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Highlights

- Ciprofloxacin metabolites M_3 and M_4 were tested at sub-inhibitory concentrations
- M_3 and M_4 induced up to 8-fold MIC increases in *S. aureus* after 30 days
- Both metabolites enhanced *P. aeruginosa* biofilm; WGS identified wspA mutation A290D
- RNAseq showed 220 differentially expressed genes including quorum sensing pathways

Ciprofloxacin Metabolites Drive Resistance Development and Biofilm Changes in *Pseudomonas aeruginosa* and *Staphylococcus aureus*

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Abstract

Objectives: To investigate how two major ciprofloxacin metabolites, 2-oxo ciprofloxacin (**M_3**) and N-formyl ciprofloxacin (**M_4**), influence the resistance profiles and biofilm characteristics of *Pseudomonas aeruginosa* (PAO1) and *Staphylococcus aureus* (ATCC 25923).

Methods: Both metabolites were synthesised, structurally validated, and co-cultured with bacterial strains at sub-inhibitory concentrations (SICs) over a 30-day period. Minimum inhibitory concentration (MIC) assays, crystal violet biofilm quantification, and genomic and transcriptomic analyses (whole genome and differential RNA sequencing) were employed to assess phenotypic and molecular adaptations.

Results: Exposure to ciprofloxacin metabolites altered bacterial behaviour despite their weak intrinsic antimicrobial activity. In *S. aureus*, continuous exposure to **M_3** and **M_4** resulted in an eight and four-fold increase, respectively, in ciprofloxacin MIC values. In *P. aeruginosa*, although MIC values remained unchanged, prolonged exposure to both metabolites enhanced biofilm formation, **M_4** ($p<0.0001$) and **M_3** ($p<0.0089$)). Genomic sequencing of *P. aeruginosa* revealed a missense mutation (A290D) in the *wspA* gene following **M_3** exposure, which activates biofilm-promoting pathways via cyclic-di-GMP signalling. RNA sequencing identified 220 differentially expressed genes, including upregulation of quorum sensing regulators (*rhlI*, *pqsH*), nitric oxide cycle genes (*nir*, *norCB*), and the *pel* operon.

Conclusions: Ciprofloxacin metabolites, though less potent than the parent antibiotic, can drive adaptive responses linked to resistance and persistence. Their capacity to induce stable genetic and transcriptomic shifts underscores their potential ecological and clinical significance as underexplored factors in AMR.

1. Introduction

The discovery and widespread clinical application of antibiotics constitute one of the greatest medical achievements of the past century, underpinning critical healthcare advances such as organ transplantation, cancer therapy, joint replacement surgeries, and infectious disease management. However, the global rise in antimicrobial resistance (AMR) now poses a significant threat to these advancements. Classified by both the United Nations (UN) and the World Health Organisation (WHO) as one of the top public health threats facing humanity, AMR was responsible for ~4.95 million deaths worldwide in 2019 alone, with projections suggesting up to 39 million annual deaths attributable to AMR by 2050 if current trends persist.[1, 2] The complexity and gravity of AMR necessitates a comprehensive and collaborative approach, encapsulated by the "One Health" framework, which acknowledges the interconnectedness of human, animal, and environmental health in tackling this crisis.[1].

Since their clinical introduction in the 1940s, antibiotics have been consistently challenged by the emergence and spread of resistant bacterial strains. This challenge has intensified due to the dwindling pipeline of novel antibiotics since the late 20th century, contributing to escalating resistance levels worldwide.[3] Post-administration, antibiotics undergo hepatic metabolism, generating primary and secondary metabolites, which, alongside unmetabolised parent compounds, are excreted via urine and faeces.[4] Several of these metabolites have been shown to be bioactive.[5] Consequently, significant quantities of these compounds enter wastewater treatment plants (WWTPs) through effluents from medical facilities, households, and industrial processes. Conventional WWTP technologies are often insufficiently effective at completely removing these residues and metabolites, resulting in their release into surface waters, groundwater, and eventually drinking water supplies.[6, 7] Agricultural practices, particularly in livestock farming, further exacerbate environmental contamination, where antibiotics are frequently administered for therapeutic purposes and, in some regions, as growth promoters. Agricultural runoff thus represents an additional, significant source of antibiotics and their metabolites entering aquatic ecosystems.[8]

Globally, there is mounting evidence of environmental contamination by antibiotics, pharmaceutical residues, and their metabolites, detected widely across various water sources.[9, 10] Compounds from antibiotic classes such as tetracyclines, sulfonamides, fluoroquinolones, and macrolides have been documented across diverse geographical locations, including Europe, North America, Asia, and Africa, with recent studies highlighting the presence and persistence of antibiotic metabolites.[11] In developing nations, these contaminants pose an even greater challenge due to the limited infrastructure for wastewater treatment and insufficient regulatory frameworks, leading to higher levels of environmental exposure and potential health risks.[1]

The implications of antibiotic residues in environmental water systems extend beyond ecological impacts, posing both direct and indirect threats to human health. Although acute toxic effects from low-dose antibiotic residues through drinking water remain uncertain, chronic exposure may induce subtle physiological disturbances or allergic reactions in sensitive individuals.[6, 12] Furthermore, some antibiotic metabolites retain partial antimicrobial activity, potentially influencing bacterial ecology and resistance dynamics, while others, initially classified as biologically inactive, could still play unrecognised roles in resistance propagation.[13] More critically, environmental exposure to low-level antibiotics exerts selective pressure favouring resistant bacterial populations, facilitating the emergence and dissemination of antibiotic resistance genes (ARGs) or driving phenotypes associated with increased antibiotic tolerance such as biofilm formation. These resistant organisms, or their resistance determinants, can subsequently compromise human health by rendering infections more challenging to manage clinically.

