JBIC Journal of Biological Inorganic Chemistry (2020) 25:1153–1165
https://doi.org/10.1007/s00775-020-01831-x

ORIGINAL PAPER

Quantitative proteomic reveals gallium maltolate induces an iron-limited stress response and reduced quorum-sensing in *Pseudomonas aeruginosa*

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Received: 25 May 2020 / Accepted: 21 October 2020 / Published online: 30 October 2020

Abstract

Gallium-based drugs have been repurposed as antibacterial therapeutic candidates and have shown significant potential as an alternative treatment option against drug resistant pathogens. The activity of gallium (Ga^{3+}) is a result of its chemical similarity to ferric iron (Fe^{3+}) and substitution into iron-dependent pathways. Ga^{3+} is redox inactive in typical physiological environments and therefore perturbs iron metabolism vital for bacterial growth. Gallium maltolate (GaM) is a well-known water-soluble formulation of gallium, consisting of a central gallium cation coordinated to three maltolate ligands, $[Ga(Maltol_{-1H})_3]$. This study implemented a label-free quantitative proteomic approach to observe the effect of GaM on the bacterial pathogen, *Pseudomonas aeruginosa*. The replacement of iron for gallium mimics an iron-limitation response, as shown by increased abundance of proteins associated with iron acquisition and storage. A decreased abundance of proteins associated with quorum-sensing and swarming motility was also identified. These processes are a fundamental component of bacterial virulence and dissemination and hence suggest a potential role for GaM in the treatment of *P. aeruginosa* infection.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00775-020-01831-x) contains supplementary material, which is available to authorized users.

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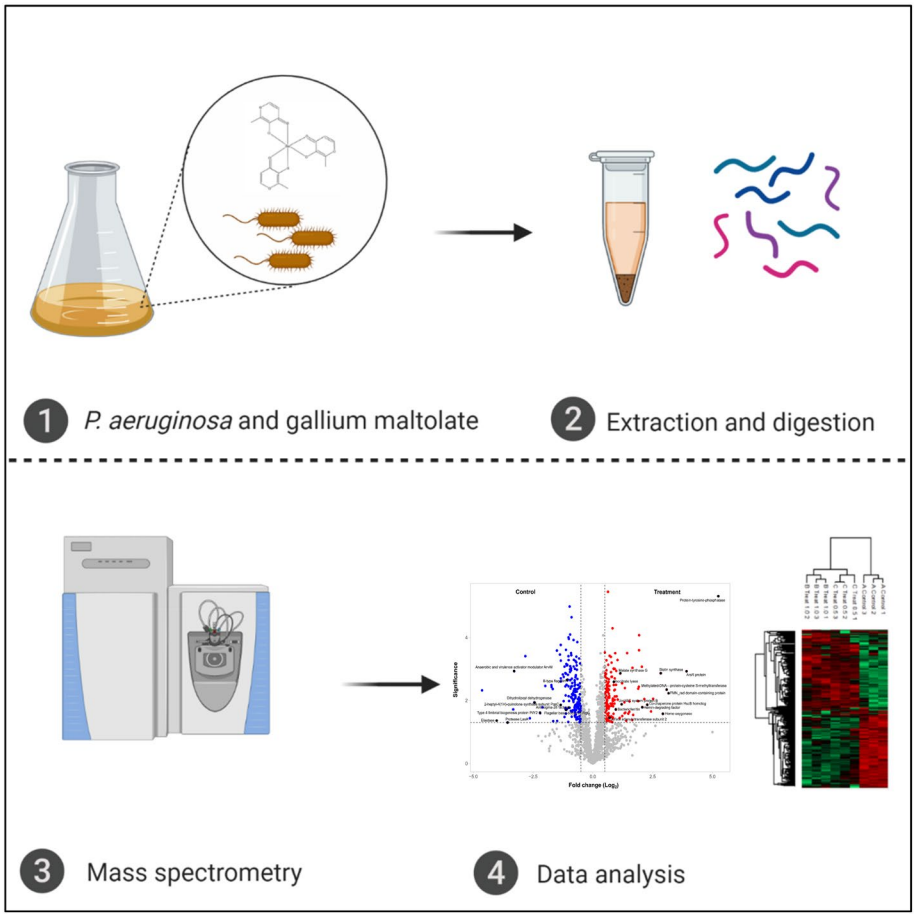
Graphic abstract



Keyword Antimicrobial · Galleria · Gallium · Iron · Proteomics · Pseudomonas · Stress

Abbreviations

AMR	Antimicrobial resistance
GaM	Gallium maltolate
PBS	Phosphate buffered saline
QS	Quorum sensing
PQS	*Pseudomonas* Quinolone signal
SSDA	Statistically significant and differentially abundant

Introduction

Antimicrobial resistance (AMR) is a growing concern globally and has become problematic for the treatment of Gram-negative infections [1]. The presence of an outer membrane in the bacterial envelope distinguishes Gram-negative from Gram-positive bacteria, and can confer resistance by inhibiting the entry and/or retention of antimicrobial agents [2, 3]. A significant contributor to increased AMR incidences is the Gram-negative bacterium *Pseudomonas aeruginosa*—a pathogen among immunocompromised individuals that can cause urinary tract infections and bacteraemia in nosocomial and community-based settings [4–6]. The presence of the pathogen is a particular threat for cystic fibrosis patients and often indicates poor clinical outcome due to the ability to persist for long periods of time and drastically affect lung function [7, 8]. The adaptability and versatility of the pathogen is accounted for by a relatively large genome (5–7 Mb) that allows the expression of many virulence genes and regulatory enzymes involved in metabolism, efflux of organic compounds and resistance and enables the pathogen to withstand hostile environments [9, 10]. Moreover, Gram-negative bacteria can increase expression of efflux systems and modify outer membrane proteins and/or drug targets (e.g. penicillin binding proteins) to render many broad-spectrum antibiotics ineffective; frequent use of which can accelerate the development of these mechanisms [11–13].

The advent of AMR necessitates the production of novel treatments with unique modes of action. Interference with bacterial iron metabolism has proven effective as an alternative method to eliminate infection since iron is a vital nutrient for the growth, survival and virulence of many bacteria [14–17]. A fully functioning immune system and iron sequestration (for redox homeostasis, respiration and DNA synthesis and repair) are protective strategies of the human body to limit access to pathogens [18–20]. Successful pathogens such as *P. aeruginosa* can overcome this through multiple iron uptake systems. Pyochelin and pyoverdine are among two siderophores that act as iron scavengers whilst the Feo system utilises phenazines to reduce insoluble ferric iron (Fe^{3+}) to soluble ferrous iron (Fe^{2+}) that can readily diffuse into the cells [21–23].

The semi-metallic element, gallium, shares chemical properties with iron that allow it to act as an iron mimetic in biological environments [15]. Transport of gallium in the blood closely resembles that of iron, whereby gallium forms a complex with the iron transporter transferrin, and with the aid of transferrin receptor 1, enters cells via endocytosis [24, 25]. Approximately 2/3 of transferrin is unbound in physiological conditions, leaving it vacant for the attachment of gallium (and other metals) [26, 27].

The medicinal application of gallium commenced with the use of gallium-based radiopharmaceuticals to detect and monitor cancerous tissue. The concentration of gallium incorporated into cells is directly proportional to the metabolic and proliferative activity, hence these scans can reveal the severity of some cancers such as lymphomas [28, 29]. Use later expanded to the development of gallium-based complexes as potential anti-cancer drugs [29–31]. Gallium limits the availability of iron to malignant cell lines [32–34] and compromises mitochondrial function through the stimulation of calcium efflux and consequent initiation of apoptosis [35]. Significantly, the gallium-based complex, tris(8-quinolinolato)gallium(III), KP46, (Fig. 1), has recently reached a phase I/II clinical trial as a potential anticancer agent [36].

The simple gallium salt, gallium nitrate, showed promising antineoplastic and antimicrobial activity as a first-generation compound [37]. Gallium maltolate (GaM), a Ga(III) coordination complex of maltol, $[Ga(Maltol_{-1H})_3]$, first reported by Finnegan et al., as a neutral water-soluble complex of "medical interest" is a second-generation gallium-based compound which has potentially enhanced bioavailability as an oral and topical treatment [26, 38–40].

More recently, the physiological and pathological requirements of iron in bacteria have sparked interest in the repurposing of gallium as an antimicrobial agent. The inability of bacterial cells to differentiate between iron and gallium results in the detrimental incorporation of gallium into the cell [41]. Gallium is unable to alternate from a trivalent to divalent form under normal physiological conditions and therefore disrupts bacterial iron metabolism and inhibits cell growth [38].

Studies on antineoplastic and antimicrobial applications of gallium have described similar modes of action whereby gallium interferes with iron-dependent ribonucleotide reductase enzymes to prevent DNA synthesis [16, 42–46]. Myette et al., explored the synergistic effect of gallium nitrate with ribonucleotide reductase inhibitors gemcitabine and hydroxyurea on leukemic cells and with the help of previous findings, revealed the role of gallium in replacing iron in the M2 subunit of ribonucleotide reductase to cease DNA synthesis and cell proliferation [42, 47, 48]. CCRF-CEM cells were highly susceptible to a combination of gallium nitrate and gemcitabine due to the suspected cytotoxic effect and incorporation of gallium into DNA, preventing strand elongation [42].

