Vanillin inhibits PqsR-mediated virulence in *Pseudomonas aeruginosa*

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Abstract:

Reduced efficacy of antibiotics in bacterial diseases is a global concern in clinical settings. Development of anti-virulence compounds which disarm bacterial virulence is an attractive therapeutic agent for complementary antibiotics usage. One potential target for anti-virulence compounds is quorum sensing (QS), the intercellular communication system in most pathogens, such as *Pseudomonas aeruginosa*. QS inhibitors (QSIs) can inhibit QS effectively, attenuate QS-mediated virulence, and improve host clearance in infections. While studies focused on developing homoserine-based *las* QSI, few targeted the quinolone-based *pqs* QS, which implicated host cytotoxicity and biofilm formation. It is imperative to develop novel anti-*pqs*-QS therapeutics for combinatorial antibiotic treatment of microbial diseases. We employed a *gfp*-based transcriptional *pqs* biosensor to screen a natural compounds library and identify vanillin (4-hydroxy-3-methoxybenzaldehyde), the primary phenolic aldehyde of vanilla bean. The vanillin inhibited *pqs* expression and its associated phenotypes, namely pyocyanin production and twitching motility in *P. aeruginosa*. Molecular docking results revealed that vanillin binds to the active site of PqsR, the PQS-binding response regulator. Combinatorial treatment of vanillin with antimicrobial peptide (colistin) inhibited biofilm growth *in vitro* and improved treatment in the *in vivo C. elegans* acute infection model. We demonstrated that vanillin could dampen *pqs* QS and associated virulence, thus providing novel therapeutic strategies against *P. aeruginosa* infections.

Keywords:

Vanillin; Quorum sensing; Pseudomonas Quinolone Signal (PQS); Biofilm; Virulence; *Pseudomonas aeruginosa*

1. Introduction

Antibiotic resistance poses a catastrophic threat to human health, contributing to 700,000 deaths annually and potentially costing 10 million lives by 2050 [1]. The Infectious Diseases Society of America had determined six bacterial pathogens to be heavily involved in multidrug resistance (MDR) - ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* species) [2]. Natural compounds with the ability to inhibit MDR offer advantages over synthetic drugs due to availability in food, high safety levels and lesser side effects.

Other than current antibiotics, anti-virulence therapy has great potential to inhibit pathogenic bacteria and show potential for controlling pathogen infections [3, 4]. At present, the development and application of anti-virulence agents have become a hotspot in the research of food and medicine. Using anti-virulence therapy will delay the rise of resistant mutants as it exerts lower selective pressure than that by conventional antibiotics [5, 6]. Furthermore, anti-virulence agents can be used together with antibiotics synergistically to treat bacterial infections [7].

A promising target for the development of anti-virulence therapeutics is quorum sensing (QS), which is the intercellular communication system employed in most bacteria to coordinate gene expression on virulence factor production and biofilm formation [3]. For instance, the nosocomial pathogen *P. aeruginosa* which commonly cause intestinal and lung infections [8, 9], contains three interconnected QS systems (*las*, *rhl*, and *pqs*) to control social behaviors and fine-tune its expression of virulence, such as motility, biofilm formation and secretion of virulence factors [10]. The synthases (LasI, RhlI, PqsA-D) are involved in biosynthesis of the QS signaling molecules (OdDHL, BHL, and PQS respectively), while LasR, RhlR, and PqsR act as response regulators which bind to QS signaling molecules and coordinate downstream response. The homoserine lactone-based *las* and *rhl* QS systems regulate elastase and rhamnolipid production respectively. The quinolone-based *pqs* system regulates production of pyocyanin which is highly cytotoxic to mammalian cells and extracellular DNA (eDNA) release for biofilm formation [11, 12].

Hence, it is crucial to employ safe naturally occurring compounds with the specific aim of inhibiting *pqs* QS-mediated virulence. Several natural extracts from plants have previously shown to inhibit quorum sensing, such as gingerol from ginger and sulforaphone from broccoli could inhibit *las* QS [13, 14]. In view of the lack of *pqs* QS inhibitors from food products, we screened for *pqs* QS inhibitors from the in-house compound library derived from natural sources, leading to the identification of 4-hydroxy-3-methoxybenzaldehyde (vanillin), a phenolic aldehyde which is commonly found in vanilla bean. While

vanillin had been previously correlated to quorum quenching of homoserine lactone-based QS in *Aeromonas hydrophilia* for treatment of biofouled reverse osmosis membranes [15], we showed that 4 vanillin acts as an inhibitor of the *pqs* QS, probably via the PqsR receptor. Inhibition of *pqs* QS was reflected in concentration-dependent reduction of twitching motility and pyocyanin production by vanillin.

To show that vanillin could be deployed together with antibiotics, we employed a combinatorial treatment of vanillin and colistin against *P. aeruginosa*. Colistin is an antimicrobial peptide which is an antibiotic of last resort against Gram-negative bacteria [16]. The combinatorial treatment reduced the minimal inhibitory concentration (MIC) of colistin dose by 2-fold, thus boosting the eradication of biofilm formation and in an *in vivo C. elegans* infection model. *C. elegans* possesses many characteristics that allows it to be an excellent research model for infection, including intestinal infection, and presence of innate immunity. It is also frequently employed a model organism for antibacterial drug discovery [17, 18]. Our results supported the potential of vanillin as an anti-virulence agent, which can be used together with antibiotics in eradicating bacterial infections.

2. Results:

2.1 Inhibition assay with the P. aeruginosa pqsA-gfp(ASV) strain by vanillin:

From an in-house natural compounds library, we performed a preliminary screen by using the *P. aeruginosa* wild-type PAO1/p_{pqsA}-*gfp* transcription fusion biosensor [19]. PqsA is involved in the synthesis of PQS, the autoinducer of *pqs* QS [20]; thus the transcriptional fusion biosensor is a suitable indicator for measuring the expression of *pqs* QS. We identified and determined vanillin to be an inhibitor of *pqs* operon. With increasing concentrations of vanillin, we observed growth inhibition of PAO1, where minimal inhibitory concentration (MIC) is 16 mM (Figure 1a).

Next, we incubated PAO1/p_{pqsA}-*gfp* with vanillin, where we observed a dose-dependent inhibition of *pqsA-gfp* (Figure 1b) without affecting growth. Hence, we determined that the IC_{50} value of vanillin in inhibiting expression of p_{pqsA}-*gfp* was 0.81 mM (Figure 1c). Vanillin is typically used at edible concentrations (usually 2% or equivalent of 131 mM) in the food industry [21, 22]. The concentrations that we had used in this project were significantly lower than that of commercial products, which referred to the potential utility of vanillin. To effectively employ vanillin as an anti-*pqs* inhibitor, we used 2 mM 4-hydroxy-3-methoxybenzaldehyde for our downstream experiments, as it did not have significant effect on bacterial growth but possessed inhibitory effect against *pqs* QS expression.