While the environmental fate of the parent antibiotics is well documented, the biological effects of their metabolites remain largely unexamined. For example, ciprofloxacin (a second-generation fluoroquinolone antibiotic) has been used for decades in both human and veterinary medicine, and it is on the WHO list of medically important antimicrobials. It is known to be metabolised in humans to 4 major metabolites (desethylene ciprofloxacin (**M_1**), *N*-sulfo

ciprofloxacin (**M₂**), 2-oxo ciprofloxacin (**M₃**), and *N*-formyl ciprofloxacin (**M₄**), all of which have high structural similarity to the parent ciprofloxacin (Figure 1).[4, 5] Environmental concentrations, biological activities at sub-minimum inhibitory concentrations (sub-MIC), and health implications of these metabolites remain poorly characterised. This study investigates the impact of exposure to sub-inhibitory concentrations (SICs) of two of these antibiotic metabolites (**M₃** and **M₄**) on biofilm formation and susceptibility profiles (MIC values) in clinically relevant bacteria such as *Pseudomonas aeruginosa* (PAO1) and *Staphylococcus aureus* (SA), with a view to understanding their potential role in promoting AMR and tolerance. Antibiotic-driven AMR can be promoted not only by parent compounds but also by transformation products and metabolites, which may retain biological activity and selective pressure. This is supported by evidence that tetracycline degradation products rapidly enrich resistant *E. coli* mutants,[14] alongside growing artificial intelligence/machine learning capability to identify and predict environmental transformation products and their fate.[15]

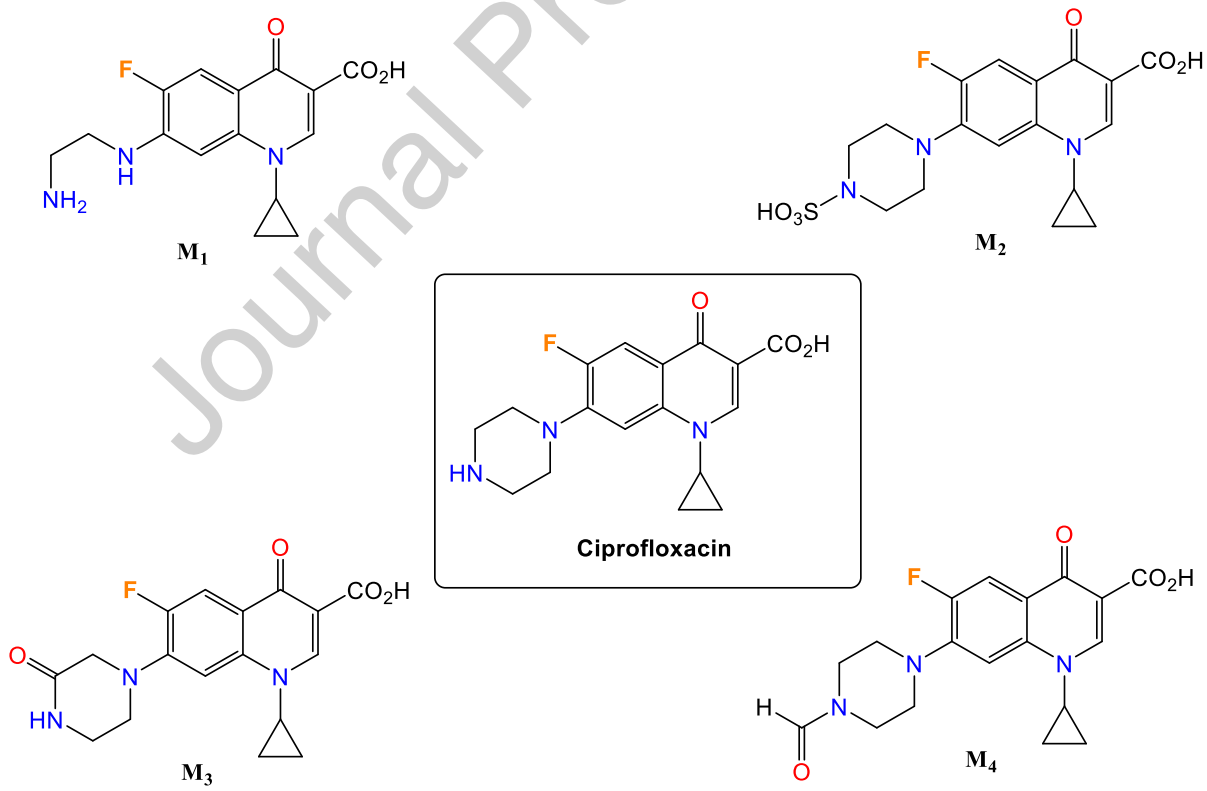


Figure 1.

Figure 1: Ciprofloxacin and its 4 main human metabolites

This study addresses this critical gap by examining how ciprofloxacin metabolites influence adaptive resistance mechanisms.

