

Magnesium modulates phospholipid metabolism to promote bacterial phenotypic resistance to antibiotics

Reviewed Preprint

v2 • December 4, 2024

Revised by authors

Reviewed Preprint

v1 • August 13, 2024

Hui Li, Jun Yang, Su-fang Kuang, Huan-zhe Fu, Hui-ying Lin, Bo Peng 

State Key Laboratory of Bio-Control, School of Life Sciences, Guangdong Province Key Laboratory for Pharmaceutical Functional Genes, Southern Marine Science and Engineering Guangdong Laboratory (Zhuhai), Sun Yat-sen University, Higher Education Mega Center, Guangzhou, China • Laboratory for Marine Biology and Biotechnology, Marine Fisheries Science and Food Production Processes, Qingdao Marine Science and Technology Center, Qingdao, China

 https://en.wikipedia.org/wiki/Open_access
 Copyright information

eLife Assessment

The study explored the influence of magnesium on phenotypic antibiotic resistance in two strains of *Vibrios*: *V. alginolyticus* ATCC33787 and *V. parahaemolyticus* VP01. This research is **fundamental** for revealing the phenotypic antibiotic resistance mechanism utilized by the specified model bacteria in elevated levels of magnesium. The study produced **convincing** evidence indicating that in high concentrations of magnesium, the efficacy of selected antibiotics was diminished due to decreased biosynthesis of unsaturated fatty acids and PE, along with an increase in the biosynthesis of PG.

<https://doi.org/10.7554/eLife.100427.2.sa3>

Abstract

Non-inheritable antibiotic or phenotypic resistance ensures bacterial survival during antibiotic treatment. However, exogenous factors promoting phenotypic resistance are poorly defined. Here, we demonstrate that *Vibrio alginolyticus* are recalcitrant to killing by a broad spectrum of antibiotics under high magnesium. Functional metabolomics demonstrated that magnesium modulates fatty acid biosynthesis by increasing saturated fatty acid biosynthesis while decreasing unsaturated fatty acid production. Exogenous supplementation of unsaturated and saturated fatty acids increased and decreased bacterial susceptibility to antibiotics, respectively, confirming the role of fatty acids in antibiotic resistance. Functional lipidomics revealed that glycerophospholipid metabolism is the major metabolic pathway remodeled by magnesium, where phosphatidylethanolamine (PE) biosynthesis is reduced and phosphatidylglycerol (PG) production is increased. This process alters membrane composition, increasing membrane polarization, and decreasing permeability and fluidity, thereby reducing antibiotic uptake by *V. alginolyticus*. These findings suggest the presence of a previously unrecognized metabolic mechanism by which bacteria escape antibiotic killing through the use of an environmental factor.

Introduction

Non-inheritable antibiotic or phenotypic resistance represents a serious challenge for treating bacterial infections. Phenotypic resistance does not involve genetic mutations. Phenotypic resistance does not involve genetic mutations and is transient, allowing bacteria to resume normal growth. Biofilm and bacterial persisters are two phenotypic resistance types that have been extensively studied (Brandis *et al.*, 2023; Corona & Martinez, 2013). Biofilms have complex structures, containing elements that impede antibiotic diffusion, sequestering and inhibiting their activity (Ciofu *et al.*, 2022). Biofilm-forming bacteria and persisters also have distinct metabolic states that significantly reduce their antibiotic susceptibility (Yan & Bassler, 2019). These two types of phenotypic resistance share the common feature in their retarded or even cease of growth in the presence of antibiotics (Corona & Martinez, 2013). However, specific factors that promote phenotypic resistance and allow bacteria to proliferate in the presence of antibiotics remain poorly defined.

Metal ions have a diverse impact on the chemical, physical, and physiological processes of antibiotic resistance (Booth *et al.*, 2011; Lu *et al.*, 2020; Poole, 2017). This includes genetic elements that confer resistance to metals and antibiotics (Poole, 2017) and metal cations that directly hinder (or enhance) the activity of specific antibiotic drugs (Zhang *et al.*, 2014). The metabolic environment can also impact the sensitivity of bacteria to antibiotics (Jiang *et al.*, 2023; Lee & Collins, 2012; Peng *et al.*, 2015; Zhang *et al.*, 2020; Zhao *et al.*, 2021). Light metal ions, such as magnesium, sodium, and potassium, can behave as cofactors for different enzymes (Du *et al.*, 2016) and influence drug efficacy. Heavy metal ions, including Cu^{2+} and Zn^{2+} , confer resistance to antibiotics (Yazdankhah *et al.*, 2014; Zhang *et al.*, 2018). Recent reports suggest that sodium negatively regulates redox states to promote the antibiotic resistance of *Vibrio alginolyticus* (Yang *et al.*, 2018), while actively growing *Bacillus subtilis* cope with ribosome-targeting antibiotics by modulating ion flux (Lee *et al.*, 2019). In Gram-negative bacteria, by contrast, zinc enhances antibiotic efficacy by potentiating carbapenem, fluoroquinolone, and β -lactam-mediated killing (Isaei *et al.*, 2016; Zhang *et al.*, 2014). Magnesium influences bacterial structure, cell motility, enzyme function, cell signaling, and pathogenesis (Wang *et al.*, 2019). This mineral also modulates microbiota to harvest energy from the diet (Garcia-Legorreta *et al.*, 2020), allowing *Bacillus subtilis* to cope with ribosome-targeting antibiotics by modulating ion flux (Lee *et al.*, 2019). However, the role of magnesium in promoting phenotypic resistance is less well understood.

Vibrios inhabit seawater, estuaries, bays, and coastal waters, regions full of metal ions such as magnesium (Kumarage *et al.*, 2022). Magnesium is the second most dissolved element in seawater after sodium. At a salinity of 3.5‰ seawater, the magnesium concentration is about 54 mM (Potis, 1968), and in deep seawater, can be as high as 2,500 mM (Wang *et al.*, 2024). *Vibrio parahaemolyticus* and *V. alginolyticus* are two representative *Vibrio* pathogens that infect humans and aquatic animals, resulting in illness and economic loss, respectively (Grimes, 2020). (Fluoro)quinolones such as balofloxacin are used to treat *Vibrio* infection, however, resistance has emerged due to overuse (Suyamud *et al.*, 2024). Indeed, (fluoro)quinolones are one of China's two primary residual chemicals associated with aquaculture (Liu *et al.*, 2017). *Vibrio* can develop quinolone resistance through mutations in the DNA gyrase gene or through plasmid-mediated mechanisms (Dutta *et al.*, 2021). Thus, the use of *V. parahaemolyticus* and *V. alginolyticus* as bacterial representatives, and balofloxacin as a quinolone-based antibacterial representative, can help to define novel magnesium-dependent phenotypic resistance mechanisms of pathogenic *Vibrio* species.

The current study evaluated whether magnesium induces phenotypic resistance in *Vibrio* species and defined the molecular/genetic basis for this resistance. Genetic approaches, GC-MS analysis of metabolite and membrane remodeling upon antibiotic exposure, membrane physiology, and extensive antimicrobial susceptibility testing were used for the evaluations.

Results

Mg²⁺ promotes phenotypic resistance to antibiotics

Marine environments and agriculture, where antibiotics are commonly used, are rich in magnesium. To investigate whether this mineral impacts antibiotic activity, the minimal inhibitory concentration (MIC) of *V. alginolyticus* ATCC33787 and *V. parahaemolyticus* VP01, which we referred as ATCC33787 and VP01 afterwards, isolated from marine aquaculture, to balofloxacin (BLFX) in Luria-Bertani medium (LB medium) plus 3% NaCl as LBS medium and “artificial seawater” (ASWT) medium that included the major ion species in marine water (Wilson, 1975) (LB medium plus 210 mM NaCl, 35 mM Mg₂SO₄, 7 mM KCl, and 7 mM CaCl₂) were assessed (Suppl. Table 1). The MICs of ATCC33787 to BLFX were 54 and 1 µg/mL in ASWT and LBS medium, respectively. Similarly, the MICs to VP01 were 25 and 3 µg/mL in ASWT and LBS medium, respectively (Fig 1A). The role of exogenous NaCl in antibiotic resistance was investigated previously (Yang *et al.*, 2018). The current study found that the MIC for BLFX was the same in LB and M9 minimal medium (M9 medium) plus 7 mM KCl or 7 mM CaCl₂ (Suppl. Fig 1). However, the MIC for BLFX was higher in ASWT medium supplemented with Mg₂SO₄ or MgCl₂ than in LB medium (Fig 1B). And Mg₂SO₄ or MgCl₂ had no difference on MIC, suggesting it is Mg²⁺ not other ions contribute to the MIC change. The MIC for BLFX increased at higher concentrations of MgCl₂ in ASWT medium. Specifically, adding 50 mM or 200 mM MgCl₂ increased the MIC for BLFX by 200- or 1600-fold, respectively (Fig 1C). At balofloxacin doses of 1.56, 3.125, 6.25, and 12.5 µg, the zone of inhibition decreased with increasing MgCl₂ (Fig 1D). Exogenous MgCl₂ also increased the MICs for other quinolone (*e.g.* nalidixic acid, levofloxacin, ciprofloxacin, ofloxacin, and moxifloxacin) (Fig 1E) and non-quinolone antibiotics including antibacterial peptides (colistin), macrolides (roxithromycin), tetracyclines (oxytetracycline), β-lactams (ceftriaxone, ceftazidime), and aminoglycosides (amikacin, kanamycin, and gentamicin) (Fig 1F). Notably, magnesium had a reduced effect on ceftriaxone and gentamicin than other antibiotics. Importantly, exogenous MgCl₂ also increased MICs of clinic isolates, carbapenem-resistant *Escherichia coli*, carbapenem-resistant *Klebsiella pneumoniae*, carbapenem-resistant *Pseudomonas aeruginosa* and carbapenem-resistant *Acinetobacter baumannii* to balofloxacin (Fig 1G). These findings indicate that Mg²⁺ promotes phenotypic resistance.

MgCl₂ affects bacterial metabolism

To investigate how magnesium regulates phenotypic resistance, other effects of magnesium, including its ability to quench balofloxacin activity and induce efflux pump expression and LPS biosynthesis, were excluded (Suppl. Text; Suppl. Fig 2). While Mg²⁺ is a co-factor for enzymes (Garfinkel and Garfinkel, 1985), whether this mineral can promote antibiotic resistance independent of this function remains unknown.

To better understand how magnesium affects bacterial metabolism, *V. alginolyticus* was cultured in M9 medium in the presence of various amounts of MgCl₂ (0, 0.78, 3.125, 12.5, 50, or 200 mM) and the levels of 54 metabolites were assessed by GC-MS. Five biological and two technical replicates were evaluated for each treatment (Suppl. Fig 3). The levels of 41 metabolites were differential (*p* < 0.05) and are presented as Z-values (Fig 2A; Suppl. Fig 4). Orthogonal partial least square discriminant analysis (OPLS-DA) was conducted that separated the six treatments into three groups: Group 1 included 0 mM, 0.78 mM, and 3.125 mM MgCl₂; Group 2 was a singlet of 12.5

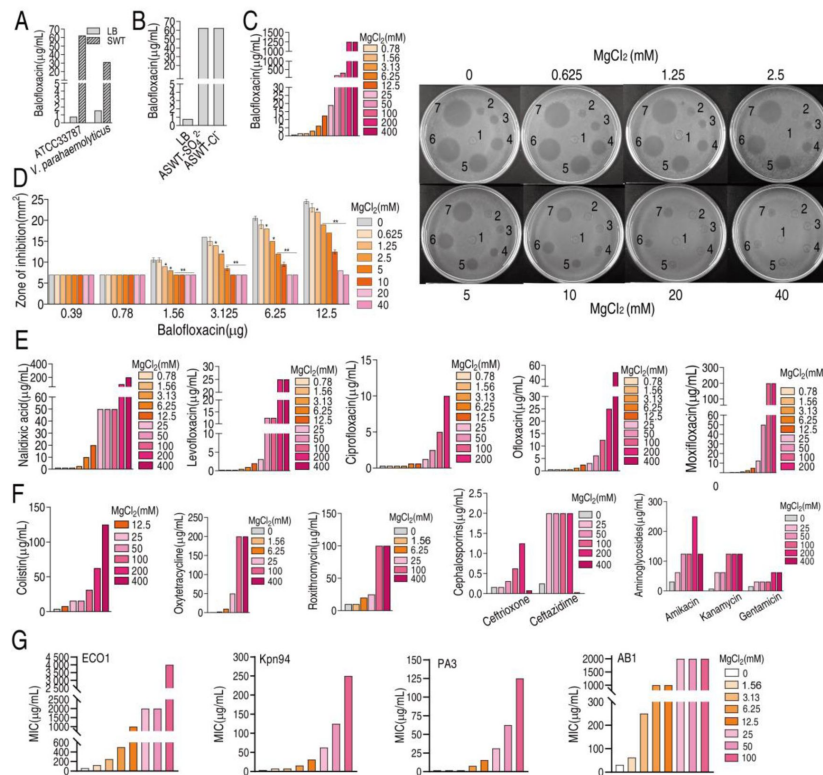


Figure 1.

Magnesium promotes bacterial resistance to antibiotics.

A. MIC of ATCC33787 and *V. parahaemolyticus* to BLFX in ASWT or LB medium as determined by the microtitre-dilution method. **B.** MIC of ATCC33787 to BLFX in ASWT with additional MgSO_4 or MgCl_2 as determined by the microtitre-dilution method. **C.** MIC of ATCC33787 to BLFX in ASWT at the indicated concentrations of MgCl_2 as determined by the microtitre-dilution method. **D.** MIC of ATCC33787 to BLFX at the indicated concentrations of BLFX and MgCl_2 as determined by the Oxford cup test. The numbers 1, 2, 3, 4, 5, 6, and 7 represent 0, 0.39, 0.78, 1.56, 3.125, 6.25, and 12.5 μg BLFX, respectively. **E.** MIC of ATCC33787 to other quinolones in ASWT at the indicated concentrations of MgCl_2 as determined by the microtitre-dilution method. **F.** MIC of ATCC 33787 to other classes of antibiotics in ASWT at the indicated concentrations of MgCl_2 as determined by the microtitre-dilution method. **G.** MIC of carbapenem-resistant *Escherichia coli*, carbapenem-resistant *Klebsiella pneumoniae*, carbapenem-resistant *Pseudomonas aeruginosa*, and carbapenem-resistant *Acinetobacter baumannii* isolates to BFLX at the indicated concentrations of MgCl_2 .

mM; Group 3 included 50 mM and 200 mM MgCl_2 . Component t[1] distinguishes Group 1 from Groups 2 and 3; component t[2] distinguishes Group 3 from Groups 1 and 2 (**Fig 2B** [↗](#)). Discriminating variables are shown as S-plot, where cut-off values are ≥ 0.05 absolute value of covariance p and 0.5 for correlation p(corr). Six biomarkers/metabolites were selected from component t[1] and p[1]. More specifically, the abundance of cadaverine, urea, palmitic acid, aminoethanol, and fumaric acid were increased, but pyroglutamic acid and glutamic acid were decreased (**Fig 2C** [↗](#); **Suppl. Fig 5A** [↗](#)). Pathway enrichment analysis suggests that twelve pathways are involved. Notably, one of the pathways is biosynthesis of unsaturated fatty acids (terminology in the software. Actually, it is biosynthesis of fatty acids) (**Fig 2D** [↗](#)), where the abundance of palmitic acid and stearic acid were increased in an Mg^{2+} dose-dependent manner (**Fig 2E** [↗](#); **Suppl. Fig 6** [↗](#)). Moreover, palmitic acid was a crucial biomarker (**Suppl. Fig 6** [↗](#)). The increase in fatty acid biosynthesis could be partially explained by an imbalanced pyruvate cycle/TCA cycle, in which fumarate levels increased at higher Mg^{2+} while succinate levels increased at lower Mg^{2+} (**Suppl. Fig 5B** [↗](#)). These findings indicated that glycolysis fluxes into fatty acid biosynthesis rather than the pyruvate cycle/TCA cycle. The relevance of fatty acids and BLFX was demonstrated by the observation that exogenous palmitic acid increased bacterial resistance to balofloxacin (**Fig 2F** [↗](#)). These results suggest that fatty acid metabolism may be critical to magnesium-based phenotypic resistance.

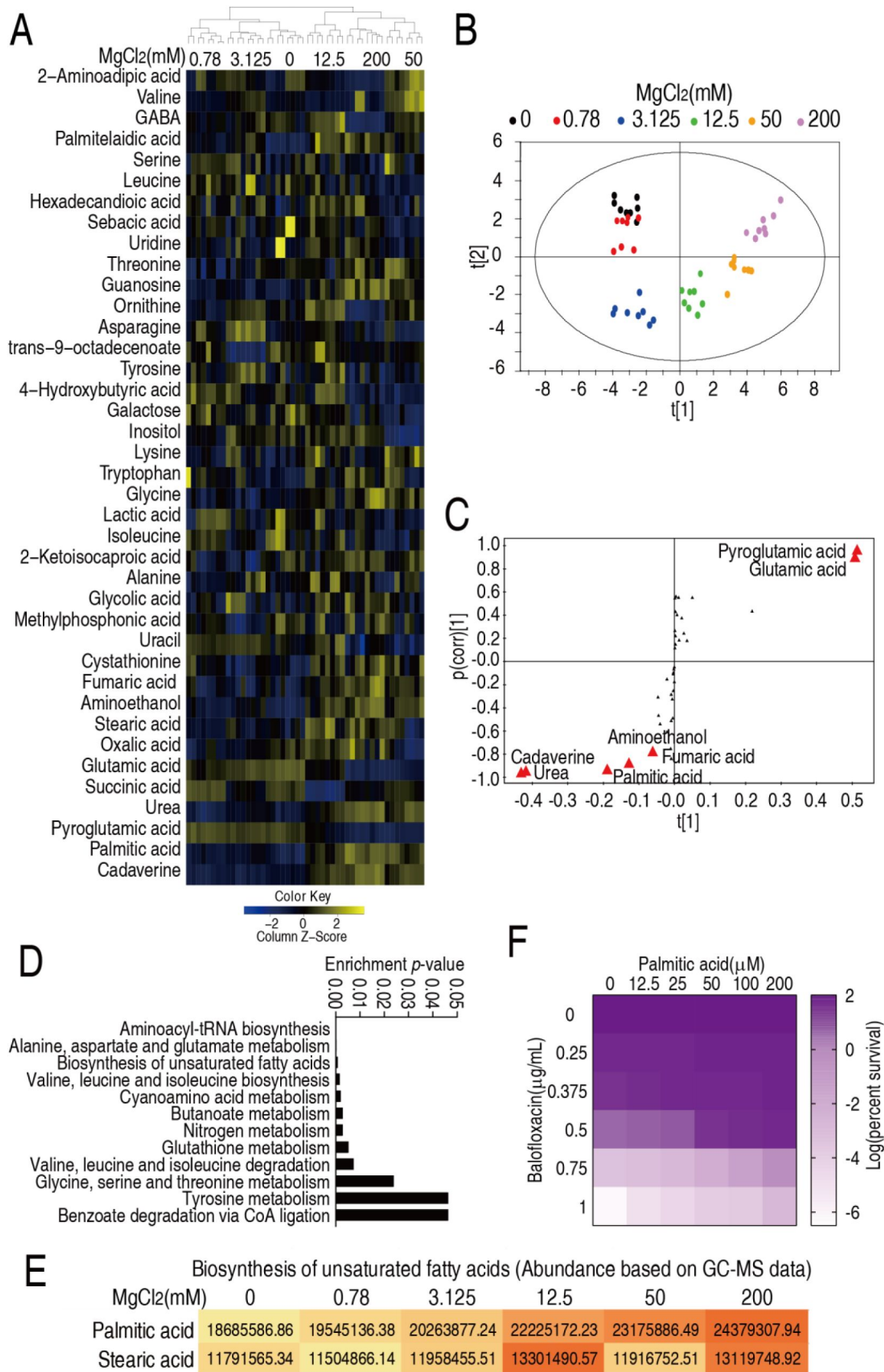


Figure 2.

Mg²⁺-induced metabolomic change.

A. Differential metabolomes in the absence or presence of the indicated concentrations of MgCl₂. The yellow and blue colors indicate an increase or decrease in metabolite levels relative to the median metabolite level, respectively (see color scale). Euclidean distance calculations were used to generate a heatmap that shows clustering of the biological and technical replicates of each treatment. **B.** OPLS-DA analysis of different MgCl₂-induced metabolome concentrations. Each dot represents a technical replicate of samples in the plot. **C.** S-plot generated from OPLS-DA. Predictive component p [1] and correlation p(corr) [1] differentiate 0, 0.78, 3.125 mM MgCl₂ from 12.5, 50, 200 mM MgCl₂. Predictive component p[2] and correlation p(corr)[2] separate 0, 0.78, 50, 200 mM MgCl₂ from 3.125, 12.5 mM MgCl₂. The triangle represents metabolites in which candidate biomarkers are marked. **D.** Enriched pathways by differential abundances of metabolites. **E.** Areas of the peaks of palmitic acid and stearic acid generated by GC-MS analysis. **F.** Synergy analysis for BFLX with palmitic acid for *V. alginolyticus*. Synergy was performed by comparing the dose needed for 50% inhibition of the synergistic agents (white) and non-synergistic (i.e., additive) agents (purple).

