Potential use of dimethyl sulfoxide in treatment of infections caused by *Pseudomonas aeruginosa*

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ABSTRACT

Dimethyl Sulfoxide (DMSO) is commonly used as a solvent to dissolve water-insoluble drugs or other test samples in both *in vivo* and *in vitro* experiments. It was observed during our experiment that DMSO at non-inhibitory concentrations could significantly inhibit pyocyanin production in human pathogen *Pseudomonas aeruginosa.* Pyocyanin is an important pathogenic factor whose production is controlled by cell-density dependent quorum-sensing (QS) system. Investigation of the effect of DMSO on QS shows that DMSO has significant QS antagonistic activities, and micromolar range concentrations of DMSO attenuated a battery of QS-controlled virulence factors, including rhamnolipids, elastase, LasA protease and biofilm formation. Further study indicates that DMSO inhibition of biofilm formation and pyocyanin production is attained by reducing the production of autoinducer molecule of the *rhl* QS system, N-butanoyl-L-homoserine lactone (C4-HSL). In a mouse model of burn wound infected with *P. aeruginosa*, treatment with DMSO decreased mouse mortality significantly compared with the control group. The capacity of *P. aeruginosa* pathogenicity attenuation points to the potential use of DMSO in treatment of *P. aeruginosa* infection as an anti-pathogenic agent. As a commonly used solvent, however, DMSO's impact on bacterial virulence calls for cautionary attention in its usage in biological, medicinal and clinical studies.

Keywords: Dimethyl sulfoxide; *Pseudomonas aeruginosa*; Quorum sensing; Infection; Biofilm formation, Anti-pathogenic agent

Pseudomonas aeruginosa is a prevalent opportunistic pathogen capable of causing various infections in humans, including pneumonia and urinary tract infection, bloodstream infection, and infection in burn patients (1). Chronic infection caused by *P. aeruginosa* and associated pulmonary inflammation are ultimately responsible for the majority of mortality of patients with cystic fibrosis (2). The ability of *P. aeruginosa* to cause diverse infections is attributed to its myriad of virulence factors and biofilm-forming capability, which are controlled by the intercellular communication system, quorum sensing (QS) (3-5).

P. aeruginosa has two acyl-homoserine lactone (AHL)-mediated QS systems, known as the *las* and *rhl* QS systems. The *las* and *rhl* systems consist of the transcriptional activators LasR and RhlR, and the signal synthases LasI and RhlI, respectively. The major signals in the *las* and *rhl* systems are N-(3-oxododecanoyl)-HSL (3-oxo-C12-HSL) and N-butyryl-HSL (C4-HSL), respectively (6, 7). *P. aeruginosa* employs these QS systems to control a wide range of extracellular virulence factors including pyocyanin, elastase and rhamnolipid (8-13). The AHL-mediated QS systems also play a crucial role in biofilm formation by *P. aeruginosa*, a common cause of resistance to antibiotics and difficulties in infection treatment (14). The *las* system influences the activation of *pel* and the biofilm matrix formation accordingly (15), and the *rhl* system contributes to the maintenance of biofilm architecture through production of rhamnolipid surfactants (16, 17). Due to the crucial role of QS systems in regulating virulence and biofilm formation, inhibition of the QS systems therefore provides an alternative therapeutic approach for treating *P. aeruginosa* infections. Extensive studies have focused on searching for drugs capable of blocking QS and attenuating pathogenicity.

63 DMSO is an important polar aprotic solvent that is frequently used as a vehicle in both *in vivo*
64 and *in vitro* experiments, including in quorum sensing inhibitor (QSI) screening studies (18-21).
65 It was observed unexpectedly during our experiments that DMSO could significantly inhibit the
66 production of pigmented compound pyocyanin, an important pathogenic factor in *P. aeruginosa*
67 that is tightly regulated by the QS systems. Besides its cryoprotective and tissue penetration
68 enhancing actions, DMSO has been used to treat numerous conditions and ailments in
69 pre-clinical research and, in some cases, clinical situations. The conditions treated include
70 dermatologic diseases (22), pain (23), chronic prostatitis (24), gastrointestinal disorders (25-28),
71 wound healing (29), pulmonary (30, 31), and interstitial cystitis (IC) (32), even though in most
72 cases the pharmacological mechanisms are unknown.
73 In this study, we investigated the effect of DMSO on the pathogenicity of *P aeruginosa*. We
74 present data showing that DMSO exhibited significant antagonistic activities on QS-associated
75 virulence factors in *P. aeruginosa*, such as rhamnolipids, elastase, LasA protease and biofilm
76 formation. The mechanism of DMSO attenuation of virulence factors was found to be reducing
77 the production of N-butanoyl-L-homoserine lactone (C4-HSL), the signal molecule of the *rhl*
78 system. Using a burned-mouse model the use of DMSO in treating *P. aeruginosa* infection was
79 examined.

80 **MATERIALS AND METHODS**

81 **Materials, plasmids and culture conditions**

82 The bacterial strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* PAO1

and its derivatives were routinely grown at 37°C on LB (Luria-Bertani) agar plates or in LB broth with orbital shaking at 200 rpm. Where appropriate, antibiotics were used at the following concentrations: for *E. coli*, kanamycin (Kn) was used at 50 µg/ml; for *P. aeruginosa*, tetracycline (Tc) was used at 300 µg/ml in *Pseudomonas* isolation agar (PIA) and trimethoprim (Tmp) at 300 µg/ml in LB broth. Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as indicated.

Monitoring gene expression

The plasmid pMS402 containing a promoterless *luxCDABE* reporter gene cluster was used to construct promoter-reporter fusions of virulence related genes as described previously (33). Virulence gene expression in liquid cultures was monitored based on light production from the promoterless *luxCDABE* operon downstream of the gene promoter (34). Overnight cultures of the reporter strains were diluted to an optical density of 0.2 at 600 nm (OD_{600}) and cultivated for an additional 2 h before use as inoculants. The cultures were inoculated into parallel wells in a 96-well black plate with a transparent bottom. Aliquots of a fresh culture (5 µl) were inoculated into the wells, which contained 95 µl of medium with or without DMSO (2% v/v). To prevent evaporation during the assay, 50 µL of filter-sterilized mineral oil (Sigma) was added. Both luminescence and bacterial growth (OD_{600}) were measured every 30 min for 24 h in a Victor^3 Multilabel Counter (PerkinElmer, USA). The light production measured in counts per second (cps) is proportional to the level of gene expression. The light production values were then normalized to bacterial growth. The level of gene expression is presented by relative

luminescence unit (RLU) cps/OD_{600}, the normalized cps values.

To monitor the gene expression on solid medium, the reporter strains were plated onto soft-top LB agar. Overnight cultures of the reporter strains were diluted to an optical density of 0.2 at 600 nm (OD_{600}). The upper layer of medium (LB medium with 0.7% agar), cooled to 40°C, was mixed with 100 µl of the diluted cultures supplemented with DMSO (2% v/v) or 100 µl of the diluted cultures alone (as control), and spread onto the lower layer of medium (LB medium with 1% agar). The plates were incubated overnight in 37°C, and imaging was performed using the LAS3000 imaging system (Fuji Corp.).