Treatment of *Mycobacterium tuberculosis* with gallium nitrate reduced ribonucleotide activity by a maximum of 60%. The Ib class of ribonucleotide reductases in *M. tuberculosis* is vital for cell growth and a likely target of gallium

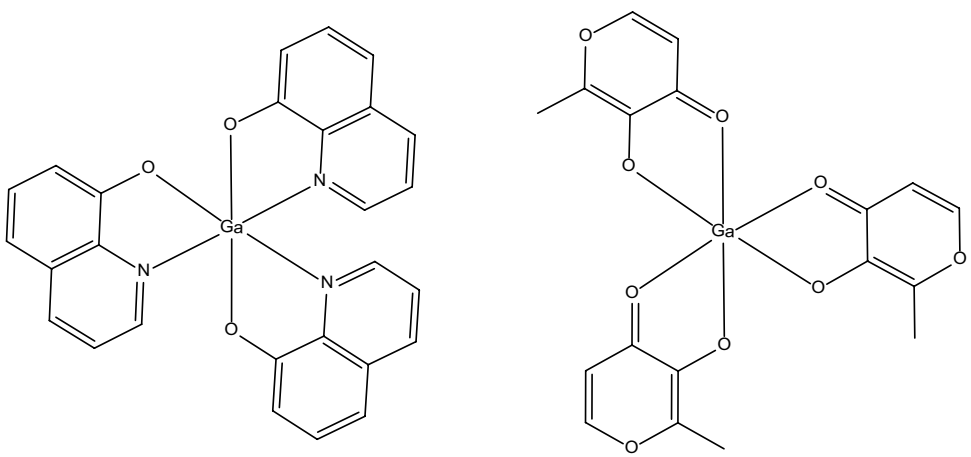


Fig. 1 Chemical structures of KP46 (left) and GaM (right)

[43, 49]. These findings were highly comparable with those shown in *P. aeruginosa*, where gallium was presumably targeted by one class of ribonucleotide reductase enzyme [14].

Unsurprisingly, rapidly proliferating cancer cells demand more iron [50, 51] and therefore exposure to gallium increases concentration at these target sites [52, 53]. This is further enhanced in the cases of lymphomas and bladder cancer due to increased transferrin receptor 1 expression [54, 55].

Chitambar [29] summarised the progress of human clinical trials on a range of gallium compounds. FDA-approved gallium nitrate has been used for the treatment of Non-Hodgkin's lymphoma and bladder cancer, with minimal safety issues when administered with caution. The optimum dosing regimen involves intravenous infusion of the drug over a number of days [56]. Doses ranging between 100 and 500 mg showed no adverse effects on healthy individuals [40] while additional studies revealed reductions in tumor mass and symptoms in a hepatocellular carcinoma patient [44]. Gallium-containing compounds have generally been well tolerated in human subjects although some side-effects including nausea, vomiting and anemia have been reported [57]. Alternatively, the use of gallium as an antimicrobial has shown promising results with no adverse toxicity as demonstrated in phase 1 clinical trials on CF patients with *P. aeruginosa* infection. Intravenous therapy improved lung function and was comparable with existing antibiotics [14].

The importance of iron and limited host supply opens a window of opportunity to target the nutritional demands of bacteria. This novel approach along with an abundance of literature documenting the therapeutic capability of gallium (and GaM), have encouraged these studies to obtain additional insight on the mode of action [16, 58–62].

The aim of the work presented here was to assess the ability of GaM to inhibit the growth of *P. aeruginosa* in vivo and in vitro and using label free mass spectrometry to uncover the response of this bacterium to this metal.

Methods

P. aeruginosa culture conditions

P. aeruginosa PAO1 was cultured for 24 h in nutrient broth (Oxoid, UK) at 37 °C in an orbital shaker (200 rpm). Stocks were kept on nutrient agar (Oxoid).

Gallium maltolate synthesis

GaM was synthesised as previously reported by Finnegan et al. [39].

Bacterial toxicity assays

P. aeruginosa PAO1 was cultured for 24 h in nutrient broth at 37 °C to the stationary phase and samples were diluted to 1/100 in nutrient broth (overnight growth produced cultures with an optical density of approximately 1.0 at 600 nm (OD_{600}) representing 3×10^8 CFU/ml of bacteria). Aliquots (100 µl) were added to serially diluted GaM (0.997–250 µg/ml) and nutrient broth in a 96-well plate (Sarstedt, Germany). Plates were incubated at 37 °C for 24 h and growth was measured at 600 nm.

Galleria mellonella larvae viability assays

Sixth instar larvae of the greater wax moth, *Galleria mellonella*, (Livefoods Direct Ltd., Sheffield, UK) were stored at 15 °C prior to inoculation. Ten healthy larvae (i.e. no appearance of melanisation), weighing 250 ± 50 mg were selected and stored in 9 cm petri dishes containing wood shavings.

Bacterial cells were diluted in PBS to give concentrations of 3×10^0, 3×10^1, 3×10^2, 3×10^3 CFUs/ml and 20 µl aliquots of bacterial suspension were injected into larvae via the last left pro-leg using U-100 insulin syringe (Terumo Europe, N.V., Belgium). Aliquots (20 µl) of 500 or 1000 µg/ml GaM treatment and PBS as a control were injected into the last right pro-leg 30 min post bacterial infection. Larvae were incubated at 37 °C for all studies. Larval survival was based on the level of melanisation and/or response to touch.

Determination of hemocyte density

G. mellonella larvae were inoculated with 20 µl aliquots of 500 and 1000 µg/ml GaM solutions or PBS and incubated at 37 °C. Larvae, ($n=3$, per sample), were bled to extract a total of 90 µl of hemolymph. Hemolymph was diluted in 100 µl sterile PBS and *N*-phenylthiourea to prevent melanisation in pre-chilled microcentrifuge tubes. Hemocyte density was calculated using a hemocytometer and determined as number of cells per ml.

P. aeruginosa protein extraction and purification

P. aeruginosa cultures grown overnight to the stationary phase were split (50/50) and re-grown in fresh media supplemented with GaM (500 µg/ml and 1000 µg/ml) at 37 °C for an additional 6 h (at which stage early stationary phase was reached, Fig S1B). Proteins were extracted using 6 M urea, 2 M thiourea and a selection of protease inhibitors (PMSF (50 mM), aprotinin, leupeptin, pepstatin A, and TLCK (1 mg/ml), Sigma). Cell debris was pelleted by centrifugation at 9000×*g* for 5 min. Proteins were quantified via the Bradford protein assay and acetone precipitated (100 µg)

overnight at −20 °C. The acetone was removed and proteins were re-suspended in 25 µl of resuspension buffer (6 M Urea, 2 M Thiourea, 0.1 M Tris–HCl (pH 8.0) dissolved in deionised water). The Qubit™ protein quantification system (Invitrogen) was used to quantify 2 µl aliquots of protein samples. Ammonium bicarbonate (50 mM) was added to the remaining samples and proteins were reduced with 0.5 M dithiothreitol (DTT) (Sigma-Aldrich) at 56 °C for 20 min and alkylated with 0.5 M iodoacetamide (IAA) (Sigma-Aldrich) in the dark at room temperature for 15 min. Proteins were digested with Sequence Grade Trypsin (0.5 µg/ml) (Promega) and incubated overnight at 37 °C. Trifluoroacetic acid (1 µl of 100%) (Sigma-Aldrich) was added to inhibit tryptic digestion. Following 5 min incubation at room temperature, samples were centrifuged at 13,000×*g* for 10 min. Peptides were purified using C-18 spin columns (Pierce) to yield a total of approximately 30 µg of protein and dried in a SpeedyVac concentrator (Thermo Scientific Savant DNA120) at 39 °C for 2 h. Samples were resuspended in 2% acetonitrile and 0.05% trifluoroacetic acid and sonicated in a water bath for 5 min followed by centrifugation at 15,500×*g* for 5 min. The supernatant was extracted and used for mass spectrometry.

Mass spectrometry

Digested *P. aeruginosa* protein samples (0.75 µg) were loaded onto a QExactive Mass Spectrometer (ThermoFisher Scientific) connected to a Dionex Ultimate™ 3000 (RSLC-nano) chromatography system. Purified hemolymph proteins were loaded in the same manner. An acetonitrile gradient was used to separate peptides in a BioBasic™ C18 Pico-Frit™ column (100 mm in length, 75 mm inner diameter) using a 65 min reverse phase gradient at a flow rate of 250 nL/min. The mass spectrometer was operating in an automatic dependent switching mode to acquire all data. A high resolution MS scan (300–2000 Dalton) was performed using the Orbitrap to select the 15 most intense ions prior to MS/MS.

Protein identification and LFQ normalisation of MS/MS data was carried out using MaxQuant version 1.6.6.0 (https://maxquant.org/) following established procedures outlined previously [63]. The Andromeda search engine in MaxQuant matched MS/MS data against a UniProt-SWISS-PROT database for *P. aeruginosa* PAO1 [64].

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [65] partner repository with the dataset identifier PXD019265.