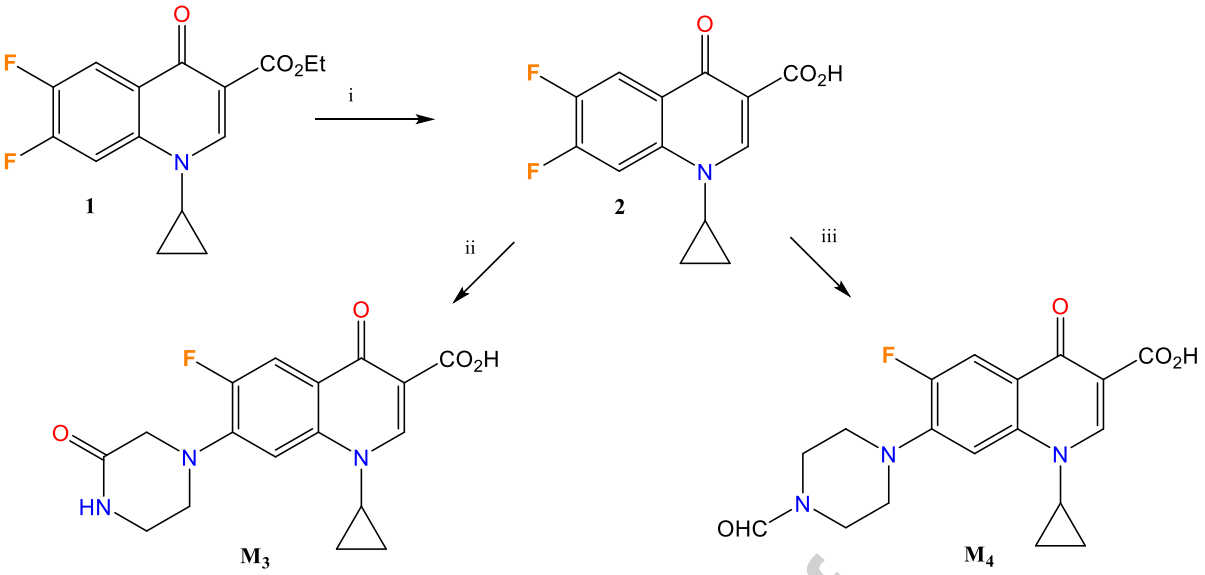
2. Materials and methods – see Supporting Information file

3. Results

3.1 Synthesis of Ciprofloxacin metabolites and validating their structure

Ciprofloxacin is widely prescribed in human medicine and remains a frontline option for diverse bacterial infections, including urinary tract infections, respiratory tract infections, skin and soft-tissue infections and gastrointestinal infections.[16] Due to its broad clinical use substantial amounts pass through the body unmetabolised, with the remainder converted into four main metabolites in humans (Figure 1), yet their biological activity is poorly characterised. We selected metabolites **M_3** and **M_4** for further assessment because **M_3** is the most abundantly observed human metabolite,[4] while both **M_3** and **M_4** are more synthetically accessible than **M_1** and **M_2**. This makes them suitable candidates for evaluating whether ciprofloxacin's metabolites influence the behaviour of *P. aeruginosa* and *S. aureus*. By adaptation of Petersen's method, we prepared **M_3** & **M_4** in >100 mg quantities, (Figure 2,[4, 5]) to study their

effects on the development of bacterial resistance. Hydrolysis of the ester **1**, under acidic conditions, gave the difluoro carboxylate **2** in 93% yield. Originally Zeiler and Gau used a thermal aromatic nucleophilic substitution (S_NAr reaction), which involved heating in DMSO solution.[4, 5] Microwave assisted organic synthesis (MAOS) has been used extensively to shorten reaction times and improve isolated yields for many organic transformations.[17] On a 50 mg scale of carboxylate **2**, reaction with 2-oxo piperazine, in DMSO at 130 ^oC, using the thermal method, gave $\mathbf{M_3}$ in a poor yield of 34%, which improved to a 73% yield on a 200 mg scale. In comparison, the corresponding MAOS method (170 ^oC for 20 mins), gave yields of 49% and 54% for the 50 mg and 200 mg scale reactions, respectively. In a similar manner, for the preparation of the $\mathbf{M_4}$, using N-formyl piperazine, gave yields of 37% (thermal) and only 24% (MAOS) on a 50 mg scale, which again increased to 60% (thermal) and 52% (MAOS) on the 200 mg scale. The metabolites $\mathbf{M_3}$ and $\mathbf{M_4}$ were fully characterised by IR and NMR spectroscopy, HRMS and microanalysis (see supporting information for details). With >100mg quantities in hand, microbiological studies were undertaken to ascertain their effects, if any, on the development of bacterial resistance in two bacterial strains, to the parent antibiotic ciprofloxacin.



Conditions: i) 10% HCl (aq)/EtOH, reflux, 3h; ii) 2-oxopiperazine, DABCO, DMSO, 170 ^{o}C, 1.5 h; iii) *N*-formylpiperazine, DABCO, DMSO, 170 ^{o}C, 1 h

Figure 2.

Figure 2: Synthesis of the 2-oxo ciprofloxacin (**M3**) and N-formyl ciprofloxacin (**M4**) human metabolites of ciprofloxacin

3.2 Microbiological studies

To establish baseline activity, we determined the minimum inhibitory concentrations (MICs) of ciprofloxacin, and **M3** and **M4**, against *P. aeruginosa* (PAO1) and *S. aureus* (SA). As shown in Table 1, PAO1 showed a substantial change in the sensitivity compared to ciprofloxacin, while SA showed no biologically significant change in MIC values for both metabolites compared to ciprofloxacin.

Table 1.

Table 1. Minimum inhibitory concentrations (MICs) of ciprofloxacin and its metabolites M_4 and M_3 against PAO1 and SA. MIC values are expressed in **$\mu g.mL^{-1}$**. Values shown as ">" indicate that no inhibition was observed at the highest concentration tested. Data represents results from three independent experiments.

Bacterial strain	Ciprofloxacin ($\mu g.mL^{-1}$)	*N*-Formyl ciprofloxacin (M_4) ($\mu g.mL^{-1}$)	2-oxo ciprofloxacin (M_3) ($\mu g.mL^{-1}$)
PAO1	0.0625	>0.25	>0.25
SA	0.25	0.5	0.5

Growth in the presence of Ciprofloxacin metabolites drives resistance in *S. aureus* but not in *P. aeruginosa*

Both species were subcultured for 30 days in the presence of SICs of **M_3** or **M_4**. After this, all adapted strains were re-tested against ciprofloxacin to determine whether prolonged exposure to the metabolites altered susceptibility. Appropriate controls included unadapted strains and strains subcultured without antimicrobial exposure.