2.2 vanillin inhibited pqs response, but not biosynthesis:

The *pqs* QS is dictated by the symphony of biosynthesis of PQS (*pqsA-D* and *pqsH*) and response to PQS (*pqsR*) [23]. To determine whether vanillin could inhibit the biosynthesis or response genes, we used a Δ*pqsA*/p_{pqsA}-*gfp* mutant, which could not synthesize its own PQS but still could respond via PqsR, and incubated it with exogenously added 10 µM PQS and varying levels of vanillin. In the presence of exogenous PQS only, Δ*pqsA*/p_{pqsA}-*gfp* retained its response activity, but increasing levels of vanillin could reduce expression of *pqsA-gfp* (Figure 2a).

To further confirm that vanillin indeed inhibited PqsR, but not the *pqs* biosynthesis proteins, we first employed the Δ*pqsR* mutant, Δ*pqsR*/p_{lac}-*pqsR* complementation strain and Δ*pqsR*/p_{lac}-*pqsABCDE* mutant which possessed overexpression of *pqs* operon but could not respond to PQS. For simplicity sake, we presented the peak p_{pqsA}-*gfp* expression values of each strain treated with 0, 1, 2, and 4 mM vanillin at 300 mins (Figure 2b). As expected, the Δ*pqsR*/p_{pqsA}-*gfp* mutant did not respond to increasing levels of vanillin, albeit possessing lower GFP expression (Figure 2b). PqsR complementation to Δ*pqsR*/p_{lac}-*pqsR*/p_{pqsA}-*gfp* restored its sensitivity to vanillin (Figure 2b). Finally, vanillin did not have significant effect on Δ*pqsR*/p_{lac}-*pqsABCDE*/p_{pqsA}-*gfp*, which had overproduction of PQS but could not respond in the absence of PqsR (Figure 2B). This meant that vanillin could inhibit *pqs* QS via PqsR.

2.3 Molecular docking revealed vanillin bound to PqsR active site:

As we had shown that vanillin could inhibit PqsR in the previous section, we employed molecular docking with Autodock Vina to show that vanillin (yellow-colored molecule) could bind to PqsR's active site at a similar location like the ligand NHQ (cyan-colored molecule) (Figure 3a). It was previously reported that NHQ binds to PqsR through various active site residues, such as I186 and L208 [24]. Here, we demonstrated this by comparing NHQ binding to active sites residue L208 (Figure 3b) and vanillin binding to I186 (Figure 3c). Our analysis also showed that vanillin binds to PqsR with a binding affinity of -4.0 kcal/mol, which was similar to the binding affinity of NHQ (-6.1 kcal/mol).

2.4 Vanillin inhibits pqs QS-mediated phenotypes:

Since vanillin could inhibit PqsR, we next showed that vanillin could impose an inhibitory effect on *pqs* QS–controlled virulence in *P. aeruginosa*. Firstly, as PqsR induced PQS biosynthesis in a positive feedback loop [25], we observed that vanillin could reduce PQS concentrations in the *P. aeruginosa* planktonic cultures (Figure 4a). Next, the production of pyocyanin by *P. aeruginosa* was significantly inhibited by 1mM vanillin (Figure 4b).

Lastly, as PqsR also controlled the pili-mediated twitching motility [26], we next showed that 2mM vanillin could significantly reduce *P. aeruginosa*'s twitching motility (Figure 4c and 4d). These results implied that vanillin is an effective inhibitor of *pqs*–mediated motility and virulence via PqsR inhibition.

2.5 Combinatorial treatment of vanillin and colistin against planktonic P. aeruginosa:

As vanillin was intended as an anti-virulence agent for combinatorial therapy with antibiotics, we evaluated the effectiveness of the combinatorial therapy of vanillin and colistin, a commercial peptide-based antibiotic of last resort. Firstly, to evaluate the colistin susceptibility of *P. aeruginosa* under planktonic conditions, we used 1 mM vanillin with varying colistin concentrations. The MIC of colistin monotherapy of planktonic *P. aeruginosa* was 1 μg ml^{-1}, while combinatorial therapy with vanillin saw a 2-fold reduction in colistin MIC to 0.5 μg ml^{-1} in planktonic cells (Figure 5a). As for the expression of *pqs* QS, we observed that colistin could significantly induce p_{pqsA}-*gfp* expression in *P. aeruginosa* at sub-MIC levels (0.5 μg ml^{-1}). This observation was probably attributed to the ability of most antibiotics to induce QS and biofilm formation in many bacterial species at sub-MIC levels [27, 28]. However, addition of 1 mM vanillin could negate this effect (Figure 5b).

2.6 Combinatorial treatment of vanillin and colistin against biofilm P. aeruginosa:

We then asked if vanillin could work with colistin in combination to eradicate biofilms. As biofilms are more resistant to antimicrobial treatment than planktonic cells, the minimal biofilm eradication concentration (MBEC) is usually higher than MIC, thus posing a significant problem in treating biofilm infections. Although *P. aeruginosa* was previously known to tolerate higher than 10 μg ml^{-1} colistin [29], we observed that addition of 1 mM vanillin to colistin treatment could significantly reduce biofilm biomass and colony-forming units (CFU ml^{-1}) (Figure 5c and 5d). Hence, the MBEC of colistin in biofilm treatment was significantly reduced to 1 μg ml^{-1}, implying that the combinatorial treatment of vanillin and colistin could improve the eradication of *P. aeruginosa* biofilms significantly.

We then evaluated the effects of vanillin and colistin on the biofilms by performing epifluorescence microscopy and showing significant reduction in biofilm biomass with combinatorial treatment of vanillin and colistin (Figure 6a-b). Furthermore, the live/dead ratio, as determined by GFP levels of live biofilm and propidium iodide (PI) staining of dead cells, significantly decreased with combinatorial treatment of vanillin and colistin, implying the increased ability of colistin to kill biofilms under the presence of vanillin (Figure 6c).

2.7 In vivo evaluation of vanillin and colistin against P. aeruginosa in C. elegans model of infection:

To confirm that our *in vitro* observations are translatable in treating *in vivo* infections, we evaluated the efficacy of vanillin and colistin in monotherapy and combinatorial therapy. Since PQS-mediated virulence was previously reported to be detrimental to *C. elegans* survival [30, 31], we employed the *C. elegans* model of acute infection [31-34].

Without colistin treatment, it was apparent that *P. aeruginosa* could kill *C. elegans* within 16 hrs (Figure 7). While colistin could reduce *P. aeruginosa* cytotoxicity, combinatorial therapy of colistin and vanillin provided a significantly higher survival rate for *C. elegans* during *P. aeruginosa* infection. Hence, our results revealed that vanillin could be used as an anti-virulence agent which could be used in synergy with antibiotics to treat infections.

3. Discussion:

In the face of rising antibiotic resistance threat, it is increasingly viable to employ drugs which inhibit virulence instead of bacterial survival and growth, to reduce the selection pressure on pathogens. Without the selective pressure on bacterial survival, anti-virulence agents did not cause the emergence of resistant mutants in *in vitro* experimental evolution assays [35]. Furthermore, the specificity of anti-virulence agents to target pathogens conferred a narrow spectrum of activity which would not affect the native microbiota [36].