Data analysis

General procedures for data processing and graphic generation were performed on Perseus v.1.6.6.0 (https://maxquant.org/) as described by Deslyper et al. [66]. Proteins not identified in at least two out of the three replicates were removed. Imputation of the data replaced missing values with values that mimic low abundance proteins randomly selected from a distribution specified by a downshift of 1.8 times the mean standard deviation (SD) of all measured values and a width of 0.3. Two sample *t* tests were carried out with a cut-off of $p < 0.05$. For additional analysis, protein names and functions were obtained from searching the Uniprot ID from the Uniprot Knowledgebase (www.uniprot.org).

Results

Analysis of the in vitro and in vivo effect of GaM against *P. aeruginosa*

The growth of *P. aeruginosa* PAO1 when exposed to a range of GaM concentrations in vitro, was measured after 24 h at 37 °C (Fig. 2). A concentration of 62.5 µg/ml inhibited growth by approximately 50% and the maximum concentration used (250 µg/ml) inhibited growth by 70%.

Larvae of *G. mellonella* were administered GaM doses of 125, 250, 500 and 1000 µg/ml in order to measure toxicity in the host. Larvae administered GaM showed no reduction in viability (Fig S2) and there was an increase in the hemocyte (immune cell) density at 24 h in those larvae that received a dose of 500 or 1000 µg /ml (Fig S3). The ability of GaM to act in the host and prolong survival of larvae infected with *P. aeruginosa* was assessed. Treatment of larvae infected with 3×10^2 and 3×10^3 CFUs of *P. aeruginosa* (doses that result in 100% mortality at 24 h) with doses of 500 and 1000 µg/ml GaM 30 min post-infection

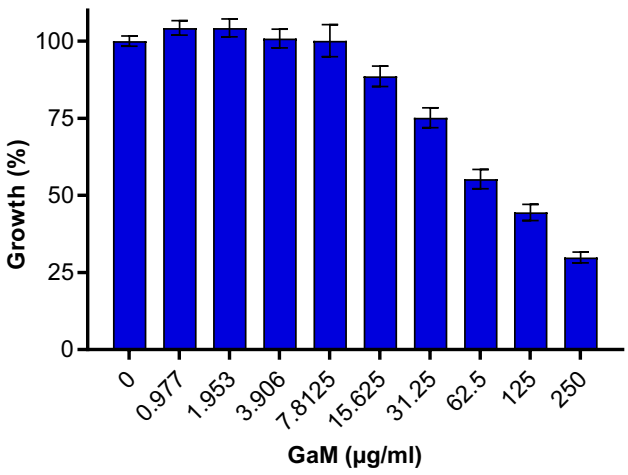


Fig. 2 Growth of *P. aeruginosa* treated with GaM in vitro. Cultures grown overnight were subjected to GaM and incubated at 37 °C and evaluated after 24 h. All values are the mean ± S.E of eight samples

significantly increased larval survival at 24 h. Treatment of larvae infected with 3×10^2 CFUs *P. aeruginosa* with a dose of 1000 µg/ml GaM post infection gave 95 ± 5% survival at 24 h. Furthermore larvae infected with 3×10^3 CFUs of *P. aeruginosa* and administered a dose of 1000 µg/ml post-infection showed 90 ± 10% survival at 24 h, (Fig. 3).

Proteomic analysis of the response of *P. aeruginosa* following exposure to GaM

Proteomic analysis allows a comparison of alterations in the whole proteome of an organism in response to a stress or treatment and can be used as a tool to identify potential modes of action of compounds on cells or whole organisms. The effect of GaM on *P. aeruginosa* was analysed via label-free quantitative mass spectrometry and visual representation of acquired data allowed for the identification of proteins and their associated pathways affected by GaM.

To investigate the proteomic response of *P. aeruginosa* to GaM in vitro, LFQ proteomics was performed on whole cell lysates. *P. aeruginosa* was grown in the presence of GaM (500 and 1000 µg/ml) for 6 h to the early stationary phase. An exposure time of 6 h was chosen to ensure adequate incorporation of the compound into the cell in order to elicit a pronounced proteomic change.

A total of 2606 proteins were initially identified and 1748 remained after filtering out contaminants and peptides identified by site from the initial data matrix. A total of 1673 proteins were present in all samples, 56 of which were exclusive to both treatment samples and 6 were exclusive to control samples (Dataset S3). Post-imputation, 389 were deemed statistically significant and differentially abundant (SSDA), ANOVA $p < 0.05$) with a fold change of > 1.5 (Dataset S1).

A distinct difference between the proteomes of treated and control samples can be observed in the principal component analysis (PCA; Fig. 4) of filtered proteins. The overlap between 500 µg/ml and 1000 µg/ml treatment samples indicates the limited effect of increasing concentrations of GaM on the *P. aeruginosa* proteome. Three replicates of treatment and control samples were resolved through hierarchal clustering of z-score normalised intensity values for all SSDA proteins, ($n = 389$), and visualised via a heatmap (Fig S4). The heatmap generated on Perseus statistical software grouped proteins based on similar median expression trends and identified two major protein clusters, A and B, representing three replicates in each sample group (Dataset S2).

The volcano plot displays 1748 filtered proteins with altered abundance levels of treated samples relative to controls. Among the top SSDA proteins increased in abundance in *P. aeruginosa* treated with 1000 µg/ml GaM in comparison to non-treated control samples include *arsC*-encoded protein-tyrosine-phosphatase (37.99-fold), arsenic-responsive ArsR protein (15.13-fold) FMN_red domain-containing protein associated with oxidation–reduction (8.98-fold), methylated-DNA–protein-cysteine methyltransferase associated with DNA-repair (8.5-fold), heme oxygenase HemO (7.6-fold), biotin synthase (7.14-fold), co-chaperone protein HscB homolog for maturation of iron-sulfur cluster-containing proteins (4.83-fold) and hemin degrading factor (4.19-fold). SSDA proteins decreased in abundance in GaM-treated samples

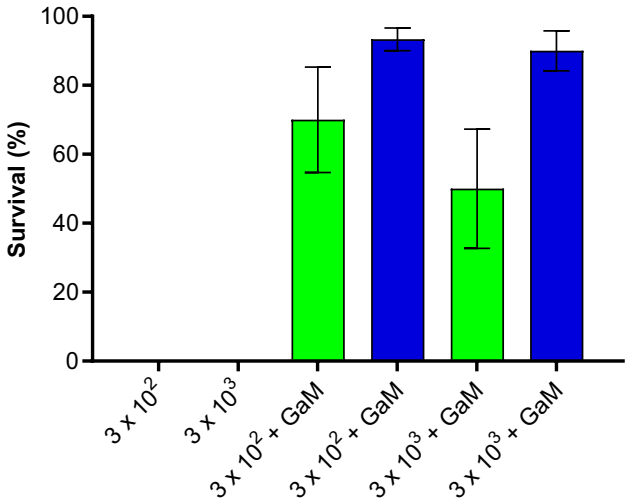


Fig. 3 Survival of *G. mellonella* larvae inoculated with *P. aeruginosa* and a single 20 µl dose of GaM (500 µg/ml (green) and 1000 µg/ml (blue)). Larvae ($n = 10$, per group) were inoculated with 3×10^2 and 3×10^3 CFUs of *P. aeruginosa* prior to GaM administration. Control groups include infected larvae injected with PBS. Larvae were observed after 24 h at 37 °C. Mean values ± S.E. were obtained from three independent experiments

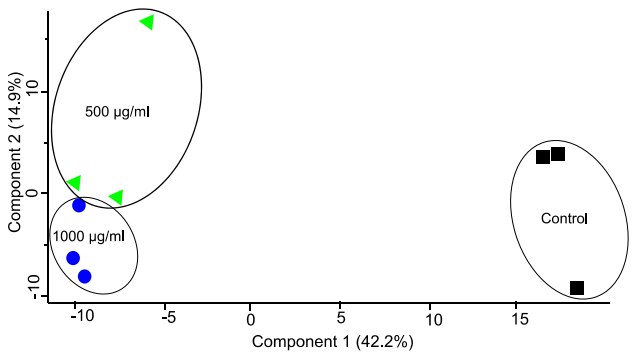


Fig. 4 Principal component analysis (PCA) of untreated *P. aeruginosa* (black), *P. aeruginosa* treated with 500 µg/ml (green) and 1000 µg/ml (blue) GaM. A contrast is shown between both treated groups and untreated controls

relative to the control group include elastase (16.35-fold) and protease A (11.83-fold) quorum-sensing enzymes, anaerobic and virulence activator-modulator AnvM (9.75-fold), dihydrolipoyl dehydrogenase involved in pathogenesis and cell redox (7.07-fold) and Type 4 fimbrial biogenesis protein PilY2 (4.6-fold), (Fig. 5).

The STRING database was used to identify biological pathways and protein networks between SSDA proteins increased and decreased in abundance versus control samples. Pathways associated with stress response, DNA damage/repair, iron-sulfur clusters and heme storage were upregulated in GaM-treated *P. aeruginosa* (Fig. 6a). Quorum sensing, flagellar motility and cell-redox pathways were downregulated (Fig. 6b).

Discussion

The survival of virtually all bacterial pathogens is heavily dependent upon the presence of iron within their host and an ability to access this metal [67]. GaM targets this weakness to disrupt iron metabolism. Although numerous studies have described mechanisms of action [26, 41, 58, 61, 68], little is known about the proteomic response of bacteria to GaM. Analysis of protein expression changes in *P. aeruginosa* in response to GaM can further inform potential modes of action.

