Antibacterial Activities of Ga(III) against E. coli Are Substantially Impacted by Fe(III) Uptake Systems and Multidrug Resistance in Combination with Oxygen Levels

Antibacterial activities of gallium Ga(III) against *E. coli* are substantially impacted by ferric Fe(III) uptake systems and multidrug resistance (MDR) in combination with oxygen levels

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ABSTRACT

The continued emergence and spread of antimicrobial resistance (AMR) – particularly multidrug resistant (MDR) bacteria – is an increasing threat driving the search for additional and alternative antimicrobial agents. WHO has categorised bacterial risk levels and includes *Escherichia coli* among the highest priority, making this both a convenient model bacterium as well as a clinically highly relevant species on which to base investigations of antimicrobials. Among many compounds examined for use as antimicrobials, Ga(III) complexes have shown promise. Nonetheless, spectrum of activities, susceptibility of bacterial species, mechanisms of antimicrobial action and bacterial characteristics influencing antibacterial actions are far from being completely understood – these are important considerations for any implementation as an effective antibacterial agent. In this investigation, we show that alteration in growth conditions to physiologically-relevant lowered oxygen (anaerobic) conditions substantially increases minimum inhibitory concentrations (MIC) of Ga(III) required to inhibit growth for 46 wild-type *E. coli* strains. Several studies have implicated a Trojan horse hypothesis wherein bacterial Fe uptake systems have been linked to promotion of Ga(III) uptake and resultant enhanced antibacterial activity. Our studies show that, conversely, carriage of accessory Fe uptake systems (Fe_acc) significantly increased the concentrations of Ga(III) required for antibacterial action. Similarly, it is shown that MDR strains are more resistant to Ga(III). Increased tolerance of Fe_acc/MDR strains was apparent under anaerobic conditions. This phenomenon of heightened tolerance has not previously been shown although the mechanisms remain to be defined. Nonetheless, this further highlights the significant contributions of bacterial metabolism, fitness and AMR characteristics and their implications in evaluating novel antimicrobials.

KEYWORDS

Gallium citrate, Antibiotics, Anaerobiosis, Multidrug resistance, Siderophore

Amongst emergent infectious disease issues, antimicrobial resistance (AMR) is recognised as a major growing concern to the extent that development and application of alternative anti-infective agents has been prioritised by World Health Organisation (WHO) [1]. Bacteria categorised as Priority 1 – Critical are all Gram-negative bacteria, a bacterial group for which identification of new antibacterial agents has diminished over recent years although some new agents are among those undergoing clinical evaluation (reviewed in [2]). The heightened concerns around this area are further exemplified through a number of very recent research papers describing identification of efficacious drugs with potential clinical utility [3-7]. Antibiotics of the advanced generation cephalosporin and carbapemen classes are particularly important and these agents include both mainstay and last-resort antibiotics. High priority target organisms are carbapenem-resistant *Acinetobacter baumannii*, carbapenem-resistant *Pseudomonas aeruginosa*, and ESBL (extended spectrum lactamase)-producing & carbapenem-resistant *Enterobacteriaceae* (primarily *Escherichia coli, Klebsiella* and *Enterobacter*). Resistance to these antibiotics continues to emerge rapidly through acquisition of ESBLs such as CTX-M and carbapenemases such as NDM & KPC, amongst others. The rapid emergence of resistant strains is facilitated by lax antibiotic stewardship, carriage of resistances on plasmids and other mobile genetic elements, and on the ease of travelling internationally, all of which is exemplified by *E. coli* ST131 [8] and *K. pneumoniae* ST258 [9]. Hence, additional options to allow for retaining utility and efficacy of existing antibacterial agents as well as development of novel antibacterials are of major importance.

Metal ions, notably gallium (Ga^{3+}), are among alternative compounds that are under assessment as novel antibacterials. For some bacteria in the WHO Critical organisms category (*P. aeruginosa*, *A. baumannii* and *K. pneumoniae*), minimum inhibitory concentrations (MIC) of gallium have been reported over ranges comparable to those of conventional antibiotics [10-16]. To date, less emphasis has been placed towards *E. coli* among the WHO Priority 1 Critical bacteria although available publications [17-20] have

provided highly varied Ga^{3+} MIC for *E. coli* including indications that it may exceed that in other high priority Gram-negative bacteria, being as much as approx. 1000-fold. Extrapolation across these data must be cautious as direct comparison between evaluations of Ga^{3+} MIC is difficult because different studies have considered different strains, assay conditions and Ga^{3+} compounds.

Several Ga^{3+} compounds have been evaluated for antibacterial activities, including two forms (nitrate and citrate) which are the subject of this investigation. Both gallium nitrate and citrate are currently in use for other medical applications, specifically hypercalcaemia [21] or tissue imaging for tumour using radioactive $^{67}Ga/^{68}Ga$ with SPECT/PET scan [22] which support the biocompatibility of gallium. Gallium is also available in DOTA and maltolate forms which have also been approved for selected medical uses [23,24]. Gallium chelated to carboxymethyl cellulose as a delivery system also shows potent growth inhibitory activities for both planktonic and surface-adherent (biofilm) bacteria [25]. Recently, there has been notable increase in assessing other gallium complexes such as acetate [26], and more particularly gallium complexed with chelators such as desferrioxamine [18,27,28], and protoporphyrin [13,29,30] in addition to citrate. It has been reported that these complexes may take advantage of Trojan Horse delivery of Ga^{3+} to intracellular compartments via bacterial ferric iron uptake systems thus enhancing antibacterial actions of gallium [11,17,27,31].

Many bacterial species possess high affinity uptake mechanisms for one or more of these chelators although carriage of systems for hydroxamates, haem/protoporphyrin and citrate (as well as other metal ion uptake systems) varies across genera, species and even strains within individual species. Taking the Trojan horse mode-of action as a postulate, iron acquisition systems have been a particular target as several studies (for example [10,11,32,33]) have indicated that gallium, as Ga^{3+}, competes with ferric ion, Fe^{3+}, uptake as its central antibacterial modes of action. Although interference in iron uptake appears pivotal, other targets for gallium have been reported including enzymes involved in

quenching reactive oxygen species, respiration, tricarboxylic acid cycle, nucleotide metabolism, and transcription and potentially transcriptional regulators [11,12,17,26,32]. A recent investigation with *E. coli* has indicated a multiplicity of gene products with possible roles in gallium sensitivity and resistance [33] including other transport (influx & efflux) systems.

