat bacterial infections. In particular, the promising results obtained with different Ga(III) compounds against CF pathogens, strongly support the development of suitable Ga(III) formulations, alone or in combination with antibiotics (Goss *et al.,* 2018), for future applications in CF individuals. Ga(III)-based compounds could be suitable for the setting of patient-specific therapies based on the infection phase, hence the prominent bacterial pathogen present in the CF lung. For instance, GaPPIX-based compounds might be promising in childhood to eradicate *H. influenzae* and *S. aureus* infections, which cause airways lesions, and subsequently promote *P. aeruginosa* colonization (Cigana *et al.,* 2018). Conversely, later in adulthood, when CF airways are colonized by other pathogens, including *P. aeruginosa*, Ga(III) formulations, such as GaN and GaM, are more advisable.

Conflict of Interest statement

Dr Lawrence Bernstein holds several patents for possible applications of GaM in human and veterinary medicine and is affiliated with a company (Gallixa LLC) that would like to obtain regulatory approval for topical gallium maltolate as a therapeutic agent. Dr. Bernstein did not participate in data collection for this study.

Acknowledgements

We wish to thank Prof. Mariassunta Casalino (Department of Science, University of Roma Tre, Italy) for providing *S. maltophilia* strains, Dr. Annalisa Pantosti (Department of Infectious, Parasitic and Immuno-mediated Diseases, Istituto Superiore di Sanità, Rome, Italy) for providing *S. pneumoniae* strains, Dr. Silvia Buroni (Department of Biology and Biotechnology, University of Pavia, Pavia, Italy) for providing *Burkholderia cepacia* complex strains, Dr. Marina Cerquetti (Department of Infectious Diseases, Istituto Superiore di Sanità, Rome, Italy) for providing *H. influenzae* strains, Dr. Serena Schippa (Pediatrics Department, Regional Cystic Fibrosis Center, Sapienza University Rome, Italy) for providing *A. xylosoxidans* strains and Dr. Ersinia Fiscarelli (Department of Experimental & Clinical Sciences, G. d'Annunzio University of Chieti-Pescara, Chieti, Italy) for providing *S. aureus* strains. We are also grateful to the personnel of Policlinico Umberto I, Sapienza University of Rome, for collecting blood samples from healthy donors.

This work was supported by grants from the Italian Cystic Fibrosis Research Foundation (grants FFC#21/2015 and FFC#18/2017).

References

[1] Filkins LM, O'Toole GA. Cystic Fibrosis Lung Infections: Polymicrobial, Complex, and Hard to Treat. PLoS Pathog 2015;11:e1005258.

[2] Parkins MD, Floto RA. Emerging bacterial pathogens and changing concepts of bacterial pathogenesis in cystic fibrosis. J Cyst Fibros 2015;14:293–304.

[3] López-Causapé C, Rojo-Molinero E, Mulet X, Cabot G, Moyà B, Figuerola J, et al. Clonal dissemination, emergence of mutator lineages and antibiotic resistance evolution in *Pseudomonas aeruginosa* cystic fibrosis chronic lung infection. PLoS One 2013;8:e71001.

[4] López-Causapé C, Rojo-Molinero E, Macià MD, Oliver A. The problems of antibiotic resistance in cystic fibrosis and solutions. Expert Rev Respir Med 2015;9:73–88.

[5] Lechtzin N, John M, Irizarry R, Merlo C, Diette GB, Boyle MP. Outcomes of adults with cystic fibrosis infected with antibiotic-resistant *Pseudomonas aeruginosa*. Respiration 2006;73:27–33.

[6] Nguyen D, Singh PK. Evolving stealth: genetic adaptation of *Pseudomonas aeruginosa* during cystic fibrosis infections. Proc Natl Acad Sci U S A 2006;103:8305–6.

[7] Sass A, Marchbank A, Tullis E, Lipuma JJ, Mahenthiralingam E. Spontaneous and evolutionary changes in the antibiotic resistance of *Burkholderia cenocepacia* observed by global gene expression analysis. BMC Genomics 2011;12:373.

[8] Stewart PS, Costerton JW. Antibiotic resistance of bacteria in biofilms. Lancet 2001;358:135–38.

[9] Moore JE, Mastoridis P. Clinical implications of *Pseudomonas aeruginosa* location in the lungs of patients with cystic fibrosis. J Clin Pharm Ther 2017;42:259–67.

[10] Bonchi C, Imperi F, Minandri F, Visca P, Frangipani E. Repurposing of gallium-based drugs for antibacterial therapy. Biofactors 2014;40:303–12.

[11] Minandri F, Bonchi C, Frangipani E, Imperi F, Visca P. Promises and failures of gallium as an antibacterial agent. Future Microbiol 2014;9:379–97.

[12] Rangel-Vega A, Bernstein LR, Mandujano-Tinoco EA, García-Contreras SJ, García-Contreras R. Drug repurposing as an alternative for the treatment of recalcitrant bacterial infections. Front Microbiol 2015;6:282.

[13] Runci F, Bonchi C, Frangipani E, Visaggio D, Visca P. *Acinetobacter baumannii* Biofilm Formation in Human Serum and Disruption by Gallium. Antimicrob Agents Chemother 2016;61.pii: e01563–16.

[14] Bernstein LR, Tanner T, Godfrey C, Noll B. Chemistry and pharmacokinetics of gallium maltolate, a compound with high oral gallium bioavailability. Met Based Drugs 2000;7:33–47.

[15] Chitambar CR. Medical applications and toxicities of gallium compounds. Int J Environ Res Public Health 2010;7:2337–61.

[16] Hijazi S, Visaggio D, Pirolo M, Frangipani E, Bernstein L, Visca P. Antimicrobial activity of gallium compounds on ESKAPE pathogens. Front Cell Infect Microbiol 2018;8:316.

[17] Kirchner S, Fothergill JL, Wright EA, James CE, Mowat E, Winstanley C. Use of artificial sputum medium to test antibiotic efficacy against *Pseudomonas aeruginosa* in conditions more relevant to the cystic fibrosis lung. J Vis Exp 2012;5:e3857.

[18] Stoltz DA, Meyerholz DK, Welsh MJ. Origins of cystic fibrosis lung disease. N Engl J Med 2015;37:351–62.

[19] Govan JR, Deretic V. Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Microbiol Rev 1996;60:539–74.

[20] Rogers GB, Hart CA, Mason JR, Hughes M, Walshaw MJ, Bruce KD. Bacterial diversity in cases of lung infection in cystic fibrosis patients: 16S ribosomal DNA (rDNA) length heterogeneity PCR and 16S rDNA terminal restriction fragment length polymorphism profiling. J Clin Microbiol 2003;41:3548–58.

[21] van der Gast CJ, Walker AW, Stressmann FA, Rogers GB, Scott P, Daniels TW, et al. Partitioning core and satellite taxa from within cystic fibrosis lung bacterial communities. ISME J 2011;5:780–91.

[22] Folkesson A, Jelsbak L, Yang L, Johansen HK, Ciofu O, Høiby N, et al. Adaptation of *Pseudomonas aeruginosa* to the cystic fibrosis airway: an evolutionary perspective. Nat Rev Microbiol 2012;10:841–51.

[23] Cystic Fibrosis Foundation. Patient registry 2016 annual data report. Cystic Fibrosis Foundation, Bethesda, MD. https://www.cff.org/Research/Researcher-Resources/Patient-Registry/2016-Patient-Registry-Annual-Data-Report.pdf; 2016.

[24] Sherrard LJ, Tunney MM, Elborn JS. Antimicrobial resistance in the respiratory microbiota of people with cystic fibrosis. Lancet 2014;384:703–13.