Being upregulated in *P. aeruginosa* during human infections [37], the *pqs* operon could induce production of virulence factors, such as pyocyanin and biofilm, while its mutants typically displayed attenuated virulence to the host and propensity to immune clearance during infections [38]. Furthermore, PQS itself was previously shown to be a virulence factor with the ability to suppress innate immunity via the nuclear factor-κB pathway [39]. Hence, it is clinically relevant to develop *pqs* QSI for improved treatment of *P. aeruginosa* infections.

Till date, various *pqs* QSI had been identified. For instance, analogs of the PqsA's substrate, anthranilate, had been employed to inhibit PqsA and prevent synthesis of PQS [40], while PqsD inhibitors were developed to reduce PQS biosynthesis [41, 42]. Although a few PqsR inhibitors were also identified [24, 43, 44], none of these compounds had entered clinical trials till date, probably due to weak pharmacological aspects, such as toxicity and poor solubility [45]. Hence, searching for naturally derived PqsR inhibitors

which are safe and easy to extract from natural food sources could represent a significant boost in the discovery of QSIs [46].

In this present work, we had determined that vanillin served as an effective Pqs inhibitor which subsequently caused a reduction in *pqs*-mediated quorum sensing and dampened production of virulence factors, such as biofilm formation and pyocyanin production. According to our results, we showed that vanillin potentially inhibited the response protein PqsR instead of inhibiting the biosynthesis genes. As a result of inhibiting *pqs* QS, downstream *pqs*-controlled virulence factors such as pili motility and production of virulence factor (pyocyanin) were significantly reduced. Since biofilm formation via the production of eDNA is also mediated by *pqs* QS[47], we also observed reduction in biofilm formation by *P. aeruginosa*. Furthermore, using molecular docking, the predicted affinity suggested that vanillin required equivalent energy binding to PqsR than NHQ, which highlighted vanillin's efficacy as a PqsR inhibitor.

Hence, vanillin could be developed as an anti-virulence agent which could be potentially used with antibiotics to treat infections relevant to *Pseudomonas aeruginosa*. As an anti-virulence agent which do not kill *P. aeruginosa*, sole treatment using vanillin could not rescue *C. elegans* from *P. aeruginosa* killing, thus another antibiotic must be added in combination to eradicate *P. aeruginosa* and improve survival of *C. elegans*. Since PQS is involved in outer membrane vesicles (OMVs) formation [48] and OMVs have protective roles against antimicrobial peptides such as colistin [49, 50], inhibition of *pqs* signaling by vanillin could potentially improve colistin treatment of *P. aeruginosa*. We showed that combinatorial treatment of this compound with colistin led to reduced biofilm formation and improved survival in model organisms during infections. Although the concentrations of vanillin used in this study appeared high in the mM range (0.015% w/v), it has been proven safe for food consumption at even higher concentrations (typically 2% w/v). Structure-activity Relationships (SAR) can be employed in future studies to make structural and chemical modifications to vanillin for optimizing its properties and activities.

Many compounds with QSI activity had been extracted from natural sources. For instance, gingerol from ginger has effect on other QS systems (*las* and *rhl*) of *P. aeruginosa*, with some effect on *pqs* [13], while sulforaphone from broccoli can inhibit the *las* QS system [51], thereby inhibiting the production of virulence factors such as elastase and rhamnolipids. Hence, vanillin offers a natural alternative in the specific inhibition of *pqs* QS, where it could inhibit *pqs*-mediated pyocyanin and biofilm formation via eDNA production. Taken together our work had shown the potential clinical utility of vanillin as a safe anti-*pqs*

QS agent, which could be used as novel combinatorial therapy with antibiotics against *Pseudomonas aeruginosa* infections.

4. Experimental Section:

4.1 Bacterial strains and media:

The bacterial strains used in this study are listed in Table 1. The biosensor strains contain a transcriptional fusion with *pqsA* promoter fused to GFP with C-terminal oligopeptide extension containing the amino acids ASV [*gfp*(ASV)], resulting GFP to be more susceptible to degradation by housekeeping proteases and rendered a short half-life. This unstable *gfp*(ASV) allows for real-time monitoring of temporal *pqs* QS expression [52]. Bacterial strains were inoculated in 2 ml of Luria-Bertani (LB) medium with 100 µg ml $^{-1}$ of carbenicillin for plasmid maintenance.

4.2 Molecular docking:

As described previously [53], we used the software program Autodock Vina v.1.1.2 designed by the Molecular Graphics Lab at the Scripps Research Institute. Before docking, the ligand MRD was removed from PqsR (4JVC) [24]. Autodock Tools v.1.5.6 was then used to modify the PqsR by eliminating water molecules and adding polar hydrogens. A search space was then generated around the amino acid residues involved in the binding of primary ligand NHQ to PqsR, and docking was conducted by setting the same residues as rigid and flexible. Autodock Tools was also used to detect the torsion root of ligands NHQ and 4-hydroxy-3-methoxybenzaldehyde. In each simulation, nine runs were conducted with the predicted binding affinity per run given in kcal/mol. 3D images were generated from PyMOL v.2.3.2.

4.3 Minimal inhibitory concentration (MIC) and half-maximal inhibitory concentrations (IC_{50}):

The MIC was determined from growing PAO1 in MH media containing a range of vanillin (0 – 16 mM) in triplicate wells of a 96-well plate (SPL Life Sciences, South Korea). The 96-well plate was incubated overnight in a Tecan Infinite 200 Pro plate reader (Tecan Group Ltd., Männedorf, Switzerland) microplate reader at 37 °C with the analysis of OD_{600} conducted every 15 mins. The half-maximal inhibitory concentrations (IC_{50}) was calculated using the Graphpad Prism 6 software package (GraphPad Software Inc., California, USA). Experiments were performed in triplicate, and the results were shown as the mean±s.d.

4.4 Inhibition of pqsA-ASV:

Following the determination of MIC, PAO1/p_{pqsA}-*ASV* was tested similarly from the previous section in triplicate wells. The 96-well plate was incubated overnight in a Tecan Infinite 200 Pro plate reader at 37

°C with the analysis of OD_{600}, and GFP (Ex: 495 nm; Em: 515 nm; Gain 60) conducted every 15 mins. The relative GFP/ OD_{600} was then derived from accounting for cell mass. Experiments were performed in triplicate, and the results were shown as the mean±s.d.

4.5 Determination of relative PQS concentration:

To show the correlation between GFP expression and *pqsA-gfp* to PQS concentration, we derived a concentration-response curve by using triplicate wells of Δ*pqsA/pqsA-gfp* grown in MH containing 0 uM to 20 uM of PQS. The Δ*pqsA/pqsA-gfp* mutant was unable to produce its PQS, thus reliant on exogenous PQS treatment to achieve expression of *pqsA-gfp* [54]. The 96-well plate was also incubated overnight in a Tecan Infinite 200 Pro plate reader at 37 °C with the analysis of OD_{600}, and GFP conducted every 15 mins. The dose-response curve (4-hydroxy-3-methoxybenzaldehyde concentration to GFP/ OD_{600}) was then derived by GraphPad Prism.