P. aeruginosa was susceptible to GaM in vitro and 70% growth inhibition was achieved at a concentration of 250 µg/ml. This study utilised *G. mellonella* larvae as an efficient in vivo model to further validate the efficacy of GaM within

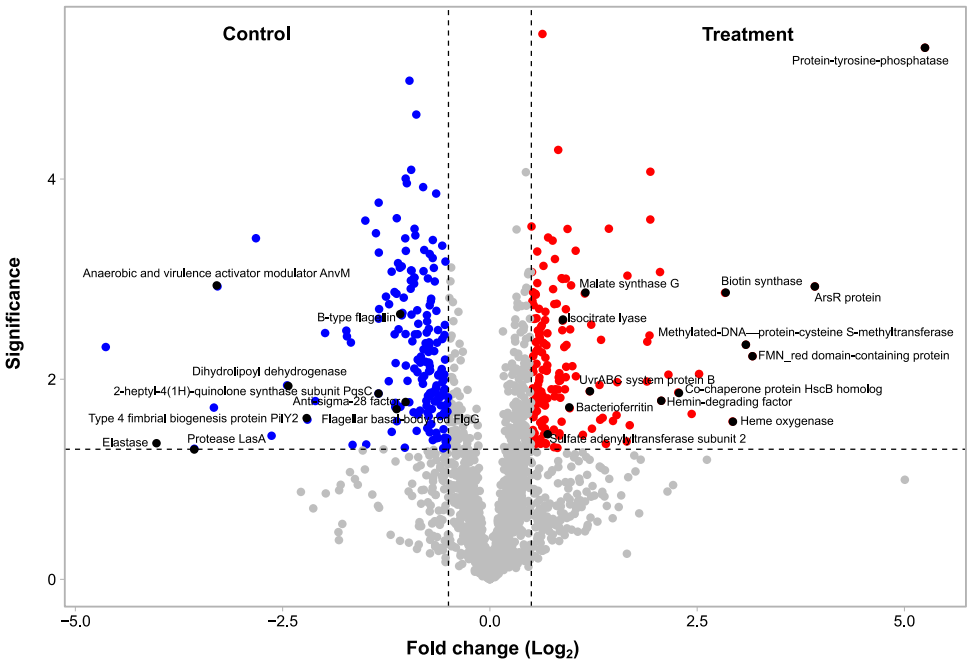


Fig. 5 Volcano plot representing differentially abundant proteins in *P. aeruginosa* treated with 1000 µg/ml GaM for 6 h. The distribution of quantified proteins is based on significance ($\log_{10} p$ value) versus the fold change (Log_2 LFQ intensity difference). Statistically significant (p value < 0.05) proteins are located above the horizontal line. Expression transcripts with relative fold changes of > 1.5 are shown with increased expression to the right (red) and decreased expression to the left (blue) of the vertical lines. SSDA proteins are annotated

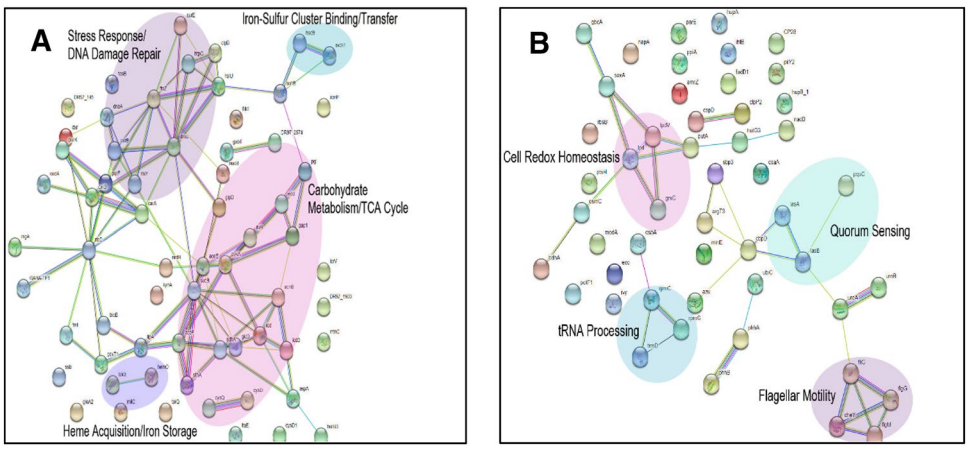


Fig. 6 Network analysis of proteins increased and decreased in abundance in *P. aeruginosa* treated with 1000 µg/ml GaM. Data obtained from the STRING database using gene lists from SSDA proteins from pair wise t tests ($p < 0.05$) shows interactions among individual proteins and associated pathways (highlighted in colour). **a** Protein pathways upregulated in GaM-treated *P. aeruginosa*. **b** Protein pathways downregulated in GaM-treated *P. aeruginosa*

a host. The insect immune system is similar to the mammalian innate immune response: the cuticle and skin are physical barriers against pathogens; blood-like hemolymph circulates hemocytes (immune cells) for phagocytosis and superoxide production, much like mammalian neutrophils [63, 69]. Despite lacking an adaptive immune response, *G. mellonella* larvae have been widely used in toxicity assays to elucidate the efficacy of both conventional and novel drugs and have provided comparable results to those from mammalian studies [69, 70]. The assessment of *G. mellonella* larvae viability revealed that GaM has growth-inhibiting activity in vivo and maintains larval survival for up to 24 h, thereafter survival rates declined. Larvae challenged with lethal concentrations of *P. aeruginosa* prior to GaM administration showed significantly increased survival in comparison to controls at 24 h. Furthermore, GaM demonstrated no toxic effects in vivo as larvae inoculated with varying concentrations of GaM showed 100% survival with no signs of melanisation for up to 24 h. To identify whether GaM induced an immunomodulatory effect, larval immune cells (hemocytes) were extracted and enumerated. Administration of GaM increased production of hemocytes, which may enhance antibacterial activity within the larvae. Determination of hemocyte density is a common criterion assessed in many *G. mellonella* studies and inoculation of antimicrobial agents has been shown to induce immune priming responses [71–74].

Quantitative mass spectrometry identified a distinct difference between the proteomes of GaM-treated *P. aeruginosa* cells versus control cells. Protein-tyrosine-phosphatase was one of the most highly upregulated proteins (37.99-fold), and although the consequence of this is uncertain, the roles of this protein in the cell stress response/resistance, secretion of polysaccharides and biofilm formation have been proposed [75–78]. Indication of a cell stress response was seen through increases in oxidation–reduction via the FMN_red domain-containing protein (8.98-fold) and DNA repair protein methylated-DNA—protein-cysteine *S*-methyltransferase (8.5-fold), which catalyzes cysteine methylation [79]. The methylation process signals for the repair of mutations in the genome induced by drug toxicity and stress [80–82]. Additional counteraction of GaM-induced DNA damage occurred as part of an SOS response via upregulation of UvrABC system protein B (2.3-fold). This component of the UvrABC repair system scans and cleaves abnormalities within DNA [83–85]. Furthermore, upregulation of the heme oxygenase (HemO) component of the heme acquisition system (7.6-fold), biotin synthase (that catalyzes biotin production through sulfur insertion; 7.14-fold) and co-chaperone HscB protein homolog associated with maturation of iron-sulfur clusters (4.83-fold) are possibly indicative of cellular mechanisms employed to counteract inadequate iron supplies in the cell [21, 86–90]. Interestingly, proteomic studies on the response of *P. aeruginosa* to iron-limited conditions also showed upregulation of heme acquisition components (such as HemO) [91], whereas a previous study utilised gallium porphyrins to disrupt heme uptake pathways and inhibit growth in *P. aeruginosa* [92]. Hemoproteins like haemoglobin provide an additional source of iron and can be degraded and liberated via HemO in the heme acquisition pathway [92, 93]. With the exception of IscU, iron-limitation also resulted in upregulation of iron-sulfur cluster proteins HscAB, IscR and IscX [91]. In contrast, GaM induced expression of the iron storage protein bacterioferritin B (1.95-fold) and TCA cycle component malate synthase GlcB (2.21-fold), both of which were downregulated in iron-limited conditions [91]. Upregulation of isocitrate lyase AceE (1.71-fold) and cysteine biosynthesis protein sulfate adenylyltransferase subunit 2 (1.63-fold; encoded by *cysD*) following GaM exposure coincided with the effects of iron-limitation [91].