EUCAST figures report MIC breakpoints for *Staphylococcus spp*.,as being >2 $\mu g.mL^{-1}$ and for *Pseudomonas aeruginosa* as >0.5 $\mu g.mL^{-1}$. For SA exposure to SICs of **M_4** and **M_3** over 30 days resulted in four- or eight-fold MIC breakpoint increase respectively, indicating reduced susceptibility (Figure 3). Specifically, **M_4** exposure increased the ciprofloxacin MIC value from 0.25 to 1 $\mu g.mL^{-1}$, and **M_3** from 0.25 to 2 $\mu g.mL^{-1}$.

The results (Figure 3) show that exposure to ciprofloxacin metabolites drove resistance development in SA but not in PAO1. The most plausible explanation, consistent with the baseline MIC data, is that the metabolites retain considerable inhibitory activity against SA. Daily exposure therefore imposes a selective pressure similar to low-dose ciprofloxacin, promoting stepwise resistance. In contrast, PAO1 is not inhibited by **M_3** and **M_4** over the concentration range tested, so exposure to metabolites does not create meaningful selective pressure and therefore does not drive resistance. This differential response reflects the distinct baseline susceptibility profiles of the two species and supports the conclusion that ciprofloxacin metabolites can contribute to resistance development where their inhibitory activity is maintained. Contrastingly, no significant MIC change was observed in PAO1 subcultures exposed to SICs of **M_4** or **M_3** over 30 days.

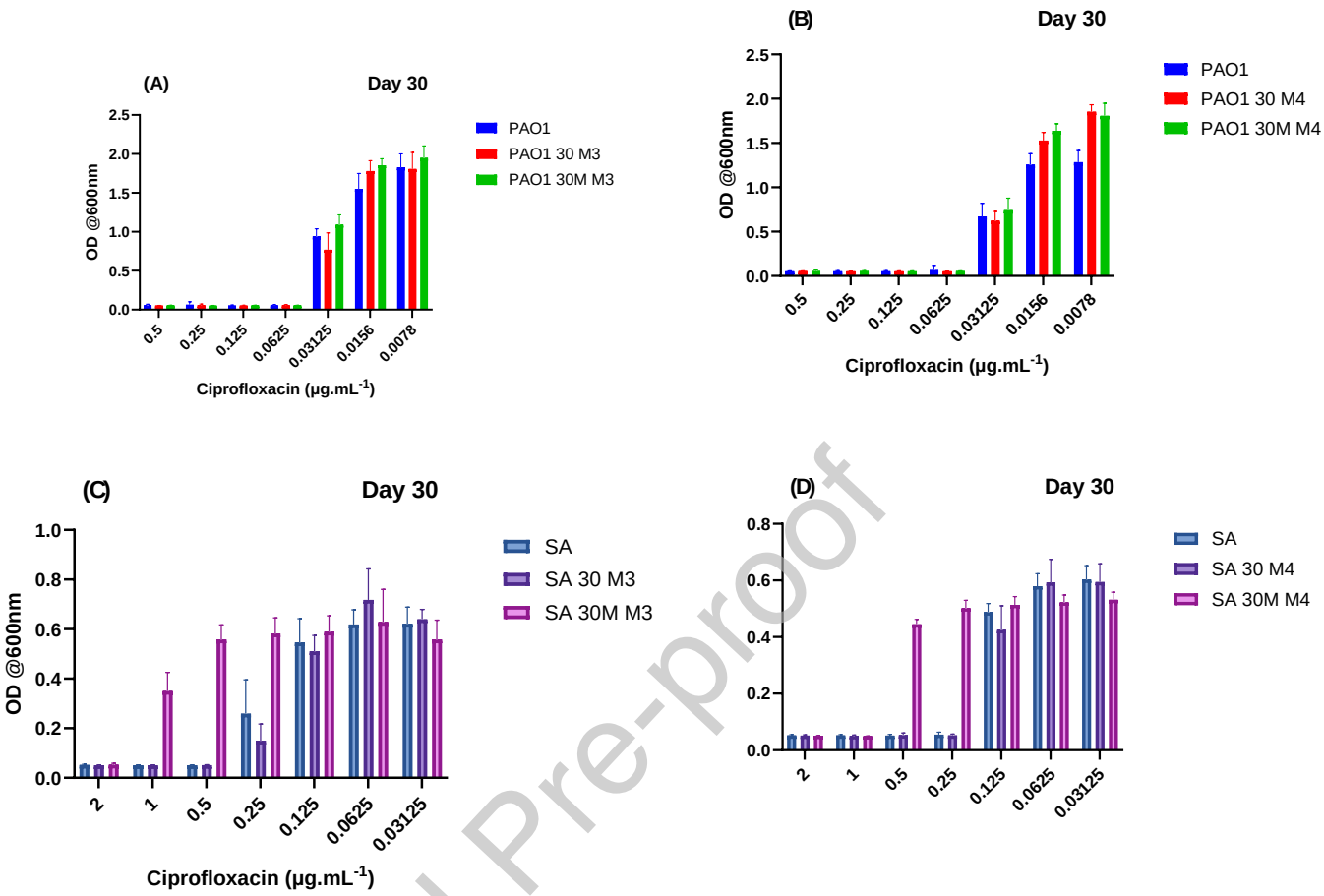


Figure 3.

Figure 3. Minimum inhibitory concentration (MIC) assay results for ciprofloxacin against PAO1 (panels A and B) and SA (panels C and D) following 30 days of daily subculturing in the absence or presence of SICs of ciprofloxacin metabolites. Metabolites tested were **M_3** (panels A and C) and **M_4** (panels B and D). Each panel compares: (i) freshly cultured strains (PAO1 and SA), (ii) strains subcultured for 30 days without metabolite exposure (PAO1 30 M3, PAO1 30 M4, SA 30 M3 and SA 30 M4), and (iii) strains subcultured for 30 days in the presence of the relevant metabolite (PAO1 30M M3, PAO1 30M M4, SA 30M M3 and SA 30M M4). All groups were subsequently exposed to ciprofloxacin at organism-relevant

concentrations. Exposure to sub-inhibitory metabolite levels increased the MIC of SA eight-fold for **M_3** (0.25 to 2 µg mL^{-1}) and four-fold for **M_4** (0.25 to 1 µg mL^{-1}), whereas PAO1 showed no meaningful change. Data represent mean OD600 ± SD from at least three independent experiments.