[25] Andrews SC, Robinson AK, Rodríguez-Quiñones F. Bacterial iron homeostasis. FEMS Microbiol Rev 2003;27:215–37.

[26] Reid DW, Anderson GJ, Lamont IL. Role of lung iron in determining the bacterial and host struggle in cystic fibrosis. Am J Physiol Lung Cell Mol Physiol 2009;297:795–802.

[27] Ballouche M, Cornelis P, Baysse C. Iron metabolism: a promising target for antibacterial strategies. Recent Pat Antiinfect Drug Discov 2009;4:190–205.

[28] Foley TL, Simeonov A. Targeting iron assimilation to develop new antibacterials. Expert Opin Drug Discov 2012;7:831–47.

[29] Bernstein LR. Mechanisms of therapeutic activity for gallium. Pharmacol Rev. 1998;50:665–82.

[30] Collery P, Keppler B, Madoulet C, Desoize B. Gallium in cancer treatment. Crit Rev Oncol Hematol 2002;42:283–96.

[31] Chitambar CR. The therapeutic potential of iron-targeting gallium compounds in human disease: From basic research to clinical application. Pharmacol Res 2017;115:56–64.

[32] Goss CH, Kaneko Y, Khuu L, Anderson GD, Ravishankar S, Aitken ML, et al. Gallium disrupts bacterial iron metabolism and has therapeutic effects in mice and humans with lung infections. Sci Transl Med 2018;10. pii: eaat7520.

[33] Goss CH, Hornick DB, Aitken ML, Anderson G, Caldwell E. Phase 1 pharmacokinetic and safety study of intravenous Ganite™ (Gallium nitrate) in CF. Pediatric Pulmunol 2012; 47,303.

[34] Kaneko Y, Thoendel M, Olakanmi O, Britigan BE, Singh PK. The transition metal gallium disrupts *P. aeruginosa* iron metabolism and has antimicrobial and antibiofilm activity. J Clin Invest 2007;117:877–88.

[35] Bonchi C, Frangipani E, Imperi F, Visca, P. Pyoverdine and proteases affect the response of *Pseudomonas aeruginosa* to gallium in human serum. Antimicrob. Agents Chemother 2015; 59:5641–46.

[36] Hijazi S, Visca P, Frangipani E. Gallium-Protoporphyrin IX Inhibits *Pseudomonas aeruginosa* Growth by Targeting Cytochromes. Front Cell Infect Microbiol 2017;7:12.

[37] Marshall KJ, Musher DM, Watson D, Mason EO. Jr. Testing of *Streptococcus pneumoniae* for resistance to penicillin. J. Clin. Microbiol 1993;31,1246–50.

[38] Montañez GE, Neely MN, Eichenbaum Z. The streptococcal iron uptake (Siu) transporter is required for iron uptake and virulence in a zebrafish infection model. Microbiology 2005;151:3749–57.

[39] Sriramulu DD, Lünsdorf H, Lam JS, Römling U. Microcolony formation: a novel biofilm model of *Pseudomonas aeruginosa* for the cystic fibrosis lung. J Med Microbiol 2005;54:667–76.

[40] Clinical and Laboratory Standards Institute. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically. 10th edn. Wayne, PA: CLSI (Approved standard M07–A10), 2015.

[41] Stojiljkovic I, Kumar V, Srinivasan N. Non-iron metalloporphyrins: potent antibacterial compounds that exploit haem/Hb uptake systems of pathogenic bacteria. Mol Microbiol 1999; 31:429–42.

[42] White DC, Granick S. Hemin biosynthesis in *Haemophilus*. J Bacteriol 1963;85:842–50.

[43] Cunrath O, Geoffroy VA, Schalk IJ. Metallome of *Pseudomonas aeruginosa*: a role for siderophores. Environ Microbiol 2016;18:3258–67.

[44] O'Toole G, Kaplan HB, Kolter R. Biofilm formation as microbial development. Annu Rev Microbiol 2000;54:49 –79.

[45] Hasan AA, Holland J, Smith A, Williams P. Elemental iron does represstransferrin, haemopexin and haemoglobin receptor expression in *Haemophilus influenzae*. FEMS Microbiol Lett 1997;150:19–26.

[46] Banin E, Lozinski A, Brady KM, Berenshtein E, Butterfield PW, Moshe M, Chevion M, Greenberg EP, Banin E. The potential of desferrioxamine-gallium as an anti-*Pseudomonas* therapeutic agent. Proc Natl Acad Sci U S A 2008;105:16761–6.

[47] Rutter WC, Burgess DR, Burgess DS. Increasing Incidence of Multidrug Resistance Among Cystic Fibrosis Respiratory Bacterial Isolates. Microb Drug Resist 2017;23:51–5.

[48] Tyrrell J, Callaghan M. Iron acquisition in the cystic fibrosis lung and potential for novel therapeutic strategies. Microbiology 2016;162:191–205.

[49] Rajan S, Saiman L. Pulmonary infections in patients with cystic fibrosis. Semin Respir Infect 2002;17:47–56.

[50] Wandersman C, Delepelaire P. Bacterial iron sources: from siderophores to hemophores. Annu Rev Microbiol 2004;58:611–47.

[51] Wright EA, Fothergill JL, Paterson S, Brockhurst MA, Winstanley C. Sub-inhibitory concentrations of some antibiotics can drive diversification of *Pseudomonas aeruginosa* populations in artificial sputum medium. BMC Microbiol 2013;13:170.

[52] Schneider-Futschik EK, Paulin OKA, Hoyer D, Roberts KD, Ziogas J, Baker MA, et al. Sputum Active Polymyxin Lipopeptides: Activity against Cystic Fibrosis *Pseudomonas aeruginosa* Isolates and Their Interactions with Sputum Biomolecules. ACS Infect Dis 2018;4:646–55.

[53] Cigana C, Bianconi I, Baldan R, De Simone M, Riva C, Sipione B, et al. *Staphylococcus aureus* Impacts *Pseudomonas aeruginosa* Chronic Respiratory Disease in Murine Models. J Infect Dis 2018;217:933–42.