Measurement of pyocyanin production

Pyocyanin was extracted from the supernatants of overnight cultures as described previously (35) with minor modifications. Briefly, PAO1 cultures were grown in LB medium supplemented with or without DMSO (2% v/v) (as control). 3 ml of chloroform was added to 5 ml of the culture supernatant. After extraction, the chloroform layer was transferred to a fresh tube and mixed with 1 ml of 0.2 M HCl. After centrifugation, the top layer (0.2 M HCl) was removed, and the absorption was measured at 520 nm. The obtained concentrations, expressed as micrograms of pyocyanin produced per ml of culture supernatant, were calculated using an extinction coefficient of 17.072 at 520 nm (36).

Elastase quantitation

The production of elastase in *P. aeruginosa* cultures was determined by elastin Congo red assay with minor modifications (37). PAO1 cultures were grown in LB medium in the presence or in

the absence of DMSO (2% v/v) (as control) to an OD_{600} of 2.0. The cells were harvested through centrifugation, and the supernatants were filter sterilized (TPP syringe filter; pore size, 0.22 µm). The filter-sterilized supernatants or LB medium were mixed 2:1 with phosphate buffer (0.1 M, pH 6.3), and 2 mg/ml elastin Congo red (Sigma) was then added. The mixture was incubated at 37°C with shaking (200 rpm) for 3 h. After centrifugation, the absorbance of the supernatant was measured at 495 nm in a spectrophotometer. Elastin-Congo red sample incubated with medium alone was used as the blank. The experiment was performed in triplicate with supernatants from three independent experiments.

LasA staphylolytic activity assay

LasA protease activity was determined by measuring the ability of culture supernatants to lyse boiled *S. aureus* cells (38). Briefly, overnight culture of *S. aureus* was boiled for 10 min and centrifuged for 10 min at 10,000 g. The resulting pellet was resuspended in 10 mM Na_2PO_4 (pH 7.5) to an OD_{600} of approximately 0.8. A 100 µl aliquot of supernatant of *P. aeruginosa* overnight culture treated with or without DMSO was added to 900 µl of a boiled *S. aureus* suspension. Medium alone and medium with DMSO were used as the blank controls respectively. The OD_{600} was determined after 0, 5, 10, 20, 30, 45, and 60 min.

Rhamnolipid detection

Rhamnolipid was detected in *P. aeruginosa* culture fluids as described previously (39). Cells from mid-exponential-phase cultures grown in Peptone Tryptone Soya Broth (5% Peptone; 0.25% Tryptone Soya Broth, pH 7.0) were washed and resuspended in modified GS medium (Medium

Group) (40) supplemented with or without DMSO at an OD_{660} of 0.2 and then incubated at 37°C with shaking. The cultures were incubated for a total of 80 h and then centrifuged at 16,000 g for 5 min. The supernatants were sterilized by passing through 0.22 μm pore-size filters, and the filtrates were extracted twice with 2 volumes of diethyl ether. The pooled ether extracts were then extracted once with 20 mM HCl, and the ether phase was evaporated to dryness. Finally, the residue was dissolved in water, and the rhamnolipid content in each sample was determined through comparison with rhamnose standards via duplicate orcinol assays (41), where 1.0 mg rhamnose corresponded to 2.5 mg rhamnolipid.

Quantification of biofilm formation

Biofilm formation was measured in a static system as described previously (42) with minor modifications. Cells from overnight cultures were standardized to OD_{600} of 0.2 and inoculated at 1:10 dilution. The fresh cultures were supplemented with DMSO (2% v/v) or without DMSO as control in 96-well polystyrene microtiter plates (Costar). After 24 h of incubation at 37°C without agitation, crystal violet solution (1% w/v) was added to each sample well at a volume equal to one-fourth of the culture volume, and the mixture was incubated at room temperature for 15 min. The wells were washed twice with distilled water and filled with 200 μl of 95% ethanol to solubilize the crystal violet. A 125 μl aliquot of this solution was transferred to a new polystyrene microtiter plate, and the absorbance was measured at 570 nm. All experiments were independently repeated 3 times, and 8 replicate samples were included in each experiment.

Bioassay of AHL activity

163 The autoinducer of the *rhl* QS system, C4-HSL, was measured using the *rhlA* promoter-based *P.*
164 *aeruginosa* strain, PDO100 (pKD-*rhlA*) (43). PDO100 is an *rhlI* mutant that is deficient in signal
165 molecule C4-HSL production and consequently lacks *rhlA* expression unless exogenous C4-HSL
166 is present in the medium. The plasmid pKD-*rhlA* in PDO100 carries the C4-HSL-responsive *rhlA*
167 promoter fused upstream of a promoterless *luxCDABE*. The light production of the reporter is
168 proportional to the amount of C4-HSL present in the medium. In the assay, two microlitres of test
169 bacterial culture (OD_{600}=1.0) of wild-type PAO1, PAO1 with DMSO, and the *rhlI* mutant
170 (PDO100) or LB medium alone was inoculated onto the seeded bioassay plate respectively.
171 These plates were incubated at 37 °C for 24 h. The bright halo zone around bacterial colonies
172 indicates AHL activity. The assays were also carried out in LB broth using the same reporter
173 fusions. Briefly, overnight cultures of the reporter strains were diluted 1:300 in LB medium, and
174 90 µL of these solutions was added to the wells of a 96-well plate. Ten microliters of
175 filter-sterilized culture supernatants containing either DMSO (2% v/v) or LB medium (as control),
176 were added to the wells, and the luminescence (measured in cps, counts per second) and OD_{600}
177 values were measured every half-hour for a total of 24 h using a Victor^3 Multilabel Plate Reader
178 (Perkin-Elmer), and the relative levels of C4-HSL were calculated from the maximal cps. values.

179 **Animal studies using a burned mouse infection model**

180 Experimental animals: Female C57BL/6J mice 8 to 10 weeks of age were purchased from the
181 Experimental Animal Center of the Fourth Military Medical University, China. Animals were
182 housed in ventilated cages in a pathogen-free facility operated with 12-h light-dark cycles, at

23°C (SD, 2°C), and 30% to 60% humidity. All mice were allowed free access to a standard laboratory rodent chow and water. Individual animals were anesthetized intraperitoneally with 1% pentobarbital (50 mg/kg body weight) and subjected to surgical procedures or sample collection. The experimental protocols were approved by Experimental Animal Committee of the Fourth Military Medical University.