3.3 Growth in the presence of Ciprofloxacin metabolites drives biofilm formation in *P. aeruginosa*

Biofilm formation is a well-recognised adaptive response to antibiotic stress, allowing bacteria to increase persistence and tolerate antimicrobials [18]. To determine whether ciprofloxacin metabolites could trigger a similar response, we assessed biofilm formation of PAO1 after the 30-day adaptation period. Importantly, biofilm assays were performed *de novo* after adaptation, not during the 30-day exposure.

Following daily subculturing in the presence of SICs of **M_3** or **M_4**, adapted cultures were inoculated into fresh medium and biofilm biomass was quantified by crystal violet staining. Control groups consisted of strains subcultured for 30 days without antimicrobial exposure.

The results (Figure 4) show that following PAO1 exposure to these metabolites we saw a significant increase in biofilm formation after 30 days (Figure 4). Particularly, exposure to **M_4** markedly enhanced biofilm formation capability ($P < 0.0001$), while **M_3** exposure resulted in a similar increase ($P = 0.0089$). These findings suggest that although the metabolites did not induce a direct shift in MIC values, they significantly altered bacterial behaviour, potentially enhancing persistence through increased biofilm formation.

To address this, biofilm formation was assessed under two conditions:

1. **Biofilm formed during continued exposure to each metabolite** (equivalent to the original Figure 4A), and
2. **Biofilm formed in the absence of metabolites** (equivalent to the original Figure 4B), where strains adapted for 30 days to **M_3** or **M_4** were grown in fresh medium without metabolites present.

The figure shows that exposure to **M_3** or **M_4** during biofilm formation increased biomass relative to controls; however, only the effect of **M_3** was maintained in the absence of the metabolite. The **M_4**-associated increase disappeared in the absence of the metabolite. These data indicate two mechanistically distinct phenomena:

- **M_3 induces a stable, likely genetic adaptation that enhances biofilm production**
- **M_4 triggers only a transient, exposure-dependent response**

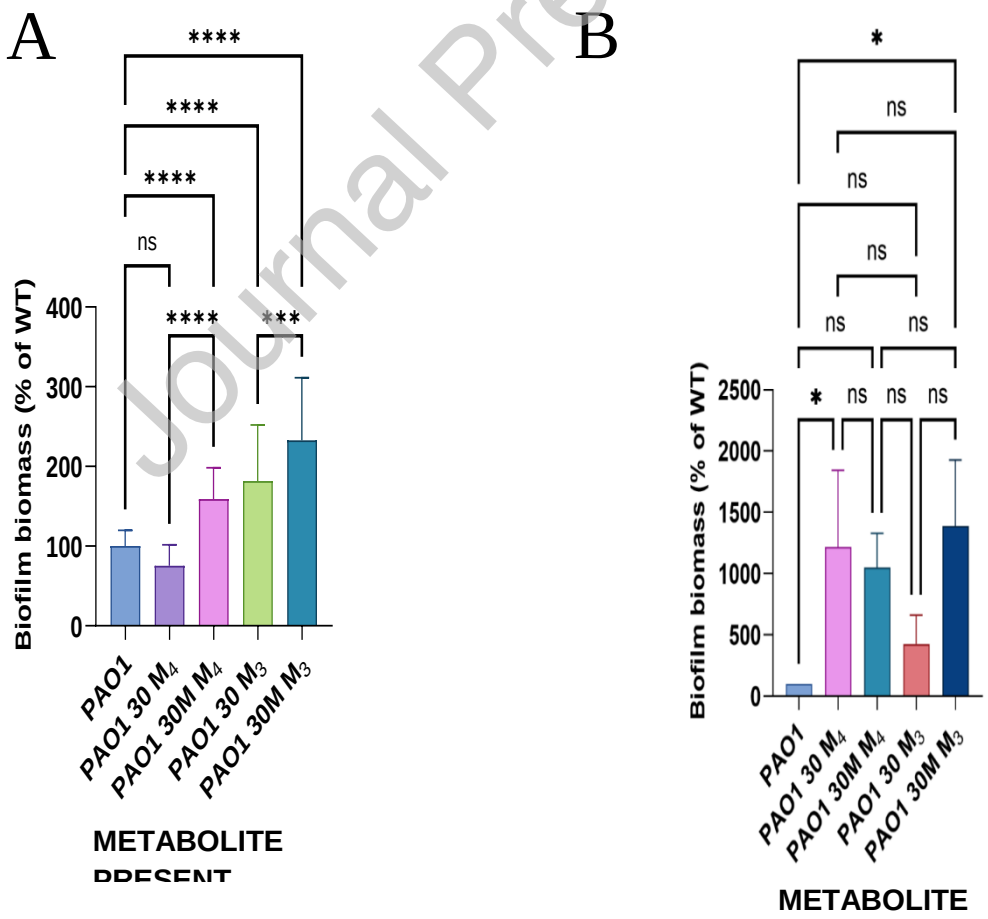


Figure 4.

Figure 4. Effect of 30-day exposure to ciprofloxacin metabolites on PAO1 biofilm formation in the presence and absence of metabolites.

Panel A (Metabolite Present) shows biofilm formation when PAO1 strains previously adapted for 30 days to **M_3** or **M_4** were assayed in the continued presence of the respective metabolite. Panel B (Metabolite Absent) shows biofilm formation when the same adapted strains were assayed in the absence of the metabolites, using fresh medium in the absence of **M_3** or **M_4**. In both panels, PAO1 represents the freshly cultured isolate, PAO1 30 **M_3** and PAO1 30 **M_4** represents the strain subcultured for 30 days without antimicrobial exposure, while PAO1 30M **M_3** and PAO1 30M **M_4** represent strains subcultured for 30 days in the presence of **M_3** or **M_4**, respectively. Biofilms were grown for 24 hours and quantified by crystal violet staining. Mann–Whitney tests were used to assess statistical significance. Panel A and B were independently assayed in different labs at different times. Data represent mean ± SD from at least three independent experiments.