Supplementary material

Supplementary Table S1
List of bacterial strains used in this study

Bacterial species	Strain	Country	Year	Source	Resistance	Reference
Achromobacter xylosoxidans^{T}	ATCC 27061	Japan	ns	ear discharge	ns	[1]
A. xylosoxidans	CF-2	Italy	2008-2010	respiratory secretion of CF patient	MDR	[2]
A. xylosoxidans	CF-3	Italy	2008-2010	respiratory secretion of CF patient	MDR	[2]
A. xylosoxidans	CF-4	Italy	2008-2010	respiratory secretion of CF patient	MDR	[2]
Burkholderia cenocepacia^{T}	LMG 16656	UK	1989	sputum of CF patient	ns	[3]
B. cenocepacia	FFC 0076	UK	ns	CF patient	ns	unpublished
Burkholderia dolosa^{T}	LMG 18943	USA	ns	sputum of CF patient	ns	[4]
B. dolosa	FFC0305	USA	ns	CF patient	ns	unpublished
Burkholderia multivorans^{T}	LMG 31010	Belgium	1992	CF patient	ns	[5]
B. multivorans	454	Czech Republic	2002	CF patient	ns	unpublished
Haemophilus influenzae	ATCC 49247	USA	1984	sputum of a pneumonia patient	ns	ATCC
H. influenzae	ATCC 9833	USA	ns	CF of patient with meningitis	ns	ATCC
H. influenzae	FC 89	Italy	2004-2009	CF patient	AMP, IPM	[6]
H. influenzae	FC 104	Italy	2004-2009	CF patient	ns	[6]
Pseudomonas aeruginosa	ATCC 15692 (PAO1)	ns	ns	infected wound	ns	ATCC
P. aeruginosa	TR1	Italy	2009	respiratory secretion of CF patient	ns	[7]
P. aeruginosa	FM12	Italy	ns	respiratory secretion of CF patient	ns	[8]
P. aeruginosa	FM13	Italy	ns	respiratory secretion of CF patient	ns	[8]
Staphylococcus aureus	ATCC 25923	USA	1945	human clinical	ns	ATCC
S. aureus	BG-1	Italy	2016	CF patient	ns	unpublished
S. aureus	BG-6	Italy	2016	CF patient	MRSA	unpublished
S. aureus	BG-7	Italy	2016	CF patient	MRSA	unpublished
Stenotrophomonas maltophilia^{T}	ATCC 13637	ns	ns	OP region of patient with mouth cancer	ns	ATCC
S. maltophilia	K279a	UK	ns	blood of non-CF patient	MDR	[9]
S. maltophilia	OBGTC23	Italy	2003-2005	sputum of CF patient	MDR	[10]
S. maltophilia	OBGTC26	Italy	2003-2005	sputum of CF patient	MDR	[10]
Streptococcus pneumoniae^{T}	ATCC 33400	ns	ns	unknown	ns	ATCC
S. pneumoniae	PFC-01	Italy	2010	CF patient	ns	[11]
S. pneumoniae	PFC-02	Italy	2010	CF patient	ns	[11]
S. pneumoniae	PFC-04	Italy	2010	CF patient	ns	[11]

Abbreviations: ATCC, American type culture collection; AMP, ampicillin; CF, cystic fibrosis; CSF, cerebrospinal fluid; IPM, imipenem; MDR, multi-drug resistant; MRSA, methicillin-resistant *Staphylococcus aureus*; OP, Oropharyngeal; PCN, penicillin; ns, not specified.
^{T} type strain.

Supplementary Table S2
List of CF *P. aeruginosa* clinical isolates used in this study

Strain	Source	Reference
AA2	respiratory secretion	[7]
AA43	respiratory secretion	[7]
BT2	respiratory secretion	[7]
BT73	respiratory secretion	[7]
FM17	respiratory secretion	[8]
KK1	respiratory secretion	[7]
KK27	respiratory secretion	[7]
KK28	respiratory secretion	[7]
KK71	respiratory secretion	[7]
TR66	respiratory secretion	[7]

References

[1] Yabuuchi E, Oyama A. *Achromobacter xylosoxidans* n. sp. from human ear discharge. Jpn J Microbiol 1971;15:477–81.

[2] Trancassini M, Iebba V, Citerà N, Tuccio V, Magni A, Varesi P, et al. Outbreak of *Achromobacter xylosoxidans* in an Italian Cystic fibrosis center: genome variability, biofilm production, antibiotic resistance, and motility in isolated strains. Front Microbiol 2014;5:138:1–8.

[3] Govan JRW, Brown PH, Maddison J, Doherty CJ, Nelson JW, Dodd M, et al. Evidence for transmission of *Pseudomonas cepacia* by social contact in cystic fibrosis. Lancet 1993;342:15–19.

[4] Vandamme P, Holmes B, Vancanneyt M, Coenye T, Hoste B, Coopman R, et al. Occurrence of multiple genomovars of *Burkholderia cepacia* in cystic fibrosis patients and proposal of *Burkholderia multivorans* sp. nov. Int J Syst Bacteriol 1997;47:1188–200.

[5] Coenye T, LiPuma JJ, Henry D, Hoste B, Vandemeulebroecke K, Gillis M, et al. *Burkholderia cepacia* genomovar VI, a new member of the *Burkholderia cepacia* complex isolated from cystic fibrosis patients. Int J Syst Evol Microbiol 2001;51:271–79.

[6] Cardines R, Daprai L, Giufrè M, Torresani E, Garlaschi ML, Cerquetti M. Genital carriage of the genus *Haemophilus* in pregnancy: species distribution and antibiotic susceptibility. J Med Microbiol 2015;64:724–30.

[7] Bragonzi A, Paroni M, Nonis A, Cramer N, Montanari S, Rejman J, et al. *Pseudomonas aeruginosa* microevolution during cystic fibrosis lung infection establishes clones with adapted virulence. Am J Respir Crit Care Med 2009;180:138–45.

[8] Massai F, Imperi F, Quattrucci S, Zennaro E, Visca P, Leoni L. A multitask biosensor for micro-volumetric detection of N-3-oxo-dodecanoyl-homoserine lactone quorum sensing signal. Biosens Bioelectron 2011;26:3444–9.

[9] Avison MB, Higgings CS, von Heldreich CJ, Bennett PM, Walsh TR. Plasmid location and molecular heterogeneity of the L1 and L2 β-lactamase genes of *Stenotrophomonas maltophilia*. Antimicrob Ag Chemother 2001;45:413.

[10] Di Bonaventura G, Prosseda G, Del Chierico F, Cannavacciuolo S, Cipriani P, Petrucca A, et al. Molecular characterization of virulence determinants of *Stenotrophomonas maltophilia* strains isolated from patients affected by cystic fibrosis. Int J Immunopathol Pharmacol 2007;20:529–37.

[11] Pimentel de Araujo F, D'Ambrosio F, Camilli R, Fiscarelli E, Di Bonaventura G, Baldassarri L, et al. Characterization of *Streptococcus pneumoniae* clones from paediatric patients with cystic fibrosis. J Med Microbiol 2014;63:1704–15.

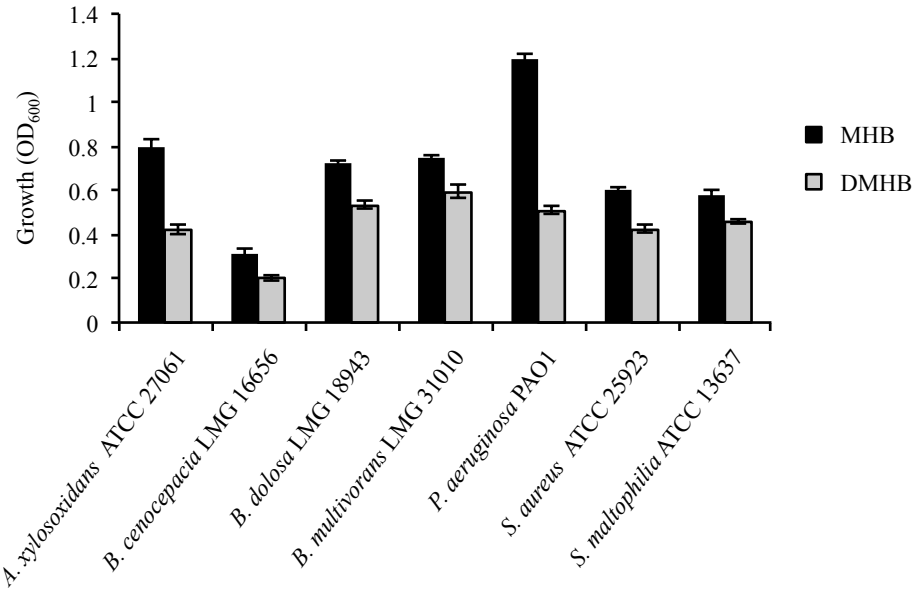


Fig. S1. Growth of CF reference-strains in MHB and DMHB. Bacterial strains were inoculated (5 x 10^5 CFU/ml) into 96-well microtiter plates containing MHB (dark bars) or DMHB (grey bars). The OD_{600} was determined after 24 h of incubation at 37 °C. Data are the means of triplicate experiments ± standard deviation (SD).