Exposure of *P. aeruginosa* to GaM downregulated quorum-sensing (QS), an important cell-to-cell communication system that relies upon a range of signalling molecules that enable bacteria to coordinate the expression of virulence genes in response to cell density [94]. The *las* and *rhl* systems and their respective transcriptional activators, LasR and RhlR, are regulated via *N*-acyl homoserine lactone signalling molecules [95]. These systems mediate a multitude of virulence factors responsible for tissue damage, nutrient acquisition, evasion of host immunity and enhanced dissemination of infection [96]. These include *lasA* (protease A) and *lasB* (elastase), both of which showed significant decreases in abundance (11.83-fold and 16.35-fold, respectively) in GaM-treated *P. aeruginosa*. The alternative QS system, *Pseudomonas* quinolone signal (PQS) system, employs alkyl quinolone signals mediated by *pqsB* and *pqsC* genes [97]. The latter gene, which encodes the 2-heptyl-4(1*H*)-quinolone synthase subunit PqsC protein and is responsible for the synthesis of quinolone signalling molecules 2-heptyl-4(1*H*)-quinolone and 2-heptyl-3(1*H*)-quinolone [98], was decreased in abundance by 2.54-fold (also shown on STRING analysis). Inhibition of PQS genes has been linked to reduced biofilm, elastase, pyocyanin and siderophore virulence factor synthesis [99, 100]. Repression of the transcriptional regulator *pvdS* and hence biosynthesis of pyoverdine has been suggested as one mode of action adopted by GaM in disrupting iron metabolism [14]. Direct inhibition of pyoverdine synthesis was not explicitly identified in this study, however, the association between QS and pyoverdine regulation has previously been outlined [101, 102]. LasR plays roles in the regulation of *lasA* and *lasB* genes and has been shown to mediate pyoverdine biosynthesis [102, 103]. It has been proposed that cell density can play a role in siderophore regulation in the Gram-negative pathogen, *Vibrio vulnificus* [104]. This idea has also been

explored in *Burkholderia cepacia*, *Vibro harveyi* and *P. aeruginosa* [104] and may provide a link between QS inhibition and the suppression of iron uptake via GaM therapy.

The invasive nature and success of *P. aeruginosa* in colonising diverse environments is accounted for by twitching/swarming motilities [105, 106], that are modulated by QS pathways with the aid of flagellar movement, to sense and move towards favourable environments [107, 108]. A number of regulatory genes are required in flagellar biosynthesis including the anti-sigma-28 factor protein (encoded by *flgM*) that regulates expression of the B-type flagellin (encoded by *fliC*) [109], and *flgG*-encoded flagellar basal-body rod protein FlgG which mediates flagellar rod assembly [110, 111]. Downregulation of flagellar basal-body rod protein FlgG, the anti-sigma-28 factor protein and B-type flagellin occurred following GaM treated with fold decreases of −2.1, −1.96 and −2.09, respectively, and are also presented on the STRING network analysis (Fig. 6). In addition, the type 4 fimbrial biogenesis protein PilY2, is a surface sensor and showed a −4.6-fold decrease in abundance in GaM-treated *P. aeruginosa*. Comparisons of iron-limitation and GaM treatment have shown inconsistencies in twitching motility in *Pseudomonas* strains. Studies by [91] correlate with research showing iron-rich environments promote biofilm-forming phenotypes, whereas iron deficiency upregulates twitching motility in *P. aeruginosa* [91, 112]. Iron limitation in *Pseudomonas fluorescens* reduces expression of flagellar motor proteins FliN (fold change ($\log_2$) −1.96) and FliG (fold change ($\log_2$) −1.75) and flagellar M-ring protein FliF (fold change ($\log_2$) −1.74) as examples [113].

While the anti-*Pseudomonal* activity of GaM is well characterised, the potency of the complex in terms of its MIC_{80} value, (> 250 µg/ml), is rather low when compared to some existing antibacterial drugs [114–117]. Synergistic combination therapy with GaM may therefore be more beneficial, particularly for the treatment of resistant pathogens. A novel proteomic approach was utilised here to shed light on an alternative mechanism of action- the disruption of iron metabolism and attenuation of virulence through reduced QS and swarming ability. There is ample evidence of the physiological links between iron and QS pathways in bacteria [118–121] and the exploitation of QS as a drug target is well established [122–125]. The development of novel or re-purposed compounds are imperative to combat AMR in clinically relevant bacteria and the results presented here indicate that GaM is active in vitro and in vivo for the treatment of recalcitrant *P. aeruginosa* infection.

Acknowledgements The production of this publication was supported by funding from a research grant from Science Foundation Ireland (SFI) and is co-funded under the European Regional Development Fund under grant number 12/RC/2275_P2. Q-exactive mass spectrometer was funded under the SFI Research Infrastructure Call 2012; Grant Number: 12/RI/2346 (3).

Author contributions Funding: KK. Experimental design: MP, DG, KK. Experimental procedures: MP, KK. Manuscript draft: MP, KK, DG. Editing and approval of manuscript: MP, KK, DG.

Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare.

Ethical approval Ethical permission was not required for this work.

References

1. Exner M, Bhattacharya S, Christiansen B, Gebel J, Goroncy-Bermes P, Hartemann P, Heeg P, Ilschner C, Kramer A, Larson E, Merkens W, Mielke M, Oltmanns P, Ross B, Rotter M, Schmithausen RM, Sonntag HG, Trautmann M (2017) Antibiotic resistance: what is so special about multidrug-resistant Gram-negative bacteria? GMS Hygiene Infect Control. https://doi.org/10.3205/dgkh000290
2. Ghai I, Ghai S (2018) Understanding antibiotic resistance via outer membrane permeability. Infect Drug Resist 11:523–530. https://doi.org/10.2147/IDR.S156995
3. Breijyeh Z, Jubeh B, Karaman R (2020) Resistance of gram-negative bacteria to current antibacterial agents and approaches to resolve it. Molecules (Basel, Switzerland) 25(6):1340. https://doi.org/10.3390/molecules25061340
4. El Zowalaty ME, Al Thani AA, Webster TJ, El Zowalaty AE, Schweizer HP, Nasrallah GK, Marei HE, Ashour HM (2015) Pseudomonas aeruginosa: arsenal of resistance mechanisms, decades of changing resistance profiles, and future antimicrobial therapies. Future Microbiol 10(10):1683–1706. https://doi.org/10.2217/fmb.15.48
5. Wang T, Hou Y, Wang R (2019) A case report of community-acquired Pseudomonas aeruginosa pneumonia complicated with MODS in a previously healthy patient and related literature review. BMC Infect Dis 19(1):130. https://doi.org/10.1186/s12879-019-3765-1
6. Bassetti M, Vena A, Croxatto A, Righi E, Guery B (2018) How to manage *Pseudomonas aeruginosa* infections. Drugs Context 7:212527. https://doi.org/10.7573/dic.212527
7. Faure E, Kwong K, Nguyen D (2018) *Pseudomonas aeruginosa* in Chronic lung infections: How to adapt within the host? Front Immunol 9:2416. https://doi.org/10.3389/fimmu.2018.02416
8. Sordé R, Pahissa A, Rello J (2011) Management of refractory Pseudomonas aeruginosa infection in cystic fibrosis. Infect Drug Resist 4:31–41. https://doi.org/10.2147/IDR.S16263
9. Moradali MF, Ghods S, Rehm BH (2017) *Pseudomonas aeruginosa* lifestyle: a paradigm for adaptation, survival, and persistence. Front Cell Infect Microbiol 7:39. https://doi.org/10.3389/fcimb.2017.00039
10. Frimmersdorf E, Horatzek S, Pelnikevich A, Wiehlmann L, Schomburg D (2010) How Pseudomonas aeruginosa adapts to various environments: a metabolomic approach. Environ Microbiol 12(6):1734–1747. https://doi.org/10.1111/j.1462-2920.2010.02253.x
11. Melander RJ, Zurawski DV, Melander C (2018) Narrow-spectrum antibacterial agents. Med Chem Commun 9(1):12–21. https://doi.org/10.1039/C7MD00528H
12. Pachori P, Gothalwal R, Gandhi P (2019) Emergence of antibiotic resistance *Pseudomonas aeruginosa* in intensive care unit; a critical review. Genes Dis 6(2):109–119. https://doi.org/10.1016/j.gendis.2019.04.001