3.4 Exploring the altered transcriptional and genomic landscape in ciprofloxacin metabolite-exposed *P. aeruginosa* and the link to altered biofilm levels

Since **M_3** generated a persistent biofilm phenotype, we sought to identify the underlying mechanism. Given that the **M_3**-adapted strain retained its enhanced biofilm phenotype even

in the absence of **M_3**, we sought to determine whether this represented a heritable genetic change rather than a transient physiological response. This question is important in the context of the One Health agenda, because stable genetic adaptations that enhance persistence or biofilm formation have the potential to disseminate within microbial populations across clinical, environmental and agricultural settings, potentially contributing to the emergence of more recalcitrant pathogens and complicate treatment strategies.

We next examined whether the increased biofilm phenotype observed in PAO1 following exposure to ciprofloxacin metabolites depended on the ongoing presence of each metabolite or whether the bacteria had undergone a stable heritable shift (Figure 4). The objective was to distinguish a real-time transcriptional response from a metabolite-induced genetic adaptation. For these reasons, we investigated the basis of the stable **M_3**-associated phenotype using a combined genomic and transcriptomic approach. Whole-genome sequencing (WGS) and differential RNA sequencing (dRNA-seq) were performed to identify mutations and regulatory changes associated with prolonged exposure to **M_3**. WGS revealed 164 polymorphic sites in **M_3**-adapted cells compared with 181 sites in unexposed controls (Supplementary Table 1). Notably, a single-nucleotide polymorphism was detected in *wspA*, substituting guanosine with thymidine at position 869 (G869T). This SNP led to Alanine substitution with Aspartic acid at amino acid 290 (A290D) causing a missense non-conservative mutation. A290D mutation is in the 280-307 aa region, which deletion constitutively activates WspA.[19] The Wsp signal transduction system is a central regulator of biofilm formation in PAO1.[20] Therefore, it is highly likely that this SNP in *wspA* contributes to the observed increased biofilm phenotype. This mutation leads to an A290D amino-acid change located within a region previously shown to drive constitutive activation of the Wsp chemosensory system, a central regulator of biofilm formation in PAO1.[19]

Additionally, the dRNA-seq showed that 220 genes were differentially expressed following **M_3** exposure compared to control non-treated cells (Supplementary Table 2). Within this subset of differentially regulated genes, several linked to biofilm formation were identified. The

quorum sensing (QS) genes *rhlI* and *pqsH* were upregulated by 2 and 1.8 log_2(FC) respectively, while the negative PQS QS regulator *pqsA* was downregulated by 4.5 log_2(FC) (Figure 5). The cbb3-type cytochrome c oxidase encoding genes *ccoP2*, *ccoO2* and *ccoQ2*, were upregulated by 1.6, 1.2 and 1.3 log_2(FC), respectively. The expression of the genes involved in nitric oxide (NO) cycle *fhp*, *nir*, *nar* and *nos* operons, as well as *norCB*, was increased by 1.3 – 3.7 log_2(FC). Moreover, there was a 1.8-2.3 log_2(FC) increase in the expression of *pel* operon, which encodes the machinery to produce Pel polysaccharide.

Together, these genomic and transcriptional alterations are consistent with a metabolite-induced stable biofilm-enhancing phenotype driven specifically by **M_3**.