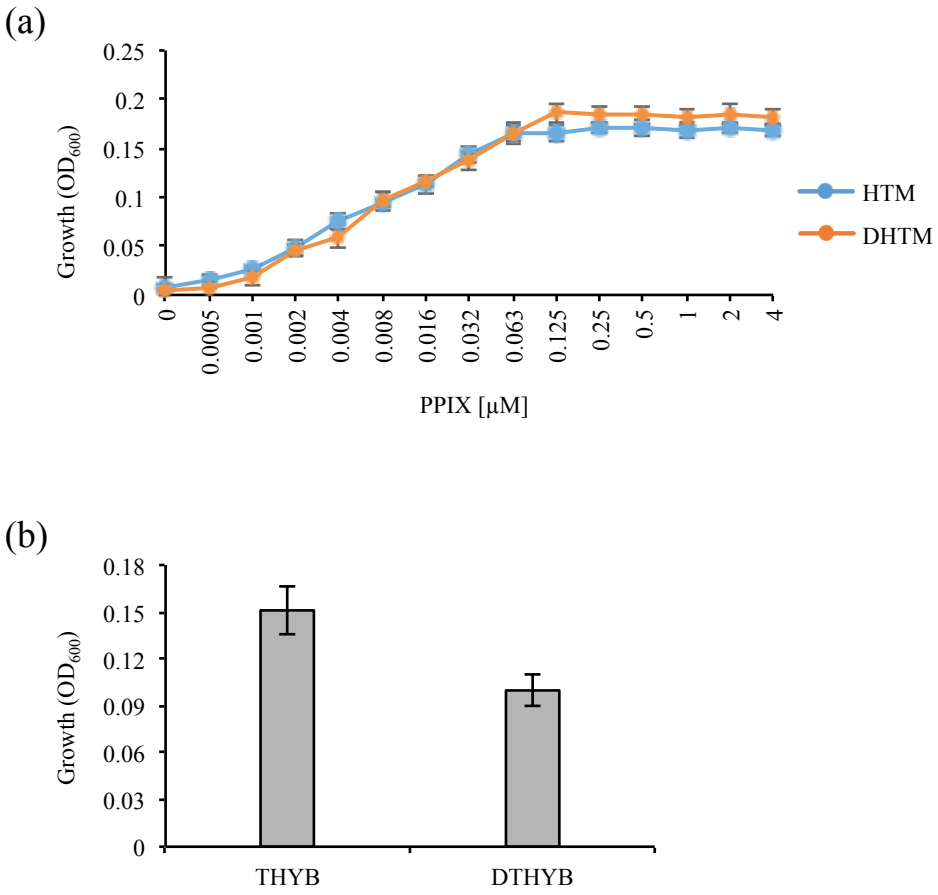


Fig. S2. Growth of *H. influenzae* and *S. pneumoniae* in standard media. (a) Growth of *H. influenzae* strains ATCC 49247 in HTM and DHTM in the presence of increasing concentrations of PPIX (from 0.0005 µM to 4 µM). (b) Growth of *S. pneumoniae* ATCC 33400 in THYB or in the Chelex-treated DTHYB. Bacteria were inoculated (5 x 10^5 CFU/ml) into 96-well microtiter plates. The OD_{600} was determined after 24 h of incubation at 37 °C in the presence of 5% CO_2. Data are the means of triplicate experiments ± SD.

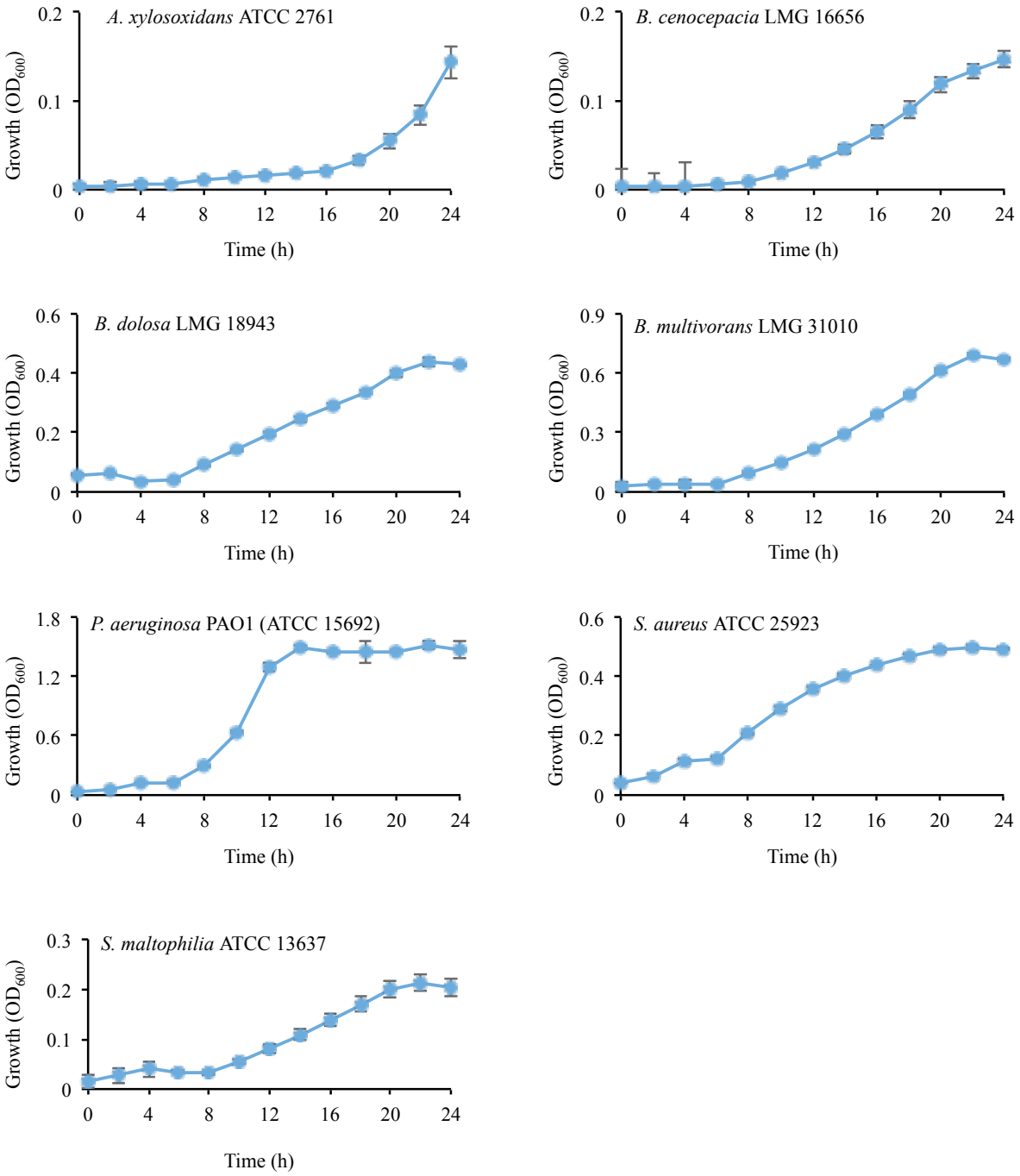


Fig. S3. Growth profiles of non-fastidious CF reference strains in ASM. Bacterial strains were inoculated (5 x 10^5 CFU/ml) into 96-well microtiter plates containing ASM. OD_{600} was monitored periodically for up to 24 h. Data are the means of triplicate experiments ± SD.