The apparent pleiotropy of gallium's antibacterial activities has potential usefulness over and above those of conventional antibiotics which each largely target single or closely-related targets. For instance, the multiplicity of targets implies that evolution of acquisition of resistance or tolerance might be delayed or avoided, especially in the context of other selective pressures required for adaptation and survival *in vivo*. In this paper, we examine several relevant aspects that have not been given sufficient consideration to date in respect of antibacterial actions of gallium. Using *E. coli* as a relevant, representative pathogen, particular attention is given to the influence of reduced oxygen levels (anaerobiosis which typifies *in situ* infection conditions) and to the influence of significant fitness capabilities of high affinity Fe^{3+} siderophore uptake systems and multidrug resistance (MDR) on antibacterial activities of Ga^{3+}. This work highlights the significant contribution of bacterial characteristics and physiological conditions as well as the importance of assessing a spectrum of clinically-relevant isolates in ascertaining antibacterial activities of novel agents such as gallium.

RESULTS

Growth conditions and counterion influence antibacterial activity of gallium

In addition to widely used aerobic conditions, we also employed anaerobic conditions (as representative of physiological conditions) in preliminary investigations of antibacterial activities of gallium using a single representative *E. coli* wild-type strain P4 [34]. MICs for Ga^{3+} in the presence of nitrate and chloride counterions were identical whereas citrate

showed higher MIC. Sodium and iron (III) nitrate, chloride and citrate salts across the same concentration range were non-inhibitory for growth. Notably, MICs for gallium (III) nitrate, gallium chloride and citrate-supplemented gallium nitrate (henceforth termed $Ga(NO_3)_3$+Cit) all increased under anaerobic conditions.

Our preliminary MIC assessments also confirmed that Fe^{3+} at concentrations of 1 or 10 μM substantially increased the MIC values for gallium. This MIC uplift was evident under both aerobic and anaerobic conditions although particularly notable under aerobic conditions in which MICs are generally lower. In all cases a gallium excess of at least 60-fold over Fe^{3+} was required to inhibit growth.

Although bactericidal activities (i.e. reduction in viable bacteria numbers, not merely prevention of growth) have been reported in the range of 5-65 μM for *P. aeruginosa* and *A. baumannii* [11,13,29], to our knowledge, MBC for gallium against *E. coli* has not been reported previously. In our assays MBC was shown to be in excess of 100 mM. Specifically, concentrations of 100 mM and 200 mM $Ga(NO_3)_3$ were required for bactericidal activity under aerobic and anaerobic conditions respectively; for $Ga(NO_3)_3$+Cit, the corresponding bactericidal concentrations were 700 mM and >700 mM respectively.

Reduced oxygen (anaerobic) conditions substantially affect (reduce) antibacterial activity of gallium

Based on our preliminary results which were broadly comparable with others', assessments of MIC were extended to evaluate 46 *E. coli* strains which were all wild-type and included carriage of a variety of accessory iron uptake systems and antibiotic resistances as summarised in Supporting Table S1. The distribution of MIC values of $Ga(NO_3)_3$ and $Ga(NO_3)_3$+Cit under aerobic and anaerobic conditions for these *E. coli* strains are presented in Figure 1 and the MIC ranges and MIC50 and MIC90 values are summarised in Table 1. Supporting Table S3 presents strain by strain MIC result under each condition tested.