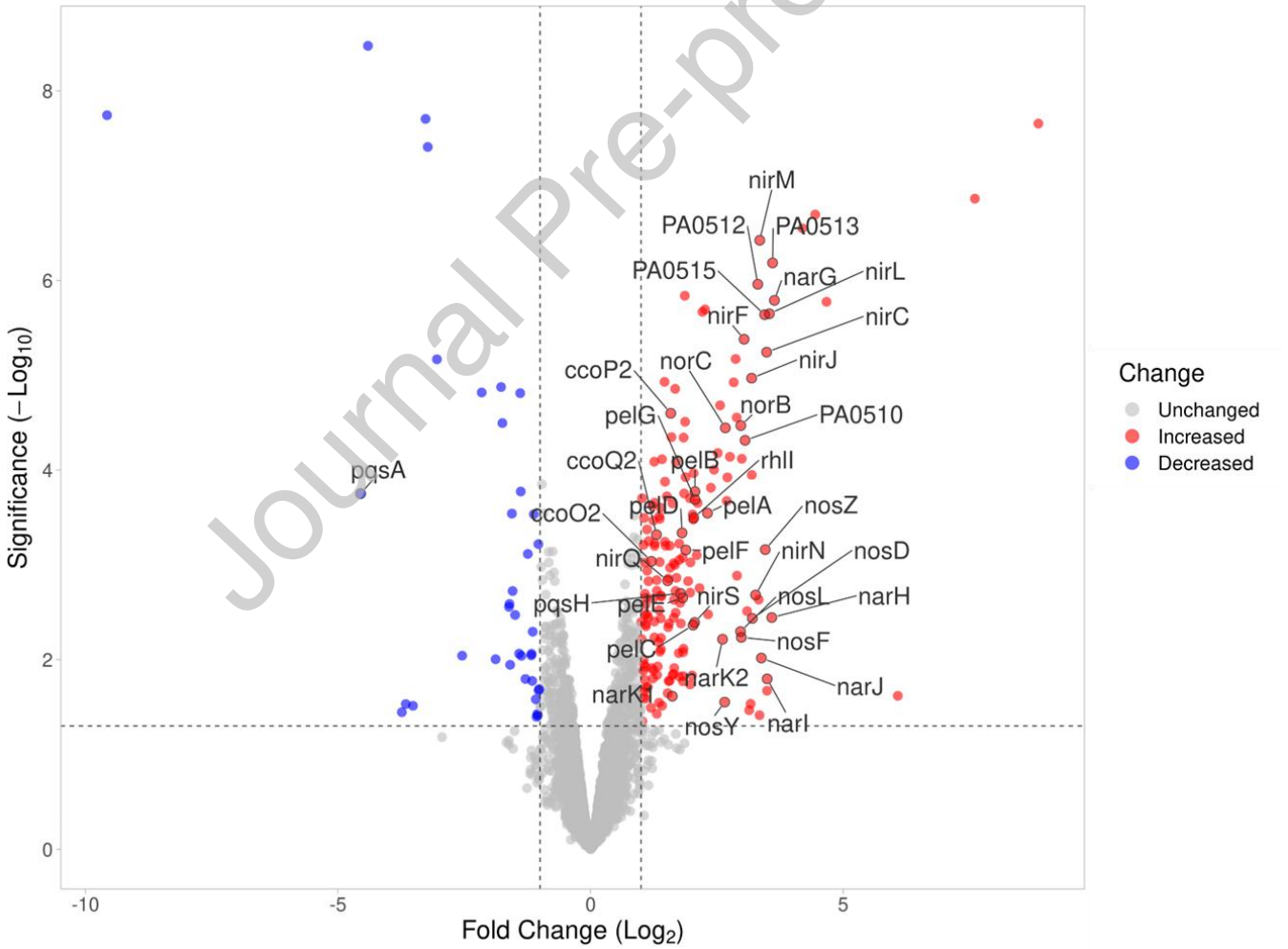


Figure 5.

Figure 5. Volcano plot representing dRNA-seq data of PAO1 exposed to M_3 vs non-exposed control cells. 38 genes were downregulated (blue dots) and 182 genes were upregulated (red dots). Highlighted are the downregulated pqsA and the upregulated pqsH genes of the PQS quorum sensing (QS) system, and the rhlI autoinducer synthase of the RhlRI QS system, as well as the genes encoding cbb3-type cytochrome c oxidases (ccoP2, ccoQ2 and ccoO2), nitric oxide cycle enzymes fhp, nar, nir (including PA0512 and PA0515), nor and nos gene clusters and the Pel polysaccharide synthesis machinery (pel operon).

4. Discussion

In the 1980s, Zeiler studied the antibacterial activity of the known human metabolites of ciprofloxacin, including **M_3** and **M_4**, against a range of Gram-positive and Gram-negative bacterial species, including PAO1 and SA strains.[5] Both compounds showed much reduced antibacterial activity compared to ciprofloxacin, with **M_3** having a MIC value of 4-16 $\mu g.mL^{-1}$ and 0.5-1 $\mu g.mL^{-1}$ against PAO1 and SA species, respectively, and **M_4** was found to be more active than **M_3** with MIC values of 1-4 $\mu g.mL^{-1}$ and 0.25-0.5 $\mu g.mL^{-1}$, respectively. Ciprofloxacin had MIC values of 0.125-0.25 $\mu g.mL^{-1}$ and 0.25-0.5 $\mu g.mL^{-1}$, respectively. Our findings show elevated resistance in SA to both metabolites, while PAO1 had the same MIC values for both metabolites as to ciprofloxacin (Table 1). Our baseline MIC values therefore confirm that **M_3** and **M_4** are either similar or weak antimicrobials under standard conditions. Nonetheless, despite their limited direct activity, prolonged exposure to metabolite SICs produced measurable adaptive responses in both bacterial species, underscoring their potential biological relevance in the environment and clinical settings. In SA, continuous exposure to

M_3 and **M_4** over 30 days resulted in an eight to four-fold increase, respectively, in ciprofloxacin MICs, suggesting the development of an adaptive resistance phenotype (Figure 3). Although data on antibiotic metabolites remain limited, our results imply that these weakly active derivatives may sustain selective pressure sufficient to maintain resistant subpopulations or selecting for phenotypes linked to increased antibiotic persistence and reduced susceptibility, particularly in environmental reservoirs where exposure is chronic and concentrations are sub-therapeutic.

For PAO1, MIC values remained unchanged after metabolite exposure, yet biofilm formation increased significantly, particularly following treatment with **M_3** (Figure 3 and 4). This is consistent with evidence that fluoroquinolone SICs can stimulate biofilm formation in PAO1 through modulation of quorum sensing and cyclic-di-GMP signalling pathways.[21, 22] Our data extends this by showing that even structurally similar ciprofloxacin metabolites can drive stable adaptive phenotypes in PAO1. In particular, the **M_3**-associated biofilm increase persists even in the absence of the metabolite, consistent with a conserved, genetically-encoded adaptation rather than a transient stress response. This stable shift in biofilm behaviour indicates that environmental residues of ciprofloxacin metabolites have the potential to select for more persistent phenotypes. Such metabolite-driven adaptations may contribute to the long-term survival and tolerance of PAO1, traits that underpin both its pathogenic success and its environmental resilience. Taken together, the MIC and biofilm data show that ciprofloxacin metabolites exert species-specific effects. In SA, where **M_3** and **M_4** retain inhibitory activity comparable to ciprofloxacin, prolonged exposure increases ciprofloxacin MICs, indicating a classic selective-pressure–driven resistance response, in contrast to PAO1 which shows no shift in MICs. The reasons for this divergence are not fully defined, but several possibilities merit consideration. One is that fluoroquinolone metabolites may traverse the Gram-negative outer membrane more efficiently than the thicker peptidoglycan-rich envelope of SA, resulting in different intracellular concentrations and stress signatures. Alternatively, the two species differ in fluoroquinolone target hierarchy: SA is more dependent on topoisomerase IV inhibition, whereas PAO1 relies predominantly on DNA gyrase. If the metabolites vary slightly

in their affinity for these targets, the resulting stress responses and adaptive pathways could diverge. These hypotheses remain speculative but offer plausible biological routes for the contrasting phenotypes observed.