To quantify PQS concentrations in our samples, we grew a 6-hr shaking MH culture of *P. aeruginosa* containing various vanillin concentrations (0 – 4 mM) at 37 °C and agitated at 200 rpm. The supernatant of the cultures was filter-sterilized and transferred to a 96-well plate. The Δ*pqsA/pqsA-gfp* mutant was then added to triplicate wells at equal volumes with the supernatant. The 96-well plate was incubated overnight in a Tecan Infinite 2000 Pro plate reader at 37 °C with the analysis of OD_{600}, and GFP conducted every 15 mins. The relative GFP/ OD_{600} readings obtained from the samples were compared to the GFP/ OD_{600} readings from the PQS dose-response curve to determine the absolute PQS concentration. Experiments were performed in triplicate, and the results are shown as the mean±s.d.

4.6 Relative Pyocyanin quantification:

The PAO1 was cultured in 10 ml MH media in varying vanillin concentrations in 50 ml centrifuge tubes at 37 °C, 200 rpm agitation. The cultures were centrifuged at 13000 g for 3 mins, and the supernatant from each sample was transferred to fresh tubes. As previously described [55], 1.8 ml of chloroform was added to each tube and vortexed. The bottom layer of chloroform was then carefully extracted from the immiscible solution and transferred to fresh tubes. The 100 µl of 0.2 M HCl was added and vortexed. The HCl layer containing the pyocyanin was then added to a 96 well plate and measured once at OD_{520} in a Tecan Infinite 200 Pro plate reader. Experiments were performed in triplicate, and the results are shown as the mean±s.d.

4.7 Twitching motility assay:

As previously described, we evaluated the impact of vanillin on the twitching motility of *P. aeruginosa*. Briefly, we prepared 1.5% LB with varying vanillin concentrations (0 and 2 mM) in triplicates. A PAO1 colony was picked with a sterile toothpick and stabbed through the center of the agar to the bottom of the plate. The inoculated plates were incubated for 48 hrs at 37 °C. Following incubation, the agar was carefully removed to prevent the disruption of the twitching ring. The twitching ring on the plate was then stained carefully with 0.1% crystal violet solution and washed three times with sterile deionized water. A representative image of the twitching rings was captured by color camera. To quantify the motility ring stained by crystal violet, 1ml 70% ethanol was added to solubilize the crystal violet. The crystal violet was then added to a 24 well plate and measured once at OD_{595} in a Tecan Infinite 200 Pro plate reader. Experiments were performed in triplicate, and the results are shown as the mean±s.d.

4.8 Combinatorial treatment of vanillin and colistin

The planktonic PAO1 was grown in varying combinations of vanillin (0 – 8 mM) and colistin (0 – 8 µg ml^{-1}) in a checkerboard assay (2-fold dilution of each compound) in a 96-well plate (SPL Life Sciences, South Korea). The 96-well plate was incubated overnight in a Tecan Infinite 200 Pro plate reader at 37 °C with the analysis of OD_{600} conducted every 15 mins. Experiments were performed in triplicate, and the results are shown as the mean±s.d.

4.9 Biofilm growth and crystal violet quantification assay:

The cultivation of biofilm culture and crystal violet staining of biofilms were conducted as described previously [56]. PAO1 biofilms were cultivated in triplicate wells of 24-well plate (SPL Life Sciences, South Korea) containing 1 ml LB per well with vanillin (0 mM and 1 mM) and colistin (0, 0.5, 1 and 2 µg ml^{-1}). The plate was incubated at 37 °C for 16 hrs under static conditions. The planktonic cells were then discarded, and the wells were washed with 0.9% NaCl for three times. Each well was then stained with 0.1% crystal violet for 5 mins. The crystal violet was discarded, and the wells were washed three times with 0.9% NaCl to remove residual crystal violet. The stained biofilm biomass was dissolved with 100% EtOH. The plate was then analyzed under the Tecan Infinite 200 Pro plate reader at OD_{595}. Experiments were performed in triplicate, and the results are shown as the mean±s.d.

4.10 Quantification of Colony-forming units (CFU) in biofilms:

Biofilms were grown in a similar manner as described in previous section. Following the removal of the planktonic cells and washing, the biofilm grown on the walls of each well were scraped with a cell scraper and resuspended vigorously in 1 ml of 0.9% NaCl solution. An aliquot of cell suspension was diluted serially in 0.9% NaCl solution. The diluted samples were then transferred to LB agar plates in 5

replicates and incubated overnight at 37 °C. The colonies grown on the agar plate were then enumerated, with the CFU ml^{-1} tabulated by colony number X dilution factor X volume factor. Experiments were performed in triplicate, and the results are shown as the mean±s.d.

4.11 Epifluorescence imaging of biofilms and data processing:

For biofilm growth, PAO1/Tn7-gfp were cultivated at 1:100X dilution in 300ul liquid media with various treatments (no treatment as control, 1 mM vanillin, 1 μg ml^{-1} colistin, and 1 mM vanillin + 1 μg ml^{-1} colistin) in a 8-well chamber (μSlide, ibiTreat, Ibidi, Germany) at 37℃ for 18 hrs. The spent media containing with planktonic cells was removed and the biofilms adhered to the bottom of the chamber were washed by 0.9% NaCl twice. Propidium iodide (PI) (final concentration = 1 μM) was added into all wells for staining dead cells.

For imaging of biofilms, all microscopy images (GFP and PI) were captured and acquired with Z-stack by using Nikon Eclipse Ti2-E Live-cell Fluorescence Imaging System with a 40× objective. At least 5 images were captured for every triplicate well.

To analyze the biomass and Live/dead ratio of biofilms, the images were processed by using the Nikon Imaging System (NIS) and ImageJ software. For the fluorescence density calculation as previously described [57], the biofilm's parts and background parts in images were selected by ImageJ and measured the Integrated Density, Mean fluorescence and Area values. The corrected total cell fluorescence (CTCF) was calculated in by the formula:

CTCF= Integrated Density – (Area of selected cell × Mean fluorescence of background readings)

4.12 In vivo C. elegans infection assay:

The *in vivo* infection assay was conducted with *C. elegans* using the acute infection assay, as previously described [58]. The PAO1 was grown in liquid media (comprises of 2.5 g peptone, 5.0 g tryptone, 2.5 g yeast extract, 5 μg ml^{-1} cholesterol, 4.0 g NaCl, 7.5 ml glycerol and ddH_2O in 1 liter) containing 1 mM vanillin only; 1 μg mL^{-1} colistin only; and 1 mM vanillin with 1 μg mL^{-1} colistin, in 96-well microplates. Thirty L3-stage animals were transferred into triplicate wells. The co-cultures were incubated at 25 °C and observed for live/dead nematodes under a stereomicroscope (Zeiss) at 0, 3, 6 and 16 hrs. The number of worms that were still alive after treatment were tabulated as % of nematodes alive. Experiments were performed in triplicate, and the results are shown as the mean±s.d.

4.13 Statistical analysis:

Where applicable, One-way-ANOVA and Student's T-test were used. Experiments were performed in triplicates and results were shown as mean ± standard deviation.

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Competing interests:

The authors declare no competing financial interests.