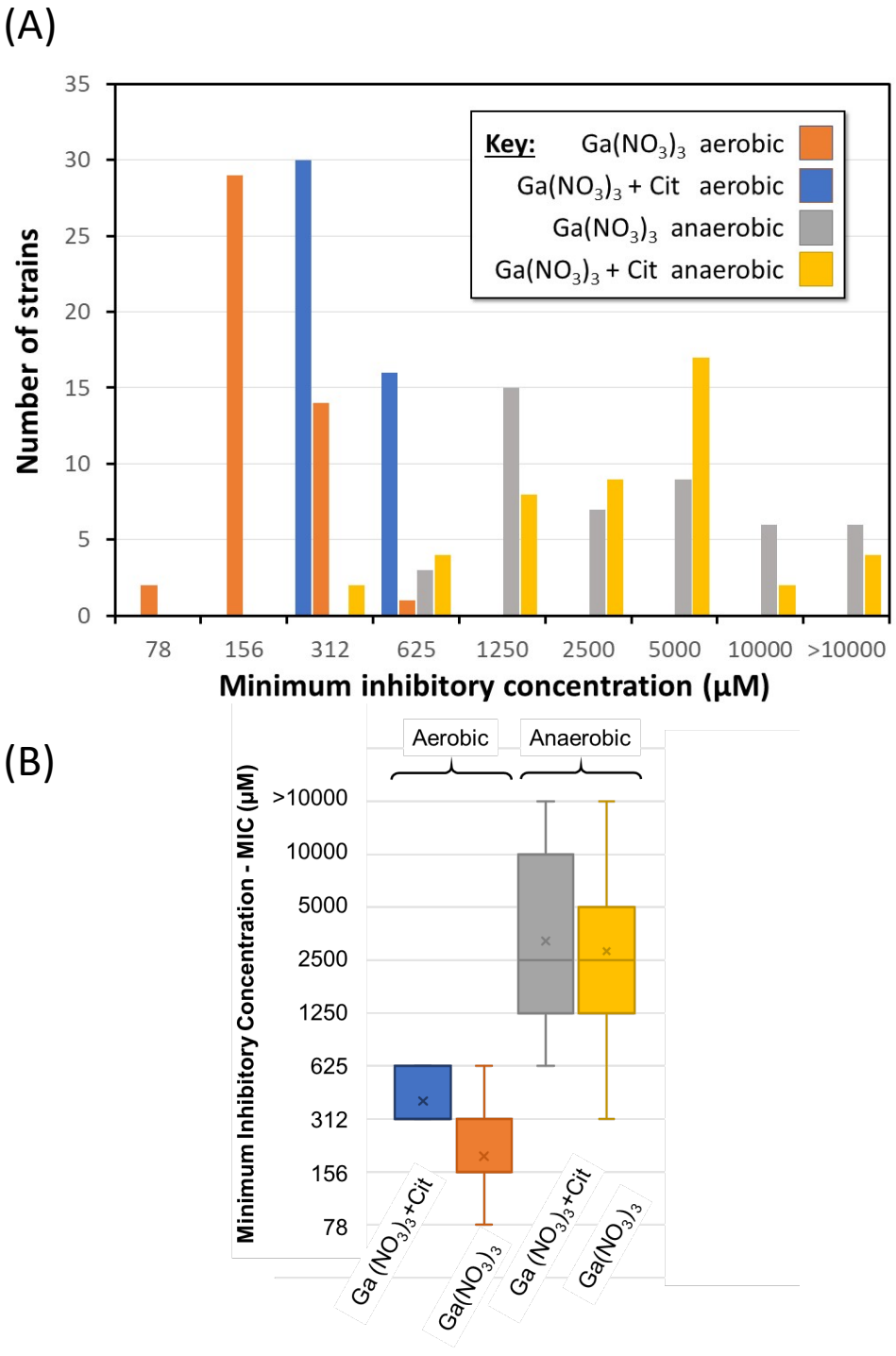

Figure 1 – Distribution of MICs of $Ga(NO_3)_3$ and $Ga(NO_3)_3$+Cit of 46 E. coli isolates exposed under aerobic and anaerobic conditions. (A) x-axis indicates MIC value and y-axis the number of isolates for indicated MIC values. Aerobic/anaerobic and $Ga(NO_3)_3$/$Ga(NO_3)_3$+Cit are indicated in insert panel. (B) box and whisker plot of MIC values.

As can be seen in Figure 1, MIC distribution varies both with counterion and with aerobic/anaerobic conditions. Under aerobic conditions, MIC50 and MIC90 values were similar for both $Ga(NO_3)_3$ and $Ga(NO_3)_3$+Cit (Table 1), being two-fold higher value in the latter; nonetheless, the MICs for $Ga(NO_3)_3$ and $Ga(NO_3)_3$+Cit showed statistical significance ($z > 6$; $p < 0.01$). Culture under anaerobic conditions resulted in substantial increases in MIC50 and MIC90 values for $Ga(NO_3)_3$+Cit of 8-fold and >16-fold while for $Ga(NO_3)_3$ of up 16-fold and 32-fold, this MIC uplift under anaerobic conditions showed statistical significance for both citrate and nitrate ($z > 7$; $p < 0.01$). Under anaerobic conditions, there was substantial overlap in MIC ranges for $Ga(NO_3)_3$ and $Ga(NO_3)_3$+Cit for which there was no statistical significant difference.

Table 1: *Summary of MIC values for 46 wild-type E. coli isolates including MIC50, MIC90 values and MIC ranges. Data for individual strains is presented in Supporting Table S1.*

	Aerobic		**Anaerobic**	
	$Ga(NO_3)_3$+Cit	$Ga(NO_3)_3$	$Ga(NO_3)_3$+Cit	$Ga(NO_3)_3$
MIC50 (μM)	312	156	2500	2500
MIC90 (μM)	625	312	>10000	10000
Range (μM)	312 - 625	78 – 625	625 - >10000	312 - >10000

A strain-by-strain plot of MIC (ranked from low to high values) are presented in Figure 2. Within this plot, a general correspondence can be discerned in MICs observed under aerobic conditions (top two rows), i.e. as MIC to $Ga(NO_3)_3$ increases, MIC to $Ga(NO_3)_3$+Cit shows similar distribution pattern. A similar trend is apparent when comparing $Ga(NO_3)_3$ and $Ga(NO_3)_3$+Cit MICs under anaerobic conditions (bottom two rows).

Plots of MIC ranked from low to high values are presented in Supporting Figures S1 and S2 from which it can be discerned that there are strain-dependent trends, with corresponding increases in MICs to both $Ga(NO_3)_3$ and $Ga(NO_3)_3$+Cit under both aerobic and anaerobic conditions. Supporting Figures S1 and S2 also make evident the major difference in MICs

imposed by anaerobic growth conditions. Indeed, multiple strains which show lower MIC ranking under aerobic conditions show higher MIC ranking under anaerobic conditions and vice versa (Supporting Figure S1). This near inversion of MICs between aerobic and anaerobic conditions (from row 2 to row 3 in Supporting Figure S2) was unexpected.

MIC of gallium is influenced by carriage of ferric (Fe^{3+}) uptake loci in *E. coli*

The implied strain-dependent influences emerging from Figure 2 and Supporting Figure S1 were investigated further, targeting two major distinguishing characteristics. Uptake of transition metals (particularly ferric ions Fe^{3+}) by Gram-negative bacteria such as *E. coli* is dependent on high affinity transport systems, which have been implicated to facilitate Ga^{3+} internalisation [27,33].

The high affinity enterobactin/enterochelin siderophore system is a core component in *E. coli* as are other ferric and ferrous iron receptor/uptake systems (ferric – FhuACDB, FhuE, FhuF, Fiu, CirA; ferrous – FeoABCD, EfeUOB). In addition, *E. coli* strains are variable in possession of accessory iron uptake systems including siderophore systems (salmochelin, aerobactin, yersiniabactin), haem system (*chu*) and ferrous iron uptake systems (SitABCD, EitABCD). Among the isolate panel examined, 19 carried one or more accessory iron uptake systems (full details in Supporting Table S2). Figure 2 presents the MIC distributions of strains based on absence/presence of accessory iron uptake system; MIC range, MIC50 and MIC values are summarised in Supporting Table S4. Statistical evaluation versus the strains lacking accessory systems showed significantly ($z < -2$; $p<0.05$) greater MIC to both $Ga(NO_3)_3$ and $Ga(NO_3)_3$+Cit under anaerobic conditions among strains carrying any of the accessory systems. Targeting evaluation to strains carrying siderophore systems (13 strains) showed additional significance ($z < -2$; $p=0.023$) for higher MIC of $Ga(NO_3)_3$ under aerobic growth conditions.