The effect of DMSO on *P. aeruginosa* PAO1 pathogenicity was examined by using the burned-mouse model as described previously with minor modification (44, 45). The mice were anesthetized and shaved on their back. Thermal injury was induced by scalding about 10% of the body surface and the mice were burned throughout the full thickness of skin (III°) using 95°C to 98°C water steam for 5 seconds. Immediately after burning, the mice were administered intraperitoneally with 0.3 mL of sterile saline to prevent dehydration. PAO1 were sub-cultured and grown at 37°C to an optical density of approximately 0.9 at 600 nm. Cells were then pelleted and serially diluted in saline. A sterilized filter paper was placed on the burn eschar and 50 μL of bacterial suspension was permeated though the filter paper.

For dissemination tests, 3 groups of mice with 9 per group were used. The *P. aeruginosa* concentration in the inoculant suspension used on the burn eschar was $1x10^5$ CFU/mL. The mice were divided randomly to DMSO treatment group, antibiotic treatment group (2% silver sulfadiazine cream) and negative control group (saline). Administration of drugs or saline started 24 h post-infection. 200 μL of 10% DMSO solution in 0.9% saline was applied to the burned skin of animals in the DMSO treatment group. Animals in the antibiotic group were given 200 μL of sliver sulfadiazine at 2% (w/v) and animals in the negative control group were given 200 μL of

saline. The administration of drugs and saline was carried out twice a day for 3 days.

At 3 days post burn infection, the mice were euthanized by intracardial injection of 0.2 ml of Pentobarbital. The livers of the animals in the negative control, antibiotic and DMSO treated groups were removed, weighed, and homogenized in PBS (46). A 100 µL aliquot of each homogenate was plated on LB agar plates to determine post burn/infection bacterial load. The number of bacteria from each liver was calculated as CFU per gram of tissue.

For lethality tests, the burned mouse infection procedures were the same as stated above except that different inoculation dosage of *P. aeruginosa* was used. A sterilized filter paper was placed on the burn eschar and 50 µL of *P. aeruginosa* suspension at 1 x 10^8 CFU/mL was permeated though the filter paper.

The experiment was performed on four groups of mice with 11 per group. Mice were divided randomly to DMSO treatment (low-dose) group, DMSO treatment (high-dose) group, antibiotic control group (imipenem), and negative control group (saline). In accordance with a previous study (47), the DMSO concentration of low-dose group was limited to 1.5 ml/kg body weight, and the concentration of large-dose group was two times that of low-dose group. Accordingly, animals in DMSO treatment (low-dose) group and DMSO treatment (high-dose) group received intraperitoneal administration of 10% DMSO solution in saline at an amount of 15 µL/g and 30 µL/g DMSO per day respectively. Animals in the antibiotic control group were injected with imipenem at 200 mg/kg daily. The final volumes of the injections were adjusted to 0.4 ml, and animals in the negative control group were given 0.4 mL of saline. The administration of drugs and saline started 2 h post infection and was carried out twice a day for 5

days, and the mortality was recorded during this period of time.

Statistical analysis

Student's T-test was used to analyze the data in all the experiments except animal studies. Mann-Whitney U test was used for analysis of nonparametric data to determine the significant differences between two groups of mice for the CFU. Kaplan-Meier test was used to determine the significance of the mortality differences between the different mouse groups. All statistical analyses were performed by SPSS software of the 16.0 version. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of DMSO on the expression of QS-related genes and global regulators

It was observed during a QSI screening that the pigmented phenazine production in *P. aeruginosa* PAO1 was greatly reduced in the presence of 2% (v/v) DMSO alone, resulting in a clear difference in the color of the cultures (Fig. 1a). To examine whether such an effect was related to bacterial growth, the impact of DMSO on the growth of *P. aeruginosa* PAO1 was determined with increasing concentrations of DMSO. The result indicated that there was no difference in PAO1 growth in the presence of DMSO at the concentrations below 2.5% (v/v) (Fig. S1).

The impact of 2% (v/v) DMSO on the expression of phenazine biosynthesis gene clusters, *phzA1* and *phzA2*, was then determined. As shown in (Fig. 1b, c and d), DMSO significantly

repressed the expression of *phzA1* and *phzA2*, which probably explain the reduced phenazine production with the presence of DMSO in the medium.

Since phenazine compounds including pyocyanin are products controlled by QS system in *P. aeruginosa* (48, 49), we tested whether the expression of genes associated with QS system were influenced by DMSO. The results (Table 2) indicate that the expression of QS related genes, including *lasI*, *lasR*, *rhlI*, *rhlR*, *lasB* and *rhlA* was all inhibited by DMSO. Examination of the effect of DMSO on other regulatory systems, such as GacA–GacS two component system and the *Pseudomonas* Quinolone Signal (PQS) system, which also play important roles in regulating phenazine production (50, 51) were also carried out. In addition to the AHL mediated QS system, DMSO inhibited the expression of *gacA*, *rpoS*, *pqsR* and *vfr* to different extents, while activated the expression of *pqsA,* which is involved in the synthesis of the Pseudomonas quinolone signal (PQS), and *exoS, exoT* and *exoY,* the effector genes of the type III secretion system (T3SS) (Table 2). The T3SS injects the effectors into host cells where the effectors exert different effects that promote infection and suppress host immune response. The activation of *exoS, exoT* and *exoY* was not significant under T3SS inducing condition but was more pronounced in non-inducing condition. These results demonstrated that DMSO could influence the expression of some of the important globe regulators and the effector genes of the T3SS in *P. aeruginosa*, together with the QS system.

Decreased production of QS-regulated extracellular virulence factors and biofilm formation by DMSO

The two zinc metalloproteasese LasB and LasA are believed to play important roles in *P. aeruginosa* pathogenesis (52). Moreover, the involvement of rhamnolipids, synthesized by enzymes encoded by *rhlA*, in biofilm formation is well documented. They are involved in maintaining open channels by affecting cell-cell interactions and in the attachment of bacterial cells to surfaces (53). As shown in Fig. 2 and Table 2, DMSO dramatically reduced the expression of *rhlA lasB*, and *lasA* . To confirm such effects, the production of elastase, LasA protease and rhamnolipids was compared in LB broth supplemented with or without DMSO.

In agreement with the gene expression data, there were significant decreases in the production of rhamnolipids (P=0.0037), elastase (P=0.0088), and LasA protease (P=0.00056) in the presence of 2% (v/v) DMSO (Fig. 3a, b and c).

Biofilm formation, a QS-controlled phenomenon in *P. aeruginosa* prompts increased antibiotic resistance and contributes to the severity of the infection in the lungs of patients with cystic fibrosis (54). Since DMSO repressed a range of QS-controlled genes which involved in biofilm formation, we tested whether DMSO inhibited the biofilm formation. As shown in Fig. 3d, the presence of DMSO greatly repressed the biofilm formation of *P. aeruginosa* (P=0.00027).