Mg²⁺ regulates fatty acid biosynthesis

Acetyl-CoA carboxylase (ACC) catalyzes the conversion of acetyl-CoA to malonyl-CoA, the first step of fatty acid synthesis. Fatty acid biosynthesis, which includes saturated and unsaturated forms, shares common biosynthetic pathways with acetyl-CoA to enoyl-ACP, and is sequentially mediated by *fabD* or *fabH*, *fabB/F*, *fabG*, and *fabA/Z*. The enzyme, enoyl-ACP, produces unsaturated fatty acids or is metabolized to acyl-ACP via *fabV* to produce saturated fatty acids. Unsaturated fatty acids produce saturated fatty acids via *tesA/B* and *yciF* (**Fig 3A**). qRT-PCR was used to quantify the expression of involved genes in bacteria treated with different concentrations of MgCl₂. The expression of 18 genes increased following treatment with 50 and/or 200 mM MgCl₂ (**Fig 3B**). The expression of *tesA*, *tesB*, and *yciA*, which convert unsaturated to saturated fatty acids, increased at 50 or/and 200 mM MgCl₂ (**Fig 3C**). *FabA* and *FabF* play key roles in unsaturated fatty acid and fatty acid biosynthesis, respectively (Feng & Cronan, 2009; Lee *et al.*, 2013). Western blot analysis showed that *FabA* levels declined and *FabF* levels increased at higher MgCl₂ concentrations (**Fig 3D**). Oxazole-2-amine and triclosan inhibit the synthesis of fatty acids and decrease bacterial survival in the presence of balofloxacin (**Fig 3E** and **3F**). These results indicate that Mg²⁺ inhibits the synthesis of unsaturated fatty acids and promotes the synthesis of saturated fatty acids.

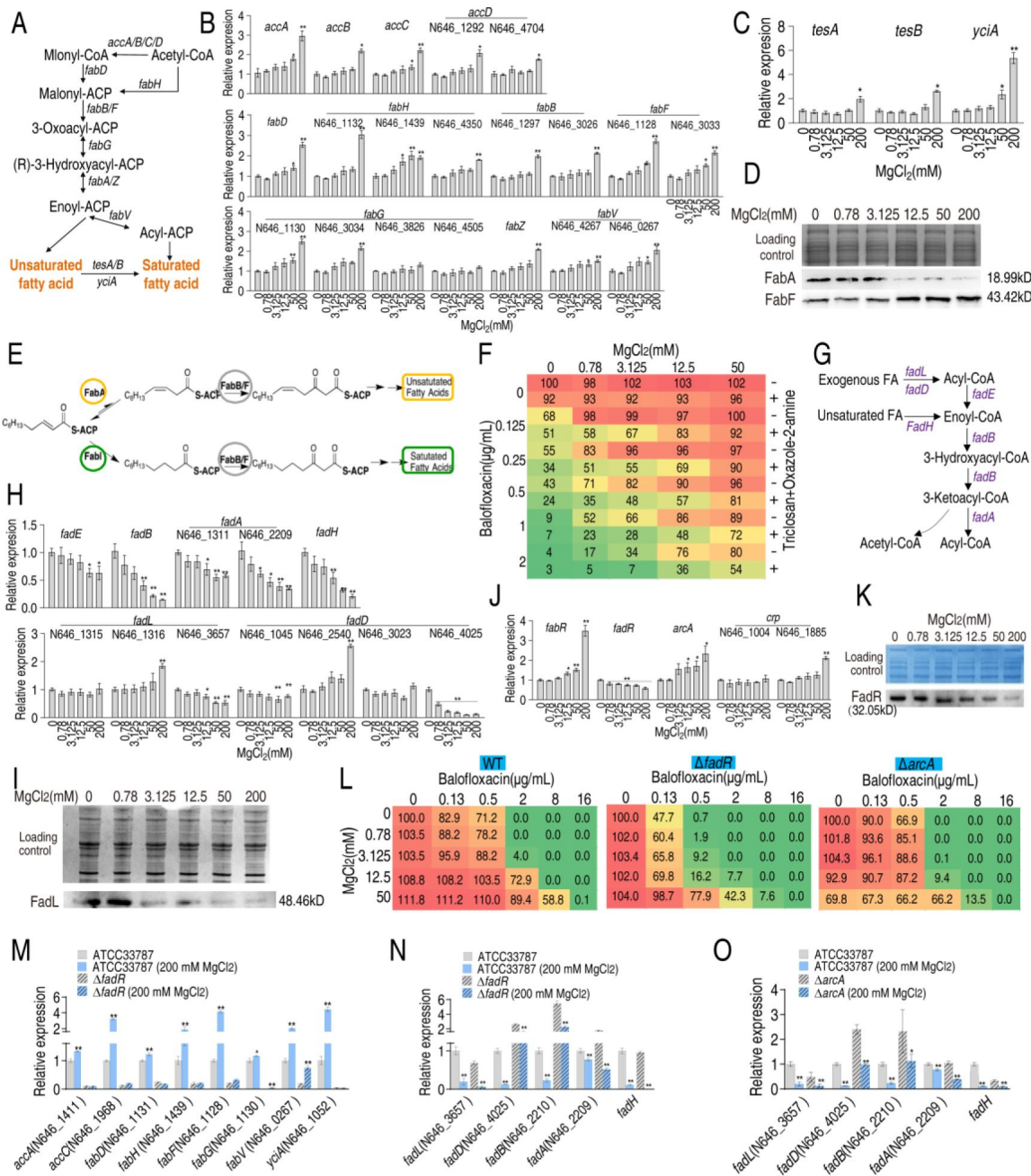


Figure 3.

Mg²⁺ promotes fatty acid biosynthesis.

A. Diagram of fatty acid biosynthesis. **B.** qRT-PCR for expression of fatty acid biosynthesis genes in the absence or presence of indicated concentrations of MgCl₂. **C.** qRT-PCR for expression of genes involved in converting unsaturated fatty acids to saturated fatty acids in the absence or presence of indicated MgCl₂ levels. **D.** Western blot for the abundance of proteins responsible for converting unsaturated fatty acids to saturated fatty acids in the absence or presence of indicated concentrations of MgCl₂. **E.** Diagram of saturated and unsaturated acid biosynthesis. **F.** Synergy analysis of balofloxacin with triclosan + oxazole-2-amine for ATCC33787. The percentage of bacterial survival was quantified in the presence of indicated MgCl₂ levels and/or balofloxacin plus triclosan and oxazole-2-amine and used to construct the isobologram. Synergy is represented using a color scale or an isobologram, which compares the dose needed for 50% inhibition of synergistic agents (blue) and non-synergistic (i.e., additive) agents (red). **G.** Diagram of fatty acid degradation. **H.** qRT-PCR for the expression of genes encoding fatty acid degradation in the absence or presence of the indicated concentrations of MgCl₂. **I.** Western blot for the abundance of FadL in the absence or presence of the indicated concentrations of MgCl₂. **J.** qRT-PCR for the expression of fatty acid biosynthesis regulatory genes in the absence or presence of the indicated concentrations of MgCl₂. **K.** Western blot for the abundance of FadR in the absence or presence of the indicated concentrations of MgCl₂. **L.** Synergy analysis for MgCl₂ with BLFX for ATCC33787 (WT), Δ *fadR*, and Δ *arcA*. Synergy is represented using a color scale or an isobologram, which compares the dose needed for 50% inhibition of the synergistic agents (blue) and non-synergistic (i.e., additive) agents (red). **M, N,** and **O** qRT-PCR for expression of genes involved in fatty acid biosynthesis (**M**) and degradation (**N** and **O**) in ATCC33787, Δ *fadR* or/and Δ *arcA* (**O**) in the presence or absence of 200 mM MgCl₂. Whole cell lysates resolved by SDS-PAGE gel was stained with Coomassie brilliant blue as loading control (**D**), (**I**) and (**K**).

In addition, we also quantified gene expression during fatty acid degradation to determine whether Mg²⁺ affects this process (Fig 3G and 3H). Interestingly, the expression of genes involved in unsaturated fatty acid degradation decreased in an MgCl₂ dose-dependent manner (Fig 3H). Western blot analysis confirmed that FadL levels decreased with increasing MgCl₂ (Fig 3I). FadH degrades unsaturated fatty acids and influences the ratio of unsaturated to saturated fatty acids. Together, these results suggest that MgCl₂ inhibits fatty acid degradation.

FabR, FadR, ArcA, and cAMP/CRP are transcriptional factors involved in regulating fatty acid biosynthesis. FabR inhibits unsaturated fatty acid biosynthesis, FadR promotes fatty acid biosynthesis and inhibits degradation, and ArcA and cAMP/CRP inhibit and promote fatty acid degradation, respectively (Feng and Cronan, 2010; Fujita et al., 2007). The expression of *fabR* and *arcA* increased, while the expression of *fadR* decreased. The expression of N646_1885, which encodes CRP, increased only in the presence of 200 mM MgCl₂ (Fig 3J). Western blot analysis confirmed that FadR levels declined with increasing MgCl₂ concentration (Fig 3K). Viability of Δ *fadR* and Δ *arcA* was reduced in the presence of MgCl₂, balofloxacin, or both in a dose-dependent manner (Fig 3L). Thus, FadR and ArcA may play roles in bacterial resistance to balofloxacin, presumably by a mechanism dependent on the abundance of saturated/unsaturated fatty acids.

To investigate whether Mg²⁺ functions through FadR and ArcA, expression of eight fatty acid biosynthetic genes (*accA*, *accC*, *fabD*, *fabH*, *fabR*, *fabG*, *fabV*, and *yciA*) and five fatty acid degradation genes (*fadL*, *fadD*, *fadB*, *fadA*, and *fadH*) were quantified in wildtype, Δ *fadR* or/and Δ *arcA* bacterial strains. As expected, fatty acid biosynthetic gene expression was reduced in Δ *fadR* cells (Fig 3M). While higher expression of *fadD*, *fadB*, and *fadA* (elevated *fadA* only for Δ *fadR* without 200 mM MgCl₂) was detected in Δ *fadR* or Δ *arcA* strains, the expression of these genes was

negatively regulated by 200 mM MgCl_2 (Fig 3N and 3O). These results indicate that Mg^{2+} promotes fatty acid biosynthesis by positively regulating *fadR*, and inhibits the degradation of fatty acids through *fadR* and *arcA*.

MgCl_2 affects 16-carbon and 18-carbon fatty acid metabolism

To investigate the effect of Mg^{2+} on the biosynthesis of saturated and unsaturated fatty acids, LC-MS was used to quantify the levels of 16-carbon and 18-carbon fatty acids, the main precursors of lipid biosynthesis (Zhang and Rock, 2008b). The abundance of the saturated fatty acid, palmitic acid (C16:0), was higher while the abundance of five unsaturated fatty acids, palmitoleic acid (C16:1), linoelaidic acid (C18:2), linoleic acid (C18:2), alpha-linolenic acid (C18:3), and stearidonic acid (C18:4) was reduced with increasing MgCl_2 concentration (Fig 4A). The levels of stearic acid (C16:0) and vaccenic acid (C18:1) remained unaffected. Total saturated fatty acid levels increased and total unsaturated fatty acid levels decreased with increasing Mg^{2+} (Fig 4B). These results suggest that Mg^{2+} upregulates saturated fatty acid biosynthesis but downregulates unsaturated fatty acid biosynthesis.

Direct exposure to palmitic acid also inhibited balofloxacin-mediated killing (Fig 2F) while linolenic acid promoted balofloxacin-mediated killing in a dose-dependent manner (Fig 4C). Increasing exogenous palmitic acid and linolenic acid increased intracellular palmitic acid and linolenic acid levels, respectively (Fig 4D). When cells were co-treated with palmitic acid, linolenic acid, and balofloxacin, the two fatty acids appeared to antagonize each other, as demonstrated by the Bliss model (Fig 4E and 4F). Furthermore, magnesium had a minimal effect on the antagonistic effect of palmitic acid, linolenic acid, and balofloxacin (Fig 4G), suggesting that this mineral functions through lipid metabolism. These results indicate that saturated and unsaturated fatty acids have an antagonistic effect on balofloxacin resistance.

Mg^{2+} promotes phospholipid biosynthesis

Fatty acids are biosynthetic precursors of lysophosphatidic acid (LPA) and phosphatidic acid (PA), two key cell membrane components (Zhang and Rock, 2008a). Thus, LC-MS was used to assess the effect of Mg^{2+} on membrane lipid composition (Suppl. Fig 7A). Higher Mg^{2+} levels increased the percentage of lipids from 61 to 67%, and saturated fatty acids from 24 to 26%, but decreased the percentage of unsaturated fatty acids from 15 to 8% (Suppl. Fig 7B). The abundance of 11, 32, and 53 lipids was increased in 3.125, 50, and 200 mM MgCl_2 -treated bacteria, respectively, while the abundance of 26, 52, and 107 lipids was decreased in 3.125, 50, and 200 mM MgCl_2 -treated bacteria, respectively (Suppl. Fig 7C). Saturated fatty acids and lipids increased and unsaturated fatty acids decreased in an Mg^{2+} dose-dependent manner (Suppl. Fig 7D). A total of 52 lipids were quantified, including 17 high-abundance lipids (Suppl. Fig 7E).

Phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) had the first and second highest abundance (Suppl. Fig 7E) and PE levels declined while PG levels increased with increasing Mg^{2+} (Fig 5A and 5B). A similar effect was observed for the unsaturated derivatives of PE and PG (Fig 5C). Principal component analysis (PCA) showed that component t [1] differentiated 0 and 3.125 mM Mg^{2+} from 50 and 200 mM Mg^{2+} , while component t [2] separated 50 mM Mg^{2+} from 0 and 200 mM Mg^{2+} and variants in 3.125 mM of Mg^{2+} treatment (Fig 5D). S-plot analysis showed that behenic acid, PG[16:1(9Z)/16:1(9Z)], PG[18:2 (9Z, 12Z)/16:0], PA [14:0/22:6 (4Z, 7Z, 10Z, 13Z, 16Z, 19Z)], and lysoPE(16:0/0:0) were upregulated while linoelaidic acid, palmitoleic acid, PE-NMe(24:0/24:0), PE[16:0/14:1(9Z)], and lysoPA(i-12:0/0:0) were downregulated (Fig 5E). Of these, the abundance of PG[16:1(9Z)/16:1(9Z)], PG[18:2(9Z,12Z)/16:0], and behenic acid increased, while the levels of linoelaidic acid, palmitoleic acid, PE[16:0/14:1(9Z)], and lysoPA decreased with increasing Mg^{2+} levels (Fig 5F). Thus, altered phospholipid abundance may play a role in the effect of Mg^{2+} on antibiotic resistance.

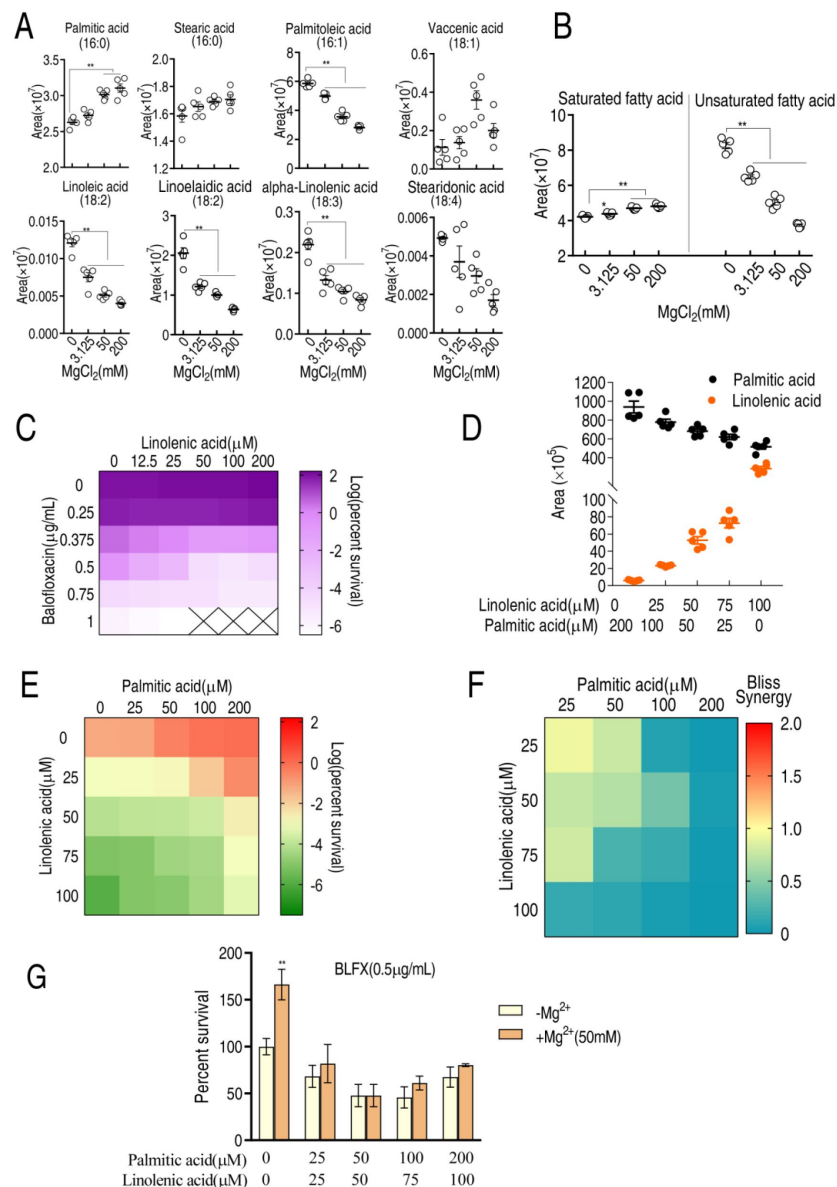


Figure 4.

LC-MS targeted 16-carbon and 18-carbon fatty acids and the role of palmitic acids and linolenic acids in BLFX resistance.

A. Scatter plots of 16-carbon and 18-carbon fatty acids, detected by LC-MS. The area indicates the area of the peak of the metabolite in total ion chromatography using GC-MS. **B.** Scatter plots of total saturated fatty acids and unsaturated fatty acids with 16-carbon and 18-carbon (**A**). **C.** Synergy analysis for BLFX with linolenic acid for ATCC 33787. Synergy is represented using a color scale or an isobologram, which compares the dose needed for 50% inhibition of synergistic agents (while) and non-synergistic (i.e., additive) agents (purple). **D.** LC-MS for the abundance of intracellular linolenic acid and palmitic acid of ATCC33787 in synergy with the indicated exogenous linolenic acid and palmitic acid. **E.** Synergy analysis of linolenic acid and palmitic acid in BLFX-mediated killing to ATCC33787. Synergy is represented using a color scale or an isobologram, which compares the dose needed for 50% inhibition of synergistic agents (blue) and non-synergistic (i.e., additive) agents (red). **F.** Bliss analysis (**E**). **G.** Percent survival of ATCC33787 in the presence of linolenic acid, palmitic acid, and BLFX with or without 50 mM $MgCl_2$.

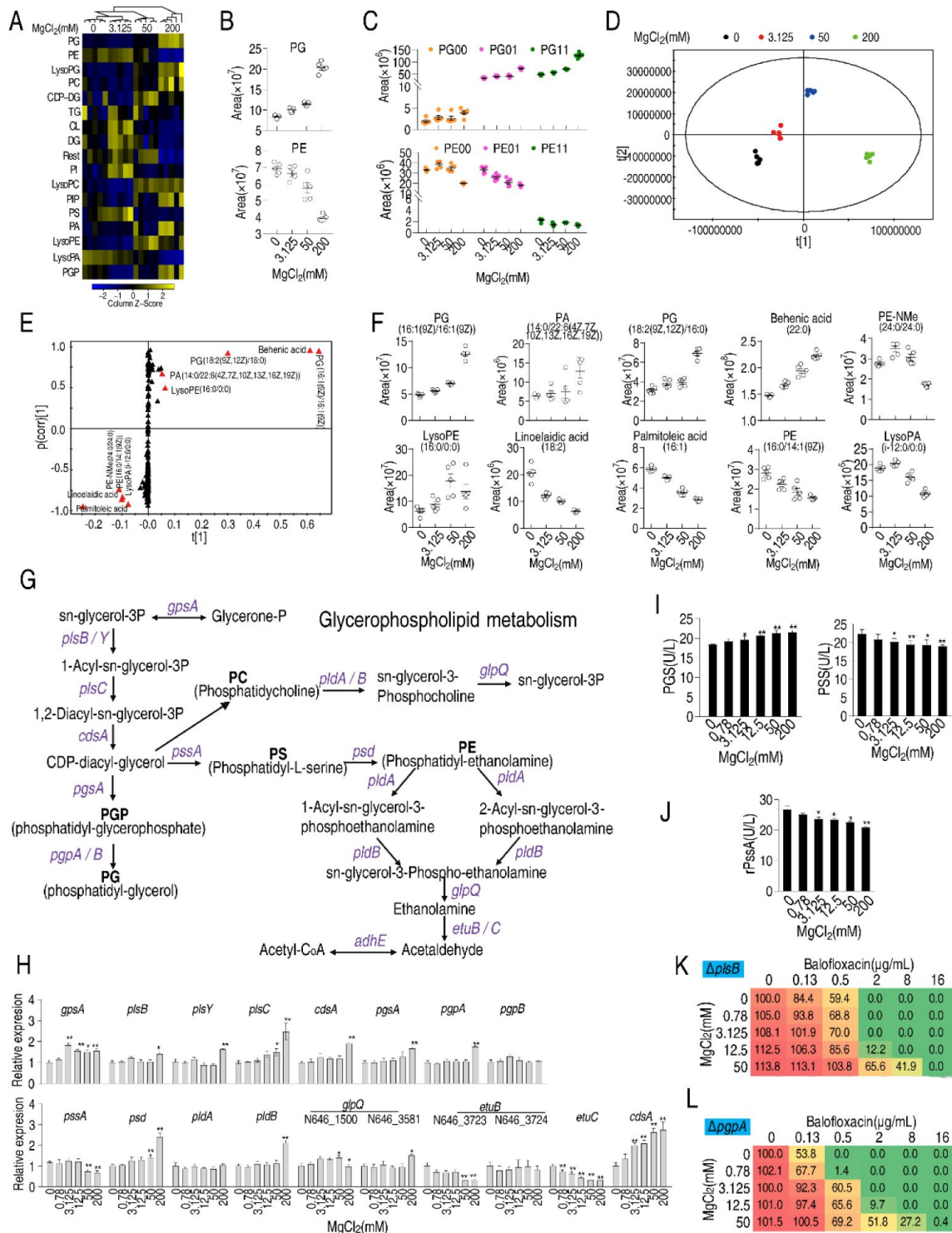


Figure 5.

Effect of Mg²⁺ on phospholipid biosynthesis.