13. Beceiro A, Tomás M, Bou G (2013) Antimicrobial resistance and virulence: a successful or deleterious association in the bacterial world? Clin Microbiol Rev 26(2):185–230. https://doi.org/10.1128/CMR.00059-12
14. 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 et al (2018) Gallium disrupts bacterial iron metabolism and has therapeutic effects in mice and humans with lung infections. Sci Trans Med 10(460):eaat7520. https://doi.org/10.1126/scitranslmed.aat7520
15. 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. https://doi.org/10.1016/j.phrs.2016.11.009
16. Kaneko Y, Thoendel M, Olakanmi O, Britigan BE, Singh PK (2007) The transition metal gallium disrupts Pseudomonas aeruginosa iron metabolism and has antimicrobial and antibiofilm activity. J Clin Investig 117(4):877–888. https://doi.org/10.1172/JCI30783
17. Cherayil BJ (2011) The role of iron in the immune response to bacterial infection. Immunol Res 50(1):1–9. https://doi.org/10.1007/s12026-010-8199-1
18. Ganz T, Nemeth E (2006) Regulation of iron acquisition and iron distribution in mammals. Biochem Biophys Acta 1763(7):690–699. https://doi.org/10.1016/j.bbamcr.2006.03.014
19. Puig S, Ramos-Alonso L, Romero AM, Martínez-Pastor MT (2017) The elemental role of iron in DNA synthesis and repair. Metallomics 9(11):1483–1500. https://doi.org/10.1039/c7mt00116a
20. Caza M, Kronstad JW (2013) Shared and distinct mechanisms of iron acquisition by bacterial and fungal pathogens of humans. Front Cell Infect Microbiol 3:80. https://doi.org/10.3389/fcimb.2013.00080
21. 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. https://doi.org/10.3389/fcimb.2013.00075
22. Lau CK, Krewulak KD, Vogel HJ (2016) Bacterial ferrous iron transport: the Feo system. FEMS Microbiol Rev 40(2):273–298. https://doi.org/10.1093/femsre/fuv049
23. Dumas Z, Ross-Gillespie A, Kümmerli R (2013) Switching between apparently redundant iron-uptake mechanisms benefits bacteria in changeable environments. Proc Biol Sci 280(1764):20131055. https://doi.org/10.1098/rspb.2013.1055
24. Chitambar CR, Zivkovic-Gilgenbach Z (1990) Role of the acidic receptosome in the uptake and retention of 67Ga by human leukemic HL60 cells. Can Res 50(5):1484–1487
25. Chikh Z, Ha-Duong NT, Miquel G, El Hage Chahine JM (2007) Gallium uptake by transferrin and interaction with receptor 1. J Biol Inorg Chem 12(1):90–100. https://doi.org/10.1007/s00775-006-0169-7
26. Chitambar CR (2016) Gallium and its competing roles with iron in biological systems. Biochem Biophys Acta 1863(8):2044–2053. https://doi.org/10.1016/j.bbamcr.2016.04.027
27. Harris WR, Pecoraro VL (1983) Thermodynamic binding constants for gallium transferrin. Biochemistry 22(2):292–299. https://doi.org/10.1021/bi00271a010
28. van Amsterdam JA, Kluin-Nelemans JC, van Eck-Smit BL, Pauwels EK (1996) Role of 67Ga scintigraphy in localization of lymphoma. Ann Hematol 72(4):202–207. https://doi.org/10.1007/s002770050161
29. Chitambar CR (2012) Gallium-containing anticancer compounds. Future Med Chem 4(10):1257–1272. https://doi.org/10.4155/fmc.12.69
30. Hayes RL (1978) The medical use of gallium radionuclides: a brief history with some comments. Semin Nucl Med 8(3):183–191. https://doi.org/10.1016/s0001-2998(78)80027-0
31. Timerbaev AR (2009) Advances in developing tris(8-quinolinolato)gallium(iii) as an anticancer drug: critical appraisal and prospects. Metallomics 1(3):193–198. https://doi.org/10.1039/b902861g
32. Hara T (1974) On the binding of gallium to transferrin. Int J Nuclear Med Biol 1(3):152–154. https://doi.org/10.1016/0047-0740(74)90021-7
33. Sephton RG, Harris AW (1975) Gallium-67 citrate uptake by cultured tumor cells, stimulated by serum transferrin. J Natl Cancer Inst 54(5):1263–1266. https://doi.org/10.1093/jnci/54.5.1263
34. Chitambar CR, Seligman PA (1986) Effects of different transferrin forms on transferrin receptor expression, iron uptake, and cellular proliferation of human leukemic HL60 cells. Mechanisms responsible for the specific cytotoxicity of transferrin-gallium. J Clin Investig 78(6):1538–1546. https://doi.org/10.1172/JCI112746
35. Chitambar CR, Wereley JP, Matsuyama S (2006) Gallium-induced cell death in lymphoma: role of transferrin receptor cycling, involvement of Bax and the mitochondria, and effects of proteasome inhibition. Mol Cancer Ther 5(11):2834–2843
36. Kubista B, Schoefl T, Mayr L, van Schoonhoven S, Heffeter P, Windhager R, Keppler BK, Berger W (2017) Distinct activity of the bone-targeted gallium compound KP46 against osteosarcoma cells—synergism with autophagy inhibition. J Exp Clin Cancer Res 36(1):52. https://doi.org/10.1186/s13046-017-0527-z
37. Hart MM, Smith CF, Yancey ST, Adamson RH (1971) Toxicity and antitumor activity of gallium nitrate and periodically related metal salts. J Natl Cancer Inst 47(5):1121–1127
38. Giacani L, Bernstein LR, Haynes AM, Godornes BC, Ciccarese G, Drago F, Parodi A, Valdevit S, Anselmi L, Tomasini CF, Baca AM (2019) Topical treatment with gallium maltolate reduces *Treponema pallidum* subsp. pertenue burden in primary experimental lesions in a rabbit model of yaws. PLoS Neglect Trop Dis 13(1):e0007076. https://doi.org/10.1371/journal.pntd.0007076
39. Finnegan M, Lutz T, Nelson W, Smith A, Orvig C (1987) Neutral water-soluble post-transition-metal chelate complexes of medical interest: aluminum and gallium tris(3-hydroxy-4-pyronates). Inorg Chem 26(13):2171–2176. https://doi.org/10.1021/ic00260a033
40. 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(1):33–47. https://doi.org/10.1155/MBD.2000.33
41. 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(4):1331–1337. https://doi.org/10.1128/AAC.01330-08
42. Myette MS, Elford HL, Chitambar CR (1998) Interaction of gallium nitrate with other inhibitors of ribonucleotide reductase: effects on the proliferation of human leukemic cells. Cancer Lett 129(2):199–204. https://doi.org/10.1016/s0304-3835(98)00104-9
43. Olakanmi O, Kesavalu B, Pasula R, Abdalla MY, Schlesinger LS, Britigan BE (2013) Gallium nitrate is efficacious in murine models of tuberculosis and inhibits key bacterial Fe-dependent enzymes. Antimicrob Agents Chemother 57(12):6074–6080. https://doi.org/10.1128/AAC.01543-13
44. Bernstein LR, van der Hoeven JJ, Boer RO (2011) Hepatocellular carcinoma detection by gallium scan and subsequent treatment by gallium maltolate: rationale and case study. Anticancer Agents Med Chem 11(6):585–590. https://doi.org/10.2174/187152011796011046