The impact of DMSO on virulence was through the *rhl* system

It is known that many of the virulence factors affected by DMSO, including phenazine production and biofilm formation, are positively controlled by the C4-HSL mediated *rhl* QS system. It is possible that the altered expression of the virulence factors caused by DMSO could have resulted from DMSO's inhibition of the *rhl* QS system. To test this possibility, we

284 determined the impact of DMSO on the production of homoserine lactone signal C4-HSL using
285 an *rhlA* promoter-based *P. aeruginosa* reporter strain PDO100 (pKD-*rhlA*). The amount of
286 C4-HSL in the culture supernatant in the presence and absence of DMSO was measured as light
287 production using this reporter system. As shown in Fig. 4, the level of C4-HSL in PAO1 was
288 significantly reduced in the presence of DMSO ($P=1.75E^{-08}$), which is consistent with the
289 decreased gene expression at transcriptional level. The results indicate that DMSO influences
290 virulence factors probably by inhibiting the production of C4-HSL.

291 To further prove the connection between the *rhl* system and the effect of DMSO, we
292 examined the impact of DMSO on the production of the pyocyanin by addition of exogenous
293 C4-HSL. As shown in the Fig. 5a, the addition of C4-HSL could negate DMSO's repression on
294 pyocyanin production compared with the wild type (P=0.00013). We further assessed biofilm
295 formation in the *rhlI* mutant strain PDO100 (deficient in C4-HSL production). As presented in
296 Fig. 5b, in contrast to the wild type PAO1, no repression on biofilm formation was observed for
297 PDO100 (P=0.2638) in the presence of DMSO. The addition of extraneous C4-HSL elevated the
298 level of biofilm formation of PDO100 to the wild type level, and extraneous C4-HSL was able to
299 restore the inhibitory effect of DMSO on PDO100 biofilm formation (P=0.00021). Taken
300 together, these results indicate that DMSO inhibited the production of virulence factors and
301 biofilm formation probably via the *rhl* system.

302 **Activation of the effector genes of the type III secretion system by DMSO**

303 It has been reported that T3SS) is negatively regulated by *rhl* system (55). We determined

304 whether DMSO could affect the expression of T3SS in *P. aeruginosa*. In contrast to the above
305 examined virulence factors, the presence of DMSO at 2% (v/v) activated the expression of *exoS,*
306 *exoT,* and *exoY,* the effector genes of T3SS (Table 2). Interestingly, DMSO treatment resulted in
307 less activation in *exoS*, *exoT* and *exoY* expression in the *rhlI* mutant background (PDO100) than
308 in the wild type PAO1 (data not shown), indicating that the activation of the T3SS by DMSO is
309 partially dependent on the *rhl* QS system. Consistent with the activation of T3SS effectors,
310 response regulator gene *gacA* of the GacA/S-RsmA regulatory pathway was repressed by the
311 presence of DMSO (Table 2). It is known that the GacA/S two component system negatively
312 regulates RsmA synthesis which is required for T3SS. Clearly, more than one pathway is
313 involved in the effect of DMSO on T3SS.

314 **Treatment of *P. aeruginosa* infection by DMSO in a burned mouse model**

315 Based on the result that DMSO affected the production of a plethora of QS related virulence
316 factors at a non-inhibitory concentration (2% v/v), we employed a burned-mouse model to test
317 DMSO's effect on *P. aeruginosa* pathogenicity *in vivo*. Two different animal studies were carried
318 out, one testing bacterial dissemination and the other, lethality.

319 For the dissemination tests, the mice were divided randomly to DMSO treatment group,
320 antibiotic treatment group (silver sulfadiazine) and negative control group (saline). 200 μL of 10%
321 DMSO solution in 0.9% saline was applied to the burned skin of animals in the DMSO treatment
322 group 24 h post infection. Animals in the antibiotic group were given 2% silver sulfadiazine
323 cream and animals in the negative control group were given 200 μL of saline. At 3 days post burn

infection, the number of bacteria from each liver was determined and calculated as CFU per gram of tissue. As shown in Fig. 6, the bacterial loads in the DMSO and silver sulfadiazine treated groups were significantly lower than the untreated group. No significant difference was observed between the DMSO treated group and the silver sulfadiazine treated group. It is to be noted that high concentration of DMSO (e.g. 10%) could inhibit *P. aeruginosa* growth (Fig S2). The application of DMSO at the burn infection site might affect the bacterial growth. However, since DMSO was administrated 24 h post-infection and a relatively large number of *P. aeruginosa* cells were inoculated, such an effect probably represents a minor contributing factor.

To test the effect of DMSO on mouse survival after burn infection, four groups of mice (n =11 per group) were burned and inoculated with approximately 5×10^6 CFU (50 μL of *P. aeruginosa* suspension at 1 x 10^8 CFU/mL) of *P. aeruginosa*. The treatments started 2h post infection and the survival of the mice was followed for a total of 5 days. As shown in Fig. 7, the survival rate of the DMSO-treated (low-dose) mice was significant higher than the saline-treated group (P=0.021). Similarly, antibiotic treatment group had significantly higher survival rate than the saline-treated group (P=0.002). Enhanced survival was observed in the high-dose DMSO-treated mice compared with the saline-treated group. However, there was no statistic difference between the two DMSO treated groups (P>0.05). Taken together, the results suggest that DMSO could attenuate *P. aeruginosa* pathogenicity in burned mouse model, and hence can potentially be used for treatment of infections caused by *P. aeruginosa*.

Interaction of DMSO with antibiotics

It has been recently reported that DMSO affects antibiotic susceptibilities in *E. coli* (56). To test whether DMSO interferes with the effect of conventional antibiotics, we tested the MICs of six antibiotics from different classes against *P. aeruginosa* in the presence of different concentrations of DMSO. The result is shown in Table 3. DMSO at 1% or 2% made *P. aeruginosa* slightly more sensitive to the killing for CIP whose MIC decreased from 0.4 μg/mL to 0.2 μg/mL. However, the MICs of both CHL and CEF increased two-fold in the presence of DMSO. No changes were observed with the MICs of MEM, CAR or TET. It seems that the interaction of DMSO with antibiotics was antibiotic specific.

DISCUSSION

The success of *P. aeruginosa* as an opportunistic pathogen depends largely on its battery of virulence arsenals. In this study, we have shown that DMSO, an important polar aprotic solvent, was capable of affecting the expression of a plethora of QS-related genes at a non-inhibitory concentration (2% v/v). DMSO could significantly attenuate QS-controlled virulence factor production in *P. aeruginosa*, including biofilm formation, pyocyanin, rhamnolipids, LasA protease and elastase, all of which are known to be positively controlled by *rhl* QS system (57). DMSO inhibited the transcription of the *rhlI* gene and reduced the level of C4-HSL, and the effect of DMSO on QS controlled virulence factors was diminished in the *rhlI* mutant background (PDO100). The addition of C4-HSL to the culture of PDO100 could reverse the impact of DMSO on the expression of these virulence factors. These results indicate that the inhibitory effect of DMSO on *P. aeruginosa* QS controlled virulence factors was mediated

364 through the *rhl* system and the reduction of C4-HSL by DMSO was the main cause of its impact
365 on these virulence factors.