A. Heatmap showing changes in differential lipid levels at the indicated concentration of MgCl₂. **B.** Abundance of ATCC33787 phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) at the indicated concentrations of MgCl₂. **C.** Scatter plots of PG and PE at different saturation levels in the presence of the indicated MgCl₂ levels. **D.** PCA analysis of different concentrations of MgCl₂-induced phospholipids metabolomes. Each dot represents a technical replicate of samples in the plot. **E.** S-plot generated from OPLS-DA. Predictive component *p* [1] and correlation *p* (corr) [1] differentiated 0 and 3.125 mM MgCl₂ from 50 and 200 mM MgCl₂. **F.** Scatter plot of biomarkers in data (**E**). **G.** Diagram showing glycerophospholipid metabolism. **H.** qRT-PCR of the expression of genes encoding glycerophospholipid metabolism in the absence of or at the indicated concentrations of MgCl₂. **I.** PGS and PSS levels in the absence or presence of the indicated concentrations of MgCl₂. **J.** Activity of recombinant PSS in the absence or presence of the indicated concentrations of MgCl₂. **K** and **L** Synergy analysis for MgCl₂ with BLFX for *ΔplsB* (**K**) and *ΔpgpA* (**L**). Synergy is represented using a color scale or an isobologram, which compares the dose needed for 50% inhibition for synergistic agents (blue) and non-synergistic (i.e., additive) agents (red).

PE and PG are the two end products of phospholipid metabolism (**Fig 5G**). While expression of most genes in the phospholipid biosynthetic pathway, including *psd*, *pldB*, and *glpQ*, increased in the presence of 200 mM MgCl₂, the expression of *pssA* and *etuB*, *etuC*, encoding the first enzyme and the last enzymes in the pathway, respectively, remained the same. In addition, the expression of *gpsA*, which encodes GpsA and transforms sn-glycerol-3P to glycerone-P, increased independent of Mg²⁺ concentration (**Fig 5H**). Phosphatidylglycerol phosphate synthase (PGS), encoded by *pgsA*, and phosphatidylserine synthase (PSS), encoded by *pssA*, are critical enzymes for the biosynthesis of PG and PE, respectively. PGS and PSS levels were elevated and reduced, respectively, with increasing Mg²⁺ (**Fig 5I**). The effect of Mg²⁺ on the activity of PSS was confirmed using recombinant PSS (**Fig 5J**). Deletion of *plsB* and *pgpA*, the first and last genes involved in PG biosynthesis, respectively, lowered cell viability in the presence of balofloxacin (note: *Δpsd* could not be obtained) (**Fig 5K** and **5L**). These results indicate that phospholipids may play a role in the effect of Mg²⁺ on balofloxacin resistance; more specifically, PE may inhibit and PG may promote resistance to balofloxacin.

Mg²⁺ regulates membrane polarization, permeability and fluidity to confer balofloxacin resistance

Mg²⁺ remodels membrane composition, which may impair membrane potential and polarization, critical to membrane permeability and uptake of antibiotics (Lee *et al.*, 2019; Peng *et al.*, 2015). The voltage-sensitive dye, DiBAC4(3) showed that 12.5–200 mM MgCl₂ promoted membrane depolarization in a dose-dependent manner (**Fig 6A**). Meanwhile, MgCl₂ had a dose-dependent (**Fig 6B**) and time-dependent (**Fig 6C**) effect on proton motive force (PMF). These results suggest that Mg²⁺-dependent changes in membrane depolarization may influence antibiotic resistance.

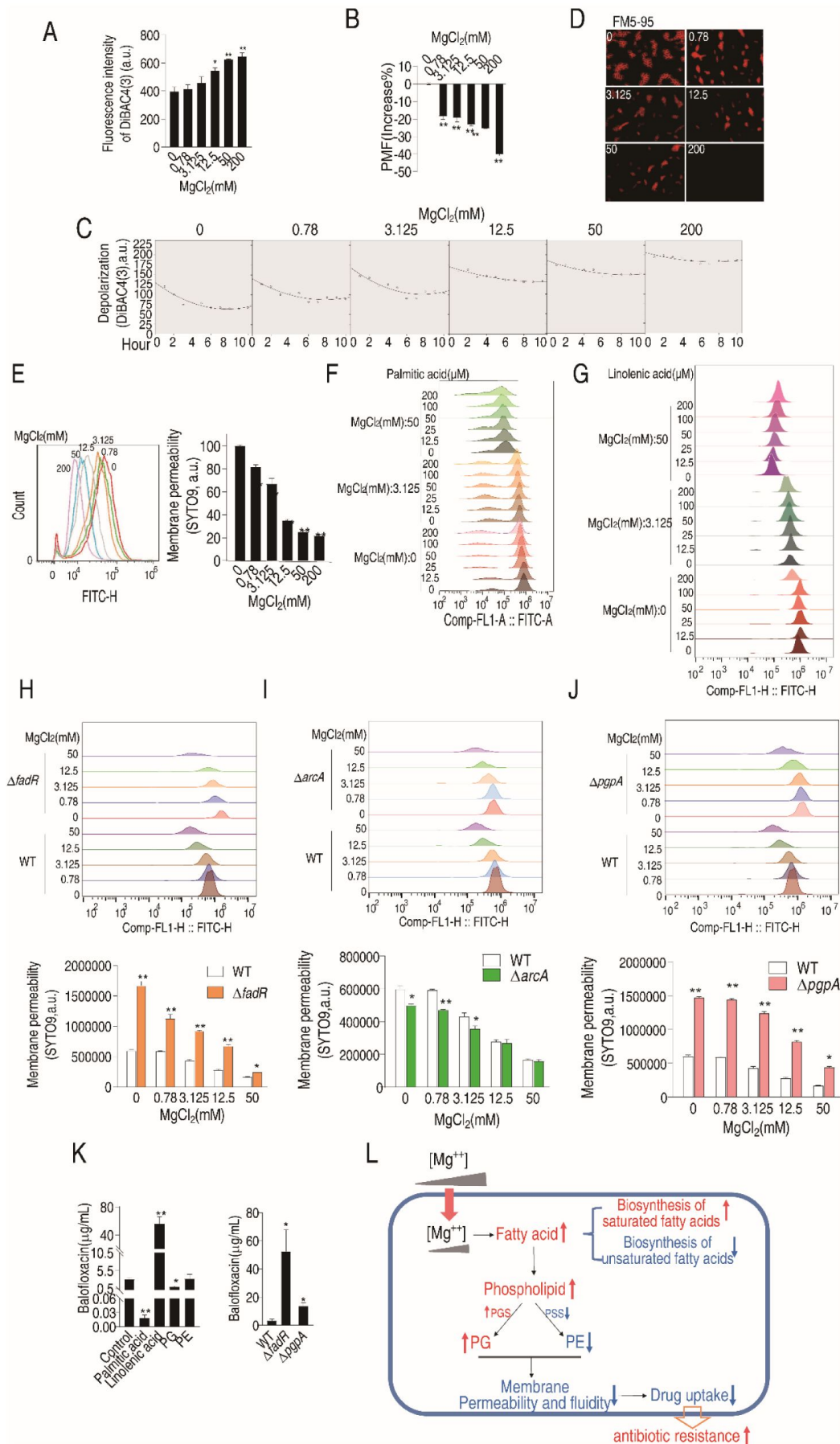


Figure 6.

Mg²⁺ regulates membrane polarization, permeability, and fluidity to confer balofloxacin resistance.

A and **B**. Depolarization (**A**) and PMF (**B**) of ATCC33787 in the absence of or at indicated concentrations of MgCl₂. **C** and **D**. Dynamic depolarization (**D**) Membrane fluidity of ATCC33787 in the absence or presence of indicated concentrations of MgCl₂, as shown by fluorescence microscopy. **E**. Membrane permeability of ATCC33787 in the absence or presence of the indicated concentrations of MgCl₂. **F** and **G**. Membrane permeability of ATCC33787 cultured in palmitic acid (**F**) or linolenic acid (**G**) at the indicated concentrations of MgCl₂. **H** and **I**. Membrane permeability of $\Delta fadR$ (**H**) and $\Delta arcA$ (**I**) in the absence or presence of the indicated concentrations of MgCl₂. **J**. Membrane permeability of $\Delta pgpA$ in the absence or presence of the indicated concentrations of MgCl₂. **K**. Intracellular BLFX of ATCC33787 in the presence of palmitic acid, linolenic acid, PG, and PE (left panel) or $\Delta fadR$ and $\Delta pgpA$ mutants (right panel). **L**. Diagram of mechanisms of Mg²⁺-mediated resistance to BLFX.

The efficacy of antibiotics is strongly influenced by bacterial membrane permeability and fluidity (Saeloh *et al.*, 2018 [↗](#); Zhao *et al.*, 2021 [↗](#)). Thus, fluorescence microscopy was used to visualize these functions after incubating *V. alginolyticus* cells with FM5-95 and various concentrations of MgCl₂. FM5-95 staining decreased with increasing concentrations of Mg²⁺, and no staining was observed in the presence of 200 mM Mg²⁺ (Fig 6D [↗](#)). SYTO9, a green fluorescent dye that binds to nucleic acid, enters and stains bacteria cells when there is an increase in membrane permeability (Lehtinen *et al.*, 2004 [↗](#); McGoverin *et al.*, 2020 [↗](#)). Staining decreased with increasing MgCl₂, indicating that bacterial membrane permeability declined in an Mg²⁺ dose-dependent manner (Fig 6E [↗](#)). These results indicate that exogenous Mg²⁺ reduces bacterial membrane permeability.

Exogenous palmitic acid also shifted the fluorescence signal peaks to the left in an MgCl₂-dependent manner while palmitic acid only slightly shifted the peaks (Fig 7F [↗](#)). In contrast, exogenous linolenic acid shifted the peak to the right in a dose-dependent manner at 50 mM MgCl₂ (Fig 6G [↗](#)). These results indicate that exogenous palmitic acid and linolenic acid decrease or increase membrane permeability, respectively. Membrane permeability was increased in $\Delta fadR$ cells than in WT cells and was reduced by Mg²⁺, remaining higher than the control (Fig 6H [↗](#)). However, $\Delta arcA$ cells had lower membrane permeability at lower MgCl₂ concentrations (Fig 6I [↗](#)). $\Delta pgpA$ cells exhibited higher membrane permeability than control cells at all MgCl₂ concentrations (Fig 6J [↗](#)). These data are consistent with the viability of the mutants described above (Figs 3L [↗](#) and 5L [↗](#)).

Relative membrane permeability appeared to correlate with relative intracellular balofloxacin and antibiotic efficacy. Exogenous palmitic acid and linolenic acid reduced and promoted the uptake of balofloxacin at 159- and 18-fold, respectively (Fig 6K [↗](#)). Loss of *fadR* and *pgpA* increased intracellular balofloxacin (Fig 6K [↗](#)). These data suggest that bacterial membrane permeability is a critical factor required for the efficacy and uptake of balofloxacin, in the presence or absence of exogenous MgCl₂.

Discussion

This study explored the effect of Mg²⁺ on the phenotypic antibiotic resistance of *V. alginolyticus*. Exogenous Mg²⁺ was found to promote saturated fatty acid biosynthesis and inhibit unsaturated fatty acid biosynthesis. Mg²⁺ was also shown to upregulate PGS activity and PG abundance while downregulating PSS activity and PE abundance, decreasing membrane permeability and antibiotic uptake (Fig 6L [↗](#)) and enabling phenotypic resistance. These findings are consistent with the

known impact of exogenous Mg^{2+} on bacterial survival in the presence of balofloxacin and other antibiotics, and the association between membrane permeability and drug efficacy. This study further showed that exogenous fatty acids influence membrane permeability and bacterial survival in the presence of balofloxacin.

Mg^{2+} is the most abundant divalent cation in cells (Pohland and Schneider, 2019; Pontes et al., 2015) playing many essential roles, including stabilizing macromolecular complexes and membranes, binding cytoplasmic nucleic acids and nucleotides, interacting with phospholipid head groups and cell surface molecules, and acting as an essential cofactor in many enzymatic reactions (Groisman et al., 2013). The present study showed for the first time that exogenous Mg^{2+} influences palmitic acid and linolenic acid levels and upregulates PGS while downregulating PSS. These findings extend our current understanding of the biological functions of Mg^{2+} .

Recent findings suggest a link between fatty acid biosynthesis and the antibiotic resistance of *E. tarda* and *V. alginolyticus* (Su et al., 2021). The current study found that Mg^{2+} had opposing effects on the abundance of saturated and unsaturated fatty acids, stimulating saturated fatty acid biosynthesis at moderately high levels, while inhibiting unsaturated fatty acids biosynthesis at ≥ 3 mM. Thus, the ratio of saturated fatty acids to unsaturated fatty acids should help in predicting antibiotic resistance.

Prior studies of Gram-positive and Gram-negative bacteria (Kumariya et al., 2015; Said et al., 1987) found that 10–20 mM Mg^{2+} disrupts *Staphylococcus aureus* membranes and kills stationary-phase *S. aureus* cells but does not influence the survival of *Escherichia coli* and *Bacillus subtilis* (Xie and Yang, 2016). Low concentrations of Mg^{2+} (≤ 10 mM) induce PmrAB-dependent modification of lipid A in wild-type *E. coli* (Herrera et al., 2010). In two mundticin KS-resistant *Enterococcus faecium* mutants, a putative zwitterionic amino-containing phospholipid increased significantly, while phosphatidylglycerol and cardiolipin levels declined (Sakayori et al., 2003). However, the impact of Mg^{2+} on antibiotic resistance by regulating PGS and PSS activity was not reported. More importantly, exogenous $MgCl_2$ was shown to target these enzymes in opposing ways to increase the PE/PG ratio, a previously unknown mechanism of Mg^{2+} -mediated antibiotic resistance.

Prior studies show that in *Stenotrophomonas maltophilia*, PhoP/Q is activated by low magnesium levels and PhoP/Q inhibition or inactivation stimulates β -lactam antibiotic uptake (Huang et al., 2021). The effects of the outer membrane permeabilizers, polymyxin B nonapeptide and EDTA, are completely abolished by 3 mM Mg^{2+} (Kwon and Lu, 2006). In response to Mg^{2+} -limited growth, enteric Gram-negative bacteria show higher lipid A acylation, which alters membrane permeability and reduces the uptake of cationic antimicrobial peptides (Guo et al., 1998). Mg^{2+} reduces the high sensitivity of rough *Salmonella typhimurium*, *S. minnesota*, and *Escherichia coli* O8 (which includes defects in the lipopolysaccharide carbohydrate core) mutants to several antibiotics (Stan-Lotter et al., 1979). Mg^{2+} (1 mM) inhibited aminoglycoside-mediated outer membrane permeabilization in *Pseudomonas aeruginosa* (Hancock et al., 1981). However, mechanisms underlying the impact of Mg^{2+} on antibiotic resistance have remained largely unknown. The current study revealed that Mg^{2+} plays a critical role in regulating the membrane permeability required for antibiotic resistance by modulating PE and PG during phospholipid metabolism.

Aquaculture is an environmental gateway to the development and globalization of antimicrobial resistance due to the excessive use of these drugs to prevent and treat bacterial contaminants (Cabello et al., 2020; Millanao et al., 2018). The current study found that Mg^{2+} increased antibiotic resistance, providing a reasonable explanation of why antibiotics are being excessively used in aquaculture and a possible solution to Mg^{2+} -induced antibiotic resistance by balancing phospholipid metabolism. This finding should promote an overall reduction in antibacterial use that will improve environment and food safety.

In summary, Mg^{2+} modulated the phenotypic resistance of *V. alginolyticus* to balofloxacin and potentially other quinolones and other classes of antibiotics by altering membrane permeability and thus reducing antibiotic uptake. These findings inform the development of methods to improve the therapeutic effect of antibiotics on magnesium-induced phenotypic resistance.

Materials and methods

Bacterial strains and culture

V. alginolyticus ATCC33787 and *V. parahaemolyticus* 01 are from our laboratory collection (Jiang et al., 2022 [↗](#); Kou et al., 2022 [↗](#)). They were cultured in 0.5% yeast broth (HuanKai Microbial, Guangdong, China) (pH 7.0) with 3% NaCl at 30 °C overnight. The cultures were diluted 1:50 (v/v) and grown in fresh 0.5% yeast broth supplemented with desired concentrations of $MgCl_2$ at 30 °C until OD₆₀₀ = 0.6. Bacteria were harvested by centrifugation at 8,000 rpm for 3 min and resuspended in corresponding concentrations of $MgCl_2$ to 0.6 of OD₆₀₀.

Measurement of the minimum inhibitory concentration (MIC) by microtitre-dilution-method

MIC using microtitre-dilution-method was performed as previously described (Zhang et al., 2019 [↗](#)). In brief, the overnight bacterial cultures in 3% NaCl were diluted at 1:100 (v/v) in fresh 0.5% yeast broth supplemented with desired concentrations of $MgCl_2$, cultured at 30 °C and collected when the cells arrived at an OD₆₀₀ of 0.6 in medium without $MgCl_2$. Then, 1×10^5 CFU cells were dispensed into each well of a 96-well microtiter polystyrene tray after which a series of 2-fold dilutions of antibiotic was added. Following incubation at 30 °C for 16 h, the MIC was defined as the lowest antibiotic concentration that inhibited visible growth. Three biological repeats were carried out.

Measurement of MIC by Oxford method

Determination of MIC by Oxford cup was performed as previously described (Liu et al., 2015 [↗](#)). Bacterial cells cultured overnight in yeast medium were diluted at 1:100, shaken at 30 °C and 200 rpm until the OD₆₀₀ nm reached at 0.6. Aliquot of 100 µL cells were spread on 0.5% yeast solid medium containing 3% NaCl and 0, 0.625, 1.25, 2.5, 5, 10, 20, or 40 mM of $MgCl_2$. Oxford cups were placed on the solid medium, and various amounts of balofloxacin (0, 0.39, 0.78, 1.56, 3.125, 6.25, and 12.5 µg) were added. After culturing at 30 °C for 12 h, diameter of the inhibition zone was measured and the inhibition area was calculated.

Measurement of intracellular balofloxacin

Measurement of intracellular balofloxacin was performed by LC-MS analysis and plate-counting assay. For LC-MS analysis, ATCC33787 cultured in the desired concentrations of $MgCl_2$ were collected and adjusted to OD 0.6. Aliquot of 50 mL bacterial cells were added into a 250 mL Erlenmeyer flask and then 300 µL of 10 mg/mL balofloxacin (final concentration is 60 µg/mL). After being cultured for 6 h at 30 °C, these bacterial cells were collected and adjusted to OD 1.0 with 3% NaCl containing corresponding concentrations of $MgCl_2$. Aliquot of 30 mL bacteria were collected and washed three times using mobile phase (acetonitrile: double distilled water containing 0.1mol/l formic acid = 35:65) for LC-MS detection. The bacterial cells were added 1 mL mobile phase, crushed in ice water bath for 10 min (crush for 2S, pause for 3S at 35% power). Following by centrifugation, supernatants were collected, filtered, and then measured by using liquid chromatography for balofloxacin. Different concentrations of balofloxacin were used for a standard curve. For plate-counting assay, ATCC33787 cells cultured overnight were diluted at 1:100, shaken at 30 °C and 200 rpm until the OD₆₀₀ nm reached at 0.6. The cells were diluted at 1:100 and spread on LB agar to preparing ATCC33787 plates. Meanwhile, ATCC33787 cells were

cultured in medium with desired MgCl_2 and at 30 °C and 200 rpm for 6 h. These cells were collected, washed and sonicated. The sonicated solution was added to Oxford cups with the ATCC33787 plates. After culturing at 30 °C for 12 h, diameter of the inhibition zone was measured. A gradient of balofloxacin was used as a standard curve for drug quantification.

Measurement of intracellular magnesium

Measurements of intracellular Mg^{2+} concentration were carried out as previously described with a modification (Yang et al., 2018 [DOI](#)). In brief, *V. alginolyticus* cultured in 0%, 0.78%, 3.125%, 12.5%, 50%, and 200% MgCl_2 were collected, washed, and resuspended in the same concentrations of MgCl_2 to an OD600 of 1.0. Aliquots of 20 mL of bacterial suspensions were centrifuged and washed by ddH_2O once. The resulting cells were weighted, which was designated wet weight. These samples were freeze-dried overnight, which was designated dry weight. Intracellular water volume was calculated using the following formula: $W \times (1 - W/D - 0.23)$ as described previously (Unemoto et al., 1973 [DOI](#)). Two hundred microliters of concentrated nitric acid was added to the dried cells and then heated in 75 °C for 20 min. After diluting the samples 20-fold, they were analyzed for Mg^{2+} concentration by inductively coupled plasma mass spectrometry (ICP-MS) (iCAP 6500, Thermo Fisher). The intracellular Mg^{2+} concentration was calculated according to the volume of intracellular water.

Western blotting

Western blotting was carried out as described previously (Yao et al., 2019 [DOI](#)). Bacterial protein samples were prepared by ultrasound treatment, resolved on a 12% SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare Life Sciences). The membranes were incubated with 1:100 of the primary mouse antibodies, followed by goat anti-mouse secondary antibodies conjugated with horseradish peroxidase. Band intensities were detected by using a chemiluminescence imaging analysis system, Tanon-5200.