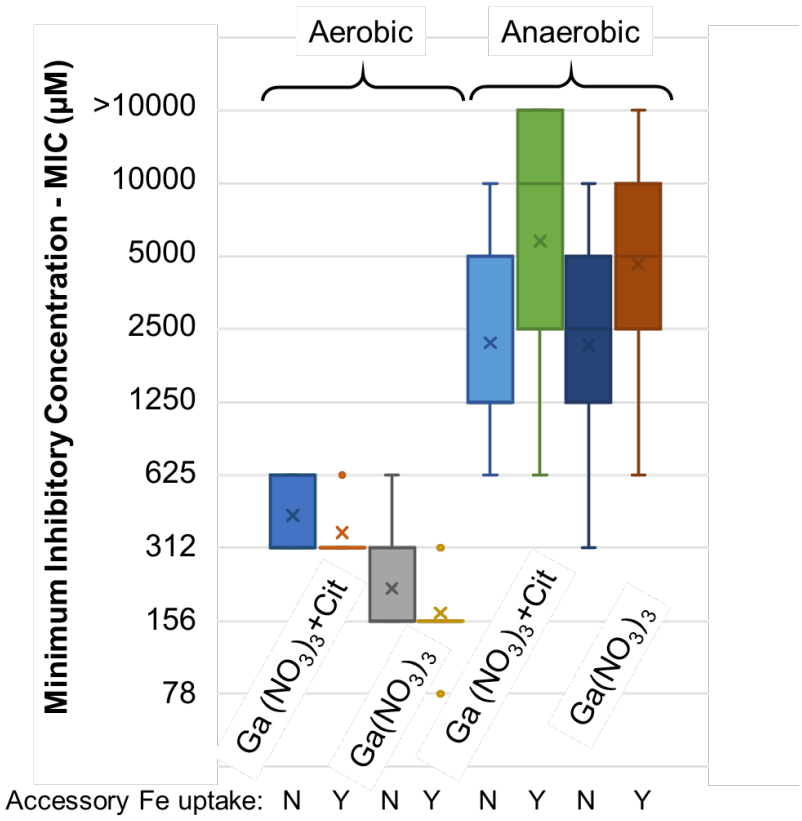

Figure 2: MIC distributions for 46 wild-type E. coli isolates categorised by accessory Fe uptake locus status. Strains naturally lacking any accessory Fe uptake locus are indicated by N; strains naturally possessing one or more accessory locus are indicated by Y. Full details of strain characteristics are as detailed in Supporting Table S2. Aerobic/anaerobic growth conditions and $Ga(NO_3)_3$/ $Ga(NO_3)_3$+Cit are annotated.

Since the gallium citrate formulation is FDA approved while *E. coli* isolates naturally vary in carriage of a ferric citrate transport system (*fecIRABCDE*; alternatively, the FEC locus) [34], we investigated whether this system showed any correspondence to MIC for either $Ga(NO_3)_3$ or $Ga(NO_3)_3$+Cit. Figure 3 summarises MIC distributions of strains based on absence/presence of FEC ferric citrate uptake system; MIC range, MIC50 and MIC values are summarised in Supporting Table S5.

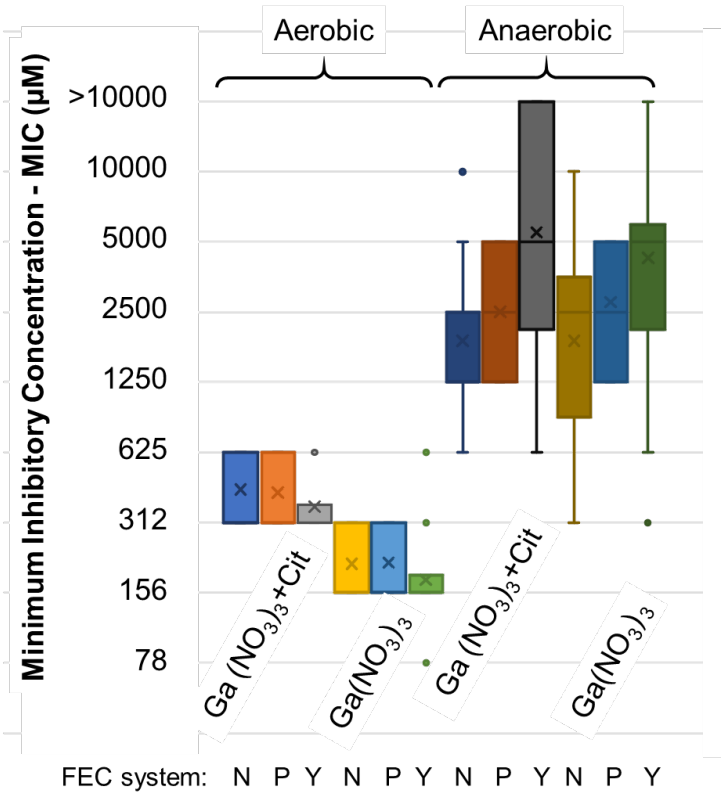

Figure 3: MIC distributions for 46 wild-type E. coli isolates categorised by FEC ferric citrate uptake locus status. Strains naturally lacking FEC locus are indicated by N; strains naturally possessing a partial locus (fecIRA) are indicated by P; strains naturally carrying the entire FEC locus (fecIRABCDE) are indicated by Y. Full details of strain characteristics are as detailed in Supporting Table S2. Aerobic/anaerobic growth conditions and $Ga(NO_3)_3$/$Ga(NO_3)_3$+Cit are annotated.

Under aerobic conditions, a modest though statistically significant higher MIC for $Ga(NO_3)_3$+Cit compared to $Ga(NO_3)_3$ was confirmed for all FEC types ($z > 2$; $p < 0.05$ in all cases). Similarly, strains of all three FEC types showed significantly higher MICs for both $Ga(NO_3)_3$ and $Ga(NO_3)_3$+Cit under anaerobic conditions compared to aerobic conditions ($z > 2$; $p < 0.01$ in all cases). Under anaerobic conditions, significance was reached when comparing FEC-positive versus FEC-negative strains for both $Ga(NO_3)_3$ and $Ga(NO_3)_3$+Cit with the FEC-positive strains showing significantly higher MIC compared to FEC-negative strains ($z > 2$; $p<0.01$). Statistical evaluation also implies that *fecIRA* may be intermediate in MIC characteristics however results did not reach statistical significance.