366 DMSO treatment activated the expression of *pqsA* involved in the synthesis of PQS. A
367 previous study has reported that the transcription of *pqsA* is repressed by the *rhl* QS system, and
368 *pqsABCDE* transcription increases accordingly in *rhlI* mutant strain PDO100 (58). It is possible
369 that the activation of *pqsA* expression by DMSO was due to the inhibition of C4-HSL by DMSO.
370 The activation of T3SS effectors by DMSO can also be partially explained by the connection
371 between *rhl* system and T3SS. However, the activation of *gacA*, *rpoS* and *pqsR* by DMSO is
372 probably also accounts for the effect on T3SS since T3SS is negatively regulated by GacA and
373 RpoS respectively (59, 60). Further investigation is required to understand the complete
374 regulatory mechanisms involved in *P. aeruginosa* in relation to the effect of DMSO.

375 The finding in this study that DMSO (2% v/v) exhibited significant QS antagonistic activities
376 is somewhat unexpected, considering DMSO is frequently used as a solvent in screening for QS
377 inhibitors. The QS system is an intriguing target for antimicrobial therapy, since it plays an
378 essential role in regulating the expression of genes involved in pathogenicity and the formation of
379 biofilms. Disrupting the QS signaling pathway is one promising way for controlling infections;
380 the production and secretion of important virulence factors in pathogens, including *P. aeruginosa*,
381 would be prevented by blocking QS. One of the best-studied examples of QS inhibitors is
382 halogenated furanone compounds from a marine alga *Delisea pulchra*. Several other compounds
383 such as chemically modified QS inhibitor (QSI) furanone C-30 and ajoene from garlic have been
384 also identified and they can attenuate infection by *P. aeruginosa in vivo* (61). The macrolide

antibiotic azithromycin has also been revealed to interfere with *P. aeruginosa* QS system to inhibit virulence factor production and biofilm formation (62, 63), which holds great promises for macrolide therapy for chronic pulmonary infection in cystic fibrosis patients. The repression of the *rhl* system and its related virulence factors makes DMSO an ideal QSI candidate for treatment of infections caused by *P. aeruginosa*.

Accordingly, we used burned/*P. aeruginosa* infection mouse model to test whether DMSO may also have practical use of significance against *P. aeruginosa* infections owing to its QS antagonistic activities. The fact that DMSO significantly reduced the bacterial load and enhanced the survival rate of the burned mice infected with *P. aeruginosa* is remarkable. DMSO has been used for many illnesses in humans, including dermatologic diseases (22), gastrointestinal disorders (25-28), wound healing (29), pulmonary (30, 31), and interstitial cystitis (IC) (64). However, its usefulness has, in most cases, been controversial and the only FDA-approved use is the treatment of interstitial cystitis. In addition, the pharmacological mechanisms of DMSO in almost all the cases are unknown. It is not known whether the usefulness, if any, of the DMSO treatment in above mentioned conditions is related to its effect on bacteria potentially involved. Interestingly, it has recently been reported that DMSO is effective for preventing *Acanthamoeba* keratitis if included in contact lens disinfectants (65). It has also been shown that DMSO reduced product dimethyl sulfide is able to inhibit *Salmonella* host cell invasion and the expression of *hilA* and pathogenicity island-associated genes (66). While the increased survival rate of the animals may be attributable to the decreased virulence factor production in the DMSO treated animals, the reason for the decreased bacterial load in the liver of the DMSO treated animals is

not straightforward, considering that DMSO has no bactericidal or growth inhibiting effect on *P. aeruginosa* at the concentrations used in this study. We speculate the effect of DMSO as QS inhibitor on *P. aeruginosa* virulence factors probably made the pathogen less pathogenic or even non-pathogenic, and hence allowed the host immune system to rid it or shifted the balance between the pathogen and the host defense mechanisms.

Compared with imipenem treatment group, the DMSO treatment was not as effective as the antibiotic treatment. Also, there is no significant increase in survival rate with the higher dose DMSO treatment group compared with the lower dose group. Considering the effect of DMSO on T3SS effectors, maximal efficacy of DMSO may require an optimal concentration of DMSO to be administrated. Besides the inhibitory effect on virulence factors of *P. aeruginosa in vivo,* adverse effect on other virulence factors may be present for DMSO treatment. However, comparing to the laboratory condition, the effect of DMSO on T3SS was minimal under T3SS inducing condition which is more similar to the host condition. The bigger effect on T3SS under laboratory condition might be due to other chemical properties of DMSO such as its nucleophilic characteristic that alter the culture conditions.

It is conceivable that DMSO could be best used in combination with antibiotics. However, possible interactions between DMSO and antibiotics also need to be addressed. It has recently reported that DMSO inhibited the action of some ROS-dependent antibiotics against *Escherichia coli* (67). In light of this report, we have tested the effect of DMSO on the efficacy of six different antibiotics against *P. aeruginosa*. Different from what reported for *E. coli*, DMSO at 1% or 2% made *P. aeruginosa* slightly more sensitive to the killing for CIP (MIC decreased from 0.4

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µg/mL to 0.2 µg/mL), but slightly less sensitive for CHL and CEF (two-fold increase). No changes were observed with the MICs of MEM, CAR or TET. It seems that the interaction of DMSO with antibiotics could be both antibiotic and bacterium species specific. Such an effect should be considered if both DMSO and antibiotics are used at the same time.

The mouse burned/infection model used in this study is in fact a sepsis model. With a high dose of *P. aeruginosa*, the burn and *P. aeruginosa* infection induced severe sepsis which progressed rapidly into systemic inflammatory response syndrome and multiple organ dysfunction syndrome (MODS) in mice (45). The ability of DMSO to lower bacterial loads when applied topically and enhance survival rate when injected intraperitoneally indicate that DMSO could potentially be used both as a systemic drug and as topically administrated agent for *P. aeruginosa* skin infections caused by burn.

DMSO is frequently used as a solvent in drug discovery research. DMSO's impact on virulence factors of bacterial pathogen complicates its usage as solvent in biological and medicinal studies. At the least, caution to such an effect is warranted when DMSO is used as a vehicle in antibiotic research.

CONFLICT OF INTEREST STATEMENT

The authors have no financial or commercial conflicts of interest with the current work or its publication.