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577 **Table 1**

Strain(s)	Description	Source or reference
P. aeruginosa strains		
PAO1	Prototypic non-mucoid wild type strain	[59]
Δ*pqsR*	*pqsR* gene knockout of PAO1 constructed by allelic exchange	[60]
PAO1/Tn7-gfp	Gm^r; Constitutively expressed GFP	[61]
PAO1/p_{pqsA}-*gfp*	Gm^r/$Carb^r$; PAO1 containing pqsA-gfp reporter fusion	[60]
Δ*pqsR*/p_{pqsA}-*gfp*	Gm^r/$Carb^r$; ΔpqsR containing pqsA-gfp reporter fusion	This study
ΔpqsR/p_{lac}-pqsR/p_{pqsA}-*gfp*	Gm^r/$Carb^r$; ΔpqsR/pqsR containing pqsA-gfp reporter fusion	This study
Δ*pqsR*/p_{lac}-*pqsABCDE*/p_{pqsA}-*gfp*	Gm^r/$Carb^r$; ΔpqsR/pqsABCDE containing pqsA-gfp reporter fusion	This study

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Figures:

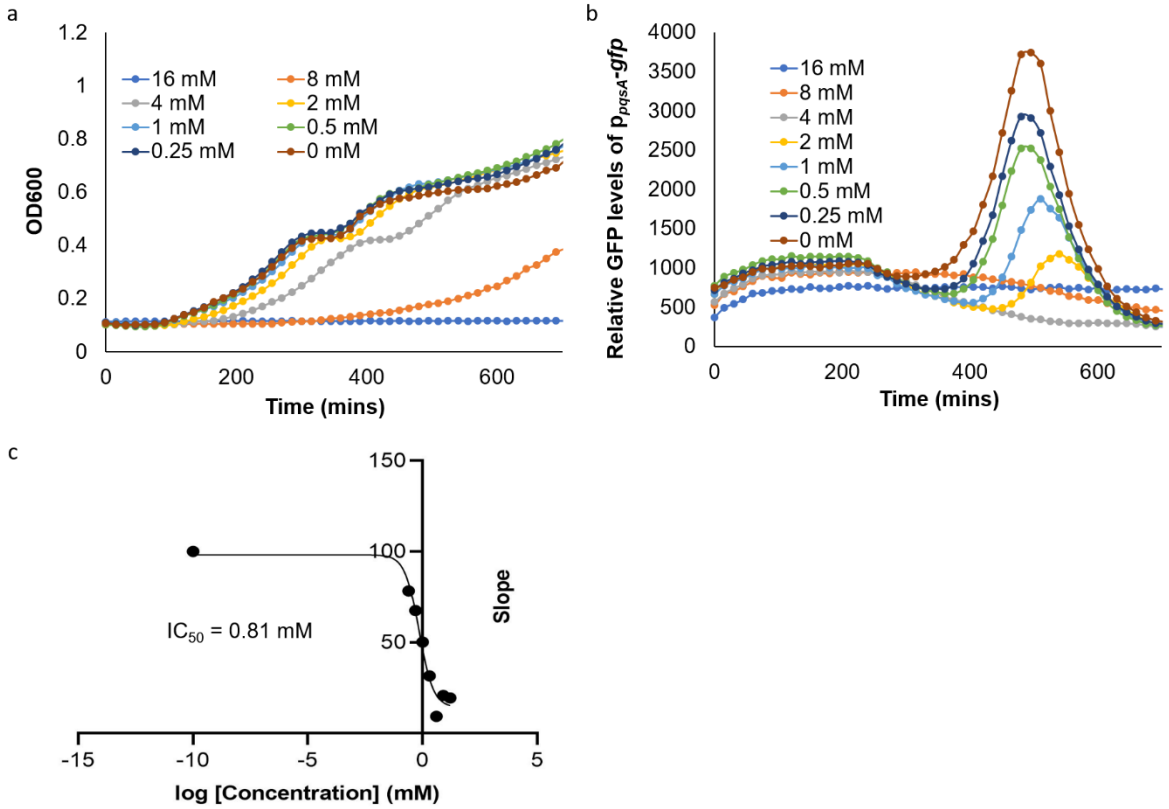


Figure 1. Inhibition of *pqs* QS expression by vanillin. (**a**) Cell density of PAO1/p_{pqsA}-*ASV* in varying concentrations of vanillin. (**b**) Inhibitory effect of various concentrations of vanillin on p_{pqsA}-*gfp* expression. (**c**) The half-maximal inhibitory concentration (IC_{50}) of cell density of p_{pqsA}-*gfp* in different concentrations of vanillin. The slope of the curve was calculated based on its respective dose-response curves and plotted against the log concentration. The slope is indicative of the biosynthesis rate of GFP due to PQS induction.

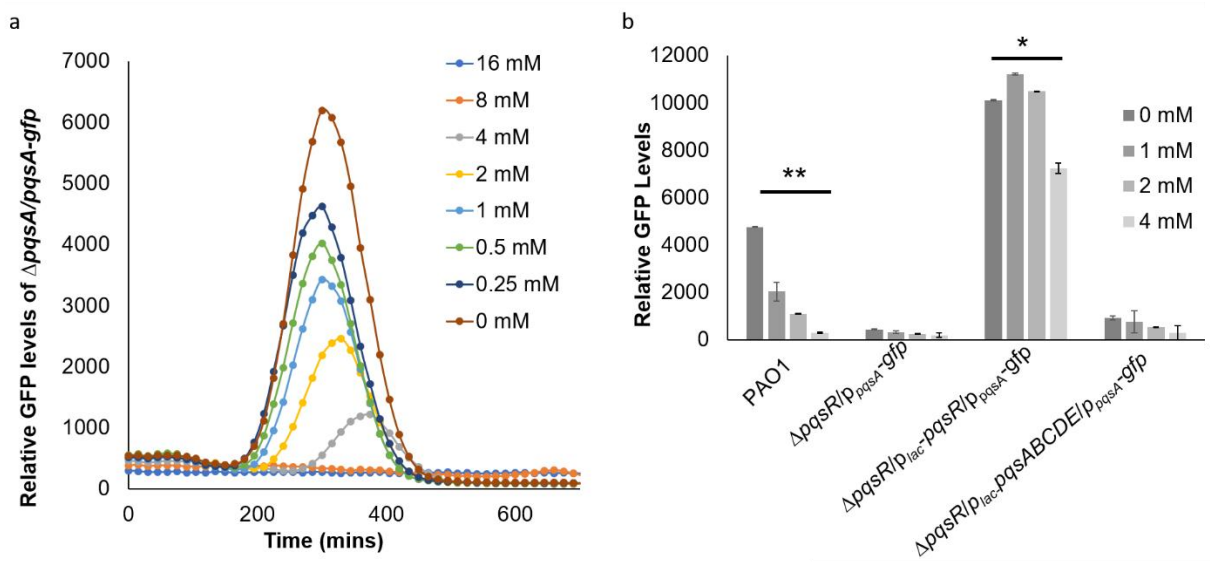


Figure 2. Inhibition of QS-controlled virulence factors by vanillin in *P. aeruginosa* mutants (**a**) Relative GFP quantification of ΔpqsA/p_{pqsA}-*gfp* with exogenously added 10 μM PQS in varying concentrations of vanillin. (**b**) The relative GFP quantification of PAO1, ΔpqsR/p_{pqsA}-gfp, ΔpqsR/p_{lac}-pqsR/p_{pqsA}-*gfp*, Δ*pqsR*/p_{lac}-*pqsABCDE*/p_{pqsA}-*gfp* in varying concentrations of vanillin. Means and s.d. from triplicate experiments are shown. *P < 0.05, **P < 0.01, One-way ANOVA.