In addition to the 46 wild-type *E. coli* strains, we also examined whether deletion of the entire FEC locus (*fecIRABCDE; δfec*) in *E. coli* strain P4 (which we produced previously [34])

affected MICs compared to the parent P4 strain. As presented in Supporting Table S3, parent strain and *δfec* showed identical MIC values for all compound-condition combinations suggesting that it may not be FEC locus *per se* but rather that co-selected characteristics may be responsible for differing MICs among FEC-positive and -negative strains.

Multidrug resistance (MDR) in *E. coli* contributes to MIC of gallium

In Enterobacteriaceae such as *E. coli*, multidrug resistance (MDR) has been defined as carriage of resistances to three or more classes of antibiotics [35]. As summarised in Supporting Table S1, seven of the strains used in this investigation can be classified as MDR – the other strains either have single resistance (6 strains) or no resistances (33 strains) and are considered together as non-MDR strains. Figure 4 summarises MIC distributions of strains based on MDR status; MIC range, MIC50 and MIC values are summarised in Supporting Table S6.

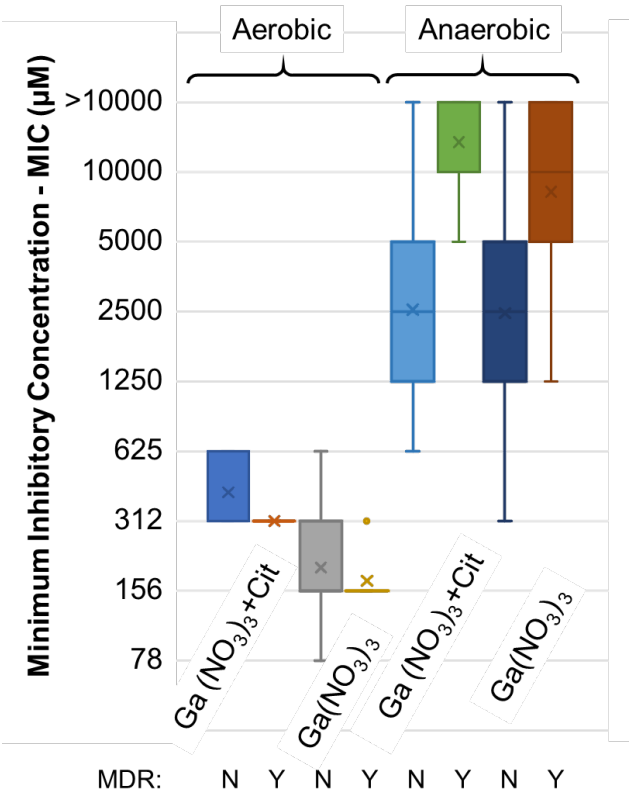

Figure 4: MIC distributions for 46 wild-type E. coli isolates categorised by multidrug-resistance (MDR) status. Strains possessing resistance to three or more classes of antibiotics are classified as MDR and are indicated by Y; other strains are indicated by N; Full details of strain characteristics are as detailed in Supporting Table S1. Aerobic/anaerobic growth conditions and $Ga(NO_3)_3$/$Ga(NO_3)_3$+Cit are annotated.

Statistical evaluation indicated significant difference between MDR and non-MDR strains for $Ga(NO_3)_3$ under anaerobic conditions ($z > 3$; $p < 0.01$) and for $Ga(NO_3)_3$+Cit under both aerobic (z 1.69; p 0.045) and anaerobic ($z > 2.5$; $p < 0.01$) conditions – in each of these cases, the MIC values for MDR strains was significantly higher.

DISCUSSION

With the continually growing concerns associated with antimicrobial resistance (AMR) there is increasing interest in development and implementation of mitigations to limit negative impacts on human health. Among options considered as non-conventional antimicrobial agents either as adjuncts or alternatives to conventional antibiotics are metal ions such as gallium, zinc, silver and among others [36]. In addition to antibacterial activities at potentially therapeutically useful concentrations, gallium (as Ga^{3+}) has proven biocompatibility and has been approved for clinical uses as a therapeutic for treatment of chemotherapy-associated hypercalcaemia [21] and for diagnostic imaging [22].

Heightened concerns around AMR has led to a prioritisation of certain pathogens as key subjects for research and development in aspects of resistance and antimicrobials. The World Health Organisation exemplifies this prioritisation in which highest priority (termed critical) is placed on the Gram-negative bacteria *P. aeruginosa*, *A. baumannii* and Enterobacteria (primarily *E. coli* and *K. pneumoniae*) [1]. Unsurprisingly, antibacterial activities of gallium against these organisms has begun to be explored. To date, most of these investigations have targeted *P. aeruginosa* and *A. baumannii* and have shown antibacterial activity in the 0.5 to 20 $mg.L^{-1}$ (ca. 7 – 280 μM) range [10-15] which is comparable to conventional antibiotics and supports the potential usefulness of gallium for these pathogens. Although less attention has been targeted towards Enterobacteria, *K. pneumoniae* appears to demonstrate similar sensitivity [13,16]. In contrast, reports on the sensitivity of *E. coli* have been inconsistent with some studies showing similar levels of

sensitivity to other Gram-negative bacteria [17] while other reports of substantially greater (approx. 1000-fold) concentrations of Ga^{3+} was required for antibacterial action against *E. coli* [17-20]. Our investigation substantially resolves the issue of *E. coli* sensitivity to Ga^{3+} through examination of an extended panel of wild-type isolates. *E. coli* is not only a highly useful model bacterium but – as noted above – is one of the globally prioritised MDR bacteria. More detailed assessment of the activities of Ga^{3+} against this bacterial species and the bacterial determinants that influence its relative activities against these bacterial species require further elucidation, hence our investigation.