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637 **Table 1. Bacterial strains and plasmids used in this study**

Strains or plasmids	Relevant characteristics	Source
Strains		
E. coli		
SM10-λpir	Mobilizing strain, RP4 integrated in the chromosome, Kn^r	Invitrogen
DH10B	F- mcrA Δ(mrr-hsdRMS-mcrBC)80dlacZ ΔM15 Δ lacX74 deoR recA1 endA1 araD139 Δ(araleu)7697 galUgalKλ-rpsLnupG	Invitrogen
P. aeruginosa		
PAO1	Wild type	
PDO100	*rhlI*mutant of PAO1	(57)
Plasmids		
pMS402	Expression reporter plasmid carrying the promoterless*luxCDABE* gene; Kn^r, Tmp^r	(33)
CTX6.1	Integration plasmid origins of plasmid mini-CTX-*lux*; Tc^r	This lab
pKD-*phzA1*	pMS402 containing *phzA1*promoter region; Kn^r, Tmp^r	(33)
pKD-*phzA2*	pMS402 containing *phzA2* promoter region; Kn^r, Tmp^r	(33)
pKD-*lasI*	pMS402 containing *lasI* promoter region; Kn^r, Tmp^r	(43)
pKD-*rhlI*	pMS402 containing *rhlI* promoter region; Kn^r, Tmp^r	(43)

pKD-*rhlA*	pMS402 containing*rhlA* promoter region; Kn^r, Tmp^r	(33)
pKD-*gacA*	pMS402 containing*gacA* promoter region; Kn^r, Tmp^r	(33)
pKD-*rpoS*	pMS402 containing *rpoS*promoter region; Kn^r, Tmp^r	(33)
pKD-*pilG*	pMS402 containing *pilG* promoter region; Kn^r, Tmp^r	(33)
pKD-*fliC*	pMS402 containing *fliC*promoter region; Kn^r, Tmp^r	(33)
pKD-*pqsA*	pMS402 containing *pqsA* promoter region; Kn^r, Tmp^r	(68)
pKD-*pqsR*	pMS402 containing *pqsR* promoter region; Kn^r, Tmp^r	(68)
pKD-*pqsH*	pMS402 containing*pqsH* promoter region; Kn^r, Tmp^r	(34)
pKD-*exo*S	pMS402 containing *exoS* promoter region; Kn^r, Tmp^r	(33)
pKD-e*xoY*	pMS402 containing *exoY* promoter region; Kn^r, Tmp^r	(33)
pKD-*exoT*	pMS402 containing*exoT* promoter region; Kn^r, Tmp^r	(33)
pKD-*exsD*	pMS402 containing *exsD* promoter region; Kn^r, Tmp^r	(33)
pKD-*exsC*	pMS402 containing *exsC* promoter region; Kn^r, Tmp^r	(33)
CTX-*exoS*	Integration plasmid, CTX6.1 with a fragment of pKD-*exoS*containing*exoS* promoter region and *luxCDABE* gene; Knr, Tmp^r, Tc^r	This lab
CTX-*exoY*	Integration plasmid, CTX6.1 with a fragment of pKD-*exoY* containing *exoY* promoter region and *luxCDABE*gene; Kn^r, Tmp^r, Tc^r	This lab
CTX-*exoT*	Integration plasmid, CTX6.1 with a fragment of	This lab

	pKD-*exoT*containing *exoT* promoter region and *luxCDABE*gene; Kn^r, Tmp^r, Tc^r	
CTX -*phzA1*	Integration plasmid, CTX6.1 with a fragment of pKD-*phzA1* containing *phzA1* promoter region and *luxCDABE* gene; Kn^r, Tmp^r, Tc^r	This lab
CTX -*phzA2*	Integration plasmid, CTX6.1 with a fragment of pKD-*phzA2* containing *phzA2* promoter region and *luxCDABE* gene; Kn^r, Tmp^r, Tc^r	This lab

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644 **Table 2. Effect of DMSO on the expression of virulence related genes in *P. aeruginosa***

Virulence factors	Function	Fold-change of gene expression levels
lasI	autoinducer synthesis protein LasI	-1.5*
lasB	elastase LasB synthesis	-5.3**
lasA	LasA protease synthesis	-2.9*
rhlA	rhamnolipid synthesis	-20.1**
rhlI	autoinducer synthesis protein RhlI	-1.4*
phzA1	phenazine biosynthesis protein	-2.6*
phzA2	phenazine biosynthesis protein	-4.7**
pqsA	anthranilate-coenzyme A ligase	+2.1*
pqsR	transcriptional regulator PqsR	-1.8*
pilG	twitching motility protein PilG	-1.5*
fliC	flagellin type B	-1.8*
flhA	flagellar biosynthesis protein FlhA	-3.1*
exoS	exoenzyme S (ADP-ribosyltransferase)	+, 1.7*$^{\triangle}$; +7.5**
exoY	adenylate cyclase ExoY	+, 1.8*$^{\triangle}$; +3.2*
exoT	exoenzyme T	+, 1.5$^{\triangle}$; +8.1**
gacA	response regulator GacA	-4.4**
rpoS	sigma factor RpoS	-3.0*

—: repression of gene expression by DMSO; +: activation of gene expression by DMSO;

$^{\triangle}$ indicates changes under calcium-depleted, T3SS-inducing condition.

The gene expression data was normalized by bacterial growth (OD_{600}). Student's T-test was used to analyze the data. * and ** indicate statistically very significant difference ($P<0.01$) and statistically highly significant difference ($P<0.001$) respectively.

650 **Table 3. Effect of DMSO on antibiotic MICs against *P. aeruginosa* PAO1.**

DMSO	MIC (µg/mL)					
	CIP	**CEF**	**MEM**	**CAR**	**CHL**	**TET**
0	0.4	1.5	4	150	15	10
1%	0.2	3	4	150	30	10
2%	0.2	3	4	150	30	10

651 CIP, Ciprofloxacin; CEF, Ceftazidime; MEM, Meropenem; CAR, Carbenicillin; CHL,

652 Chloramphenicol; TET, Tetracycline.

Figure legends:

Fig. 1. Inhibition of pyocyanin production by DMSO in *P. aeruginosa*. (a) Color changes of *P. aeruginosa* cultures in the presence of DMSO, a reflection of the effect of DMSO on phenazine production. (b) The changed expression of *phzA1* and *phzA2* on solid medium. Reporter strains was plated on soft-top LB agar (0.7% w/v) supplemented with or without DMSO, and imaging was done using a LAS3000 imaging system. The expression of *phzA1* (c) and *phzA2* (d) in LB broth supplemented with or without 2% (v/v) DMSO. These experiments were repeated three times and data shown are representatives of similar results. Relative luminescence unit (RLU): cps(counts per second)/OD_{600}.

Fig. 2. Repression of *rhlA* (a), *lasB* (b) and *lasA* (c) expression by DMSO. The experiments were repeated at least three times. The results represent the means of triplicate experiments and the error bars indicate standard deviations. Relative luminescence unit (RLU): cps(counts per second)/OD_{600}.

Fig. 3. Decreased production of virulence factors in the presence of DMSO. The production of rhamnolipids (a) LasB protease (b) and LasA protease activity (c) and biofilm formation (d). LasA protease activity was determined by measuring the ability of culture supernatants to lyse boiled *S. aureus* cells. In this assay, labels LB, LB+DMSO, PAO1 and PAO1+DMSO respectively represent LasA protease activity in LB medium (negative control 1), LB with DMSO (negative control 2), PAO1 LB culture supernatant, and PAO1 LB+DMSO culture supernatant. Results represent the means ± SD values of three independent experiments performed in triplicate.