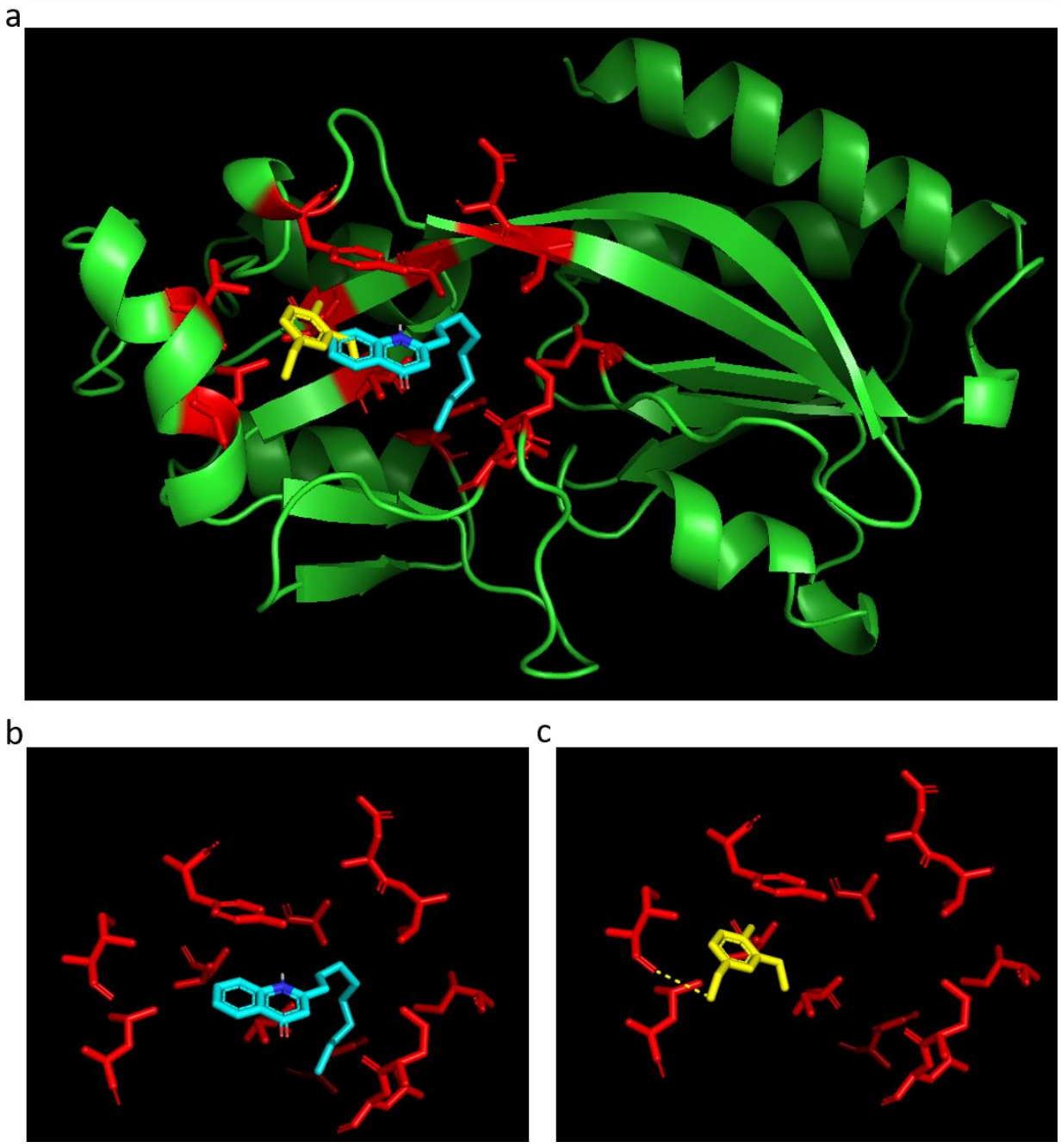


Figure 3. Molecular Docking of vanillin and NHQ (**a**) Comparison of docking NHQ (blue) and vanillin (yellow) in the active site (red) of PqsR (green). (**b**) NHQ binding to L208 in PqsR active site, with binding affinity of -6.1 kcal/mol. (**c**) vanillin binding to I186 in PqsR active site, with binding affinity of -4.0 kcal/mol. Molecular docking was performed using Autodock Vina v.1.1.2, and graphics were generated with PyMOL v.2.3.2.