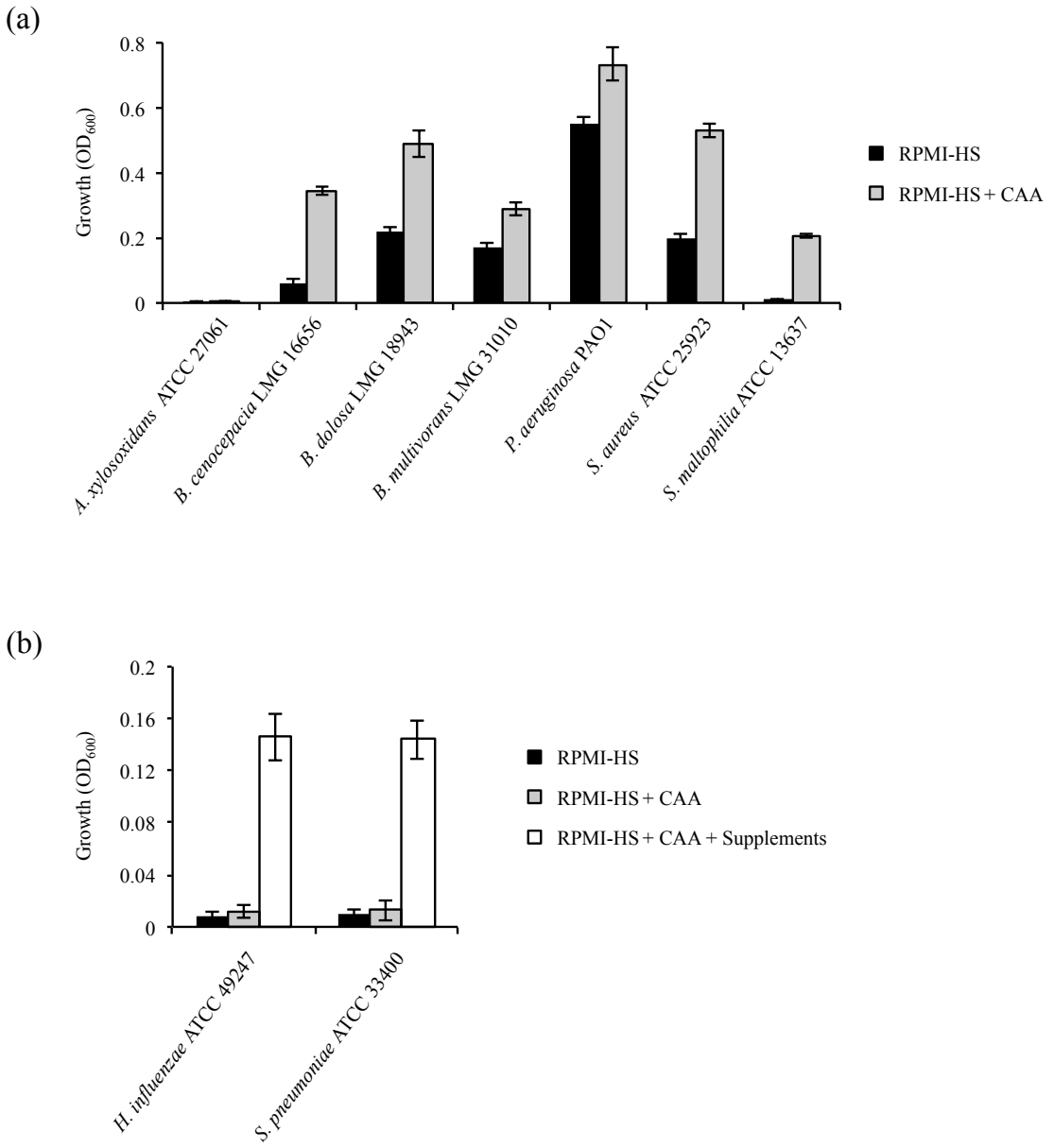


Fig. S4. Growth of CF reference-strains in modified RPMI-HS. Non-fastidious (a) and fastidious (b) bacterial strains were inoculated (5 x 10^5 CFU/ml) into 96-well microtiter plates containing RPMI-HS (black bars), RPMI-HS + CAA (grey bars), or RPMI-HS + CAA (white bars) additioned with supplements (3.3 µg/ml of hypoxanthine, 100 µg/ml of L-alanine, 55 µg/ml of L-cysteine hydrochloride, 6.6 µg/ml NAD, and 8 µg/ml uracil). OD_{600} was determined after 24 h of incubation at 37 °C. Data are the means of triplicate experiments ± SD.