In our preliminary assessments of gallium antibacterial activities against *E. coli* which were carried out under aerobic conditions as routinely used in assessments of antibacterial activities, MICs of 156μM (approx. 40 mg L^{-1}) were obtained for $Ga(NO_3)_3$ and $GaCl_3$ and 312 μM for $Ga(NO_3)_3$+Cit (approx. 80 mg L^{-1}). However, bactericidal concentrations exceeded 100mM, reaching higher than 700mM during anaerobic growth which is more than 1000-fold greater than bactericidal concentrations reported for other Gram-negative bacteria *P. aeruginosa* and *A. baumannii* [11,13,15,29]. Although the MICs we have observed for aerobically-grown *E. coli* are higher than reported for *K. pneumoniae*, *P. aeruginosa* and *A. baumannii*, they were comparable to one previous report for *E. coli* [18] yet around 100-fold greater than another study [17] and 50-fold lower than another [19,20]. Interestingly, the MBC that we observed was more comparable to the growth inhibitory levels reported by Gugala et al. [19,20]. Our observed MBC values suggest that gallium may not be applicable for use in control of *E. coli* infections however the main reported action of gallium is as a bacteriostatic agent and MIC values approach those of conventional antibiotics, at least for aerobically-growing *E. coli*. Therefore, it remains uncertain whether gallium might be adopted for treatment of *E. coli* infections and any such decision would be subject to further validation through detailed *in vitro* and *in vivo* studies, including infection studies.

The culture conditions being used in those previous studies [17-20] differed substantially from other assay protocols including our own which may contribute to the major differences

observed in inhibitory concentrations of gallium. Moreover, there were substantial differences in the panel of isolates/strains used, with ours representing the broadest range of wild-type isolates assessed to date. In addition to supporting the need for inclusion of standardised methodologies between investigations (as has been successfully applied for antibiotic susceptibility testing for many years), the differences in inhibitory activities are interpreted as strongly indicative of significant condition-dependent influences on antibacterial activities of gallium.

Of major significance was the substantial increase in gallium MICs when *E. coli* was cultured anaerobically. This observation extended to an entire panel of 46 wild-type *E. coli* isolates which included strain NCTC 12241 (ATCC 25922) which is widely-used as a reference strain in antibiotic sensitivity testing. Under aerobic conditions, there was a small but significant difference in MIC between $Ga(NO_3)_3$+Cit and $Ga(NO_3)_3$. Of far greater significance was the substantial increase in MIC for both $Ga(NO_3)_3$+Cit and $Ga(NO_3)_3$ when assays were carried out anaerobically, representing an increase in MIC90 of at least 32-fold. This observation has major relevance to infection conditions in which availability of molecular oxygen (used as a terminal electron acceptor in aerobic respiration) is diminished compared to atmospheric levels. For instance, in perfused tissues oxygen levels are typically equivalent to approx. 5% whilst at sites of inflammatory damage and in the colon, availability of oxygen is negligible (anaerobic) [37,38]. *E. coli* is facultatively anaerobic and when oxygen is limited, undergoes a substantial reprogramming in gene expression and metabolism as well as other phenotypes with many of these changes are controlled by the FNR and ArcA regulators [39,40]. In an early study [17], the importance of aerobic respiration on susceptibility to Ga^{3+} was suggested. Our results extend that observation and implicate metabolic activities under physiologically-relevant conditions as significant predicators of the antibacterial actions of Ga^{3+} and potentially other antibacterial compounds.

Reactive oxygen species (ROS) are understood to play significant roles in the actions of many antibacterial agents including conventional antibiotics [41], disinfectants [42] and

metals [43]. However, promotion of ROS is not a direct activity of gallium and its antibacterial mode-of-action is primarily via inhibition of iron metabolism [44]. Other reported targets for gallium include catalase and superoxide dismutase (SOD) [12,45], which play crucial roles in quenching of ROS, produced both through bacterial metabolism and host defences. Participation of enterobactin, the core *E. coli* siderophore system, in protection against ROS has also been reported [46]. Targeting of enterobactin and catalase/SOD by gallium might therefore enhance antibacterial actions of ROS produced by other means. As our experiments identified that growth anaerobically substantially reduced antibacterial activity of Ga^{3+}, we speculate that this may arise through reduced abundance of ROS generated under anaerobic conditions. Specific determinants and mechanisms linking gallium and ROS-mediated antibacterial actions in *E. coli* remains to be defined as does the extent to which other bacterial species differ in these.

Might our observations have implications for other high priority bacterial species? Other WHO critical priority bacteria such as *K. pneumoniae* are also facultative anaerobes and show major reprogramming under varying oxygen levels [47,48]; presumably this species would display similar alterations in MIC although this remains to be tested. *P. aeruginosa* and *A. baumannii* are termed strict aerobes, although they typically infect sites such as respiratory tract, circulatory system and well-perfused tissues (which have equivalent to approx. 5% oxygen); both *P. aeruginosa* [49,50] and *A. baumannii* [51] alter their metabolic activities under reduced oxygen (including anaerobic) conditions to enable survival and replication. Thus, our observation that oxygen availability (and, hence, bacterial metabolism under these conditions) imposes a major influence on antibacterial actions has major implications for the utility of potential inhibitory agents such as gallium. Further investigations including genus, species and strain variables in combination with growth condition variables are required to elucidate.

Within our panel of 46 *E. coli* strains there was sufficient diversity to initiate assessment of two major variables, namely accessory iron uptake systems and multidrug resistance (MDR).

Reported antibacterial actions of gallium highlight interference in Fe uptake, primarily in *P. aeruginosa* [11,52] despite other targets having been reported. Although an early reported observation of gallium inhibitory activity for *E. coli* noted interactions with iron availability [32] there has been little advancement in understanding of inhibitory actions in this bacterial species. Recently, Gugala et al [33] provided a report of determinants of gallium sensitivity in *E. coli* through which transport systems were implicated although this initial work provided no definitive indication of metal ion (Fe^{3+}) uptake systems.