Metabolomics analysis

Metabolomics analysis was performed by GC-MS as described previously (Yang et al., 2018 [DOI](#)). Briefly, ATCC33787 were cultured in 0.5% yeast broth with desired MgCl_2 . Equivalent numbers of cells were quenched with 60% (v/v) cold methanol (Sigma) and then centrifuged at 8,000 rpm at 4 °C for 5 min. One milliliter of cold methanol was used to extract metabolites. To do this, the samples were sonicated for 5 min at a 10-W power setting using the Ultrasonic Processor (JY92-IIDN, Scientz, China), followed by centrifugation at 12,000 rpm in 4 °C for 10 min. Supernatants were collected and 10 μL ribitol (0.1 mg per mL, Sigma-Aldrich, USA) was added into each sample as an internal quantitative standard. The supernatants were concentrated for metabolite derivatization and then used for GC-MS analysis. Every experiment was repeated by five biological replicates. GC-MS detection and spectral processing for GC-MS were carried out using the Agilent 7890A GC equipped with an Agilent 5975C VL MSD detector (Agilent Technologies, USA) as described previously (Unemoto et al., 1973 [DOI](#); Yang et al., 2018 [DOI](#)). Statistical difference was obtained by Kruskal Wallis test and Manne Whitney test using SPSS 13.0 and a p value < 0.01 was considered significant. Hierarchical clustering was completed in the R platform (<https://cran.r-project.org/> [DOI](#)) with the function “heatmap.2” of “gplots library”. Z score analysis was used to scale each metabolite. Multivariate statistical analysis included orthogonal partial least square discriminant analysis (OPLS-DA) implemented with SIMCA 12.0 (Umetrics, Umeå, Sweden). Control scaling was selected prior to fitting. All variables were mean centered and scaled to pareto variance of each variable. Rank-sum test and a permutation test were used to identify differentially expressed metabolites. OPLS-DA was used to reduce the high dimension of the data set. Differential metabolites to their respective biochemical pathways were outlined in the MetaboAnalyst 3.0 (<http://www.metaboanalyst.ca/> [DOI](#)). Pathways were enriched by raw p value < 0.05 .

qRT-PCR

Quantitative real time polymerase chain reaction (qRT-PCR) was carried out as described previously (Yang et al., 2020 [DOI](#)). Total RNA was extracted from *V. alginolyticus* using TRIZOL reagent (Invitrogen Life Technologies) according to the protocol. RNA Electrophoresis was carried out in 1% (w/v) agarose gels to identify quality of the extracted RNA. By using a PrimeScript™ RT reagent Kit with gDNA eraser (Takara, Japan), reverse transcription-PCR was carried out on 1 µg of total RNA and primers are listed in **Table EV2**. [DOI](#) qRT-PCR was performed in 384-well plates with a total volume of 10 µL and the reaction mixtures were run on a LightCycler 480 system (Roche, Germany). Data are shown as the relative mRNA expression compared to 0% MgCl₂ test with the endogenous reference 16S rRNA gene.

Antibiotic bactericidal assay

Antibiotic bactericidal assay was performed as described previously with a modification (Li et al., 2016 [DOI](#); Zhao et al., 2021 [DOI](#)). The cultured bacteria of ATCC33787 and its mutant strains were transferred to fresh yeast medium at a dilution of 1:1000 and dispensed into test tubes and then the indicated concentrations of MgCl₂ and balofloxacin were added. If desired, 2 mM 2-aminooxazole and 1 µg/mL triclosan were or a metabolite complemented. These mixtures were incubated at 30 °C and 200 rpm for 6 h. Cells were collected. To determine CFU per mL, 100-µL samples were 10-fold serially diluted with 900 µL M9 buffer and an aliquot of 5 µL of each dilution was spotted onto the LB agar plates and cultured at 30 °C for 8 h.

Construction of gene-deleted mutants

Construction of gene-deleted mutants was carried out as described previously (Kuang et al., 2021 [DOI](#)). Primers were designed as shown in **Table EV3** [DOI](#) using CE Design V1.03 software. To construct gene-deleted mutants, upstream and downstream 500-bp fragments were amplified from the genome using two pairs of primers (primers P1 and P2, primers P3 and P4), and then merged into a 1,000-bp fragment by overlap PCR using a pair of primers (primers P1 and P4). After the fragments were digested by *Xba*I, they were ligated into the pDS132 vector digested by the same enzymes, transformed into MC1061 competent cells. Conversion products were coated on LB plate containing 25 µg/mL chloramphenicol and cultured at 37 °C overnight. The plasmids from colony growing on the plate were identified by PCR using a pair of primers (primers P1 and P4) and sequenced. The sequenced plasmids were transformed into MFD λ pir competent cells as donor. MFD λ pir and recipient bacterium ATCC 33787 were cultured to an optical density (OD) of 1.0 and then mixed at a ratio of 4:1. After centrifugation, the pellets were resuspended with 50 µL LB medium including DAP (100 µg/mL), dropped onto sterilized filter paper on LB medium including DAP (100 µg/mL), and cultured for 16-18 h at 37 °C.

All bacteria rinsed from the filter paper with LB medium were smeared onto the LB plate with chloramphenicol (25 µg/mL) and Ampicillin (100 µg/mL). After cultured for 16-18 h at 37 °C, bacteria were screened by LB plate with above two antibiotics. The bacteria were identified by plasmid PCR using a pair of primers P1 and P4 and sequencing and then were cultured and smeared onto the LB plates with 20% sucrose. The clones were cultured and smeared onto the LB plates with 20% sucrose again. The clones, which did not grow on the LB plates with chloramphenicol but grew on the LB plates with 20% sucrose, were identified by PCR using primer P7P8 (primer P7 is set at about 250 bp upstream of P1, and primer P8 is set at about 250 bp downstream of P4), P4P7, and P5P6 (for amplification of the target gene).

LC-MS analysis for lipidomics

ATCC33787 were cultured in medium with desired concentrations of MgCl₂, washed by 3% NaCl with the desired concentrations of MgCl₂, and adjusted to OD 1.0. Aliquot of 30 mL the cultures was centrifuged each sample and bacterial cells were collected. Aliquot of 800 µL distilled water

was added and treated for 5 min at 100 °C to inactivate phospholipase C. Protein concentration was determined with BCA kit. Sample solution with 3 mg protein was transferred into a 10 mL centrifuge tube, and supplemented with distilled water to 800 µL. Then all samples were processed as follows: 1 mL chloroform and 2 mL methanol (to make the volume ratio of chloroform: methanol: water = 1:2: 0.8) were added and cholic acid was as an internal standard; These mixtures were vortexed for 2 min and then 1 mL chloroform was add and vortex for 30s; 1 mL 10% NaCl solution was add, vortexed for 30s, and placed at room temperature overnight. After the solution was layered, a 2.5 mL syringe was used to suck the lowest layer (chloroform layer) to a new EP tube. Solution of the chloroform layer was evaporated in the rotary evaporator for 2 h and then 1 mL mobile phase (50% and 50%B. A, 25 mM ammonium acetate / methanol (30: 70); B, methanol) was added for analysis of lipids by LC-MS. Database (<https://hmdb.ca>) was used for lipid identification.

Measurement of enzyme activity

Activity of pyruvate dehydrogenase (PDH), α-ketoglutarate dehydrogenase (KGDH) and succinate dehydrogenase (SDH) was carried out as previously described (Jiang et al., 2020). Cells cultured in medium were collected and washed three times with saline. The bacterial cells were suspended in Tris-HCl (pH 7.4) and disrupted by sonic oscillation for 6 min (200 W total power with 35% output, 2 s pulse, 3 s pause over ice). After centrifugation, supernatants were collected. The protein concentration of the supernatant was determined using Bradford assay (Beyotime, P0009). Then, 200 µg proteins were used for determination of pyruvate dehydrogenase (PDH), α-ketoglutarate dehydrogenase (KGDH) and succinate dehydrogenase (SDH) activity. Levels of PGS and PSS were quantified by ELISA kits according to manufacture's instruction (Shanghai Fusheng Industrial Co., Ltd., China)

Measurement of proton motive force

Measurement of the transmembrane voltage delta PSI (δPSI), which is the electrical component of the PMF, was performed as previously described (Cheng et al., 2018). Bacteria cultured in medium with desired concentrations of MgCl₂ were collected at centrifugation and labeled by DiO2(3). Approximately 1 × 10⁷ CFU were added to a flow cytometry tube containing 1 mL buffer with 10 µM DiOC2(3) (Sigma) and incubated in the dark for 30 min at 30 °C. Samples were assayed with BD FACSCalibur flow cytometer with a 488 nm excitation wavelength. Gates for bacterial populations were based on the control population by using forward versus side scatter and red versus green emission. Size and membrane potential determined the intensity of Red (488 nm excitation, 610 nm emission) fluorescence. The diverse ratios of red and green indicated fluorescence intensity values of the gated populations. Computational formula of membrane

potential: $\text{Log}(10^{3/2} \times (\frac{\text{red fluorescence}}{\text{green fluorescence}}))$.

Measurement of depolarization

The membrane potential was estimated by measuring the fluorescence of the potential-dependent probe DiBAC4(3) (Saint-Ruf et al., 2016). DiBAC4(3) is a voltage-sensitive probe that penetrates depolarized cells, binding intracellular proteins or membranes exhibiting enhanced fluorescence and red spectral shift. ATCC33787 were cultured in medium with desired concentrations of MgCl₂, collected and then adjusted to OD 0.6. Aliquot of 100 µL bacterial cells were diluted to 1 mL, and 2 µL of 5 mM DiBAC4(3) was added. After incubated for 15 min at 30 °C without light and vibration, these samples were filtered and detected by BD FACSCalibur.

Measurement of fluidity by fluorescence microscopy

Measurement of membrane fluidity is performed as previously described (Wen et al., 2022). Briefly, ATCC33787 were cultured in medium with indicated concentrations of MgCl₂, collected and then adjusted to OD 0.6. Aliquot of 100 µL bacteria cells of each sample were diluted to 1 mL and

10 μ L (10 mg/mL) FM5-95 (Thermo Fisher Scientific, USA) was added. FM5-95 is a lipophilic styryl dye that insert into the outer leaflet of bacterial membrane and become fluorescence. This dye preferentially bind to the microdomains with high membrane fluidity (Wen et al., 2022 [DOI](#)). After incubated for 20 min at 30 °C at vibration without light, the sample was centrifuged for 10 min at 12,000 rpm. The pellets were resuspended with 20 μ L of 3% NaCl. Aliquot of 2 μ L sample was dropped on the agarose slide, and take photos under the inverted fluorescence microscope.

Measurement of membrane permeability

Measurement of membrane permeability was carried out as described previously (Su et al., 2021 [DOI](#)). ATCC33787 were cultured in medium with desired concentrations of $MgCl_2$, collected and then adjusted to OD 0.6. Aliquot of 100 μ L bacteria cells of each sample were diluted to 1 mL and 2 μ L 10 mg/mL SYTO9 was added. After incubated for 15 min at 30 °C at vibration without light, the mixtures were filtered and measured by flow cytometry (BD FACSCalibur, USA).

Acknowledgements

This work was sponsored by the International Cooperation and Exchange program of National Natural Science Foundation of China (32273177) (to P.B.), Innovation Group Project of Southern Marine Science and Engineering Guangdong Laboratory (Zhuhai) (311020006) (to P.B.), Natural Science Foundation of Guangdong Province (2022A1515012079) (to L.H.), the Science and Technology Planning Project of Guangdong Province (2023B1212060028), and Fundamental Research Funds for the Central Universities, Sun Yat-sen University (24lgzy004).

Additional information

Author contributions

B. Peng conceived the idea, designed the experiments, and supervise the projects. H. Li, J. Yang, and S.F. Kuang conducted the experiments and interpreted the results.

Declaration of interest statement

Authors declare that they have no competing interests.

Supplementary text

Magnesium and balofloxacin binding only partially contributes to phenotypic resistance

Prior studies suggest that the chelation of antibiotics by magnesium ions inhibits antibiotic uptake (Deitchman et al., 2018 [DOI](#); Lunestad and Goksøyr, 1990 [DOI](#)). To investigate whether magnesium binds to balofloxacin, balofloxacin was pre-incubated with magnesium, and zone of inhibition (ZOI) analysis was conducted. Six different concentrations of balofloxacin were separately incubated with six different concentrations of $MgCl_2$, and then spotted on filter paper so that a defined amount of balofloxacin could be used for ZOI. While lower concentrations of $MgCl_2$ (0.78, 3.125, or 12.5 mM) did not alter the ZOI, higher concentrations, including 50 and 200 mM $MgCl_2$, decreased the ZOI (Suppl. Fig 2A [DOI](#)), suggesting that even high doses of magnesium had only a partial effect on balofloxacin through direct binding. For example, at 200 mM $MgCl_2$ and 5 or 10 μ g/mL balofloxacin, the balofloxacin ZOI was 53.2 and 70.3% of the ZOI at 0 mM $MgCl_2$, suggesting that $\geq 50\%$ of the antibiotics were still functional. Intracellular BLFX also decreased with increasing

MgCl₂ (Suppl. Fig 2B [↗](#)), while exogenous Mg²⁺ increased intracellular Mg²⁺ levels in a dose-dependent manner. For example, exogenous 50 and 200 mM MgCl₂ increased intracellular Mg²⁺ levels to 1.21 and 1.31 mM, respectively (Suppl. Fig 2C [↗](#)). The relationship between TolC, an efflux pump that transports quinolones from bacterial cells, and Mg²⁺ was also assessed (Kobylka *et al.*, 2020 [↗](#); Song *et al.*, 2020 [↗](#)). The expression of TolC/*tolC* was unaffected by Mg²⁺ (Suppl. Fig 2D [↗](#)). Magnesium is critical for LPS stability. LPS levels increased at 200 mM Mg²⁺ (Suppl. Fig 2E [↗](#)), however, the loss of *waaF*, *lpxA*, and *lpxC*, three key genes involved in LPS biosynthesis, did not influence balofloxacin sensitivity/resistance in the presence of Mg²⁺ (Suppl. Fig 2F [↗](#)). These findings suggest that magnesium-induced LPS biosynthesis does not contribute directly to BLFX resistance and demonstrate that Mg²⁺ influx is involved in balofloxacin resistance.

Supplementary tables

	LBS	ASWT
Tryptone(%)	1	1
Yeast extract(%)	0.5	0.5
NaCl(mM)	171	210
MgSO ₄ (mM)	0	35
KCl(mM)	0	7
CaCl ₂ (mM)	0	7

Table 1

Comparison in components in LBS and ASWT

Table 2

Primes used in the present study

gene		KEGG entry	Sequences (5'-3')	gene		KEGG entry	Sequences (5'-3')
<i>accA</i>	Forward	N646_1411	AAAGGTCGTGAAACCAAAGA	<i>fadD</i>	Forward	N646_4025	CATTATTTACTGCGATCCTCG
	Reverse		AGCCGTGTCGATAAATGTG		Reverse		CCATATTGAGATATTGTCTGGG
<i>accB</i>	Forward	N646_1967	TCAAGCACCAATTCACACG	<i>fadE</i>	Forward	N646_1395	TATCCCACTGATGTCAAAGG
	Reverse		ACTTCTGACCCACTTCTACG		Reverse		CAACCGACAGACACTCAACC
<i>accC</i>	Forward	N646_1968	AGAAGTGAAGTGGTGTGTGG	<i>fadB</i>	Forward	N646_2210	ATCTACTAAACGCCAGAAAA
	Reverse		GGATAGTGTCTGCCTTTGGAC		Reverse		GCTGAAGCCGCCAAAGTA
<i>accD</i>	Forward	N646_1292	CGAACTTGAGCCACAGGATA	<i>fadA</i>	Forward	N646_1311	CAGATTAGCGGCCAGTTTCT
	Reverse		ACGACTGAACCCATTGAACC		Reverse		TCAAGCGCGGTAAATGT
<i>accD</i>	Forward	N646_4704	AAGTTTGTCCGAGCAGTTGA	<i>fadA</i>	Forward	N646_2209	ATTGCGGGCTGCGATC
	Reverse		GAGACGCCACCATAAGTTTG		Reverse		GCACACGGCAGAGACTGC
<i>fabD</i>	Forward	N646_1131	CTCGCTGGTCAAGTCGT	<i>fadH</i>	Forward	N646_1258	AAGTGCTGCCCTGCTCG
	Reverse		TGCTGGCTTCATAAGTGCA		Reverse		ATGATGGCGTCGAGTTAATG
<i>fabH</i>	Forward	N646_1132	GACCGTTCAACCATCATCCT	<i>fabR</i>	Forward	N646_2038	TTTGCTACTACGCCAAGTATGCGT
	Reverse		GCCATTTATCCGCATCTTG		Reverse		CGCTCACGCAACAACAACGAA
<i>fabH</i>	Forward	N646_1439	GACGCTCCAACATGGCTAG	<i>fadR</i>	Forward	N646_1147	AATGCTAGGCGCTGGAA
	Reverse		AGTTTCGCTGGTGACAATAAGA		Reverse		TGAATGCGGCTTCTCTG
<i>fabH</i>	Forward	N646_4350	GGAAGACAACGGTTACTGGG	<i>arcA</i>	Forward	N646_1744	TGGGTTGAATGGTTTAGTGATG
	Reverse		TGCGGAATCACAACATCG		Reverse		TGAAGCCAGTGACGGTGAA
<i>fabB</i>	Forward	N646_1292	TTGACAGTGCGCCATC	<i>crp</i>	Forward	N646_1004	AAGGTGCTGCTACTTGCTGA
	Reverse		TGTCTTGCTACGCCATTTAAGA		Reverse		GCTTCGGATTGGTAATGGTT
<i>fabB</i>	Forward	N646_3036	CAACATTAATGCTGCGGCT	<i>crp</i>	Forward	N646_1885	CAAAACAACAGACGCAATG
	Reverse		TGAGCGGCCACATTAC		Reverse		CTCAGGAGAACAGCCAACAAT
<i>fabF</i>	Forward	N646_1128	GGTGTGCTATCGGCTCA	<i>gpaA</i>	Forward	N646_1922	GTGCGGGTATGTCTGATGG
	Reverse		ATGTTTACAATGGTCGAAGGA		Reverse		ATAAAGGTTTCTGGCTGTGC
<i>fabF</i>	Forward	N646_3033	AACCTGCCAAAGCACTACAAGC	<i>plsB</i>	Forward	N646_2043	GCTTAGTTCGCTTTAGTCCC
	Reverse		TGATGAGCCGTATGCGATAC		Reverse		CACTTGCGGTTGTGGC
<i>fabG</i>	Forward	N646_1130	CAACCTAACGCCAATCTACC	<i>plsY</i>	Forward	N646_2566	CGCCAAGAATGATAGGGTC
	Reverse		CCACGAGACGCAACTTCA		Reverse		TTATTAGGTTTCGGTTTCCAGT
<i>fabG</i>	Forward	N646_3034	GACCGAAGAAGATGGGATG	<i>plsC</i>	Forward	N646_1749	CGATTTCGCTCGGTCAA
	Reverse		TGCGCTGTAGTTAGTTGACC		Reverse		TACTGTTTCTGCGGCTGT
<i>fabG</i>	Forward	N646_3826	AGTAAAGGCGGCGTCATC	<i>cdsA</i>	Forward	N646_1423	TTGCTGCCCTTGTGCC
	Reverse		TCTGGCAGGTTCTTGGTCA		Reverse		GCCAAGATTTCGTCGTTTT
<i>fabG</i>	Forward	N646_4505	CCCAAGCGTTATCATCA	<i>pgsA</i>	Forward	N646_1026	GTTTTGGCGCGTTTATT
	Reverse		TTCGGGTTTCATTTGCTCT		Reverse		TTTCTCGCGCAATCATC
<i>fabZ</i>	Forward	N646_1416	GAGAAGTACCTTCACGCGAT	<i>pgpA</i>	Forward	N646_2838	ATGAGTTTGGCGGCTTT
	Reverse		CTGTTGCCCTGAGCCATTG		Reverse		TCCCGCCACGATATCAT
<i>fabV</i>	Forward	N646_4267	AGTAATGGGCGGCGAAG	<i>pgpB</i>	Forward	N646_3562	TTTCTTTGGCGGGTTGT
	Reverse		TGGGTGCGTAATGTCAGG		Reverse		CTTCAGGGCGGTGCAT
<i>fabV</i>	Forward	N646_0267	GGTGGTTTACTCACTGGCATC	<i>pssA</i>	Forward	N646_2008	GATAAAACCGCCAACGA
	Reverse		TCTTCAACGCCCATACC		Reverse		TTACGTGCGGCGATATT
<i>tesA</i>	Forward	N646_4268	AGCATTTCTGGCGATACAAC	<i>psd</i>	Forward	N646_1916	CACTCTGCGCCAAATG
	Reverse		GGAGGGAAACCTTTAGGC		Reverse		ACGAGGCGGAGTGATC
<i>tesB</i>	Forward	N646_0021	CTTTGGATTCTGCTAATGG	<i>pldA</i>	Forward	N646_4183	CTTTTATAGTCCGAGAAC
	Reverse		ATAGAGTGGTCAATGGTGCC		Reverse		CATAACCCGCTGAGTATTGT
<i>yciA</i>	Forward	N646_1052	TGTCTCAGCTCGACTTAGCG	<i>pldB</i>	Forward	N646_2069	GATCTCTACGCCAAGGT
	Reverse		GGGTTTGACCCATACTTCCA		Reverse		AGTGCGCGATGATGAAG
<i>fadL</i>	Forward	N646_1315	CTAAGTATTGGCGCAACGTACG	<i>glpQ</i>	Forward	N646_1500	CTATCCAACCCGATTCCC
	Reverse		GCCGCCGACTTGTTCTG		Reverse		ACCAAGGCGCTTTAATTT

<i>fadL</i>	Forward	N646_1316	CTGCCTTCGCCGATGAT	<i>glpQ</i>	Forward	N646_3581	AAAAGTCCAATGCCGCA
	Reverse		GAAATTCGCGATTGGTTTG		Reverse		GTAACCGGCTTGTGGAG
<i>fadL</i>	Forward	N646_3657	TTATCTGCGGGTGCCTCT	<i>glpQ</i>	Forward	N646_3723	CACAAATGGCGCTATCTG
	Reverse		GTCACGCCCGTCACCAT		Reverse		CCATCGGCTTCTTCACTC
<i>fadD</i>	Forward	N646_1045	TGTTCTTGAGCTGGTGAAA	<i>glpQ</i>	Forward	N646_3724	GACTTGTGAAGCCCGC
	Reverse		AACACGCAATGACGAGGTAT		Reverse		AGATACTCAGGGCCAATAAAC
<i>fadD</i>	Forward	N646_2540	GCATCGGTCGATTCTTCC	<i>enuC</i>	Forward	N646_3725	CTTTAAGCGCTGATGATGA
	Reverse		CGCCAATCTTAACCTGTGC		Reverse		GTTAGACCGGGTCTCTCTC
<i>fadD</i>	Forward	N646_3023	CTCTGATGATGCCAAACCTAC	<i>adhE</i>	Forward	N646_1240	AGCGATGCTGCACGAA
	Reverse		ACGAGGAGTGTAAGTGGGTT		Reverse		CCTGTAACTAATATGGCTGAAC