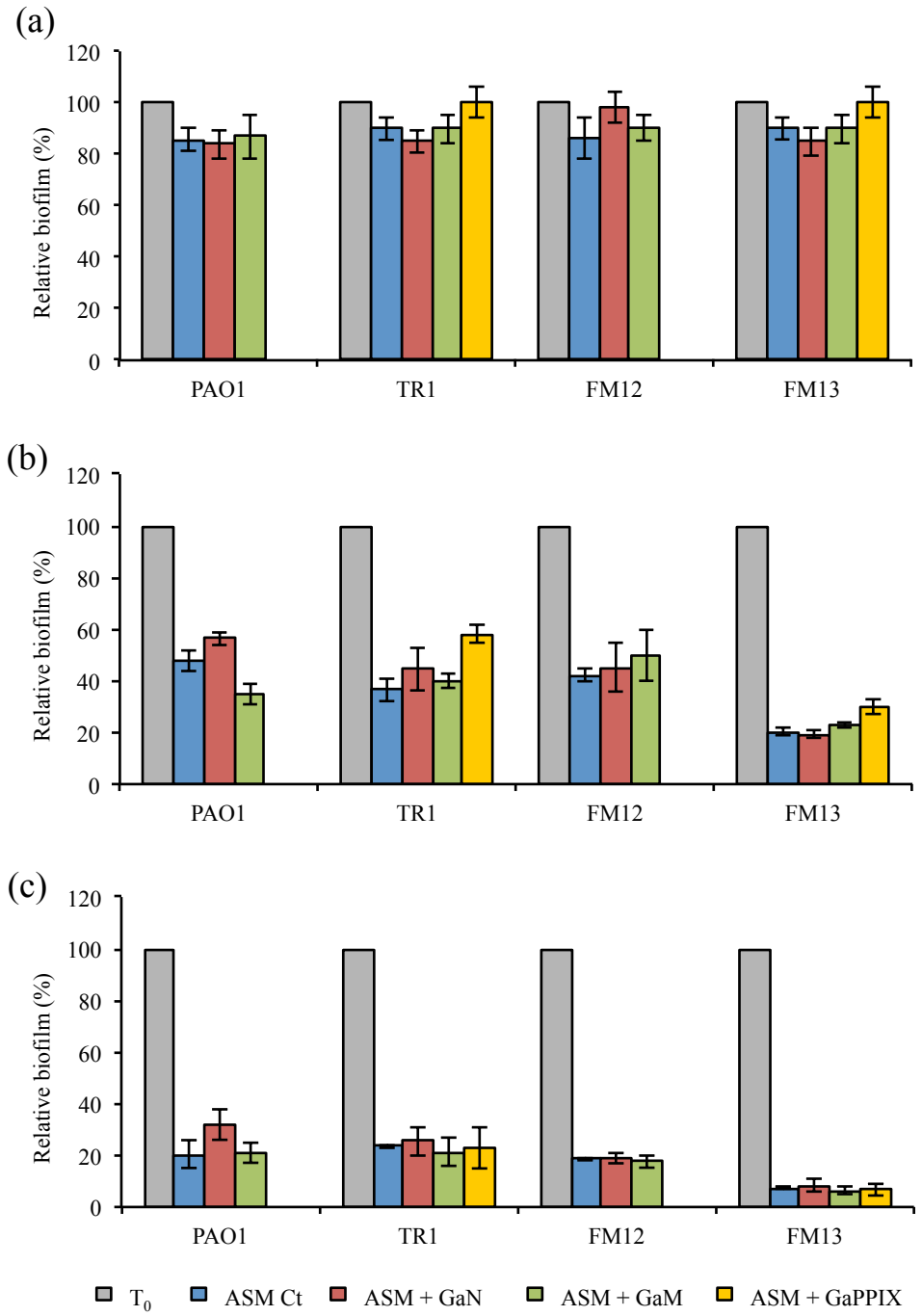


Fig. S5. Activity of Ga(III)-based compounds against *P. aeruginosa* preformed biofilm. Preformed 24 h *P. aeruginosa* biofilms were challenged with GaN, GaM and GaPPIX at 32 µM, or fresh ASM medium as control. Biofilm disruption was quantified by crystal violet microtiter plate assay after 24 h (a) 48 h (b) and 72 h (c) of incubation at 37 °C. The values are expressed as the percentage relative to the 24 h preformed biofilm (T_0) before Ga(III) challenge, and represent the means of two experiments in triplicate ± SD.

Chapter 5

Concluding remarks and future perspectives

Chapter 5

Concluding remarks and future perspectives

Healthcare practitioners are facing the decline of the "golden era" of antibiotics, since the therapeutic success of conventional antibiotics is vanished by the dramatic increase of antibiotic resistance. This holds true particularly for ESKAPE pathogens (*E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and *Enterobacter* species (Deplano *et al.,* 2005; Elemam *et al.,* 2009; Nowak *et al.,* 2017), which are responsible of worryingly high morbidity and mortality worldwide (Boucher *et al.,* 2009). While resistance to existing antibiotics is constantly increasing, the antibiotic discovery pipeline is running dry. For this reason, the development of novel strategies to treat infections sustained by MDR pathogens has become a matter of vital importance. Given the essential role of iron in host-pathogen interactions, many efforts have been put forth to inhibit iron-dependent bacterial metabolism (Ballouche, 2009; Foley and Simeonov, 2012). However, some strategies, including the use of iron chelators, originally developed to treat iron overload in humans, and the use of inhibitory compounds to block siderophores biosynthesis, have so far been unsuccessful: *i)* chelators may behave as Janus-faced molecules, by promoting bacterial growth instead of displaying an antibacterial effect (Kontoghiorghes *et al.,* 2010; Visca *et al.,* 2013), and *ii)* the redundancy, multiplicity, and diversity of siderophore biosynthethic gene clusters make blocking siderophore biosynthesis ineffective (Foley and Simeonov, 2012) (Chapter 1). Recent studies have explored the possibility to use iron mimetics as novel therapeutics; such drugs would interfere with bacterial iron metabolism likely displaying pleiotropic effects. One such iron mimetic, which holds promise as an antibacterial agent, is the post-transition metal gallium Ga(III) (Minandri *et al.,* 2014; Arivett *et al.,* 2015; Goss *et al.,* 2018). However, the lack of screening methods and comparative data on the activity of Ga(III) in standard conditions represents a pitfall for the use of Ga(III) as a last-resort antibacterial agent.

In this PhD thesis, we have comparatively investigated the possibility to hijack bacterial iron metabolism, using three Ga(III)-based compounds, namely, Ga(III)-nitrate (GaN), Ga(III)-maltolate (GaM) (Bernstein *et al.,* 2000), and Ga(III)-protoporphyrin IX (GaPPIX) (Chitambar, 2017), belonging to the first-, second-, and third- generations of Ga(III) formulations, respectively, in standard and RPMI-1640 supplemented with 10% complement-free human serum (RPMI-HS) media (Hijazi *et al.,* 2018 – Chapter 3). We have defined suitable test conditions, represented by RPMI-HS, as the presence of HS provides a more realistic milieu to assess the antibacterial activity of Ga(III) compounds *in vitro*. We found that both GaN and GaM were able to inhibit the most

relevant ESKAPE pathogens, including *K. pneumoniae, P. aeruginosa* and *A. baumannii*, being GaM was more potent than GaN. This is probably due to the solubility of GaM in both water and lipids that allows the penetration of cell walls and membranes, as opposed to the lack of lipophylicity of GaN (Bernstein *et al.,* 2000; DeLeon *et al.,* 2009). In contrast, we found that GaPPIX lost its antibacterial activity in RPMI-HS, likely due to the presence of human serum albumin (HSA), the most abundant plasma protein, which binds GaPPIX due to its similarity with heme, and counteracts its inhibitory effect (Chapter 3). Further direct experimental evidence providing the predicted GaPPIX-albumin interaction will be valuable to confirm our findings, before proposing GaPPIX as a valuable therapeutic option. On the other hand, other factors, such as the number and the type of heme-utilization systems that are variably present in bacterial strains, may positively influence GaPPIX susceptibility, as in the case of *A. baumannii* ACICU and C13-373 (Chapter 3). Similarly, GaN and GaM susceptibility varied among strains belonging to *K. pnemoniae* and *E. cloacae* species. Given this mixed efficacy among species and among strains within the same species, each Ga(III) formulation needs to be thoroughly tested *in vitro* and *in vivo* against each pathogen.

While nosocomial infections are predominantly ascribed to a single species, lung infections of patients suffering from CF, are usually caused by multispecies communities. The main bacterial pathogens are *A. xylosoxidans*, *B. cepacia* complex (*Bcc*), *H. influenzae*, *P. aeruginosa*, *S. aureus, S. maltophilia*, and *S. pneumoniae*, that coexist and evolve during infections (Folkesson *et al.,* 2012; Filkins and O'Toole, 2015; Parkins and Floto, 2015). The loss of efficacy of antibiotics to treat MDR CF-associated bacterial infections also calls for the development of novel antibacterial therapies. On this basis, the possibility to hijack bacterial iron metabolism using GaN, GaM and GaPPIX was extended to a representative panel of pathogens associated with CF (Chapter 4). In this part of this PhD thesis, the artificial sputum medium (ASM; Kirchner et *al.,* 2012), which better mimics the thick and stagnant airways mucus of CF patients, was also included, as it represents a more suitable growth medium to investigate the antibacterial proprieties of drugs against CF pathogens (Wright *et al.,* 2013; Schneider-Futschik *et al.,* 2018). Combining data obtained in all media tested, we found that Ga(III)-based compounds were effective against all CF-associated pathogens, except *S. pneumoniae* (Chapter 4). Interestingly, some species (*i.e., S. aureus* and *H. influenzae*) were extremely susceptible to GaPPIX only, suggesting a Ga(III)-formulation species specificity, which should drive the choice of the most suitable Ga(III)-based compound according to the infectious etiology. Concerning the treatment of CF-associated infections, our work suggests the use of GaPPIX in childhood to eradicate *H. influenzae* and *S. aureus* infections, which cause airways lesions, and subsequently promote *P. aeruginosa* colonization (Cigana *et al.,* 2018).