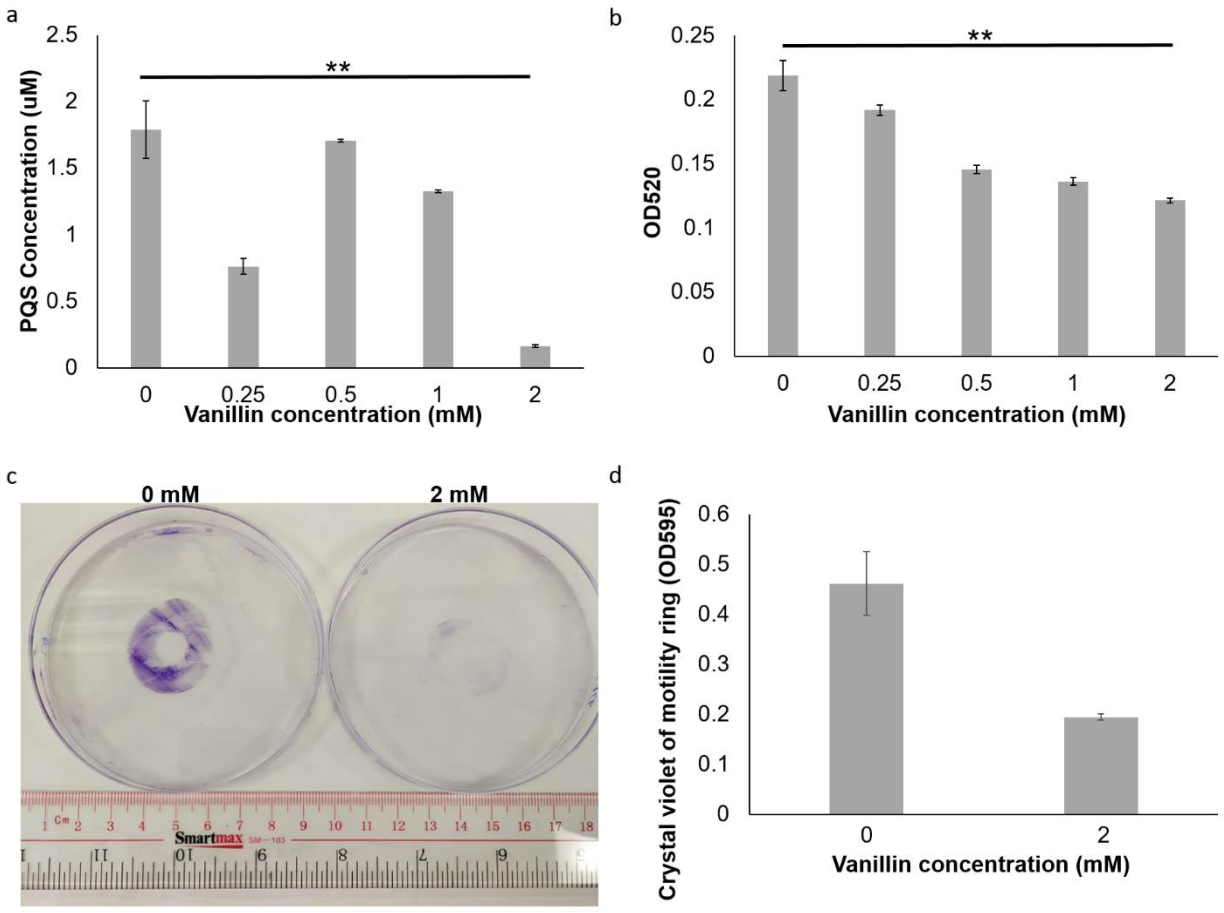
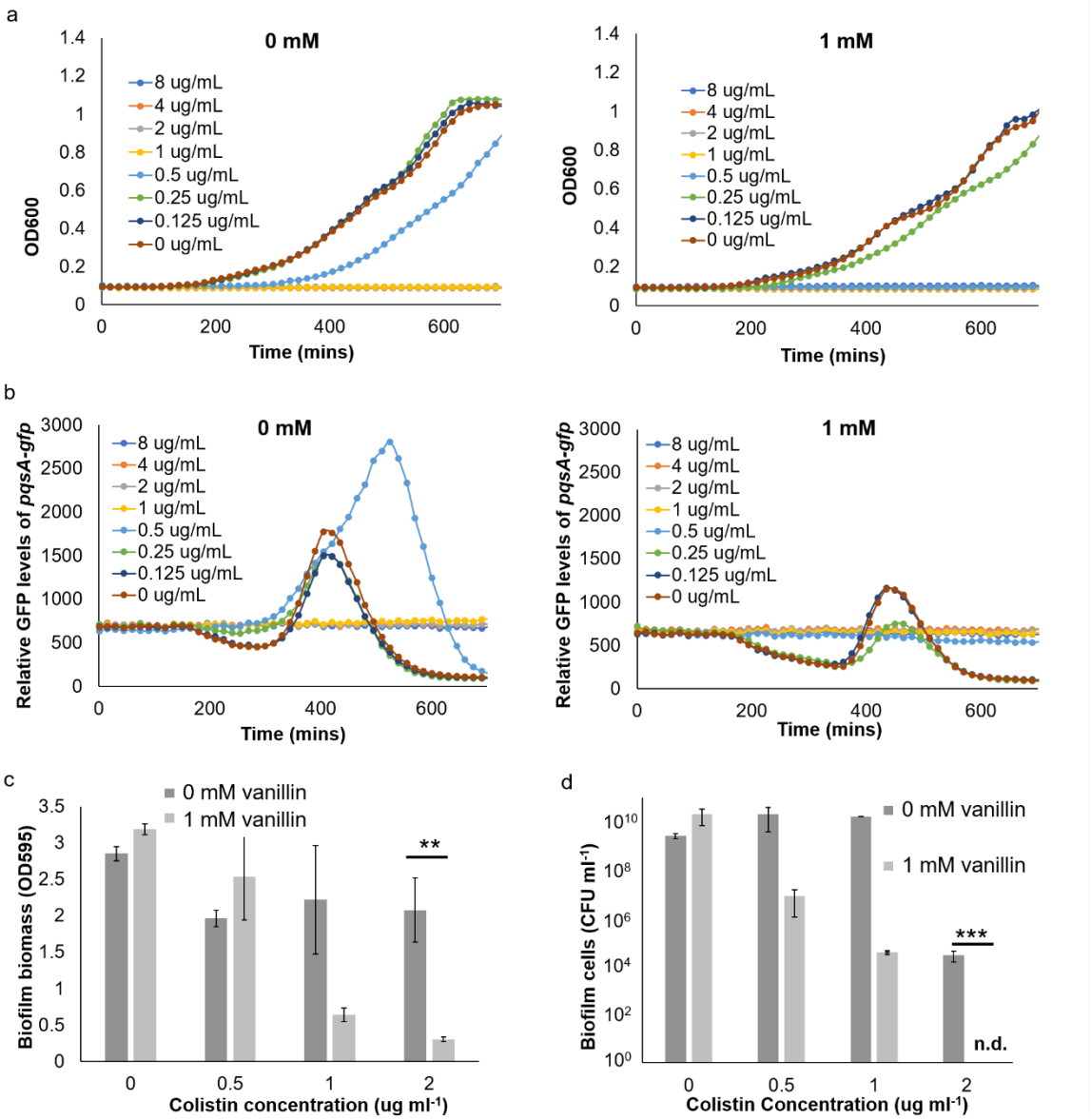


Figure 4. Inhibition of QS-controlled virulence factors by vanillin in *P. aeruginosa*. (**a**) PQS concentration in PAO1 cultures with vanillin treatment. (**b**) Pyocyanin assay of PAO1 in varying concentrations of vanillin. Means and s.d. from triplicate experiments were shown. **P < 0.01, ***P < 0.001, One-way ANOVA. (**c**) Representative images of crystal violet-stained twitching rings generated by *P. aeruginosa* with vanillin treatment. (**d**) Quantification of crystal violet for twitching rings generated by *P. aeruginosa* with vanillin treatment. Means and s.d. from triplicate experiments were shown. *P < 0.05, One-way ANOVA.



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608 **Figure 5.** Combinatorial treatment of vanillin and colistin on *P. aeruginosa*. (**a**) Growth curves of PAO1
609 in 0 mM (left panel) and 1 mM (right panel) vanillin with varying concentrations of colistin. (**b**) Relative
610 gfp quantification of p_{pqsA}-*gfp* in 0 mM (left panel) and 1 mM (right panel) vanillin with various
611 concentrations of colistin. (**c**) Crystal violet staining of PAO1 in 0 mM and 1 mM vanillin with varying
612 concentrations of colistin. (**d**) The CFU ml^{-1} of biofilm cells treated with 0 mM and 1 mM vanillin with
613 various concentrations of colistin. N.d: not detected. Means and s.d. from triplicate experiments are
614 shown. *P < 0.05, **P < 0.01, One-way ANOVA.