Table 2 (continued)

<i>gene</i>		primer (5'-3')
<i>fadR</i>	P1	aaaaaggatcgatcct TACTCCCCTGAACTCAAGCAC
	P2	TGTCAGCGCTTATCTACTTTTATCCAATATTGATTCTG
	P3	ATAAAAAAGTAGATAAGCGCTGACAAGTTGGATAAAC
	P4	atcgcatgcggtacc GCAAAGAGATGACTTGCATAATGAGTA
	P5	ATGGTCATAAAGGCGAAAAGCC
	P6	TTAGCTGTCGTCTCCGTGAAGTT
	P7	AATAGTGCTTAAGTATCTGTTGT
	P8	ATGACCATAGTGTGCAAGA
<i>arcA</i>	P1	aaaaaggatcgatcct GACCTCAAGTTACTGTATGGGTTCC
	P2	TAGCGGTTAAATTGGCGGTACCTAAATTTGTGACA
	P3	TAGGTACCGCCATAGATTTAACCGCTAATATGCAAAGGG
	P4	atcgcatgcggtacc ATTGATGGCAGGCTGTAGTTCC
	P5	ATGCAAACCCCGCAGATTCT
	P6	TGGCGATGATTCTGGCGT
	P7	CGACGGCAAAGCTTCCTC
	P8	AAAAGTTGGTTGGAACAAAGGAA
<i>plsB</i>	P1	aaaaaggatcgatcct TTAGGTGTTCTCTGCGGCAT
	P2	AAGGTAAAACGGAAAGTTCACAGCCTCAATAGGTATTC
	P3	GCTGTGAACTTTTAACCGTTTACCTTCTTCCAATAAGA
	P4	atcgcatgcggtacc AGCTATTTTATAGGCTTGGCTTTTA
	P5	ACAACGATAATGATGTCCCAACC
	P6	GAACGACCACCCTCACTGAAGTA
	P7	GCTCATCATTGGCTGCGTCT
	P8	TGCTGACCATGAACCCGC
<i>waaf</i>	P1	aaaaaggatcgatcct AGTTGTGGGTTGCGTGTGTTGA
	P2	GAACTAACATGAGCTTGTGTTTCCAGTATAGGAATT
	P3	GAAAAACAAAGCTCATGTTAGTTCGTATTGTTTATACCTT
	P4	atcgcatgcggtacc TTGGCGTAGTTCTGACACGATT
	P5	AACGCCATCCAGATAGCCAAAT
	P6	AGGTTTCAAGCCCGCCAAGA
	P7	ATATGTGGGTACTACAGTTGCTGCA
	P8	CATCTAATACCTGTTCGTCCTC
<i>lpxA</i>	P1	aaaaaggatcgatcct TACGACCTGTTGCCACGTTTG
	P2	ATTCGCCAATTAATTAATACTCTCGACGAGCACAC
	P3	GAGAGTTTTAATTAATTGGCGAATGAACCAATAAATAA
	P4	atcgcatgcggtacc ATTTTAAAGATGCGCTTCTGACG
	P5	GGCAGCGGTGGTAGAAGAGG
	P6	AACGGGTGAATCGCAGACAG
	P7	ATTGATGAGATGAATAAGCGCCT
	P8	TAACTCGCTACCACGACTGCCT
<i>lpxC</i>	P1	aaaaaggatcgatcct AGACGGTTGGTAATACAGTGAAG

Table 3

Primers used in the present study for construction of gene-deleted mutants

	P2	GCCAAGTATTTACTGCTTGCCTCTCAAAAATTGTC
	P3	AGAGGCAAGCAGTAAATACTTGGCATTAGAATTAATAA
	P4	atcgc atgcggtacc CTGGAGAAATCTTACAGCTTAAC
	P5	ATTGAATCAGACGAGCAACG
	P6	CGAATGTAGTCCACTCCCAC
	P7	AGAATGCCGTTCAAGGTATTGC
	P8	TTTACTTAAAGTGGTGACTGATT
<i>pgpA</i>	P1	aaaaaggatcgatcct AAGCACTAGAGCGAGCTCATTAC
	P2	GTTCATGGTTTAATCCTTAAACCTATACTGCTAGTTT
	P3	AGGTTTAAGGATTAAACCATGAACTAAACACTGGACGT
	P4	atcgc atgcggtacc ATACCCTCGTGGTAGCGGCAT
	P5	GGCACAATTACCATTTACGG
	P6	TAGAAACAGGGCAACACCAG
	P7	CATTTTTCGAACTGGCTGATT
	P8	GCAACGTGGTTATGACCAATT

Small letters are homologous recombination sequences with pDS132 plasmid.

Table 3 (continued)

Supplementary figures

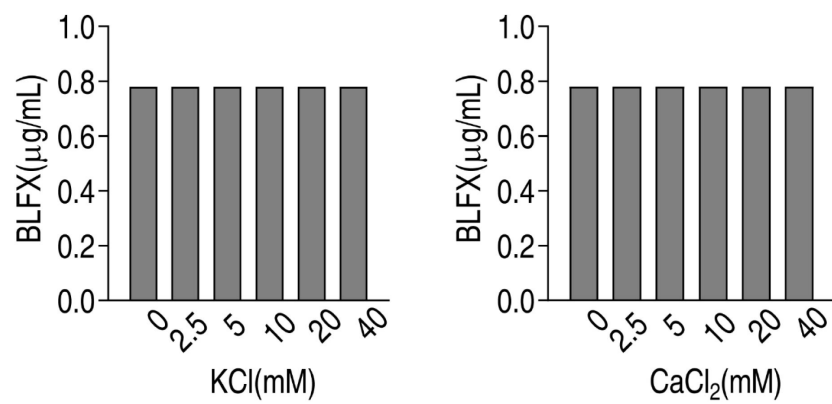


Figure 1

MIC of ATCC33787 to BLFX in the absence or presence of the indicated concentrations of KCl or CaCl₂ in M9 media.

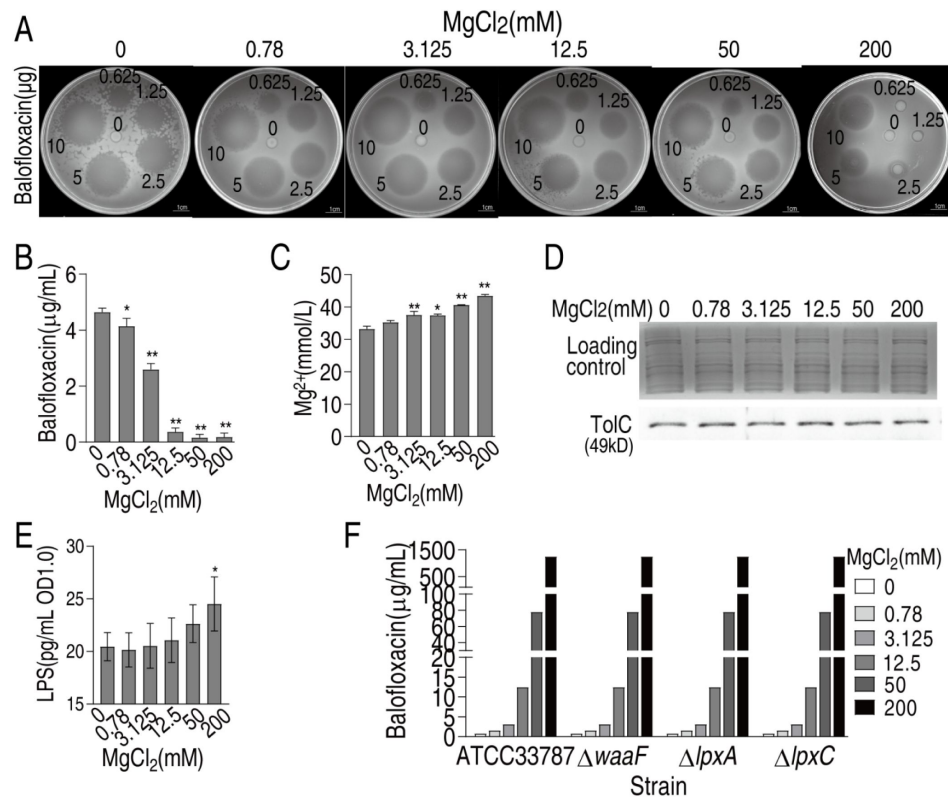


Figure 2

Mg^{2+} decreases balofloxacin uptake.

A. Oxford cup test for effect of different concentrations of Mg^{2+} on antibacterial action of different dose of BLFX to ATCC33787. To do this, 0 mM, 0.78 mM, 3.125 mM, 12.5 mM, 50 mM, and 200 mM $MgCl_2$ were individually mixed with 0 μ g/mL, 12.5 μ g/mL, 25 μ g/mL, 50 μ g/mL, 100 μ g/mL or 200 μ g/mL BLFX for 5 h. Then, 50 μ L were added into Oxford cup for antibacterial efficiency, which contained 0 μ g, 0.625 μ g, 1.25 μ g, 2.5 μ g, 5 μ g, or 10 μ g BLFX, respectively.

B. Intracellular BLFX of ATCC 33787 in ASWT with the indicated concentrations of $MgCl_2$ and 60 μ g/mL BLFX.

C. Intracellular Mg^{2+} of ATCC 33787 in ASWT with the indicated concentrations of $MgCl_2$.

D. Western blot for abundance of TolC in the presence of $MgCl_2$. Whole cell lysates resolved by SDS-PAGE gel was stained with Coomassie brilliant blue as loading control.

E. LPS quantification at indicated concentrations of $MgCl_2$.

F. MIC of ATCC 33787 and its mutants $\Delta waaF$, $\Delta lpxA$, $\Delta lpxC$ in ASWT with the indicated concentrations of $MgCl_2$, which is measured by microtitre-dilution-method.

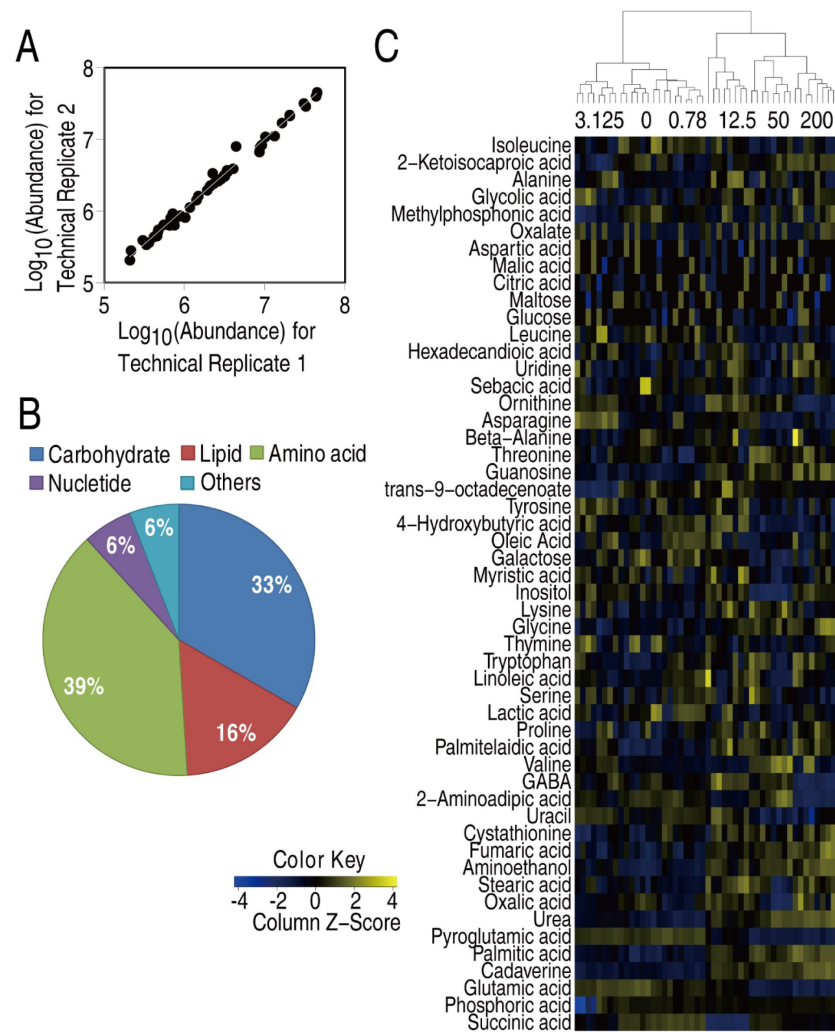


Figure 3

Metabolic profiles of *V. alginolyticus* in different concentrations of MgCl_2 .

- A.** Reproducibility
- B.** Percentage of metabolites in every category
- C.** Heatmap of metabolites

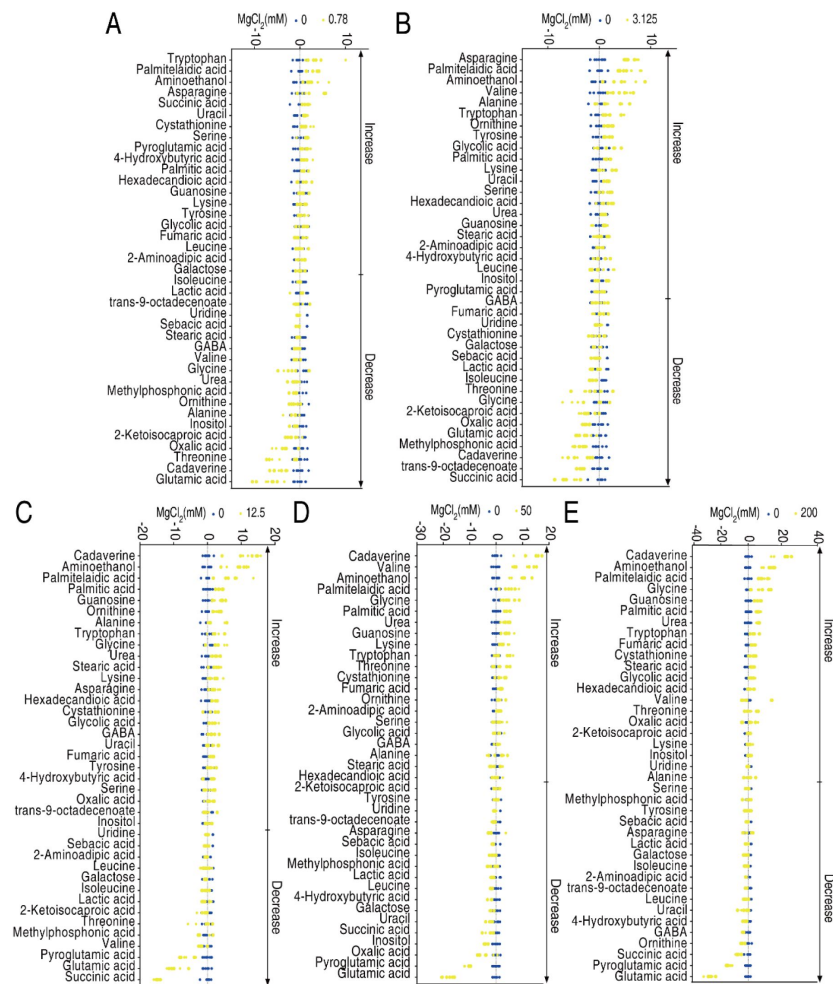


Figure 4

Heatmap and Z score plots of differential metabolites.

A. Heatmap of differential metabolites

B-F. Z score plots of differential metabolites.

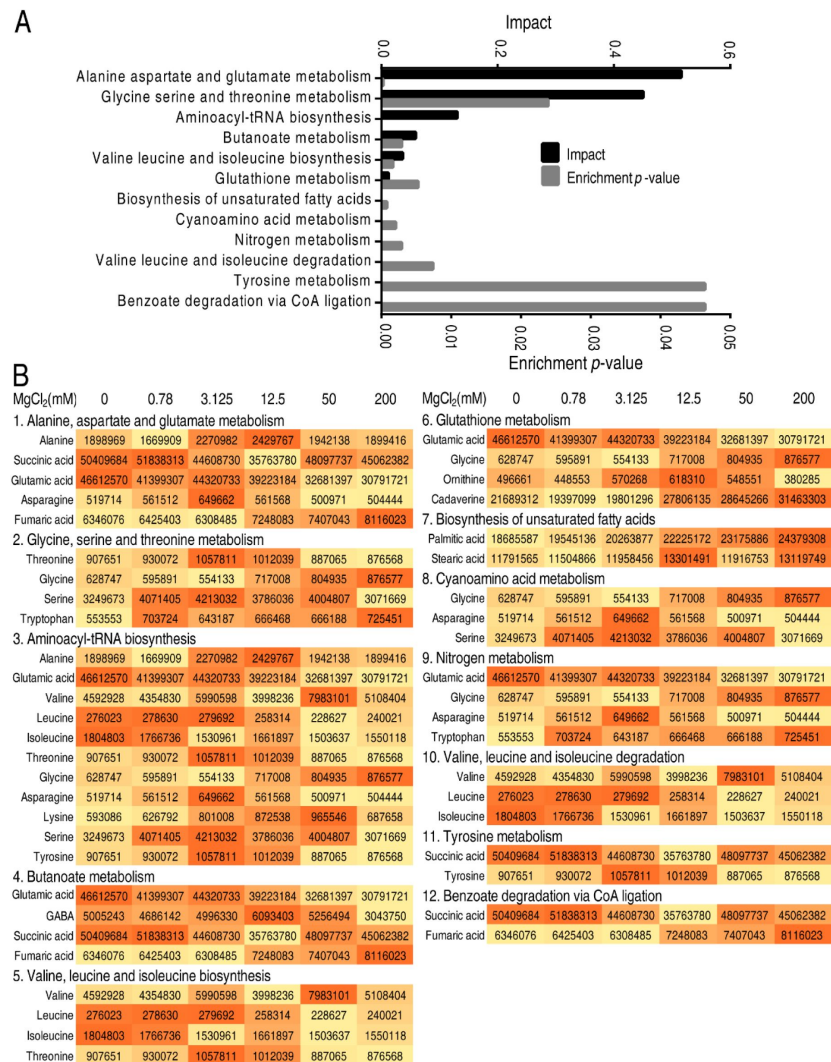


Figure 5

Pathway enrichment of differential metabolites.

- A.** Pathway enrichment of differential metabolites
B. Differential metabolites in enriched pathways

Figure 6

Scatter plots of differential metabolites identified by S-plot.

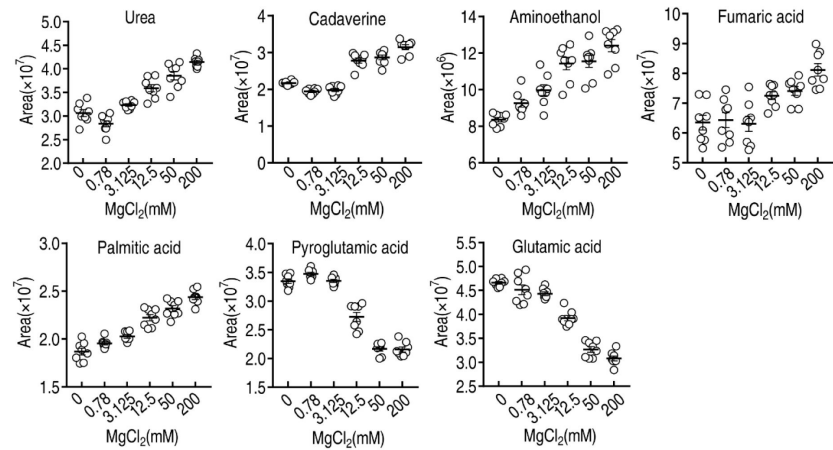
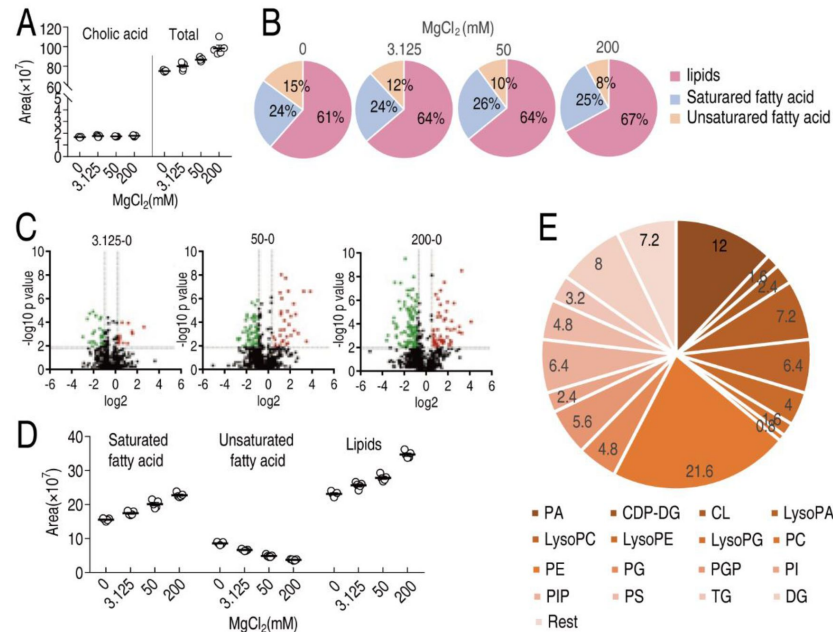


Figure 7

Lipidomes in the different concentrations of $MgCl_2$.

- A. Area of fatty acids in the presence of indicated concentrations of $MgCl_2$.
B. Percentage of lipids, saturated fatty acid and unsaturated fatty acid in the presence of indicated concentration of $MgCl_2$.
C. Volcano plots of lipidomics of indicated concentration of $MgCl_2$ as compared to non-treated control.
D. Relative abundance of saturated fatty acids, unsaturated fatty acids and lipids in the presence of indicated concentrations of $MgCl_2$.
E. Relative percentage of indicated lipids.