Conversely, later in adulthood, when CF airways are colonized by other pathogens, including *P. aeruginosa*, Ga(III) formulations, such as GaN and GaM, are advisable. Indeed, Phase 1 clinical trial on CF patients based on intravenous GaN administration (ClinicalTrial.gov, NCT01093521) has recently shown that Ga(III) improves lung function in patients with CF and *P. aeruginosa* chronic lung infection (Goss *et al.,* 2018). Moreover, phase 2 clinical trial is ongoing, to provide further evidence about the safety and clinical efficacy in adults CF patients chronically infected with *P. aeruginosa* (www.clinicaltrial.gov; NCT02354859). However, Ganite® approved by the U.S. Food and Drug Administration is no longer available from Genta, and more convenient routes of administration, for example, nebulization of inhalable formulations, should be envisaged. Moreover, administration of Ga(III) in combination with antibiotics for future applications in CF individuals should also be considered (Goss *et al.,* 2018). The interesting results obtained on CF pathogens, including, but not limited to *P. aeruginosa*, should encourage further *in vivo* studies, in order to confirm our *in vitro* data, and boost the future development of Ga(III)-based antimicrobials to treat polymicrobial CF associated-infections. Previous studies reported that Ga(III) is able to prevent biofilm formation and disrupts preformed *P. aeruginosa* biofilm (Kaneko *et al.,* 2007; Banin *et al.,* 2008). Although, in our experimental conditions (*i.e.,* growth in ASM medium and CV assay) the effect of Ga(III)-based compounds on formation and disruption of *P. aeruginosa* biofilm was not detected (Chapter 4), we cannot exclude that Ga(III)-based compounds may alter the *P. aeruginosa* biofilm tridimensional structure. Further studies, such as confocal microscopy analysis and continuous culture in flow chambers, are required to gain more insight into the anti-biofilm activity of Ga(III) compounds on *P. aeruginosa*.

Our work confirms the efficacy of both GaN and GaM against all planktonic growth *P. aeruginosa* strains, and demonstrated, for the first time, an antibacterial activity of GaPPIX against this bacterium (Hijazi *et al.,* 2017 – Chapter 2, Chapter 3, Chapter 4). In particular, we focused on the understanding of GaPPIX mechanism of action in *P. aeruginosa* (Chapter 2). It is well known that *P. aeruginosa* possess two heme-uptake systems (Ochsner *et al.,* 2000), which could be used by this bacterium to acquire heme-iron during infection (Konings *et al.,* 2013). We found that GaPPIX enters the *P. aeruginosa* cell exclusively through the heme-uptake systems, and consequently reduces bacterial growth. However, different from GaN, bacterial growth was never completely inhibited at GaPPIX concentrations up to 100 μM. This result is probably due to the different pathways taken by GaN and GaPPIX to enter *P. aeruginosa* cells and/or their different targets. GaN is known to enter *P. aeruginosa* cell through HitAB iron transport proteins (García-Contreras *et al.,* 2013) and *via* the PCH siderophore (Frangipani *et al.,* 2014), whereas GaPPIX only enters through heme-uptake systems, since the double mutant in both heme receptors was fully resistant to GaPPIX

(Chapter 2). Therefore, the susceptibility of *P. aeruginosa* to GaPPIX depends on the expression levels of heme-acquisition systems, given the fact that overexpression of both HasR and PhuR from multicopy pUCP18 plasmid made *P. aeruginosa* PAO1 extremely susceptible to GaPPIX, even at very low concentration. Different from GaN, which was recently demonstrated to inhibit essential iron-containing bacterial enzymes, such as those involved in DNA synthesis, and oxidant defense (Goss *et al.,* 2018), GaPPIX inhibits heme-containing molecules, especially cytochromes, as demonstrated in Chapter 2. Thus, the susceptibility of *P. aeruginosa* to GaPPIX could be further enhanced when the three high affinity terminal oxidases, in particular Cco-1, Cco-2 and Cio, are essential for bacterial growth. Indeed, clinical isolates (from CF and non-CF patients) were more susceptible to GaPPIX than the PAO1 prototype. This result is probably due to the adaptation of these strains to heme acquisition, since heme represents the principal iron source *in vivo* (Marvig *et al.,* 2014; Nguyen *et al.,* 2014), and/or to the relay of Cco-1, Cco-2 and Cio to sustain *P. aeruginosa* aerobic growth in the CF airways (Alvarez-Ortega and Harwood, 2007). Based on previous low toxicity effect reported for GaPPIX (Stojiljkovic *et al.,* 1999; Arivett *et al.,* 2015; Chang *et al.,* 2016), further studies should be encouraged to assess the effect of GaPPIX against *P. aeruginosa* infection *in vivo*.

In conclusion, in this PhD thesis, the possibility to hijack bacterial iron metabolism using the post-transition metal Ga(III) has been investigated. Ga(III)-based compounds therapies could be considered not only as last-resort drugs for the treatment of otherwise untreatable (pan-drug resistant) infections, but also as a first-line drug in order to delay the use of antibiotics and prevent the emergence of resistance.

Chapter 5 references

Alvarez-Ortega C, Harwood CS. (2007). Responses of *Pseudomonas aeruginosa* to low oxygen indicate that growth in the cystic fibrosis lung is by aerobic respiration. Mol. Microbiol. **65**:153-165.

Arivett BA, Fiester SE, Ohneck EJ, Penwell WF, Kaufman CM, Relich RF, Actis LA. (2015). Antimicrobial Activity of Gallium Protoporphyrin IX against *Acinetobacter baumannii* Strains Displaying Different Antibiotic Resistance Phenotypes. Antimicrob Agents Chemother. **59**:7657-7665.

Ballouche M, Cornelis P, Baysse C. (2009). Iron metabolism: a promising target for antibacterial strategies. Recent Pat Antiinfect Drug Discov. **4**:190-205.

Banin E, Lozinski A, Brady KM, Berenshtein E, Butterfield PW, Moshe M, Chevion M, Greenberg EP, Banin E. (2008). The potential of desferrioxamine-gallium as an anti-Pseudomonas therapeutic agent. Proc Natl Acad Sci U S A. **105**:16761-16766.

Bernstein LR, Tanner T, Godfrey C, Noll B. (2000). Chemistry and pharmacokinetics of gallium maltolate, a compound with high oral gallium bioavailability. Met Based Drugs. **7**:33-47.

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.

Chang D, Garcia RA, Akers KS, Mende K, Murray CK, Wenke JC, Sanchez CJ. (2016). Activity of Gallium Meso- and Protoporphyrin IX against Biofilms of Multidrug-Resistant *Acinetobacter baumannii* Isolates. Pharmaceuticals (Basel). **9**. pii: E16.

Chitambar CR. (2017). The therapeutic potential of iron-targeting gallium compounds in human disease: From basic research to clinical application. Pharmacol Res. **115**:56-64.

Cigana C, Bianconi I, Baldan R, De Simone M, Riva C, Sipione B, Rossi G, Cirillo DM, Bragonzi A. (2018). *Staphylococcus aureus* Impacts *Pseudomonas aeruginosa* Chronic Respiratory Disease in Murine Models. J Infect Dis. **217**:933-942.

DeLeon K, Balldin F, Watters C, Hamood A, Griswold J, Sreedharan S, Rumbaugh KP. (2009). Gallium maltolate treatment eradicates *Pseudomonas aeruginosa* infection in thermally injured mice. *Antimicrob Agents Chemother*. **53**:1331-1337.

Deplano A, Denis O, Poirel L, Hocquet D, Nonhoff C, Byl B. Nordmann P, Vincent JL, Struelens MJ. 2005. Molecular characterization of an epidemic clone of panantibiotic-resistant *Pseudomonas aeruginosa*. J Clin Microbiol. **43**:1198-1204.