45. Jakupec MA, Keppler BK (2004) Gallium in cancer treatment. Curr Top Med Chem 4(15):1575–1583. https://doi.org/10.2174/1568026043387449
46. Merli D, Profumo A, Bloise N, Risi G, Momentè S, Cucca L, Visai L (2018) Indium/Gallium Maltolate effects on human breast carcinoma cells: in vitro investigation on cytotoxicity and synergism with mitoxantrone. ACS Omega 3(4):4631–4640. https://doi.org/10.1021/acsomega.7b02026
47. Chitambar CR, Matthaeus WG, Antholine WE, Graff K, O'Brien WJ (1988) Inhibition of leukemic HL60 cell growth by transferrin-gallium: effects on ribonucleotide reductase and demonstration of drug synergy with hydroxyurea. Blood 72(6):1930–1936
48. Narasimhan J, Antholine WE, Chitambar CR (1992) Effect of gallium on the tyrosyl radical of the iron-dependent M2 subunit of ribonucleotide reductase. Biochem Pharmacol 44(12):2403–2408. https://doi.org/10.1016/0006-2952(92)90686-d
49. Mowa MB, Warner DF, Kaplan G, Kana BD, Mizrahi V (2009) Function and regulation of class I ribonucleotide reductase-encoding genes in mycobacteria. J Bacteriol 191(3):985–995. https://doi.org/10.1128/JB.01409-08
50. Pfeifhofer-Obermair C, Tymoszuk P, Petzer V, Weiss G, Nairz M (2018) Iron in the tumor microenvironment-connecting the dots. Front Oncol 8:549. https://doi.org/10.3389/fonc.2018.00549
51. Wang Y, Yu L, Ding J, Chen Y (2018) Iron metabolism in cancer. Int J Mol Sci 20(1):95. https://doi.org/10.3390/ijms20010095
52. Chitambar CR (2010) Medical applications and toxicities of gallium compounds. Int J Environ Res Public Health 7(5):2337–2361. https://doi.org/10.3390/ijerph7052337
53. Chitambar CR (2004a) Apoptotic mechanisms of gallium nitrate: basic and clinical investigations. Oncology (Williston Park, N.Y.) 18(13 Suppl 10):39–44
54. Başar I, Ayhan A, Bircan K, Ergen A, Taşar C (1991) Transferrin receptor activity as a marker in transitional cell carcinoma of the bladder. Br J Urol 67(2):165–168. https://doi.org/10.1111/j.1464-410x.1991.tb15101.x
55. Habeshaw JA, Lister TA, Stansfeld AG, Greaves MF (1983) Correlation of transferrin receptor expression with histological class and outcome in non-Hodgkin lymphoma. Lancet (London, England) 1(8323):498–501. https://doi.org/10.1016/s0140-6736(83)92191-8
56. Chitambar CR (2004b) Gallium nitrate for the treatment of non-Hodgkin's lymphoma. Expert Opin Investig Drugs 13(5):531–541. https://doi.org/10.1517/13543784.13.5.531
57. Malfetano JH, Blessing JA, Homesley HD, Hanjani P (1991) A phase II trial of gallium nitrate (NSC #15200) in advanced or recurrent squamous cell carcinoma of the cervix. A gynecologic oncology group study. Investig New Drugs 9(1):109–111. https://doi.org/10.1007/BF00194560
58. 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. https://doi.org/10.3389/fcimb.2018.00316
59. 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(12):5568–5580. https://doi.org/10.1128/AAC.00386-11
60. Wang Y, Han B, Xie Y, Wang H, Wang R, Xia W, Li H, Sun H (2019) Combination of gallium(iii) with acetate for combating antibiotic resistant *Pseudomonas aeruginosa*. Chem Sci 10(24):6099–6106. https://doi.org/10.1039/c9sc01480b
61. Arnold CE, Bordin A, Lawhon SD, Libal MC, Bernstein LR, Cohen ND (2012) Antimicrobial activity of gallium maltolate against *Staphylococcus aureus* and methicillin-resistant *S. aureus* and *Staphylococcus pseudintermedius*: an in vitro study. Vet Microbiol 155(2–4):389–394. https://doi.org/10.1016/j.vetmic.2011.09.009
62. Fecteau ME, Aceto HW, Bernstein LR, Sweeney RW (2014) Comparison of the antimicrobial activities of gallium nitrate and gallium maltolate against Mycobacterium avium subsp. paratuberculosis in vitro. Vet J (London, England: 1997) 202(1):195–197
63. Sheehan G, Kavanagh K (2018) Analysis of the early cellular and humoral responses of Galleria mellonella larvae to infection by Candida albicans. Virulence 9(1):163–172. https://doi.org/10.1080/21505594.2017.1370174
64. The Uniprot Consortium (2019) A worldwide hub of protein knowledge. Nucleic Acids Res 47(D1):D506–D515. https://doi.org/10.1093/nar/gky1049
65. Perez-Riverol Y, Csordas A, Bai J, Bernal-Llinares M, Hewapathirana S, Kundu D et al (2018) The PRIDE database and related tools and resources in 2019: improving support for quantification data. Nucleic Acids Res 47(D1):D442–D450. https://doi.org/10.1093/nar/gky1106
66. Deslyper G, Colgan TJ, Cooper AJ, Holland CV, Carolan JC (2016) A proteomic investigation of hepatic resistance to ascaris in a murine model. PLoS Neglect Trop Dis 10(8):e0004837. https://doi.org/10.1371/journal.pntd.0004837
67. Skaar EP (2010) The battle for iron between bacterial pathogens and their vertebrate hosts. PLoS Pathog 6(8):e1000949. https://doi.org/10.1371/journal.ppat.1000949
68. Cohen ND, Slovis NM, Giguère S, Baker S, Chaffin MK, Bernstein LR (2015) Gallium maltolate as an alternative to macrolides for treatment of presumed Rhodococcus equi pneumonia in foals. J Vet Intern Med 29(3):932–939. https://doi.org/10.1111/jvim.12595
69. Browne N, Kavanagh K (2013) Developing the potential of using Galleria mellonella larvae as models for studying brain infection by Listeria monocytogenes. Virulence 4(4):271–272. https://doi.org/10.4161/viru.24174
70. Kavanagh K, Sheehan G (2018) The use of *Galleria mellonella* Larvae to identify novel antimicrobial agents against fungal species of medical interest. J Fungi (Basel, Switzerland) 4(3):113. https://doi.org/10.3390/jof4030113
71. Taszłow P, Vertyporokh L, Wojda I (2017) Humoral immune response of *Galleria mellonella* after repeated infection with *Bacillus thuringiensis*. J Invertebr Pathol 149:87–96. https://doi.org/10.1016/j.jip.2017.08.008
72. Sheehan G, Clarke G, Kavanagh K (2018) Characterisation of the cellular and proteomic response of *Galleria mellonella* larvae to the development of invasive aspergillosis. BMC Microbiol 18(1):63. https://doi.org/10.1186/s12866-018-1208-6
73. Gandra RM, McCarron P, Viganor L, Fernandes MF, Kavanagh K, McCann M, Branquinha MH, Santos A, Howe O, Devereux M (2020) *In vivo* Activity of copper(II), manganese(II), and silver(I) 1,10-phenanthroline chelates against *Candida haemulonii* using the *Galleria mellonella* model. Front Microbiol 11:470. https://doi.org/10.3389/fmicb.2020.00470
74. Fuchs BB, Li Y, Li D, Johnston T, Hendricks G, Li G, Rajamuthiah R, Mylonakis E (2016) Micafungin elicits an immunomodulatory effect in galleria mellonella and mice. Mycopathologia 181(1–2):17–25. https://doi.org/10.1007/s11046-015-9940-z
75. Kiley TB, Stanley-Wall NR (2010) Post-translational control of *Bacillus subtilis* biofilm formation mediated by tyrosine phosphorylation. Mol Microbiol 78(4):947–963. https://doi.org/10.1111/j.1365-2958.2010.07382.x
76. Klein G, Dartigalongue C, Raina S (2003) Phosphorylation-mediated regulation of heat shock response in *Escherichia coli*. Mol Microbiol 48(1):269–285. https://doi.org/10.1046/j.1365-2958.2003.03449.x

77. Standish AJ, Morona R (2014) The role of bacterial protein tyrosine phosphatases in the regulation of the biosynthesis of secreted polysaccharides. Antioxid Redox Signal 20(14):2274–2289. https://doi.org/10.1089/ars.2013.5726
78. Musumeci L, Bongiorni C, Tautz L, Edwards RA, Osterman A, Perego M, Mustelin T, Bottini N (2005) Low-molecular-weight protein tyrosine phosphatases of *Bacillus subtilis*. J Bacteriol 187(14):4945–4956. https://doi.org/10.1128/JB.187.14.4945-4956.2005
79. Yao Q, Zhang L, Wan X, Chen J, Hu L, Ding X, Li L, Karar J, Peng H, Chen S, Huang N, Rauscher FJ 3rd, Shao F (2014) Structure and specificity of the bacterial cysteine methyltransferase effector NleE suggests a novel substrate in human DNA repair pathway. PLoS Pathog 10(11):e1004522. https://doi.org/10.1371/journal.ppat.1004522
80. Schlagman SL, Hattman S, Marinus MG (1986) Direct role of the *Escherichia coli* Dam DNA methyltransferase in methylation-directed mismatch repair. J Bacteriol 165(3):896–900. https://doi.org/10.1128/jb.165.3.896-900.1986
81. Cohen NR, Ross CA, Jain S, Shapiro RS, Gutierrez A, Belenky P, Li H, Collins JJ (2016) A role for the bacterial GATC methylome in antibiotic stress survival. Nat Genet 48(5):581–586. https://doi.org/10.1038/ng.3530
82. Ghosh D, Veeraraghavan B, Elangovan R, Vivekanandan P (2020) Antibiotic resistance and epigenetics: more to it than meets the eye. Antimicrob Agents Chemother 64(2):e02225-e2319. https://doi.org/10.1128/AAC.02225-19
83. Truglio JJ, Croteau DL, Van Houten B, Kisker C (2006) Prokaryotic nucleotide excision repair: the UvrABC system. Chem Rev 106(2):233–252. https://doi.org/10.1021/cr040471u
84. Burby PE, Simmons LA (2019) A bacterial DNA repair pathway specific to a natural antibiotic. Mol Microbiol 111(2):338–353. https://doi.org/10.1111/mmi.14158
85. Crowley DJ, Hanawalt PC (1998) Induction of the SOS response increases the efficiency of global nucleotide excision repair of cyclobutane pyrimidine dimers, but not 6–4 photoproducts, in UV-irradiated *Escherichia coli*. J Bacteriol 180(13):3345–3352. https://doi.org/10.1128/JB.180.13.3345-3352.1998
86. O'Neill M, Bhakta M, Fleming K, 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 109(15):5639–5644. https://doi.org/10.1073/pnas.1121549109
87. 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(12):2265–2276
88. Reyda MR, Fugate CJ, Jarrett JT (2009) A complex between biotin synthase and the iron-sulfur cluster assembly chaperone HscA that enhances in vivo cluster assembly. Biochemistry 48(45):10782–10792. https://doi.org/10.1021/bi901393t
89. Romsang A, Duang-Nkern J, Leesukon P, Saninjuk K, Vattanaviboon P, Mongkolsuk S (2014) The iron-sulphur cluster biosynthesis regulator IscR contributes to iron homeostasis and resistance to oxidants in *Pseudomonas aeruginosa*. PLoS ONE 9(1):e86763. https://doi.org/10.1371/journal.pone.0086763
90. Miller HK, Auerbuch V (2015) Bacterial iron-sulfur cluster sensors in mammalian pathogens. Metallomics 7(6):943–956. https://doi.org/10.1039/c5mt00012b
91. Nelson CE, Huang W, Brewer LK, Nguyen AT, Kane MA, Wilks A, Oglesby-Sherrouse AG (2019) Proteomic analysis of the *Pseudomonas aeruginosa* iron starvation response reveals PrrF small regulatory RNA-dependent iron regulation of twitching motility, amino acid metabolism, and zinc homeostasis proteins. J Bacteriol 201(12):e00754-e818. https://doi.org/10.1128/JB.00754-18
92. Choi SR, Britigan BE, Narayanasamy P (2019) Dual Inhibition of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* iron metabolism using gallium porphyrin and gallium nitrate. ACS Infect Dis 5(9):1559–1569. https://doi.org/10.1021/acsinfecdis.9b00100
93. Wegele R, Tasler R, Zeng Y, Rivera M, Frankenberg-Dinkel N (2004) The heme oxygenase(s)-phytochrome system of *Pseudomonas aeruginosa*. J Biol Chem 279(44):45791–45802. https://doi.org/10.1074/jbc.M408303200
94. Rutherford ST, Bassler BL (2012) Bacterial quorum sensing: its role in virulence and possibilities for its control. Cold Spring Harbor Perspect Med 2(11):a012427. https://doi.org/10.1101/cshperspect.a012427
95. Glessner A, Smith RS, Iglewski BH, Robinson JB (1999) Roles of Pseudomonas aeruginosa las and rhl quorum-sensing systems in control of twitching motility. J Bacteriol 181(5):1623–1629. https://doi.org/10.1128/JB.181.5.1623-1629.1999
96. Turkina MV, Vikström E (2019) Bacteria-host crosstalk: sensing of the quorum in the context of *Pseudomonas aeruginosa* infections. J Innate Immunity 11(3):263–279. https://doi.org/10.1159/000494069
97. Drees SL, Li C, Prasetya F, Saleem M, Dreveny I, Williams P, Hennecke U, Emsley J, Fetzner S (2016) PqsBC, a condensing enzyme in the biosynthesis of the *Pseudomonas aeruginosa* quinolone signal: crystal structure, inhibition, and reaction mechanism. J Biol Chem 291(13):6610–6624. https://doi.org/10.1074/jbc.M115.708453
98. Liu YC, Hussain F, Negm O, Pavia A, Halliday N, Dubern JF, Singh S, Muntaka S, Wheldon L, Luckett J, Tighe P, Bosquillon C, Williams P, Cámara M, Martínez-Pomares L (2018) Contribution of the alkylquinolone quorum-sensing system to the interaction of *Pseudomonas aeruginosa* with bronchial epithelial cells. Front Microbiol 9:3018. https://doi.org/10.3389/fmicb.2018.03018
99. Diggle SP, Winzer K, Chhabra SR, Worrall KE, Cámara M, Williams P (2003) The Pseudomonas aeruginosa quinolone signal molecule overcomes the cell density-dependency of the quorum sensing hierarchy, regulates rhl-dependent genes at the onset of stationary phase and can be produced in the absence of LasR. Mol Microbiol 50(1):29–43. https://doi.org/10.1046/j.1365-2958.2003.03672.x
100. Heeb S, Fletcher MP, Chhabra SR, Diggle SP, Williams P, Cámara M (2011) Quinolones: from antibiotics to autoinducers. FEMS Microbiol Rev 35(2):247–274. https://doi.org/10.1111/j.1574-6976.2010.00247.x
101. Whiteley M, Lee KM, Greenberg EP (1999) Identification of genes controlled by quorum sensing in *Pseudomonas aeruginosa*. Proc Natl Acad Sci USA 96(24):13904–13909. https://doi.org/10.1073/pnas.96.24.13904
102. Stintzi A, Evans K, Meyer JM, Poole K (1998) Quorum-sensing and siderophore biosynthesis in *Pseudomonas aeruginosa*: lasR/lasI mutants exhibit reduced pyoverdine biosynthesis. FEMS Microbiol Lett 166(2):341–345. https://doi.org/10.1111/j.1574-6968.1998.tb13910.x
103. Coin D, Louis D, Bernillon J, Guinand M, Wallach J (1997) LasA, alkaline protease and elastase in clinical strains of *Pseudomonas aeruginosa*: quantification by immunochemical methods. FEMS Immunol Med Microbiol 18(3):175–184. https://doi.org/10.1111/j.1574-695X.1997.tb01043.x
104. Wen Y, Kim IH, Son JS, Lee BH, Kim KS (2012) Iron and quorum sensing coordinately regulate the expression of vulnibactin biosynthesis in Vibrio vulnificus. J Biol Chem 287(32):26727–26739. https://doi.org/10.1074/jbc.M112.374165
105. Zolfaghar I, Evans DJ, Fleiszig SM (2003) Twitching motility contributes to the role of pili in corneal infection caused