References

- Cheng Z.X., Yang M.J., Peng B., Peng X.X., Lin X.M., Li H (2018) **The depressed central carbon and energy metabolisms is associated to the acquisition of levofloxacin resistance in *Vibrio alginolyticus*** *J Proteomics* **181**:83–91
- Deitchman A.N., Singh R.S.P., Rand K.H., Derendorf H (2018) **Enhanced in vitro activity of tigecycline in the presence of chelating agents** *Int J Antimicrob Ag* **51**:799–802
- Dutta D., Kaushik A., Kumar D., Bag S (2021) **Foodborne Pathogenic Vibrios: Antimicrobial Resistance** *Front Microbiol* **12**
- Feng Y.J., Cronan J.E (2010) **Overlapping Repressor Binding Sites Result in Additive Regulation of *Escherichia coli* FadH by FadR and ArcA** *J Bacteriol* **192**:4289–4299
- Fujita Y., Matsuoka H., Hirooka K (2007) **Regulation of fatty acid metabolism in bacteria** *Mol Microbiol* **66**:829–839
- Garcia-Legorreta A. *et al.* (2020) **Effect of Dietary Magnesium Content on Intestinal Microbiota of Rats** *Nutrients* **12**
- Garfinkel L., Garfinkel D (1985) **Magnesium regulation of the glycolytic pathway and the enzymes involved** *Magnesium* **4**:60–72
- Grimes D.J (2020) **The Vibrios: Scavengers, Symbionts, and Pathogens from the Sea** *Microb Ecol* **80**:501–506
- Groisman E.A., Hollands K., Kriner M.A., Lee E.J., Park S.Y., Pontes M.H (2013) **Bacterial Mg²⁺ Homeostasis, Transport, and Virulence** *Annu Rev Genet* **47**:625–646
- Guo L., Lim K.B., Poduje C.M., Daniel M., Gunn J.S., Hackett M., Miller S.I (1998) **Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides** *Cell* **95**:189–198
- Hancock R.E., Raffle V.J., Nicas T.I (1981) **Involvement of the outer membrane in gentamicin and streptomycin uptake and killing in *Pseudomonas aeruginosa*** *Antimicrob Agents Chemother* **19**:777–785
- Herrera C.M., Hankins J.V., Trent M.S (2010) **Activation of PmrA inhibits LpxT-dependent phosphorylation of lipid A promoting resistance to antimicrobial peptides** *Mol Microbiol* **76**:1444–1460
- Huang H.H., Wu B.K., Li L.H., Lin Y.T., Yang T.C (2021) **Role of the PhoPQ two-component regulatory system in the beta-lactam resistance of *Stenotrophomonas maltophilia*** *J Antimicrob Chemother* **76**:1480–1486
- Isaei E., Mansouri S., Mohammadi F., Taheritarigh S., Mohammadi Z (2016) **Novel Combinations of Synthesized ZnO NPs and Ceftazidime: Evaluation of their Activity against Standards and New Clinically Isolated *Pseudomonas aeruginosa*** *Avicenna J Med Biotechnol* **8**:169–174

- Jiang M., Chen Z.G., Li H., Zhang T.T., Yang M.J., Peng X.X., Peng B (2022) **Succinate and inosine coordinate innate immune response to bacterial infection** *Plos Pathog* **18**
- Jiang M., Kuang S.F., Lai S.S., Zhang S., Yang J., Peng B., Peng X.X., Chen Z.G., Li H (2020) **Na⁺-NQR Confers Aminoglycoside Resistance via the Regulation of L-Alanine Metabolism** *Mbio* **11**
- Kobylka J., Kuth M.S., Mueller R.T., Geertsma E.R., Pos K.M (2020) **AcrB: a mean, keen, drug efflux machine** *Ann Ny Acad Sci* **1459**:38–68
- Kou T.S., Wu J.H., Chen X.W., Chen Z.G., Zheng J., Peng B (2022) **Exogenous glycine promotes oxidation of glutathione and restores sensitivity of bacterial pathogens to serum-induced cell death** *Redox Biol* **58**
- Kuang S.F., Feng D.Y., Chen Z.G., Liang Z.Z., Xiang J.J., Li H., Peng X.X., Zhang T.T (2021) **Inactivation of Nitrite-Dependent Nitric Oxide Biosynthesis Is Responsible for Overlapped Antibiotic Resistance between Naturally and Artificially Evolved *Pseudomonas aeruginosa*** *Msystems* **6**
- Kumarage P.M., De Silva L., Heo G.J. (2022) **Aquatic environments: A potential source of antimicrobial-resistant *Vibrio* spp** *J Appl Microbiol* **133**:2267–2279
- Kumariya R., Sood S.K., Rajput Y.S., Saini N., Garsa A.K (2015) **Increased membrane surface positive charge and altered membrane fluidity leads to cationic antimicrobial peptide resistance in *Enterococcus faecalis*** *Bba-Biomembranes* **1848**:1367–1375
- Kwon D.H., Lu C.D (2006) **Polyamines increase antibiotic susceptibility in *Pseudomonas aeruginosa*** *Antimicrob Agents Ch* **50**:1623–1627
- Lee D.D., Galera-Laporta L., Bialecka-Fornal M., Moon E.C., Shen Z., Briggs S.P., Garcia-Ojalvo J., Suel G.M (2019) **Magnesium Flux Modulates Ribosomes to Increase Bacterial Survival** *Cell* **177**:352–360
- Lehtinen J., Nuutila J., Lilius E.M (2004) **Green fluorescent protein-propidium iodide (GFP-PI) based assay for flow cytometric measurement of bacterial viability** *Cytometry A* **60**:165–172
- Li W.X., Yao Z.J., Sun L.N., Hu W.J., Cao J.J., Lin W.X., Lin X.M. (2016) **Proteomics Analysis Reveals a Potential Antibiotic Cocktail Therapy Strategy for *Aeromonas hydrophila* Infection in Biofilm** *J Proteome Res* **15**:1810–1820
- Liu X.J., Zhu W.C., Su Y.B., Guo C., Zeng Z.H., Zhu H., Li H., Peng X.X (2015) **Characterization of ampicillin-stressed proteomics and development of a direct method for detecting drug-binding proteins in *Edwardsiella tarda*** *J Proteomics* **116**:97–105
- Lunestad B.T., Goksøyr J (1990) **Reduction in the antibacterial effect of oxy-tetracycline in sea water by complex formation with magnesium and calcium** *Dis. aquat. Org* **9**:67–72
- McGoverin C., Robertson J., Jonmohamadi Y., Swift S., Vanholsbeeck F (2020) **Species Dependence of SYTO 9 Staining of Bacteria** *Front Microbiol* **11**
- Peng B., Su Y.B., Li H., Han Y., Guo C., Tian Y.M., Peng X.X (2015) **Exogenous Alanine and/or Glucose plus Kanamycin Kills Antibiotic-Resistant Bacteria** *Cell Metab* **21**:249–261

- Pohland A.C., Schneider D (2019) **Mg²⁺ homeostasis and transport in cyanobacteria - at the crossroads of bacterial and chloroplast Mg²⁺ import** *Biol Chem* **400**:1289–1301
- Pontes M.H., Sevostyanova A., Groisman E.A (2015) **When Too Much ATP Is Bad for Protein Synthesis** *J Mol Biol* **427**:2586–2594
- Saeloh D., Tipmanee V., Jim K.K., Dekker M.P., Bitter W., Voravuthikunchai S.P., Wenzel M., Hamoen L.W (2018) **The novel antibiotic rhodomycinone traps membrane proteins in vesicles with increased fluidity** *Plos Pathog* **14**
- Said A.A., Livermore D.M., Williams R.J (1987) **Expression of H1 outer-membrane protein of Pseudomonas aeruginosa in relation to sensitivity to EDTA and polymyxin B** *J Med Microbiol* **24**:267–274
- Saint-Ruf C., Crussard S., Franceschi C., Orenga S., Ouattara J., Ramjeet M., Surre J., Matic I (2016) **Antibiotic Susceptibility Testing of the Gram-Negative Bacteria Based on Flow Cytometry** *Front Microbiol* **7**
- Sakayori Y., Muramatsu M., Hanada S., Kamagata Y., Kawamoto S., Shima J (2003) **Characterization of Enterococcus faecium mutants resistant to mundticin KS, a class IIa bacteriocin** *Microbiol-Sgm* **149**:2901–2908
- Song M.R., Liu Y., Huang X.Y., Ding S.Y., Wang Y., Shen J.Z., Zhu K (2020) **A broad-spectrum antibiotic adjuvant reverses multidrug-resistant Gram-negative pathogens** *Nat Microbiol* **5**
- Stan-Lotter H., Gupta M., Sanderson K.E (1979) **The influence of cations on the permeability of the outer membrane of Salmonella typhimurium and other gram-negative bacteria** *Can J Microbiol* **25**:475–485
- Su Y.B., Kuang S.F., Ye J.Z., Tao J.J., Li H., Peng X.X., Peng B (2021) **Enhanced Biosynthesis of Fatty Acids Is Associated with the Acquisition of Ciprofloxacin Resistance in Edwardsiella tarda** *Msystems* **6**
- Unemoto T., Tsuruoka T., Hayashi M (1973) **Role of Na⁺ and K⁺ in preventing lysis of a slightly halophilic Vibrio alginolyticus** *Can J Microbiol* **19**:563–571
- Wang T., Flint S., Palmer J (2019) **Magnesium and calcium ions: roles in bacterial cell attachment and biofilm structure maturation** *Biofouling* **35**:959–974
- Wen J., Vischer N.O.E., de Vos A.L., Manders E.M.M., Setlow P., Brul S. (2022) **Organization and dynamics of the SpoVAEa protein and its surrounding inner membrane lipids, upon germination of Bacillus subtilis spores** *Sci Rep* **12**
- Xie Y.T., Yang L.H (2016) **Calcium and Magnesium Ions Are Membrane-Active against Stationary-Phase Staphylococcus aureus with High Specificity** *Sci Rep-Uk* **6**
- Yang J., Zeng Z.H., Yang M.J., Cheng Z.X., Peng X.X., Li H (2018) **NaCl promotes antibiotic resistance by reducing redox states in Vibrio alginolyticus** *Environ Microbiol* **20**:4022–4036
- Yang M.J., Xu D., Yang D.X., Li L., Peng X.X., Chen Z.G., Li H (2020) **Malate enhances survival of zebrafish against Vibrio alginolyticus infection in the same manner as taurine** *Virulence* **11**:349–364

Yao Z., Guo Z., Wang Y., Li W., Fu Y., Lin Y., Lin W., Lin X (2019) **Integrated Succinylome and Metabolome Profiling Reveals Crucial Role of S-Ribosylhomocysteine Lyase in Quorum Sensing and Metabolism of *Aeromonas hydrophila*** *Mol Cell Proteomics* **18**:200–215

Zhang S., Wang J., Jiang M., Xu D., Peng B., Peng X.X., Li H (2019) **Reduced redox-dependent mechanism and glucose-mediated reversal in gentamicin-resistant *Vibrio alginolyticus*** *Environ Microbiol* **21**:4724–4739

Zhang Y.J., Boyd S.A., Teppen B.J., Tiedje J.M., Li H (2014) **Organic acids enhance bioavailability of tetracycline in water to *Escherichia coli* for uptake and expression of antibiotic resistance** *Water Res* **65**:98–106

Zhang Y.M., Rock C.O (2008) **Membrane lipid homeostasis in bacteria** *Nat Rev Microbiol* **6**:222–233

Zhang Y.M., Rock C.O (2008) **Thematic review series: Glycerolipids - Acyltransferases in bacterial glycerophospholipid synthesis** *J Lipid Res* **49**:1867–1874

Zhao X.L. *et al.* (2021) **Glutamine promotes antibiotic uptake to kill multidrug-resistant uropathogenic bacteria** *Sci Transl Med* **13**

Author information

Hui Li[†]

State Key Laboratory of Bio-Control, School of Life Sciences, Guangdong Province Key Laboratory for Pharmaceutical Functional Genes, Southern Marine Science and Engineering Guangdong Laboratory (Zhuhai), Sun Yat-sen University, Higher Education Mega Center, Guangzhou, China, Laboratory for Marine Biology and Biotechnology, Marine Fisheries Science and Food Production Processes, Qingdao Marine Science and Technology Center, Qingdao, China

[†]These authors contributed equally to this work

Jun Yang[†]

State Key Laboratory of Bio-Control, School of Life Sciences, Guangdong Province Key Laboratory for Pharmaceutical Functional Genes, Southern Marine Science and Engineering Guangdong Laboratory (Zhuhai), Sun Yat-sen University, Higher Education Mega Center, Guangzhou, China, Laboratory for Marine Biology and Biotechnology, Marine Fisheries Science and Food Production Processes, Qingdao Marine Science and Technology Center, Qingdao, China

[†]These authors contributed equally to this work

Su-fang Kuang

State Key Laboratory of Bio-Control, School of Life Sciences, Guangdong Province Key Laboratory for Pharmaceutical Functional Genes, Southern Marine Science and Engineering Guangdong Laboratory (Zhuhai), Sun Yat-sen University, Higher Education Mega Center, Guangzhou, China

Huan-zhe Fu

State Key Laboratory of Bio-Control, School of Life Sciences, Guangdong Province Key Laboratory for Pharmaceutical Functional Genes, Southern Marine Science and Engineering Guangdong Laboratory (Zhuhai), Sun Yat-sen University, Higher Education Mega Center, Guangzhou, China

Hui-ying Lin

State Key Laboratory of Bio-Control, School of Life Sciences, Guangdong Province Key Laboratory for Pharmaceutical Functional Genes, Southern Marine Science and Engineering Guangdong Laboratory (Zhuhai), Sun Yat-sen University, Higher Education Mega Center, Guangzhou, China

Bo Peng

State Key Laboratory of Bio-Control, School of Life Sciences, Guangdong Province Key Laboratory for Pharmaceutical Functional Genes, Southern Marine Science and Engineering Guangdong Laboratory (Zhuhai), Sun Yat-sen University, Higher Education Mega Center, Guangzhou, China, Laboratory for Marine Biology and Biotechnology, Marine Fisheries Science and Food Production Processes, Qingdao Marine Science and Technology Center, Qingdao, China
ORCID iD: [0000-0002-5698-6097](https://orcid.org/0000-0002-5698-6097)

For correspondence: pengb26@mail.sysu.edu.cn

Editors

Reviewing Editor

Musa Ali

Hawassa University, Hawassa, Ethiopia

Senior Editor

Bavesh Kana

University of the Witwatersrand, Johannesburg, South Africa

Reviewer #1 (Public review):

Summary:

In the manuscript entitled "Magnesium modulates phospholipid metabolism to promote bacterial phenotypic resistance to antibiotics", Li et al demonstrated the role of magnesium in promoting phenotypic resistance in *V. alginolyticus*. Using standard microbiological and metabolomic techniques, the authors have shown the significance of fatty acid biosynthesis pathway behind the resistance mechanism. This study is significant as it sheds light on the role of an exogenous factor in altering membrane composition, polarization and fluidity which ultimately leads to antimicrobial resistance.

Strengths:

Authors have used different approaches to demonstrate the effect of Mg²⁺ on drug resistance in *Vibrio alginolyticus*. The revised version of the manuscript is much improved, with a very informative introduction and a variety of methodologies with clear explanation of the experiments performed. Also, additional experiments were performed as suggested by the reviewers which certainly enhanced the quality of the paper. I believe the findings of this study will be of high impact in the bacterial community.

Weaknesses:

There are a few grammatical mistakes.

Comments on revisions:

The authors have done a comprehensive job of addressing all my concerns in their revised version.

<https://doi.org/10.7554/eLife.100427.2.sa2>

Reviewer #2 (Public review):

Summary:

In this study, the authors aimed to identify if and how magnesium affects the ability of two particular bacteria species to resist the action of antibiotics. In my view, the authors succeeded in their goals and present a compelling study that will have important implications for the antibiotic resistance research community. Since metals like magnesium are present in all lab media compositions and are present in the host, the data presented in this study certainly will inspire additional research by the community. These could include research into whether other types of metals also induce multi-drug resistance, whether this phenomenon can be observed in other bacterial species, especially pathogenic species that cause clinical disease, and whether the underlying molecular determinants (i.e. enzymes) of metal-induced phenotypic resistance could be new antimicrobial drug targets themselves.

Strengths:

This study's strengths include that the authors used a variety of methodologies, all of which point to a clear effect of exogenous Mg^{2+} on drug resistance in the targeted species. I also comment the authors for carrying out a comprehensive study, spanning evaluation of whole cell phenotypes, metabolic pathways, genetic manipulation, to enzyme activity level evaluation. The fact that the authors uncovered a molecular mechanism underlying Mg^{2+} -induced phenotypic resistance is particularly important as the key proteins should be studied further.

Weaknesses:

I thank the authors for improving their manuscript based on my previous suggestions. I still believe the Results section is long and bogs down at times.

In general, the conclusions drawn by the authors are justified by the data, except for the interpretation of some experiments. Importantly, this paper has discovered new antimicrobial resistance mechanisms and has also pointed to potential new targets for antimicrobials.

Comments on revisions:

I just wanted to thank the authors for addressing most of my previous comments.

<https://doi.org/10.7554/eLife.100427.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Public Reviews:

Reviewer #1 (Public Review):

Summary:

*In the manuscript entitled "Magnesium modulates phospholipid metabolism to promote bacterial phenotypic resistance to antibiotics", Li et al demonstrated the role of magnesium in promoting phenotypic resistance in *V. alginolyticus*. Using standard microbiological and metabolomic techniques, the authors have shown the significance of fatty acid biosynthesis pathway behind the resistance mechanism. This study is significant as it sheds light on the role of an exogenous factor in altering membrane composition, polarization, and fluidity which ultimately leads to antimicrobial resistance.*

Strengths:

- (1) *The experiments were carried out methodically and logically.*
- (2) *An adequate number of replicates were used for the experiments.*

Weaknesses:

- (1) *The introduction section needs to be more informative and to the point.*

Thank you so much for your suggestion. We have revised the introduction to make it more informative and to the point as following:

“Non-inheritable antibiotic or phenotypic resistance represents a serious challenge for treating bacterial infections. Phenotypic resistance does not involve genetic mutations and is transient, allowing bacteria to resume normal growth. Biofilm and bacterial persisters are two phenotypic resistance types that have been extensively studied (Brandis *et al.*, 2023; Corona & Martinez, 2013). Biofilms have complex structures, containing elements that impede antibiotic diffusion, sequestering and inhibiting their activity (Ciofu *et al.*, 2022). Biofilm-forming bacteria and persisters also have distinct metabolic states that significantly reduce their antibiotic susceptibility (Yan & Bassler, 2019). These two types of phenotypic resistance share the common feature in their retarded or even cease of growth in the presence of antibiotics (Corona & Martinez, 2013). However, specific factors that promote phenotypic resistance and allow bacteria to proliferate in the presence of antibiotics remain poorly defined.

Metal ions have a diverse impact on the chemical, physical, and physiological processes of antibiotic resistance (Booth *et al.*, 2011; Lu *et al.*, 2020; Poole, 2017). This includes genetic elements that confer resistance to metals and antibiotics (Poole, 2017) and metal cations that directly hinder (or enhance) the activity of specific antibiotic drugs (Zhang *et al.*, 2014). The metabolic environment can also impact the sensitivity of bacteria to antibiotics (Jiang *et al.*, 2023; Lee & Collins, 2012; Peng *et al.*, 2015; Zhang *et al.*, 2020; Zhao *et al.*, 2021). Light metal ions, such as magnesium, sodium, and potassium, can behave as cofactors for different enzymes (Du *et al.*, 2016) and influence drug efficacy. Heavy metal ions, including Cu²⁺ and Zn²⁺, confer resistance to antibiotics (Yazdankhah *et al.*, 2014; Zhang *et al.*, 2018). Recent reports suggest that sodium negatively regulates redox states to promote the antibiotic resistance of *Vibrio alginolyticus* (Yang *et al.*, 2018), while actively growing *Bacillus subtilis* cope with ribosome-targeting antibiotics by modulating ion flux (Lee *et al.*, 2019). In Gram-negative bacteria, by contrast, zinc enhances antibiotic efficacy by potentiating carbapenem, fluoroquinolone, and β -lactam-mediated killing (Isaei *et al.*, 2016; Zhang *et al.*, 2014). Magnesium influences bacterial structure, cell motility, enzyme function, cell signaling, and pathogenesis (Wang *et al.*, 2019). This mineral also modulates microbiota to harvest energy

from the diet (Garcia-Legorreta *et al.*, 2020), allowing *Bacillus subtilis* to cope with ribosome-targeting antibiotics by modulating ion flux (Lee *et al.*, 2019). However, the role of magnesium in promoting phenotypic resistance is less well understood.

Vibrios inhabit seawater, estuaries, bays, and coastal waters, regions full of metal ions such as magnesium (Kumarage *et al.*, 2022). Magnesium is the second most dissolved element in seawater after sodium. At a salinity of 3.5% seawater, the magnesium concentration is about 54 mM (Potis, 1968), and in deep seawater, can be as high as 2,500 mM (Wang *et al.*, 2024). *Vibrio parahaemolyticus* and *V. alginolyticus* are two representative *Vibrio* pathogens that infect humans and aquatic animals, resulting in illness and economic loss, respectively (Grimes, 2020). (Fluoro)quinolones such as balofloxacin are used to treat *Vibrio* infection, however, resistance has emerged due to overuse (Suyamud *et al.*, 2024). Indeed, (fluoro)quinolones are one of China's two primary residual chemicals associated with aquaculture (Liu *et al.*, 2017). *Vibrio* can develop quinolone resistance through mutations in the DNA gyrase gene or through plasmid-mediated mechanisms (Dutta *et al.*, 2021). Thus, the use of *V. parahaemolyticus* and *V. alginolyticus* as bacterial representatives, and balofloxacin as a quinolone-based antibacterial representative, can help to define novel magnesium-dependent phenotypic resistance mechanisms of pathogenic *Vibrio* species.

The current study evaluated whether magnesium induces phenotypic resistance in *Vibrio* species and defined the molecular/genetic basis for this resistance. Genetic approaches, GC-MS analysis of metabolite and membrane remodeling upon antibiotic exposure, membrane physiology, and extensive antimicrobial susceptibility testing were used for the evaluations.”