Elemam A, Rahimian J, Mandell W. 2009. Infection with panresistant *Klebsiella pneumoniae*: a report of 2 cases and a brief review of the literature. Clin Infect Dis. **49**:271-274.

Filkins LM, O'Toole GA. (2015). Cystic Fibrosis Lung Infections: Polymicrobial, Complex, and Hard to Treat. PLoS Pathog.**11**:e1005258.

Foley TL, Simeonov A. (2012). Targeting iron assimilation to develop new antibacterials. Expert Opin Drug Discov. **7**:831-847.

Folkesson A, Jelsbak L, Yang L, Johansen HK, Ciofu O, Høiby N, Molin S. (2012). Adaptation of *Pseudomonas aeruginosa* to the cystic fibrosis airway: an evolutionary perspective. Nat Rev Microbiol. **10**:841-851.

Frangipani E, Bonchi C, Minandri F, Imperi F, Visca P. (2014). Pyochelin potentiates the inhibitory activity of gallium on *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **58**:5572-5575.

García-Contreras R, Lira-Silva E, Jasso-Chávez R, Hernández-González IL, Maeda T, Hashimoto T, Boogerd FC, Sheng L, Wood TK, Moreno-Sánchez R. (2013). Isolation and characterization of gallium resistant *Pseudomonas aeruginosa* mutants. Int J Med Microbiol. **303**:574-582.

Goss CH, Kaneko Y, Khuu L, Anderson GD, Ravishankar S, Aitken ML, Lechtzin N, Zhou G, Czyz DM, McLean K, Olakanmi O, Shuman HA, Teresi M, Wilhelm E, Caldwell E, Salipante SJ, Hornick DB, Siehnel RJ, Becker L, Britigan BE, Singh PK. (2018). Gallium disrupts bacterial iron metabolism and has therapeutic effects in mice and humans with lung infections. Sci Transl Med. 1**0**. pii: eaat7520.

Hijazi S, Visaggio D, Pirolo M, Frangipani E, Bernstein L, Visca P. (2018). Antimicrobial activity of gallium compounds on ESKAPE pathogens. Front Cell Infect Microbiol. **8**:316.

Hijazi S, Visca P, Frangipani E. (2017). Gallium-Protoporphyrin IX Inhibits *Pseudomonas aeruginosa* Growth by Targeting Cytochromes. Front Cell Infect Microbiol. **7**:12.

Kaneko Y, Thoendel M, Olakanmi O, Britigan BE, Singh PK. (2007). The transition metal gallium disrupts *P. aeruginosa* iron metabolism and has antimicrobial and antibiofilm activity. J Clin Invest. **117**:877-888.

Kirchner S, Fothergill JL, Wright EA, James CE, Mowat E, Winstanley C. (2012). Use of artificial sputum medium to test antibiotic efficacy against *Pseudomonas aeruginosa* in conditions more relevant to the cystic fibrosis lung. J Vis Exp. **5**:e3857.

Konings AF, Martin LW, Sharples KJ, Roddam LF, Latham R, Reid DW, Lamont IL. (2013). *Pseudomonas aeruginosa* uses multiple pathways to acquire iron during chronic infection in cystic fibrosis lungs. Infect Immun. **81**:2697-704.

Kontoghiorghes GJ, Kolnagou A, Skiada A, Petrikkos G. (2010). The role of iron and chelators on infections in iron overload and non iron loaded conditions: prospects for the design of new antimicrobial therapies. Hemoglobin. **34**:227-239.

Marvig RL, Damkiær S, Khademi SM, Markussen TM, Molin S, Jelsbak L. (2014). Within-host evolution of *Pseudomonas aeruginosa* reveals adaptation toward iron acquisition from hemoglobin. MBio. **5**:e00966-14.

Minandri F, Bonchi C, Frangipani E, Imperi F, Visca P. (2014). Promises and failures of gallium as an antibacterial agent. Future Microbiol. **9**:379-397.

Nguyen AT, O'Neill MJ, Watts AM, Robson CL, Lamont IL, Wilks A, Oglesby-Sherrouse AG. (2014). Adaptation of iron homeostasis pathways by a *Pseudomonas aeruginosa* pyoverdine mutant in the cystic fibrosis lung. J Bacteriol. **196**:2265-2276.

Nowak J, Zander E, Stefanik D, Higgins PG, Roca I, Vila J, McConnell MJ, Cisneros JM, Seifert H, MagicBullet Working Group WP4. (2017). High incidence of pandrug-resistant *Acinetobacter baumannii* isolates collected from patients with ventilator-associated pneumonia in Greece, Italy and Spain as part of the MagicBullet clinical trial. J Antimicrob Chemother. **72**:3277-3282.

Ochsner UA, Johnson Z, Vasil ML. (2000). Genetics and regulation of two distinct haem-uptake systems, phu and has, in *Pseudomonas aeruginosa*. Microbiology. **146**:185-198.

Parkins MD, Floto RA. (2015). Emerging bacterial pathogens and changing concepts of bacterial pathogenesis in cystic fibrosis. J Cyst Fibros. **14**:293-304.

Schneider-Futschik EK, Paulin OKA, Hoyer D, Roberts KD, Ziogas J, Baker MA, Karas J, Li J, Velkov T. (2018). Sputum Active Polymyxin Lipopeptides: Activity against Cystic Fibrosis *Pseudomonas aeruginosa* Isolates and Their Interactions with Sputum Biomolecules. ACS Infect Dis. **4**:646-55.

Stojiljkovic I, Perkins-Balding D. (2002). Processing of heme and heme-containing proteins by bacteria. DNA Cell Biol. **21**:281-295.

Visca P, Bonchi C, Minandri F, Frangipani E, Imperi F. (2013). The Dual Personality of Iron Chelators: Growth Inhibitors or Promoters? Antimicrob Agents Chemother. **57**:227-239.

Wandersman C, Delepelaire P. (2004). Bacterial iron sources: from siderophores to hemophores. Annu Rev Microbiol. **58**:611-47.

Wright EA, Fothergill JL, Paterson S, Brockhurst MA, Winstanley C. (2013). Sub-inhibitory concentrations of some antibiotics can drive diversification of *Pseudomonas aeruginosa* populations in artificial sputum medium. BMC Microbiol. **13**:170.

List of publications

Hijazi S, Visca P, Frangipani E. (2017). Gallium-Protoporphyrin IX Inhibits *Pseudomonas aeruginosa* Growth by Targeting Cytochromes. Front Cell Infect Microbiol. **7**:12. doi: 10.3389/fcimb.2017.00012

Hijazi S, Visaggio D, Pirolo M, Frangipani E, Bernstein L, Visca P. (2018). Antimicrobial activity of gallium compounds on ESKAPE pathogens. Front Cell Infect Microbiol. **8**:316. doi: 10.3389/fcimb.2018.00316

Acknowledgements

I would like to thank all the people who made this thesis possible.
First of all, I would like to express my deep gratitude to Prof. Paolo Visca and Dr. Emanuela Frangipani for encouraging guidance and constructive criticism to my PhD work.
My grateful thanks go also to Dr. Daniela Visaggio who provided me her precious help and for the fruitful discussions whenever I was in need.
I would like to extend my thanks to my colleagues, Dr. Federica Runci and Mr. Massimiliano Lucidi for all the time we spent together sharing the same lab, and a special thanks for Mr. Mattia Pirolo for all the works that we achieved toghether.

I would like to express my immense gratitude to my hunsband Dr. Ali Abdallah for sharing the most beautiful and the hardest moments of my university career, for his lovely support and constant encouragement.

Finally, I would like to thank my beloved family for their unconditional love. A special mention for my lost relative Chakib Salloum for encouring me to release my dream.