673 Student's T-test was used to analyze the data. ** indicates statistically very significant differences
674 (P<0.01).

675 **Fig. 4. Inhibition of C4-HSL production by DMSO in PAO1**. (a) The relative amount of
676 C4-HSL production by PAO1 in LB broth with and without the presence of DMSO. C4-HSL in
677 the culture supernatants was measured by *rhlA* expression levels using the reporter strain
678 PDO100-*rhlA* (41). LB medium and culture supernatant of the C4-HSL non-producing strain
679 (*rhlI* deletion strain PDO100) were used as negative controls. Student's T-test was used to
680 analyze the data, and the means and standard deviations are shown. ** indicates very significant
681 differences (P<0.01) and *** indicates highly significant differences (P<0.001). The experiments
682 were repeated three times. (b) The AHL plate bioassay was performed using the reporter *P.*
683 *aeruginosa* (PDO100-*rhlA*). 2 μL of filter-sterilized culture supernatants were spotted on the
684 plate seeded with the reporter. Fresh LB and culture supernatant of the C4-HSL non-producing
685 strain PDO100 were included as controls. A halo zone around sample spot indicates the presence
686 of C4-HSL in the sample, and the size and intensity of the halo are proportional to the amount of
687 C4-HSL in the samples.

688 **Fig. 5. The *rhl* system and the effect of DMSO on pyocyanin production and biofilm**
689 **formation.** (a) Effect of DMSO on pyocyanin production with and without C4-HSL. (b) Biofilm
690 formation of PAO1, PDO100 and PDO100 with C4-HSL. Student's T-test was used to analyze
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692 differences (P<0.05); ** very significant differences (p<0.01); and *** highly significant
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694 **Fig. 6. Effect of DMSO on bacterial load in burned mouse model of the dissemination test**.
695 Samples with a log(cfu) value of zero indicate no CFU detected. Mann-Whitney U test was used
696 for analysis of nonparametric data to determine the significance of the CFU differences between
697 two groups. * indicates significant difference from the saline group (p<0.05).

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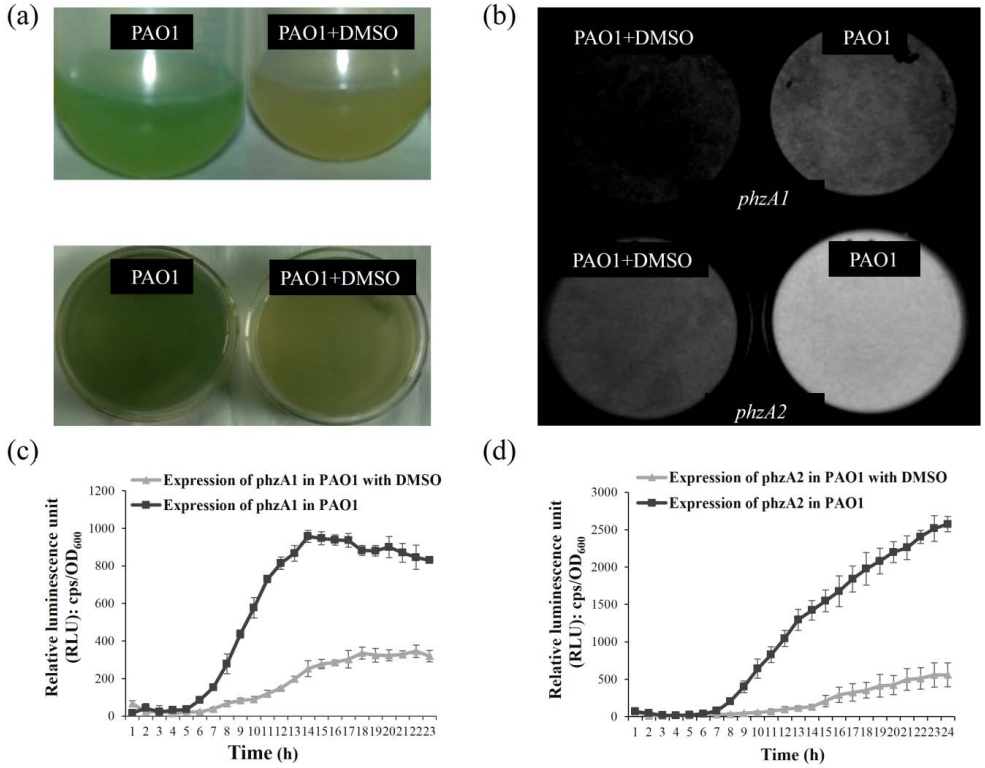

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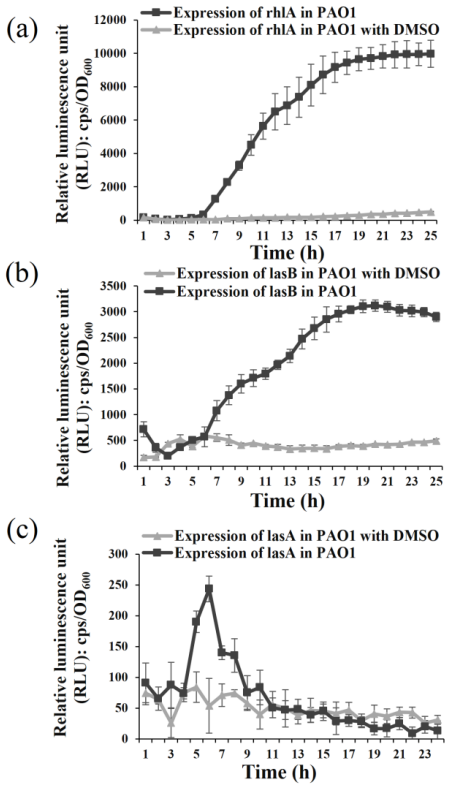

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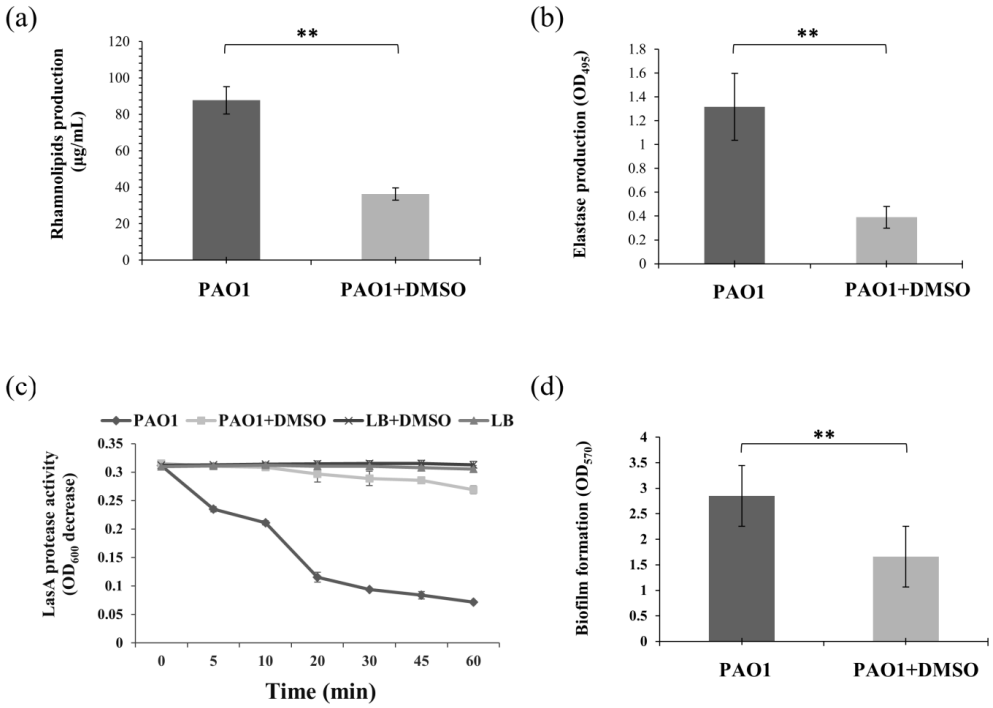