Our own investigation of the contribution of Fe uptake systems to susceptibility of *E. coli* to gallium indicated that carriage of accessory Fe uptake systems singly or in combination correspond to higher MIC values under anaerobic conditions. Considering only the accessory siderophore systems (salmochelin, aerobactin, yersiniabactin), the same anaerobiosis-dependent effect of significant uplifting MIC for $Ga(NO_3)_3$ (but not $Ga(NO_3)_3$+Cit) under aerobic conditions. Although this contrasts with observations in *P. aeruginosa* for which no role for siderophore uptake systems in gallium antibacterial action was evident [11,52], the specific questions and approaches – as well as the bacterial species targeted – was distinct. Perhaps significantly, their studies and ours (as well as others investigating gallium mode of action with other bacterial species) imply substantial organism-dependent influences in gallium susceptibility for which systematic comparison of pathogens and Fe uptake systems (and other putative targets) would be required to resolve.

As indicated above, *E. coli* may produce multiple siderophores each of which, as for *P. aeruginosa*, may play distinct roles in gallium uptake and sensitivity. For instance, the chelation of gallium by the *P. aeruginosa* siderophore pyoverdine attenuates antibacterial activity whereas the siderophore pyochelin promotes anti-pseudomonal action of gallium [53]. Although *E. coli* is typified by producing enterobactin, many wild-type strains also can produce additional siderophores as accessory fitness factors, namely salmochelin, aerobactin and yersiniabactin. There is the additional consideration of xenosiderophores which can be scavenged by these bacteria and have been assessed as carriers for gallium

[27, 28, 31]. Evidently, the contribution of distinct siderophore systems on antibacterial actions of gallium requires detailed, systematic examination similar to *P. aeruginosa* in any evaluation of potential antibacterial utility of gallium across the range of possible target pathogens.

Almost unique to *E. coli* is the ferric citrate uptake (FEC) system which comprises uptake and trans-membrane sensor-signalling relay encoded by the *fecIRABCDE* genome island [54]. This locus is present in approx. 50% of *E. coli* strains either entire or partial as *fecIRA* and – besides its role in iron acquisition – has been shown to contribute to niche adaptation [34]. This locus is also carried on plasmids present in some *K. pneumoniae* isolates [55] and many strains of *P. aeruginosa* have a system with substantial similarities [56]. Comparison of gallium in combination with citrate or nitrate counterions confirmed the slightly lower MIC of $Ga(NO_3)_3$ under aerobic growth conditions for strains irrespective of FEC locus status. Interestingly, it has recently been reported that variants in the *E. coli* FEC system, notably the outer membrane receptor/porin protein FecA, can be selected to confer heightened resistance to gallium [57] under aerobic conditions of growth. Thus sensitivity to gallium may be subject to selective pressure which adds further considerations in any prospective decision for use of this in antibacterial applications. Interestingly, a survey of more than 1000 FecA sequences from *E. coli* sourced via NCBI identifies approx 2% of these possess the N169K or G400S substitutions (as identified by Graves et al [57]; Table S7 lists protein accessions and amino acid substitutions in these positions) thus these variants may be subject to selection in natural environments. The extent to which these naturally-occurring variants may differ in sensitivity to gallium will require further evaluation in wild-type and genetically-manipulated strains. As noted above, *P. aeruginosa* has an uptake system highly similar to ferric citrate uptake [56]. However, recent investigations of gallium resistance in this species did not identify mutations in this locus, rather they identified two unrelated systems: a periplasmic iron transport system and pyochelin siderophore biosynthesis system components [58,59]. These investigations together with our own

indicate clearly that bacterial sensitivity and resistance to gallium is multifaceted and shows genus, species and even strain differences. This further emphasises that such aspects must continue to be considered in detail in evaluating novel antibacterials for their potential utility.

Interestingly, under anaerobic conditions only strains with entire FEC locus show significantly higher MIC to gallium. Under the reducing conditions of an anaerobic atmosphere Fe(II) uptake systems might be expected to predominate. However, it has been shown by multiple investigators that both Fe(II) and Fe(III) uptake systems are expressed simultaneously and are functional during infection conditions [e.g. 34,60] where oxygen levels are negligible. Thus the uptake of Ga(III) may still be facilitated by the Fe(III) siderophore uptake systems. Evidently, this is an area which requires further elucidation as it is highly relevant to the potential for usage of gallium as a therapeutic agent for infections. Further to this, it is worth noting that Ga^{3+} has other reported targets in Gram-negative bacteria including transcriptional regulator proteins, RNA polymerase, metabolic enzymes, respiration components, and catalase [11,12,26,33,45,52]. Although these systems are conserved across bacteria, these also show some divergence at genus, species and even strain levels. These observations further indicate the important contribution of both strain characteristics and environmental conditions, especially oxygen level, on levels of sensitivity to gallium and potentially specific modes of action.

The other variable that we were able to assess for possible contribution to Ga^{3+} susceptibility was multidrug resistance (MDR). Indeed, the accelerating emergence of antimicrobial resistance (AMR), notably MDR, is the major driver for evaluating alternatives such gallium. Although ours is not the only study to evaluate gallium antibacterial activities against MDR pathogens [13,20], it is the first to show that strains with MDR phenotype show significantly higher MIC values to gallium, again particularly evident under anaerobic conditions. To our knowledge this has not been yet been evaluated in other pathogens (particularly WHO priority pathogens) and further systematic evaluation is needed. Caution must be exercised in interpreting our observations, notably because MDR and carriage of accessory Fe uptake

systems are frequently coincident in *E. coli* [61]. Nonetheless, taken together, our results suggest that strains carrying accessory Fe uptake system(s) and/or multidrug resistance have advantageous levels of tolerance to gallium, a finding that may be relevant not merely for *E. coli* but also other pathogens prioritised by WHO and further exemplified by others' findings with *P. aeruginosa* [30,53,57-60]. Further examination of interplay between fitness factors (such as Fe acquisition), antibiotic resistance and gallium efficacy against a range of significant pathogen is an important goal to ensure potential utility of gallium and other alternative antimicrobials.