(2) The weakest point of this paper is in the logistics through the results section. The way authors represented the figures and interpreted them in the results section (or the figure legends) does not match. The figures are difficult to interpret and are not at all self-explanatory.

Thank you so much for your suggestion. We have followed your suggestion to check the match between result and figures. They are now revised.

(3) There are too many mislabeling of the figure panels in the main text which makes it difficult to find out which figures the authors are explaining. There should be more explanation on why and how they did the experiments and how the results were interpreted.

Thank you so much for your suggestion. We have checked the figures and main text to ensure that we make every figure clearly stated.

Reviewer #2 (Public Review):

Summary:

In this study, the authors aimed to identify if and how magnesium affects the ability of two particular bacteria species to resist the action of antibiotics. In my view, the authors succeeded in their goals and presented a compelling study that will have important implications for the antibiotic resistance research community. Since metals like magnesium are present in all lab media compositions and are present in the host, the data presented in this study certainly will inspire additional research by the community. These could include research into whether other types of metals also induce multi-drug resistance, whether this phenomenon can be observed in other bacterial species, especially pathogenic species that cause clinical disease, and whether the underlying molecular determinants (i.e. enzymes) of metal-induced phenotypic resistance could be new antimicrobial drug targets themselves.

Strengths:

This study's strengths include that the authors used a variety of methodologies, all of which point to a clear effect of exogenous Mg²⁺ on drug resistance in the targeted species. I also commend the authors for carrying out a comprehensive study, spanning evaluation of whole cell phenotypes, metabolic pathways, genetic manipulation, to enzyme activity level evaluation. The fact that the authors uncovered a molecular mechanism underlying Mg²⁺-induced phenotypic resistance is particularly important as the key proteins should be studied further.

Weaknesses:

I believe there are weaknesses in the manuscript, however. The authors take for granted that the reader is familiar with all the assays utilized, and do not properly explain some experiments, and thus I highly suggest that the authors add a brief statement in each situation describing the rationale for each selected methodology (more details are in the private review to the authors). The Results section is also quite long and bogs down at times, and I suggest that the authors reduce its length by 10 to 20%. In contrast, the Introduction is sparse and lacks key aspects, for example, there should be mention of the study's main purpose and approaches, plus an introduction to the authors' choice of species and their known drug resistance properties, as well as the drug of choice (balofloxacin). Another notable weakness is that the authors evaluated Mg²⁺-induced phenotypic resistance only against two closely related species, and thus the generalizability of this mechanism of drug resistance is not known. The paper would be strengthened if the authors could demonstrate this type of phenotypic resistance in at least one more Gram-negative species and at least one Gram-positive species (antimicrobial susceptibility evaluations would suffice), each of which should be pathogenic to humans. Demonstrating magnesium-induced phenotypic drug resistance in the WHO Priority Bacterial Pathogens would be particularly important.

In general, the conclusions drawn by the authors are justified by the data, except for the interpretation of some experiments. Importantly, this paper has discovered new antimicrobial resistance mechanisms and has also pointed to potential new targets for antimicrobials.

Thank you so much for your suggestion! We followed your idea to revise the manuscript as following:

- (1) We added a brief statement in the situation to explain the result and methodology according to your suggestion in the private review.
- (2) To make the streamline of the story more logic, we moved the whole second result to supplementary text and supplementary figure.
- (3) We revised the introduction part by adding additional information to make it informative and to the point as following:

“Non-inheritable antibiotic or phenotypic resistance represents a serious challenge for treating bacterial infections. Phenotypic resistance does not involve genetic mutations. Phenotypic resistance does not involve genetic mutations and is transient, allowing bacteria to resume normal growth. Biofilm and bacterial persisters are two phenotypic resistance types that have been extensively studied (Brandis *et al.*, 2023; Corona & Martinez, 2013). Biofilms have complex structures, containing elements that impede antibiotic diffusion, sequestering and inhibiting their activity (Ciofu *et al.*, 2022). Biofilm-forming bacteria and persisters also have distinct metabolic states that significantly reduce their antibiotic susceptibility (Yan & Bassler, 2019). These two types of phenotypic resistance share the

common feature in their retarded or even cease of growth in the presence of antibiotics (Corona & Martinez, 2013). However, specific factors that promote phenotypic resistance and allow bacteria to proliferate in the presence of antibiotics remain poorly defined.

Metal ions have a diverse impact on the chemical, physical, and physiological processes of antibiotic resistance (Booth *et al.*, 2011; Lu *et al.*, 2020; Poole, 2017). This includes genetic elements that confer resistance to metals and antibiotics (Poole, 2017) and metal cations that directly hinder (or enhance) the activity of specific antibiotic drugs (Zhang *et al.*, 2014). The metabolic environment can also impact the sensitivity of bacteria to antibiotics (Jiang *et al.*, 2023; Lee & Collins, 2012; Peng *et al.*, 2015; Zhang *et al.*, 2020; Zhao *et al.*, 2021). Light metal ions, such as magnesium, sodium, and potassium, can behave as cofactors for different enzymes (Du *et al.*, 2016) and influence drug efficacy. Heavy metal ions, including Cu²⁺ and Zn²⁺, confer resistance to antibiotics (Yazdankhah *et al.*, 2014; Zhang *et al.*, 2018). Recent reports suggest that sodium negatively regulates redox states to promote the antibiotic resistance of *Vibrio alginolyticus* (Yang *et al.*, 2018), while actively growing *Bacillus subtilis* cope with ribosome-targeting antibiotics by modulating ion flux (Lee *et al.*, 2019). In Gram-negative bacteria, by contrast, zinc enhances antibiotic efficacy by potentiating carbapenem, fluoroquinolone, and β -lactam-mediated killing (Isaei *et al.*, 2016; Zhang *et al.*, 2014). Magnesium influences bacterial structure, cell motility, enzyme function, cell signaling, and pathogenesis (Wang *et al.*, 2019). This mineral also modulates microbiota to harvest energy from the diet (Garcia-Legorreta *et al.*, 2020), allowing *Bacillus subtilis* to cope with ribosome-targeting antibiotics by modulating ion flux (Lee *et al.*, 2019). However, the role of magnesium in promoting phenotypic resistance is less well understood.

Vibrios inhabit seawater, estuaries, bays, and coastal waters, regions full of metal ions such as magnesium (Kumarage *et al.*, 2022). Magnesium is the second most dissolved element in seawater after sodium. At a salinity of 3.5‰ seawater, the magnesium concentration is about 54 mM (Potis, 1968), and in deep seawater, can be as high as 2,500 mM (Wang *et al.*, 2024). *Vibrio parahaemolyticus* and *V. alginolyticus* are two representative *Vibrio* pathogens that infect humans and aquatic animals, resulting in illness and economic loss, respectively (Grimes, 2020). (Fluoro)quinolones such as balofloxacin are used to treat *Vibrio* infection, however, resistance has emerged due to overuse (Suyamud *et al.*, 2024). Indeed, (fluoro)quinolones are one of China's two primary residual chemicals associated with aquaculture (Liu *et al.*, 2017). *Vibrio* can develop quinolone resistance through mutations in the DNA gyrase gene or through plasmid-mediated mechanisms (Dutta *et al.*, 2021). Thus, the use of *V. parahaemolyticus* and *V. alginolyticus* as bacterial representatives, and balofloxacin as a quinolone-based antibacterial representative, can help to define novel magnesium-dependent phenotypic resistance mechanisms of pathogenic *Vibrio* species.

The current study evaluated whether magnesium induces phenotypic resistance in *Vibrio* species and defined the molecular/genetic basis for this resistance. Genetic approaches, GC-MS analysis of metabolite and membrane remodeling upon antibiotic exposure, membrane physiology, and extensive antimicrobial susceptibility testing were used for the evaluations.”

(4) We examined the effect of magnesium in WHO listed priority strains, which confirmed the results as following:

“Importantly, exogenous MgCl₂ also increased MICs of clinic isolates, carbapenem-resistant *Escherichia coli*, carbapenem-resistant *Klebsiella pneumoniae*, carbapenem-resistant *Pseudomonas aeruginosa* and carbapenem-resistant *Acinetobacter baumannii* to balofloxacin (Fig 1G).”

Recommendations for the authors:

Reviewer #1 (Recommendations For The Authors):

(1) There are many grammatical mistakes to point out. The manuscript needs proofreading and editing.

We appreciate this comment! The manuscript has been revised by a native speaker.

(2) The introduction could be more informative. A little more description of magnesium - such as what it does to antibiotics and how it's known to affect the microbiome - might be helpful for the general readers. The question remains why out of all the metal ions that might affect antibiotic resistance (many of them are less explored), authors particularly decided to work on the effect of magnesium. The introduction should cover the rationale of their hypothesis. Also, the authors might want to briefly talk about the model organisms (*V. alginolyticus* and *V. parahaemolyticus*) describing how threatening they are and how they are becoming resistant to antibiotics.

We appreciate this comment! We revise the introduction by providing additional information as following:

“In Gram-negative bacteria, by contrast, zinc enhances antibiotic efficacy by potentiating carbapenem, fluoroquinolone, and β -lactam-mediated killing (Isaei *et al.*, 2016; Zhang *et al.*, 2014). Magnesium influences bacterial structure, cell motility, enzyme function, cell signaling, and pathogenesis (Wang *et al.*, 2019). This mineral also modulates microbiota to harvest energy from the diet (Garcia-Legorreta *et al.*, 2020), allowing *Bacillus subtilis* to cope with ribosome-targeting antibiotics by modulating ion flux (Lee *et al.*, 2019). However, the role of magnesium in promoting phenotypic resistance is less well understood.

Vibrios inhabit seawater, estuaries, bays, and coastal waters, regions full of metal ions such as magnesium (Kumarage *et al.*, 2022). Magnesium is the second most dissolved element in seawater after sodium. At a salinity of 3.5‰ seawater, the magnesium concentration is about 54 mM (Potis, 1968), and in deep seawater, can be as high as 2,500 mM (Wang *et al.*, 2024). *Vibrio parahaemolyticus* and *V. alginolyticus* are two representative *Vibrio* pathogens that infect humans and aquatic animals, resulting in illness and economic loss, respectively (Grimes, 2020). (Fluoro)quinolones such as balofloxacin are used to treat *Vibrio* infection, however, resistance has emerged due to overuse (Suyamud *et al.*, 2024). Indeed, (fluoro)quinolones are one of China's two primary residual chemicals associated with aquaculture (Liu *et al.*, 2017). *Vibrio* can develop quinolone resistance through mutations in the DNA gyrase gene or through plasmid-mediated mechanisms (Dutta *et al.*, 2021). Thus, the use of *V. parahaemolyticus* and *V. alginolyticus* as bacterial representatives, and balofloxacin as a quinolone-based antibacterial representative, can help to define novel magnesium-dependent phenotypic resistance mechanisms of pathogenic *Vibrio* species.

The current study evaluated whether magnesium induces phenotypic resistance in *Vibrio* species and defined the molecular/genetic basis for this resistance. Genetic approaches, GC-MS analysis of metabolite and membrane remodeling upon antibiotic exposure, membrane physiology, and extensive antimicrobial susceptibility testing were used for the evaluations.”

(3) Figure 1C is mislabeled as 1B (line 100). Line 101: The sentence is not clear and very confusing. What is meant by 15.6mM - 62.4 mM? Are they talking about the concentration of BLFX (though in the figure the concentration was shown in μ g)? Please rewrite the sentence in a simplified way. Also, the zone of inhibition was decreased with increasing $MgCl_2$, not increased.

We appreciate this comment! These have been revised, including that Fig 1B is now corrected as Fig. 1C. Line 101, which is now Line 122. The sentence was revised as following:

“At balofloxacin doses of 1.56, 3.125, 6.25, and 12.5 μg , the zone of inhibition decreased with increasing MgCl_2 (Fig 1D)”

(4) In the western blot images, it would be nice to indicate the MW of the protein bands shown. The loading control used for the experiments should be clearly mentioned in the figure legends.

We appreciate this comment! The MWs are indicated in the western-blot image throughout the manuscript.

The loading control is clearly stated in the figure legend as following:

“Whole cell lysates resolved by SDS-PAGE gel was stained with Coomassie brilliant blue as loading control.”.

(5) Figures 2 B and C: the figure legend does not explain what the authors wanted to show. It's not clear how they plotted the inhibitory curve, or the binding efficacy. These panels need an explanation of how the analysis was done.

We appreciate this comment! The figure 2 is now removed to Suppl. Fig 2, and the description of figure 2 is moved to Suppl. Text. We revise the description of the result as following, which is in Suppl. Text:

“Prior studies suggest that the chelation of antibiotics by magnesium ions inhibits antibiotic uptake (Deitchman et al., 2018; Lunestad and Goksøyr, 1990). To investigate whether magnesium binds to balofloxacin, balofloxacin was pre-incubated with magnesium, and zone of inhibition (ZOI) analysis was conducted. Six different concentrations of balofloxacin were separately incubated with six different concentrations of MgCl_2 , and then spotted on filter paper so that a defined amount of balofloxacin could be used for ZOI. While lower concentrations of MgCl_2 , (0.78, 3.125, or 12.5 mM) did not alter the ZOI, higher concentrations, including 50 and 200 mM MgCl_2 , decreased the ZOI (Suppl. Fig 2A), suggesting that even high doses of magnesium had only a partial effect on balofloxacin through direct binding. For example, at 200 mM MgCl_2 and 5 or 10 $\mu\text{g}/\text{mL}$ balofloxacin, the balofloxacin ZOI was 53.2 and 70.3% of the ZOI at 0 mM MgCl_2 , suggesting that $\approx 50\%$ of the antibiotics were still functional. Intracellular BLFX also decreased with increasing MgCl_2 (Suppl. Fig 2B), while exogenous Mg^{2+} increased intracellular Mg^{2+} levels in a dose-dependent manner. For example, exogenous 50 and 200 mM MgCl_2 increased intracellular Mg^{2+} levels to 1.21 and 1.31 mM, respectively (Suppl. Fig 2C). The relationship between TolC, an efflux pump that transports quinolones from bacterial cells, and Mg^{2+} was also assessed (Kobylka et al., 2020; Song et al., 2020). The expression of TolC/tolC was unaffected by Mg^{2+} (Suppl. Fig 2D). Magnesium is critical for LPS stability. LPS levels increased at 200 mM Mg^{2+} (Suppl. Fig 2E), however, the loss of *waaF*, *lpxA*, and *lpxC*, three key genes involved in LPS biosynthesis, did not influence balofloxacin sensitivity/resistance in the presence of Mg^{2+} (Suppl. Fig 2F). These findings suggest that magnesium-induced LPS biosynthesis does not contribute directly to BLFX resistance and demonstrate that Mg^{2+} influx is involved in balofloxacin resistance.”

(6) For the metabolomics results, it will help immensely if the authors provide a volcano plot of the identified metabolites and plot the heat map according to the -log2 metabolite intensities. In Figure 3A, it's not clear what information is conveyed through Euclidean distance calculations of the heat map. In Figure 3 B, the authors mentioned that the OPLS-DA test was conducted, although the figure shows a PCA plot, so it's not clear how these two are connected. Figure 3 E: the figure legend says scattered plot, but the panel represents color-coded numerical values, not a scattered plot. Also, it's not clear how they got those values.

We appreciate this comment! We quite agree with you that if the differential metabolites could be shown as volcano plot. However, we didn't adopt volcano plot in this study because this is a magnesium concentration-dependent metabolomes that includes 6 groups in parallel. Volcano plots may give a complex view of the comparison among different groups. We also tried to plot the heat map according to the $-\log_2$ metabolite intensities. Although this analysis cluster 200 mM and 50 mM groups better, the data of low magnesium concentrations was not consistent, which may be due to the minor metabolic change of low concentrations magnesium. Thank you for your understanding.

For Euclidean distance calculations, we explain in the figure legend as following:

"Euclidean distance calculations were used to generate a heatmap that shows clustering of the biological and technical replicates of each treatment."

In Figure 2B, which was Figure 3B in previous version, it has been replaced with OPLS-DA analysis in the revised version.

In Figure 2E, which was Figure 3E in previous version, it is revised as following:

"E. Areas of the peaks of palmitic acid and stearic acid generated by GC-MS analysis."

(7) In Figure 4, the figure legends (as well as the in the text) are not properly referred to. Please make sure to refer to the correct panel.

We appreciate this comment! The figure legends have been corrected to match the panel and text.

Figure 4F: how was the synergy analysis done? In the methods section, the authors described the antibiotic bactericidal assay protocol, but there was no clear indication of how they generated the isobologram.

We appreciate this comment! We provide additional information in the Figure 3F legend, which was Figure 4F in previous version, as following:

"Synergy analysis for BFLX with palmitic acid for *V. alginolyticus*. Synergy was performed by comparing the dose needed for 50% inhibition of the synergistic agents (white) and non-synergistic (i.e., additive) agents (purple)."

(8) Figure 5 A: the scatter plot is plotted according to the area along the Y axis: which "area" is represented here? There is absolutely no explanation, neither in the results nor in the figure legends. Using box plots might be a better option than using a scattered plot.

We appreciate this comment! "Area" has been noted in the revised manuscript as following:

"The area indicates the area of the peak of the metabolite in total ion chromatography of GC-MS."

(9) In Figure 6 A, the heat map is plotted according to the column Z scores. What is meant by "column Z score"? The corresponding figure legend says, "heat map showing differential abundance of lipid". Z scores do not represent an abundance of a variable, so the conclusion might not be appropriate here.

We appreciate this comment! In Figure 5A, which was Figure 6A in previous version, column Z score shows the abundance of metabolites analyzed, which is automatically generated in

the heat map analysis to give a sign of these metabolites tested. The legend has been revised as following:

“Heatmap showing changes in differential lipid levels at the indicated concentration of MgCl₂.”

(10) Line 313-314: it should be Figure EV6C.

We appreciate this comment! The citation has been corrected.

(11) The authors have shown that Mg²⁺ does not alter the LPS transport system, however, there was some significant increase in LPS expression at 200mM MgCl₂. It would be interesting if the authors could also check if Mg²⁺ has any effect on the outer membrane protein (OMP) integrity (by checking OMP components BamA and LptD).

We appreciate this comment! We have carefully examined the membrane permeability in Figure 7. We thus didn't perform additional experiment here to see the change of BamA and LptD. Thank you very much for your understanding.

(12) I wonder if the authors could check the effect of extracellular Mg²⁺ during the co-treatment of palmitic acid, linoleic acid, and balofloxacin. Will there still be the antagonistic effect or the presence of Mg²⁺ could change the phenotype?

We appreciate this comment! Additional experiments is performed as following:

“Furthermore, magnesium had a minimal effect on the antagonistic effect of palmitic acid, linolenic acid, and balofloxacin (Fig 4G), suggesting that this mineral functions through lipid metabolism.”

Reviewer #2 (Recommendations For The Authors):

(1) As mentioned in the Public Review, I strongly believe that the impact of this study will be more significant if magnesium-induced phenotypic drug resistance could be demonstrated in at least one other Gram-negative and one other Grampositive species, both of which should be human pathogens. The full suite of experiments would not be necessary for this suggestion; evaluation of the effect of Mg concentration in growth media on the drug resistance of other species, testing the different antibiotic types used in this study, would be sufficient.

We appreciate this comment! Additional experiments have performed to test this idea. Mg²⁺ has the similar effect on carbapenem-resistant *Escherichia coli*, carbapenem-resistant *Klebsiella pneumoniae*, carbapenem-resistant *Pseudomonas aeruginosa* and carbapenem-resistant *Acinetobacter baumannii* as the similar as on the *Vibrio* species in shown in Figure 1G. These have been described following as

“Importantly, exogenous MgCl₂ also increased MICs of clinic isolates, carbapenem-resistant *Escherichia coli*, carbapenem-resistant *Klebsiella pneumoniae*, carbapenem-resistant *Pseudomonas aeruginosa* and carbapenem-resistant *Acinetobacter baumannii* to balofloxacin (Fig 1G).”

(2) I recommend that the Introduction section be expanded. I recommend one or two sentences introducing the two *Vibrio* species selected for study. I.e. why did the authors choose these two species? What is known about their phenotypic drug resistance in the literature? Why did the authors select balofloxacin for their studies, is it a common antimicrobial used vs *Vibrios*? As well, the end of the Introduction section ends abruptly

with no transition to the present study itself. The end of the introduction should include one or two sentences introducing the main purpose of the study, its approach, and the techniques undertaken. For example, "In this study, we evaluated whether magnesium induces phenotypic resistance in Vibrio species and the molecular/genetic basis for such resistance. We used genetic approaches, GC-MS analysis of metabolite and membrane remodeling upon antibiotic exposure, membrane physiology, and extensive antimicrobial susceptibility evaluations."

We appreciate this comment! We revise the introduction by providing additional information as following:

"In Gram-negative bacteria, by contrast, zinc enhances antibiotic efficacy by potentiating carbapenem, fluoroquinolone, and β -lactam-mediated killing (Isaei *et al.*, 2016; Zhang *et al.*, 2014). Magnesium influences bacterial structure, cell motility, enzyme function, cell signaling, and pathogenesis (Wang *et al.*, 2019). This mineral also modulates microbiota to harvest energy from the diet (Garcia-Legorreta *et al.*, 2020), allowing *Bacillus subtilis* to cope with ribosome-targeting antibiotics by modulating ion flux (Lee *et al.*, 2019). However, the role of magnesium in promoting phenotypic resistance is less well understood.