Given that both SA and PAO1 displayed distinct adaptive responses to metabolite exposure, we sought to determine whether these phenotypic changes were associated with underlying genetic or transcriptional alterations. Among the two species and metabolites tested, the most compelling signal arose from the **M_3**-exposed PAO1, which maintained its elevated biofilm phenotype even in the absence of metabolites. This strongly suggested a stable, heritable shift rather than a transient, exposure-dependent response. We do acknowledge that the crystal violet staining does not distinguish live from dead cells; future studies would incorporate viable cell enumeration (CFU) and fluorescence-based live/dead staining such as resazurin, to confirm biofilm viability. Because **M_3** uniquely produced a conserved biofilm phenotype, and because PAO1 is genetically tractable with well-defined regulatory networks, this strain–metabolite combination was selected for deeper mechanistic analysis. Whole-genome sequencing (WGS) and differential RNA sequencing (dRNA-seq) were undertaken to determine whether the stable **M_3**-associated phenotype reflected underlying genomic mutations, transcriptional reprogramming or both. WspA is a chemotaxis membrane-bound protein part of the Wsp signal transduction system.[23] Activation of WspA triggers a cascade leading to phosphorylation of the response regulator and diguanylate cyclase WspR, increasing intracellular levels of the second messenger cyclic di-GMP [23]. C-di-GMP is a well-known regulator of biofilm formation in PAO1, where high levels promote sessility.[24] We show that with **M_3** exposure, a mutation in WspA (A290D) occurs in the 280-307 aa region, which upon deletion locks the protein in a constitutively active state, increasing c-di-GMP levels.[20] The Wsp system and c-di-GMP are known to affect biofilm formation via regulation of the synthesis of Pel polysaccharide, a major extracellular matrix component crucial for PAO1 biofilm formation.[19, 25-27] Consistent with this, our dRNA-seq analysis showed increased expression of the *pel* operon in the **M_3**-adapted strain (Figure 5). Together with the

identified *wspA* mutation, these findings indicate that prolonged exposure to **M_3** selected for a variant in which the Wsp pathway is constitutively activated, reflecting a metabolite-driven selection of genetic change rather than any direct interaction between **M_3** and the Wsp system. In addition to c-di-GMP, QS is also known to regulate Pel synthesis in PAO1.[22] Our results showed increased expression of the RhlRI QS system, contributing to the increased biofilm phenotype. Furthermore, exposure to **M_3** enhances the PQS QS system by downregulating the negative regulator *pqsA* and upregulating *pqsH*, which stimulates biofilm production.[28, 29] The PQS system has also been shown to inhibit NO reductase NorCB, leading to NO accumulation.[30] Our transcriptomics data showed that there was an increased expression of NorCB, as well as other NO cycle-related enzymes including NIR and NAR nitrite and nitrate reductases (Figure 5) which play central role in promoting biofilm formation via maintenance of low NO concentrations.[31] Moreover, cbb3-type cytochrome c oxidase *cco* gene cluster was upregulated, which is known to increase biofilm formation via intracellular NO accumulation.[32, 33] Despite that, low NO levels are needed for PAO1 biofilm formation, while high NO concentrations trigger dispersion of PAO1 biofilms.[34] Hence, upregulation of both PQS system and NO enzymes suggests fine-tuning of NO levels in PA cells following **M_3** exposure, resulting in enhanced biofilm formation.

This research shows that exposure to **M_3** triggers genomic and transcriptomic modifications that activate c-di-GMP and QS signalling systems, as well as fine-tuning of NO levels, resulting in enhanced Pel polysaccharide production and increased biofilm formation by PAO1, which implies enhanced virulence too. Although SA demonstrated a clear adaptive MIC response following metabolite exposure, genomic and transcriptomic analyses were not performed on this organism within the current study. We prioritised *P. aeruginosa* PAO1 as the primary genomic target given its tractable genetics and the persistence of the M3-associated biofilm phenotype. WGS and RNA-seq of SA following metabolite exposure represents a key priority for subsequent investigation and forms part of the planned next phase of this research

programme. This study highlights the need for environmental monitoring and policy frameworks addressing antibiotic metabolites as active contributors to the spread of AMR.

5. Conclusion

Our research indicates that exposure of PAO1 and SA to SICs of antibiotic metabolites can alter biofilm formation and shift MIC values, highlighting their potential to drive resistance or enhancing persistence. Exposure to antimicrobial compounds, including sub-lethal antibiotic concentrations, can unintentionally promote biofilm formation in bacteria such as PAO1.[21] Similar effects have been observed with other fluoroquinolones, which influence biofilm structure and persistence.[35] The biofilm matrix, primarily composed of polysaccharides such as Pel and Psl, enhances tolerance by limiting antibiotic penetration.[25, 27] Quorum-sensing systems, including the Pseudomonas quinolone signal (PQS), further regulate this process in response to antimicrobial stress.[22, 28] Additionally, our genetic analyses have identified concerning molecular changes, including the upregulation of biofilm-related operons (e.g., *pel*), alterations in quorum sensing pathways, and mutations influencing nitric oxide metabolism, each of which could further enhance bacterial resilience and biofilm robustness. Addressing these critical knowledge gaps surrounding the impact of antibiotic metabolites on AMR and tolerance is essential. While our studies to date have focused primarily on single metabolite exposures, investigating the combined effects of multiple metabolites, possibly derived from various antibiotics or indeed other pharmaceuticals, may reveal even greater implications. Clarifying the extent and range of these effects underscores the urgent necessity for comprehensive environmental monitoring, advanced wastewater treatment strategies, and updated regulatory frameworks to effectively mitigate risks posed by these previously overlooked contaminants.

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All dRNA-seq related datasets are available at the Gene Expression Omnibus repository (NCBI) with accession number **GSE310618** (reviewer access token: mredmegujtsvjih).

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