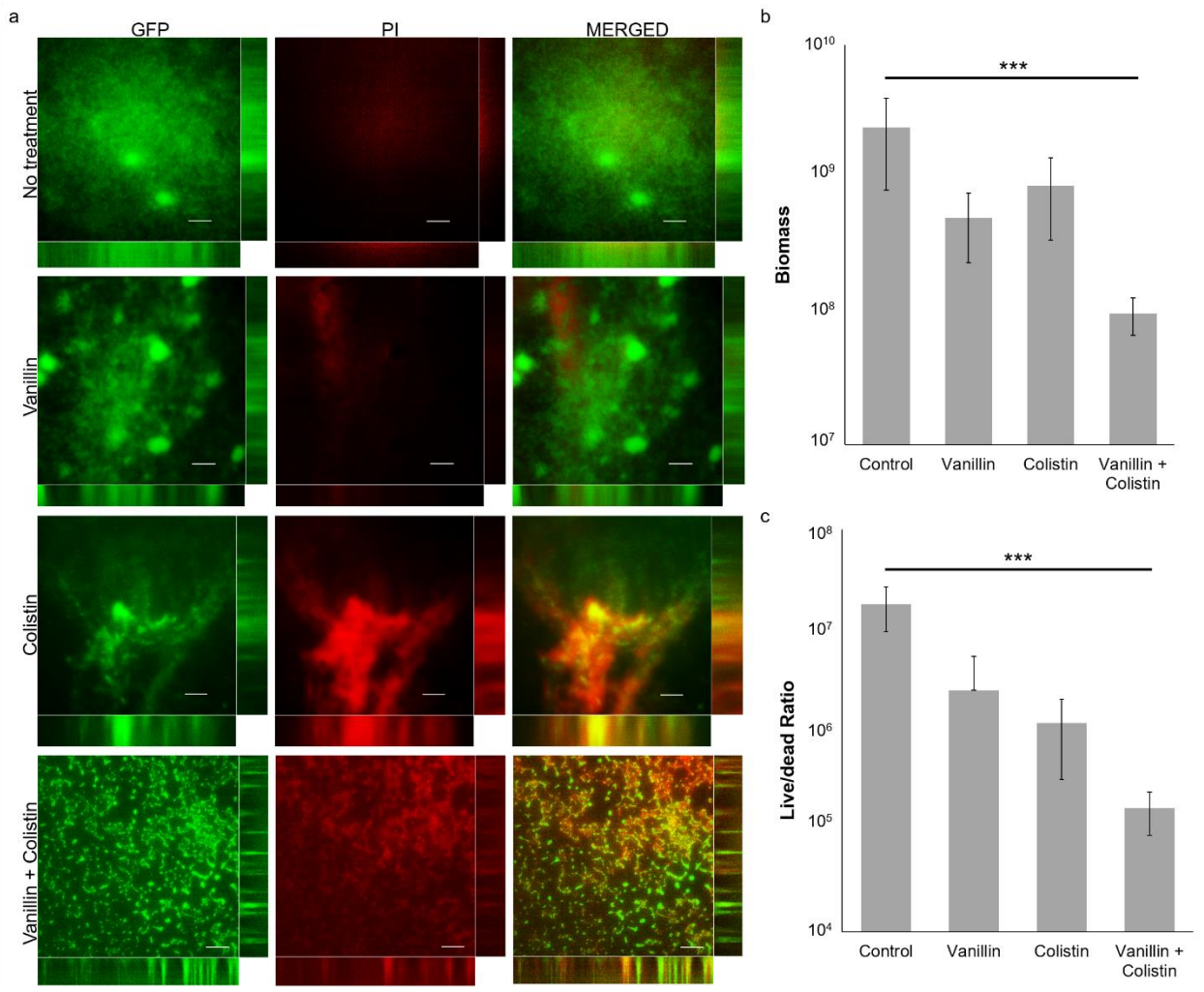
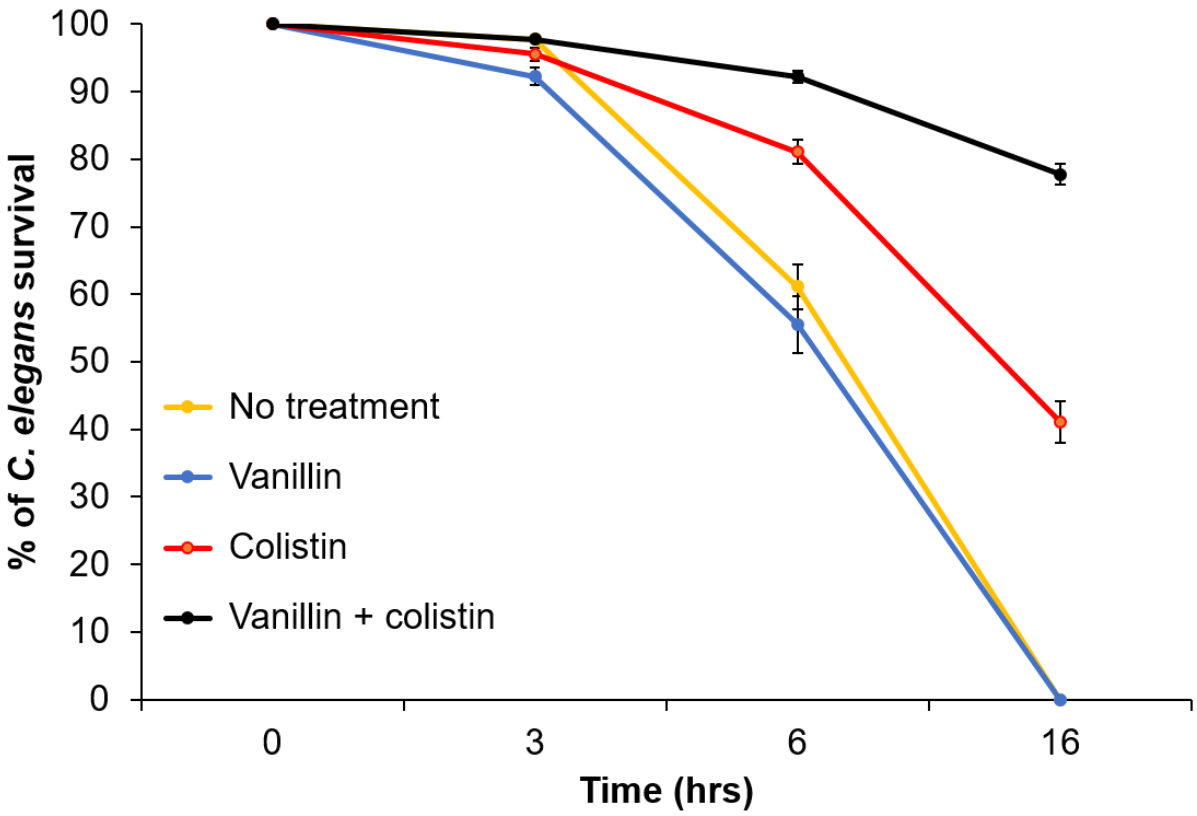


Figure 6. Combinatorial treatment of vanillin and colistin effectively eradicated biofilms. **(a)** Representative images (Scale: 50 μm), (**b**) biomass and (**c**) live/dead ratio of biofilms treated with vanillin and colistin. Means and s.d. from triplicate experiments are shown. ***P < 0.001, One-way ANOVA.



620

621 **Figure 7.** *C. elegans* survival assay in the *P. aeruginosa* acute infection model, as reflected by the
622 percentage of live nematodes. Combinatorial treatment of vanillin and colistin improved *C. elegans*
623 survival against PAO1 infection. Means and s.d. from triplicate experiments are shown. **P < 0.01,
624 ***P < 0.001, One-way ANOVA.

625