by *Pseudomonas aeruginosa*. Infect Immun 71(9):5389–5393. https://doi.org/10.1128/iai.71.9.5389-5393.2003
106. Murray TS, Ledizet M, Kazmierczak BI (2010) Swarming motility, secretion of type 3 effectors and biofilm formation phenotypes exhibited within a large cohort of *Pseudomonas aeruginosa* clinical isolates. J Med Microbiol 59(Pt 5):511–520
107. Chuang SK, Vrla GD, Fröhlich KS, Gitai Z (2019) Surface association sensitizes *Pseudomonas aeruginosa* to quorum sensing. Nat Commun 10(1):4118. https://doi.org/10.1038/s41467-019-12153-1
108. Lakshmanan D, Harikrishnan A, Jyoti K, Idul Ali M, Jeevaratnam K (2020) A compound isolated from *Alpinia officinarum* Hance. inhibits swarming motility of *Pseudomonas aeruginosa* and down regulates virulence genes. J Appl Microbiol 128(5):1355–1365
109. Frisk A, Jyot J, Arora SK, Ramphal R (2002) Identification and functional characterization of flgM, a gene encoding the anti-sigma 28 factor in *Pseudomonas aeruginosa*. J Bacteriol 184(6):1514–1521. https://doi.org/10.1128/jb.184.6.1514-1521.2002
110. Burrage AM, Vanderpool E, Kearns DB (2018) Assembly order of flagellar rod subunits in *Bacillus subtilis*. J Bacteriol 200(23):e00425-e518. https://doi.org/10.1128/JB.00425-18
111. Zhu S, Schniederberend M, Zhitnitsky D, Jain R, Galán JE, Kazmierczak BI, Liu J (2019) In situ structures of polar and lateral flagella revealed by cryo-electron tomography. J Bacteriol 201(13):e00117-e119. https://doi.org/10.1128/JB.00117-19
112. 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(2):662–671. https://doi.org/10.1128/JB.01473-07
113. Lim CK, Hassan KA, Tetu SG, Loper JE, Paulsen IT (2012) The effect of iron limitation on the transcriptome and proteome of *Pseudomonas fluorescens* Pf-5. PLoS ONE 7(6):e39139. https://doi.org/10.1371/journal.pone.0039139
114. Sader HS, Huband MD, Castanheira M, Flamm RK (2017) *Pseudomonas aeruginosa* antimicrobial susceptibility results from four years (2012 to 2015) of the international network for optimal resistance monitoring program in the United States. Antimicrob Agents Chemother 61(3):e02252-e2316. https://doi.org/10.1128/AAC.02252-16
115. Ekkelenkamp MB, Cantón R, Díez-Aguilar M, Tunney MM, Gilpin DF, Bernardini F, Dale GE, Elborn JS, Bayjanov JR, Fluit A (2020) Susceptibility of *Pseudomonas aeruginosa* recovered from cystic fibrosis patients to murepavadin and 13 comparator antibiotics. Antimicrob Agents Chemother 64(2):e01541-e1619. https://doi.org/10.1128/AAC.01541-19
116. Mustafa MH, Chalhoub H, Denis O, Deplano A, Vergison A, Rodriguez-Villalobos H, Tunney MM, Elborn JS, Kahl BC, Traore H, Vanderbist F, Tulkens PM, Van Bambeke F (2016) Antimicrobial susceptibility of *Pseudomonas aeruginosa* isolated from cystic fibrosis patients in Northern Europe. Antimicrob Agents Chemother 60(11):6735–6741. https://doi.org/10.1128/AAC.01046-16
117. Pathmanathan SG, Samat NA, Mohamed R (2009) Antimicrobial susceptibility of clinical isolates of *Pseudomonas aeruginosa* from a Malaysian Hospital. Malaysian J Med Sci 16(2):27–32
118. Oglesby AG, Farrow JM 3rd, Lee JH, Tomaras AP, Greenberg EP, Pesci EC, Vasil ML (2008) The influence of iron on *Pseudomonas aeruginosa* physiology: a regulatory link between iron and quorum sensing. J Biol Chem 283(23):15558–15567
119. Kaur AP, Lansky IB, Wilks A (2009) The role of the cytoplasmic heme-binding protein (PhuS) of *Pseudomonas aeruginosa* in intracellular heme trafficking and iron homeostasis. J Biol Chem 284(1):56–66. https://doi.org/10.1074/jbc.M806068200
120. Bollinger N, Hassett DJ, Iglewski BH, Costerton JW, McDermott TR (2001) Gene expression in *Pseudomonas aeruginosa*: evidence of iron override effects on quorum sensing and biofilm-specific gene regulation. J Bacteriol 183(6):1990–1996
121. Juhas M, Wiehlmann L, Huber B, Jordan D, Lauber J, Salunkhe P, Limpert AS, von Götz F, Steinmetz I, Eberl L, Tümmler B (2004) Global regulation of quorum sensing and virulence by VqsR in *Pseudomonas aeruginosa*. Microbiology (Reading, England) 150(Pt 4):831–841
122. Jiang Q, Chen J, Yang C, Yin Y, Yao K (2019) Quorum sensing: a prospective therapeutic target for bacterial diseases. Biomed Res Int 2019:2015978. https://doi.org/10.1155/2019/2015978
123. Asif M, Acharya M (2012) Quorum sensing: a nobel target for antibacterial agents. Avicenna J Med 2(4):97–99. https://doi.org/10.4103/2231-0770.110743
124. Suga H, Smith KM (2003) Molecular mechanisms of bacterial quorum sensing as a new drug target. Curr Opin Chem Biol 7(5):586–591. https://doi.org/10.1016/j.cbpa.2003.08.001
125. Imperi F, Massai F, Ramachandran Pillai C, Longo F, Zennaro E, Rampioni G, Visca P, Leoni L (2013) New life for an old drug: the anthelmintic drug niclosamide inhibits *Pseudomonas aeruginosa* quorum sensing. Antimicrob Agents Chemother 57(2):996–1005. https://doi.org/10.1128/AAC.01952-12