CONCLUSIONS

Our findings have implications for the use of gallium as an antibacterial either by itself or in combination with other agents. Aspects that largely have been omitted from other investigations of antibacterial activities to date but are significant considerations in evaluations include not only detailed understanding of characteristics of target genera, species and strains but also the contribution of physiologically relevant conditions. The processes by which these contribute to antibacterial actions of gallium are important to consider for any successful development and implementation of such agents in novel, sustainable antibacterial interventions. In summary, in this investigation, the major influence on gallium antibacterial activities was shown during culture under anaerobic conditions which substantially elevated MIC levels although strain-dependent characteristics have significant influence. For *E. coli* it appears that accessory Fe uptake systems including the ferric citrate system FEC and siderophore systems offer an advantage in tolerance or resistance to antibacterial actions of gallium (as nitrate or citrate salts) particularly under anaerobic conditions although any possible mechanisms are, as yet, undefined. The further potential for elevation of resistance via selective pressure [57] presents another significant issue. Similarly, MDR strains show significantly higher MIC than non-MDR strains, again exacerbated under the influence of anaerobiosis. The mechanisms underpinning these

phenotypes remain to be elucidated and have important implications for the development of gallium-based antibacterial stratagems for pathogens prioritised as targets for development of novel antimicrobial agents. The heightened MIC values observed under anaerobic conditions may be an indication of limited usefulness of gallium for certain pathogens and tissues although further investigation is an imperative to clarify many open questions regarding gallium as an alternative to conventional antibacterial agents.

METHODS

Bacterial strains, reagents and culture conditions

The *E. coli* strains used in this investigation are listed in Supporting Table S1 which provides serotype and sequence type. Other selected genotypic characteristics, namely carriage of iron uptake system genes and antibiotic resistances are also indicated. Forty three wild-type isolates of bovine origin are as previously described [32,62]; three additional wild-type strains are included for reference purposes – MG1655, NCTC 12241 (ATCC25922), NCTC11107. Also used in this investigation was an isogenic mutant of the P4 wild-type strain in which the genomic region encoding the ferric citrate uptake system was deleted [34] – this is referred to as *δfec*.

All media and reagents used in this study were purchased from Sigma-Aldrich company Ltd. (Dorset, UK) unless stated otherwise. $Ga(NO_3)_3{\cdot}H_2O$ (Alfa Aesar), $GaCl_3{\cdot}4H_2O$ (Acros), Fe-citrate, $FeCl_3$, $Fe(NO_3)_3$, NaCl, $NaNO_3$, Na_3-citrate, and citric acid solutions were all prepared as stock solution in deionised water and were sterilised by filtration through 0.22 µm syringe filter units. Since gallium citrate is not widely available commercially, $Ga(NO_3)_3$ and Na_3-citrate were combined at equimolar concentrations for some purposes in this study in a similar manner to that employed previously by others [18] – herein, this is termed $Ga(NO_3)_3$+Cit.

Routinely, M9 minimal salts medium, supplemented with 0.8% glucose as a carbon source and 100 mM HEPES buffer pH 7.2 (Gibco) was used for cultures – this medium will be referred

to as M9. Unbuffered M9 medium has been used previously for antimicrobial screening studies [10,33,63]; buffer is included as pH can substantially affect resilience characteristics of *E. coli* [64] and biologically-pertinent behaviour of Ga^{3+} ions in solution [65]. Starter cultures were grown overnight aerobically at 37°C, with shaking at 120 rpm, then diluted into fresh M9 to a final *E. coli* concentration of approx. 10^6 cfu/ml. Where required, anaerobic growth was performed in a Whitely A35 Anaerobic Workstation (Don Whitely Scientific) using anaerobic gas mixture (80% N_2, 10% H_2, 10% CO_2; supplied by BOC). As a facultative anaerobe, *E. coli* can grow robustly in M9 medium under anaerobic conditions by use of alternative terminal electron acceptors such as nitrate. fumarate, dimethyl sulfoxide, trimethylamine N-oxide) and inorganic terminal electron acceptors. Among the latter are nitrate, nitrite, ferric iron and sulphate ions.

Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) determinations

MICs were determined by broth microdilution following EUCAST guidelines, essentially as described previously [66]. A two-fold dilution series across the concentration range of 10 mM (10000 μM) to 39 μM was used for all compounds tested. Bacterial inoculum was 5 x 10^5 cfu/ml. Cultures were incubated overnight, without shaking, at 37°C, either aerobically or anaerobically, and MIC values were determined visually as the concentration where growth of the bacterium was not observable. MIC determinations were performed both aerobically and anaerobically, in quadruplicate on at least three separate occasions. The concentrations which inhibit 50% and 90% of the bacterial strains are termed MIC50 and MIC90 respectively.

MBCs were determined similarly over a concentration range of 700 mM to 10 mM for compounds under test. Following inoculation, cultures were incubated, without shaking, for 16 hours at 37°C either aerobically and anaerobically then samples of 10 μl were taken and inoculated onto LB agar which were incubated for up to a further 72 hours. MBC determinations were performed in duplicate on at least two separate occasions. MIC and MBC values are presented as μM concentration of gallium.

For assessing gallium MIC with Fe supplementation, assays were carried out as described above over using the same gallium concentration range in medium either without iron supplementation or supplemented with 1 μM, 10 μM or 100 μM Fe-citrate or $FeCl_3$ as an iron source.

Data handling, statistical evaluation and strain genomic characterisation

Datasets were managed in Microsoft Excel which was used to produce box-and-whisker plots – in all box-and-whisker figures, boxes indicate1st to 3rd quartile, X indicates the mean, horizontal lines represent median, whiskers indicate minimum and maximum, and dots represent outliers. Nonparametric Mann Whitney U-test was used for statistical evaluation as previously applied in similar evaluations of MIC in *E. coli* [67]. Calculations were performed via the online tool: https://www.socscistatistics.com/tests/mannwhitney/default2.aspx.

Survey of strain genomes for relevant gene content was carried out as described previously [62,68] and sequence resources used are further described in Supporting Materials (Table S2).

Supporting Information

The Supporting Information is available free of charge at XXXX. Strain characteristics (serotype, sequence type, MDR carriage and Fe uptake systems), minimum inhibition concentration, MIC50, MIC90 and MIC ranges against Fe uptake system and MDR characteristics, and strain plots by ascending MIC with ranking. (PDF)

Author contributions

Conceptualisation etc:	DGES, HHPY
Laboratory experimentation	CJN, SH
Data analysis	CJN, HY, TBH, RJG, ECHTL, DGES

Writing and editing DGES, HHPY

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