Vibrios inhabit seawater, estuaries, bays, and coastal waters, regions full of metal ions such as magnesium (Kumarage *et al.*, 2022). Magnesium is the second most dissolved element in seawater after sodium. At a salinity of 3.5% seawater, the magnesium concentration is about 54 mM (Potis, 1968), and in deep seawater, can be as high as 2,500 mM (Wang *et al.*, 2024). *Vibrio parahaemolyticus* and *V. alginilyticus* are two representative *Vibrio* pathogens that infect humans and aquatic animals, resulting in illness and economic loss, respectively (Grimes, 2020). (Fluoro)quinolones such as balofloxacin are used to treat *Vibrio* infection, however, resistance has emerged due to overuse (Suyamud *et al.*, 2024). Indeed, (fluoro)quinolones are one of China's two primary residual chemicals associated with aquaculture (Liu *et al.*, 2017). *Vibrio* can develop quinolone resistance through mutations in the DNA gyrase gene or through plasmid-mediated mechanisms (Dutta *et al.*, 2021). Thus, the use of *V. parahaemolyticus* and *V. alginilyticus* as bacterial representatives, and balofloxacin as a quinolone-based antibacterial representative, can help to define novel magnesium-dependent phenotypic resistance mechanisms of pathogenic *Vibrio* species.

The current study evaluated whether magnesium induces phenotypic resistance in *Vibrio* species and defined the molecular/genetic basis for this resistance. Genetic approaches, GC-MS analysis of metabolite and membrane remodeling upon antibiotic exposure, membrane physiology, and extensive antimicrobial susceptibility testing were used for the evaluations. "

(3) The authors introduce the acronym AWST but never use it again in the paper, instead they use SWT. The authors should introduce SWT only for consistency.

We appreciate this comment! We have corrected all the "SWT" to "ASWT"

(4) Line 76 is not clear: what is meant by "some of which could influence drug efficacy" - the enzymes that utilize light metal ions are co-factors? Or the metals directly?

We appreciate this comment! The information we wanted to deliver is that light metal ions can serve as cofactors to catalyze biochemical reaction. Such chemical reaction would alter the drug efficacy, e.g. the Fe-S cluster are metallocofactor for proteins which regulates redox chemistry including antibiotic-induced redox change. However, this information is not appropriate for this manuscript, so we delete this sentence.

(5) Line 90: add a reference corroborating that this chemical composition is a mimic of marine water. The NaCl concentration used in particular looks quite low.

We appreciate this comment! It was a typo error. The NaCl concentration was 210 mM as shown in Suppl. Table 1. We also provide details of the chemical composition of the marine water as following:

“Marine environments and agriculture, where antibiotics are commonly used, are rich in magnesium. To investigate whether this mineral impacts antibiotic activity, the minimal inhibitory concentration (MIC) of *V. alginolyticus* ATCC33787 and *V. parahaemolyticus* VP01, which we referred as ATCC33787 and VP01 afterwards, isolated from marine aquaculture, to balofloxacin (BLFX) in Luria-Bertani medium

(LB medium) plus 3% NaCl as LBS medium and “artificial seawater” (ASWT) medium that included the major ion species in marine water (Wilson, 1975) (LB medium plus 210 mM NaCl, 35 mM Mg₂SO₄, 7 mM KCl, and 7 mM CaCl₂) were assessed”

(6) Line 98 and Figure 1B. M9 is indicated in the text but does not appear in the figure, the figure only shows SWT. This should be checked. Line 99: based on Figure 1C, the authors are adding MgCl₂ to SWT, SWT should be mentioned in this line. Line 100: I believe this is referring to Figure 1C, which should be checked.

We appreciate this comment!

Line 98, which is now Line 118: We have corrected M9 to ASWT as following:

“However, the MIC for BLFX was higher in ASWT medium supplemented with Mg₂SO₄ or MgCl₂ than in LB medium (Fig 1B).”

Line 99, which is now Line 133: the sentence is corrected as following:

“The MIC for BLFX increased at higher concentrations of MgCl₂ in ASWT”

Line 100, which is now Line 135: we have corrected Fig 1B to Fig. 1C.

(7) Line 101: text and Figure 1D are not consistent, as Figure 1D does not show this level of precision in added MgCl₂ as indicated in the text (15.6 - 62.4 mM).

We appreciate this comment! The sentence has been corrected as following: “At balofloxacin doses of 1.56, 3.125, 6.25, and 12.5 µg, the zone of inhibition decreased with increasing MgCl₂ (Fig 1D)”.

(8) MgCl₂ clearly induces increasing levels of BLFX resistance, and to high levels, but not for every antibiotic. For example, the level of increased resistance to blactams is low (ceftriaxone) and plateaus (ceftazidime). As well, resistance to gentamicin plateaus at a lower level than the other aminoglycosides. These observations do not take away from the conclusion that Mg induces multi-drug resistance, but since the behaviour of the MICs for these drugs is different than the other drugs, they should be mentioned. Also, Figure 1F - tetracyclines (plural) is used for vertical axis label - does this refer to the tetracycline itself or the class itself, and if the class, which one was tested?

We appreciate this comment! We revise the description as following: “Notably, magnesium had a reduced effect on ceftriaxone and gentamicin than other antibiotics.”

The tetracyclines is labeled as “Oxytetracycline” in the revised manuscript.

- The magnesium chelation experiments presented in Figure 2 are not clear. The authors should briefly mention how this was done around line 128, and what data underlies the values in Figure 2C. Figure 2B is also not clear to me at all. Similarly, how the authors

measured intracellular balofloxacin and Mg²⁺ is not clear and should be mentioned briefly around lines 130-132.

We appreciate this comment! These have been rewritten following as “To investigate whether magnesium binds to balofloxacin, balofloxacin was preincubated with magnesium, and zone of inhibition (ZOI) analysis was conducted. Six different concentrations of balofloxacin were separately incubated with six different concentrations of MgCl₂, and then spotted on filter paper so that a defined amount of balofloxacin could be used for ZOI. While lower concentrations of MgCl₂, (0.78, 3.125, or 12.5 mM) did not alter the ZOI, higher concentrations, including 50 and 200 mM MgCl₂, decreased the ZOI (Suppl. Fig 2A), suggesting that even high doses of magnesium had only a partial effect on balofloxacin through direct binding. For example, at 200 mM MgCl₂ and 5 or 10 µg/mL balofloxacin, the balofloxacin ZOI was 53.2 and 70.3% of the ZOI at 0 mM MgCl₂, suggesting that ~50% of the antibiotics were still functional. Intracellular BLFX also decreased with increasing MgCl₂ (Suppl. Fig 2B), while exogenous Mg²⁺ increased intracellular Mg²⁺ levels in a dose-dependent manner. For example, exogenous 50 and 200 mM MgCl₂ increased intracellular Mg²⁺ levels to 1.21 and 1.31 mM, respectively (Suppl. Fig 2C). The relationship between TolC, an efflux pump that transports quinolones from bacterial cells, and Mg²⁺ was also assessed (Kobylka *et al.*, 2020; Song *et al.*, 2020). The expression of TolC/*tolC* was unaffected by Mg²⁺ (Suppl. Fig 2D). Magnesium is critical for LPS stability. LPS levels increased at 200 mM Mg²⁺ (Suppl. Fig 2E), however, the loss of *waaF*, *lpxA*, and *lpxC*, three key genes involved in LPS biosynthesis, did not influence balofloxacin sensitivity/resistance in the presence of Mg²⁺ (Suppl. Fig 2F). These findings suggest that magnesium-induced LPS biosynthesis does not contribute directly to BLFX resistance and demonstrate that Mg²⁺ influx is involved in balofloxacin resistance.”

- Line 135: LPS cannot be "expressed", as the authors word it here. This should be corrected. Also, the inspection of Figure 2G actually shows the levels of LPS increase with increased Mg²⁺. The authors should re-evaluate these results and change their description around this area of the Results.

We appreciate this comment! We have removed the whole Figure 2 to Supplementary Text and Supplementary Figure 2. We rewrite this part as following: “The relationship between TolC, an efflux pump that transports quinolones from bacterial cells, and Mg²⁺ was also assessed (Kobylka *et al.*, 2020; Song *et al.*, 2020). The expression of TolC/*tolC* was unaffected by Mg²⁺ (Suppl. Fig 2D). Magnesium is critical for LPS stability. LPS levels increased at 200 mM Mg²⁺ (Suppl. Fig 2E), however, the loss of *waaF*, *lpxA*, and *lpxC*, three key genes involved in LPS biosynthesis, did not influence balofloxacin sensitivity/resistance in the presence of Mg²⁺ (Suppl. Fig 2F). These findings suggest that magnesium-induced LPS biosynthesis does not contribute directly to BLFX resistance and demonstrate that Mg²⁺ influx is involved in balofloxacin resistance.”

- Section: MgCl₂ affects bacterial metabolism. Authors switched to M9 medium - why? This contrasts with other sections using SWT and should be explained. Also, I cannot evaluate whether the statistical analysis of the data here was performed correctly and was appropriate for this type of experiment. I advise the authors to move the details in lines 166-169 to the Materials and Methods and replace this section instead with a more accessible description of the statistical analysis that a non-expert would be able to appreciate. Furthermore, analysis of Figure 3A indicates that the levels of asparagine, 4-hydroxybutyric acid, uracil, cystathionine, fumaric acid, and aminoethanol have significantly changed at high MgCl₂, but these are not mentioned in the text. I suggest the authors mention these if they are relevant to the 12 enriched pathways, especially the biosynthesis of fatty acids.

We appreciate this comment!

We indicate the reason we use M9 medium as following:

“To better understand how magnesium affects bacterial metabolism” for explaining why the M9 medium was used.”

The information lines 166-169 indicated has been removed to M &M.

We have carefully examined the abundance of the metabolites and the enriched pathway. Among the listed metabolites, only fumarate is within the enriched pathways. We mention this point in our revised manuscript as following:

“The increase in fatty acid biosynthesis could be partially explained by an imbalanced pyruvate cycle/TCA cycle, in which fumarate levels increased at higher Mg²⁺ while succinate levels increased at lower Mg²⁺ (Suppl. Fig 5B). These findings indicated that glycolysis fluxes into fatty acid biosynthesis rather than the pyruvate cycle/TCA cycle. The relevance of fatty acids and BLFX was demonstrated by the observation that exogenous palmitic acid increased bacterial resistance to balofloxacin (Fig 2F). These results suggest that fatty acid metabolism may be critical to magnesium-based phenotypic resistance.”

- Line 211 appears to refer to Figure 4F and should be checked. Similarly in line 216 - appears this should be Figure 4H, and line 218 should be Figure 4H. Line 226: add a reference to Fig 4I (after *arcA* was decreased). Line 227: what are genes N646_1004 and N646_1885? Based on Fig 4J these are *crp* - authors should add to line 227. Line 228 appears to refer to Figure 4J, not Figure 4I. Line 229 - should be Figure 4K, not Figure 4I. Line 231 - should be 4L, not 4K. Line 239 - should be 4M.

We appreciate this comment! The text and figure is now matched.

- Line 312: the descriptions of "11 lipids, 32 lipids, and 53", and then "26 lipids, 52 lipids, and 107 lipids" are not clear at all and should be corrected.

We appreciate this comment! The sentence is revised as following:

“The abundance of 11, 32, and 53 lipids was increased in 3.125, 50, and 200 mM MgCl₂-treated bacteria, respectively, while the abundance of 26, 52, and 107 lipids was decreased in 3.125, 50, and 200 mM MgCl₂-treated bacteria, respectively (Suppl. Fig 7C)”

- Line 340. What is the assay the authors are using to measure the levels of the PGS and PSS enzymes? This is not mentioned or clear in this part of the Results.

We appreciate this comment! We provide the information in the manuscript as following:

“Levels of PGS and PSS were quantified by ELISA kits according to manufacture’s instruction (Shanghai Fusheng Industrial Co., Ltd., China)”

- Line 372: What is the assay for measuring membrane depolarization? This is not mentioned and I suggest it should be. Line 374: Figure 7B does not show time dependence, only dose dependence, this should be corrected, it is assumed the authors are referring to Fig 7C for the time dependence data.

We appreciate this comment! We provide the information in the result as following:

“The voltage-sensitive dye, DiBAC4(3) showed that 12.5–200 mM MgCl₂ promoted membrane depolarization in a dose-dependent manner (Fig 6A)”

We also explain how DiBAC4(3) can be used to measure membrane depolarization in the Materials and Methods section as following:

“DiBAC4(3) is a voltage-sensitive probe that penetrates depolarized cells, binding intracellular proteins or membranes exhibiting enhanced fluorescence and red spectral shift.”

To make it clear the specific figure, we revise the sentence as following:

“Meanwhile, MgCl₂ had a dose-dependent (Fig 6B) and time-dependent (Fig 6C) effect on proton motive force (PMF).”

- Line 384: mention how FM5-95 measures membrane permeability. The authors should also clarify how this reagent is used to measure membrane fluidity, and it is not clear if the data for this is presented in Figure 7 - please clarify. Regarding SYTO9 dye experiment: the authors should briefly explain the experimental design - how SYTO9 dye operates and why FACS was chosen. What is labeled with FITC?

We appreciate this comment! We clarify the reason we use FM5-95 in the Methods and Materials section as following:

“Measurement of fluidity by fluorescence microscopy

Measurement of membrane fluidity is performed as previously described (Wen et al., 2022). Briefly, ATCC33787 were cultured in medium with indicated concentrations of MgCl₂, collected and then adjusted to OD 0.6. Aliquot of 100 µL bacteria cells of each sample were diluted to 1 mL and 10 µL (10 mg/mL) FM5-95 (Thermo Fisher

Scientific, USA) was added. FM5-95 is a lipophilic styryl dye that insert into the outer leaflet of bacterial membrane and become fluorescence. This dye preferentially bind to the microdomains with high membrane fluidity (Wen et al., 2022). After incubated for 20 min at 30 °C at vibration without light, the sample was centrifuged for 10 min at 12,000 rpm. The pellets were resuspended with 20 µL of 3% NaCl. Aliquot of 2 µL sample was dropped on the agarose slide, and take photos under the inverted fluorescence microscope.”

This data is presented as micrographs in Fig. 6D, which shows the decreased FM5-95 staining with increasing concentrations of MgCl₂. We make this description clear with the following revision:

“FM5-95 staining decreased with increasing concentrations of Mg²⁺, and no staining was observed in the presence of 200 mM Mg²⁺ (Fig 6D).”

We explain the reason why we use SYTO9 as following:

“SYTO9, a green fluorescent dye that binds to nucleic acid, enters and stains bacteria cells when there is an increase in membrane permeability (Lehtinen *et al.*, 2004; McGoverin *et al.*, 2020). Staining decreased with increasing MgCl₂, indicating that bacterial membrane permeability declined in an Mg²⁺ dose-dependent manner (Fig 6E).”

We didn’t use FACS in this study, while we only analyze the fluorescence distribution with the equipment. To make it clear, we revise the sentence as following:

“After incubated for 15 min at 30 °C at vibration without light, the mixtures were filtered and measured by flow cytometry (BD FACSCalibur, USA).”

- Lines 391-397. The statement that palmitic acid shifts the peaks in Figure 7F is not supported by the data. There is essential no change in the major peak position within

each MgCl₂ concentration set with increasing palmitic acid. For the linolenic acid data, it is clear that linolenic acid increases permeability only at 50 mM MgCl₂-this should be mentioned in the text.

We appreciate this comment! We revise the sentence as following:

“Exogenous palmitic acid also shifted the fluorescence signal peaks to the left in an MgCl₂-dependent manner while palmitic acid only slightly shifted the peaks (Fig 6F). In contrast, exogenous linolenic acid shifted the peak to the right in a dose-dependent manner at 50 mM MgCl₂ (Fig 6G).”

- Line 404-405 - as mentioned earlier, the assay for the update of BLFX should be mentioned (if it is done so earlier in the text, then it does not need to be here).

We appreciate this comment! It has been mentioned in the introduction.

- Discussion: CpxA/R-OmpR pathway is mentioned here for the first time. Is this one of the pathways modified by MgCl₂ as determined during the course of the study? If so, this should be reworded to mention that. If not, the relevance of this particular pathway as it relates to light metals and phenotypic resistance should be discussed.

We appreciate this comment! Since it is not relevant to the discussion of Mg²⁺ and fatty acid biosynthesis, we delete this sentence in the revised manuscript.

-The following grammatical errors should be corrected:

-line 55 change to: "genetic mutations; instead, this type of resistance is transient, and bacteria resume normal growth"

-line 57: change to "resistance types are biofilm"

-line 61: change to "states that significantly"

-line 63: change to "resistance share the common feature in they retard or even cease in the presence"

-line 65: change to "resistance that allow bacteria to proliferate"

-line 81: change "But whether" to "Whether"

-line 178: change to "may be critical to the Mg-based phenotypic resistance"

-line 86: change to "Marine environments and agriculture are rich in magnesium, where..."

-line 93: change in to vs

-line 154: insert space after metabolism

-line 158: change 'identified' to "focused on the levels of"

-line 160: change "The levels of forty-one metabolites"

-line 198: change shared to share

-line 310: increased is duplicated, delete one

-line 451: add "the" before ratio

-line 453: gram should be capitalized

-line 462: "the regulation" should be reworded to "More importantly, the effect of exogenous MgCl targets the..."

-line 469: add dash between Mg²⁺ and limited

-line 478: change "the crucial" to "a crucial"

-there are numerous locations in the manuscript where the word "magnetism" is used when clearly the word is supposed to be magnesium - this should be corrected

We appreciate this comment! These have been corrected or revised.

Editors comments:

Page 2 line 27; Page 25 line number 426; page 27 line number 481: In the abstract and discussion, only *Vibrio alginolyticus* was mentioned, even though two *Vibrio* species were used in the study. It would be helpful to understand the rationale behind the focus on this particular species.

We appreciate this comment! We have revised the introduction to provide additional information as following:

"Vibrios inhabit seawater, estuaries, bays, and coastal waters, regions full of metal ions such as magnesium (Kumarage *et al.*, 2022). Magnesium is the second most dissolved element in seawater after sodium. At a salinity of 3.5% seawater, the magnesium concentration is about 54 mM (Potis, 1968), and in deep seawater, can be as high as 2,500 mM (Wang *et al.*, 2024). *Vibrio parahaemolyticus* and *V. alginolyticus* are two representative *Vibrio* pathogens that infect humans and aquatic animals, resulting in illness and economic loss, respectively (Grimes, 2020). (Fluoro)quinolones such as balofloxacin are used to treat *Vibrio* infection, however, resistance has emerged due to overuse (Suyamud *et al.*, 2024). Indeed, (fluoro)quinolones are one of China's two primary residual chemicals associated with aquaculture (Liu *et al.*, 2017). *Vibrio* can develop quinolone resistance through mutations in the DNA gyrase gene or through plasmid-mediated mechanisms (Dutta *et al.*, 2021). Thus, the use of *V. parahaemolyticus* and *V. alginolyticus* as bacterial representatives, and balofloxacin as a quinolone-based antibacterial representative, can help to define novel magnesium-dependent phenotypic resistance mechanisms of pathogenic *Vibrio* species."

On Page 2, line 34: The abstract contains some undefined abbreviations, such as 'PE' and 'PG', which should be explained.

We appreciate this comment! We explain the PE and PG in the revised abstract as following:

"phosphatidylethanolamine (PE) biosynthesis is reduced and phosphatidylglycerol (PG)"

On Page 2, line 31-32: For the statement "Exogenous supplementation of fatty acids confirm the role of fatty acids in antibiotic resistance..." it would be beneficial to specify whether the fatty acids were saturated or unsaturated.

Response, We appreciate this comment! We revise the sentence as following:

"Exogenous supplementation of unsaturated and saturated fatty acids increased and decreased bacterial susceptibility to antibiotics, respectively, confirming the role of fatty acids in antibiotic resistance."

The potential effects of the specific ions (SO₄ and Cl₂) present in the MgSO₄ and MgCl₂ compounds used in the study were not discussed. It would be useful to understand if these ions had any influence on the observed outcomes.

We appreciate this comment! We revise the sentence as following:

“However, the MIC for BLFX was higher in ASWT medium supplemented with MgSO₄ or MgCl₂ than in LB medium (Fig 1B). And MgSO₄ or MgCl₂ had no difference on MIC, suggesting it is Mg²⁺ not other ions contribute to the MIC change.”

On Page 8, line 141: The heading of Figure 2, "Mg²⁺ elevates intracellular Mg²⁺," seems redundant and could be revised for clarity or modified.

We appreciate this comment! Figure 2 is now moved to supplementary figure as Suppl. Fig 2. The title is revised as following:

“Figure 2. Mg²⁺ decreases balofloxacin uptake.”

On Page 4, line 91: some terms/abbreviations, such as 'LB' and 'M9,' require expansion or definition to ensure the reader's understanding.

We appreciate this comment! We include the expansion for LB and M9 in the revised manuscript as following:

“Luria-Bertani medium (LB medium)” and “M9 minimal medium (M9 medium)”

Page 4, line 92: The real seawater composition used in the experiments should be supported by a reference.

We appreciate this comment! We provide the reference in the revised manuscript as following:

““artificial seawater” (ASWT) medium that included the major ion species in marine water (Wilson, 1975) (LB medium plus 210 mM NaCl, 35 mM MgSO₄, 7 mM KCl, and 7 mM CaCl₂)”

Page 4 line, number 93: the full names of the bacterial strains (e.g., ATCC33787 and VP01) should be provided instead of just the strain numbers.

We appreciate this comment! We revised the sentence as following:

“To investigate whether this mineral impacts antibiotic activity, the minimal inhibitory concentration (MIC) of *V. alginolyticus* ATCC33787 and *V. parahaemolyticus* VP01, which we referred as ATCC33787 and VP01 afterwards,”

Finally, there appears to be a potential contradiction between the statements on page 12, lines 211-212 and 214-216, regarding the effects of Mg²⁺ on the synthesis of unsaturated fatty acids. Further explanation may be needed to reconcile these seemingly contradictory points.

We appreciate this comment! For line 221-226, which was previously line 211-212, is about the gene expression for fatty acid biosynthesis. While, Line 228 and 233, which was previously line 214-216 is about the gene expression for fatty acid degradation. We agree that the previous description is a little bit confuse. We revise the sentence to emphasize that we focus on fatty acid degradation so that the readers can tell them apart.

In the text, we revised it as following:

“In addition, we also quantified gene expression during fatty acid degradation to determine whether Mg^{2+} affects this process” In the figure legend, we also indicate that

“H. qRT-PCR for the expression of genes encoding fatty acid degradation in the absence or presence of the indicated concentrations of $MgCl_2$ ”

<https://doi.org/10.7554/eLife.100427.2.sa0>