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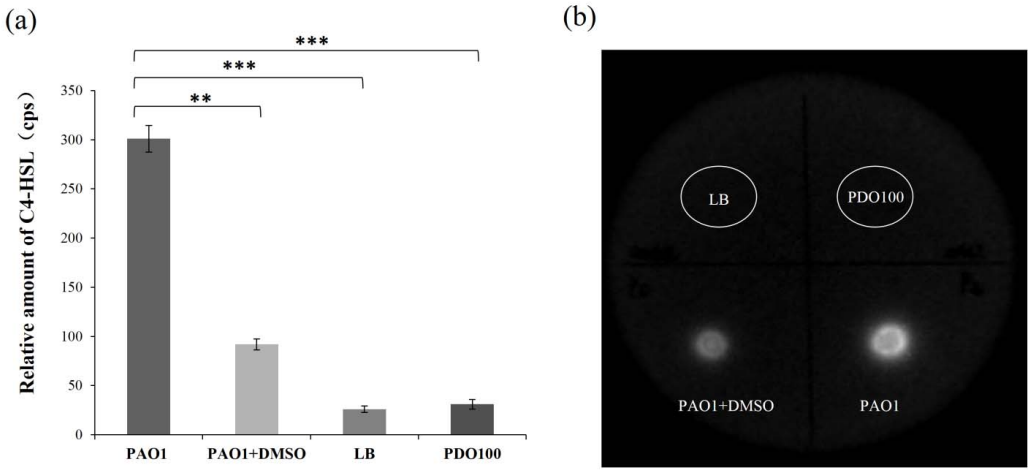

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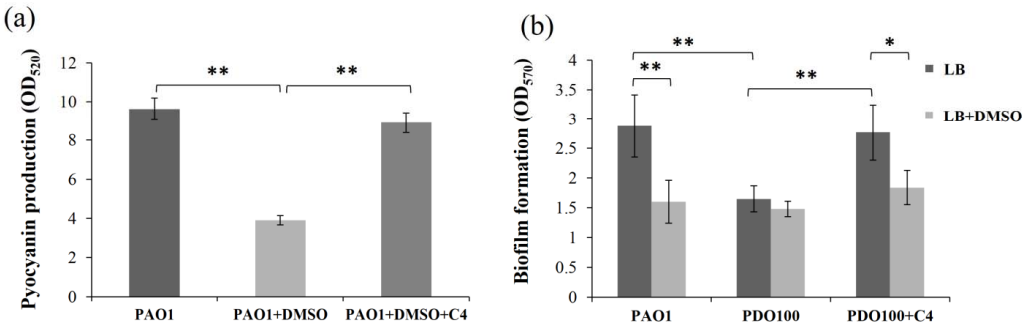

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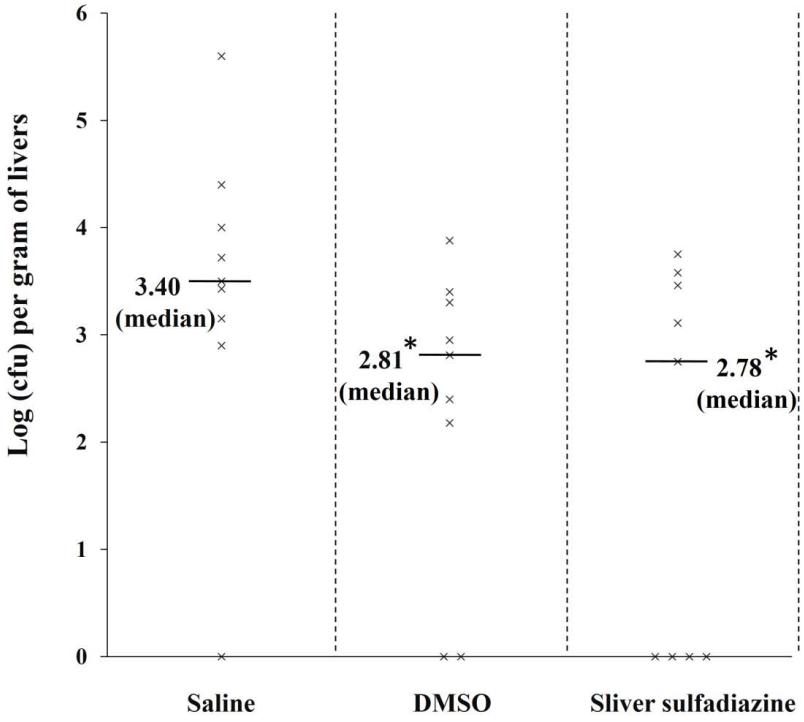

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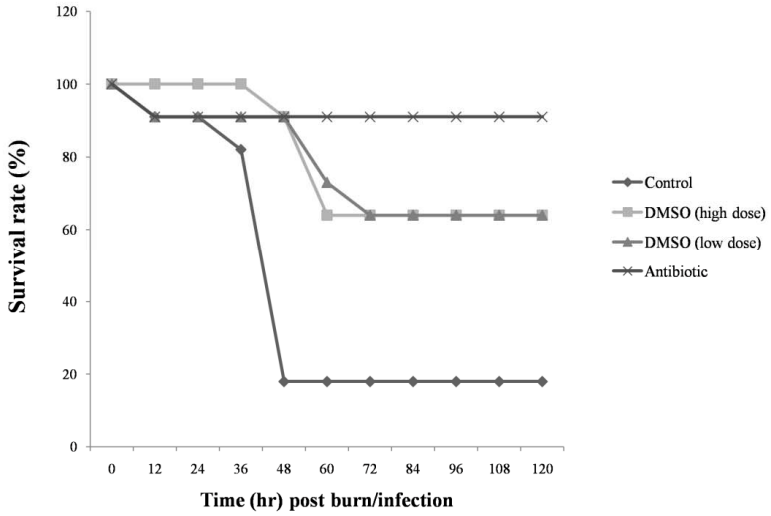

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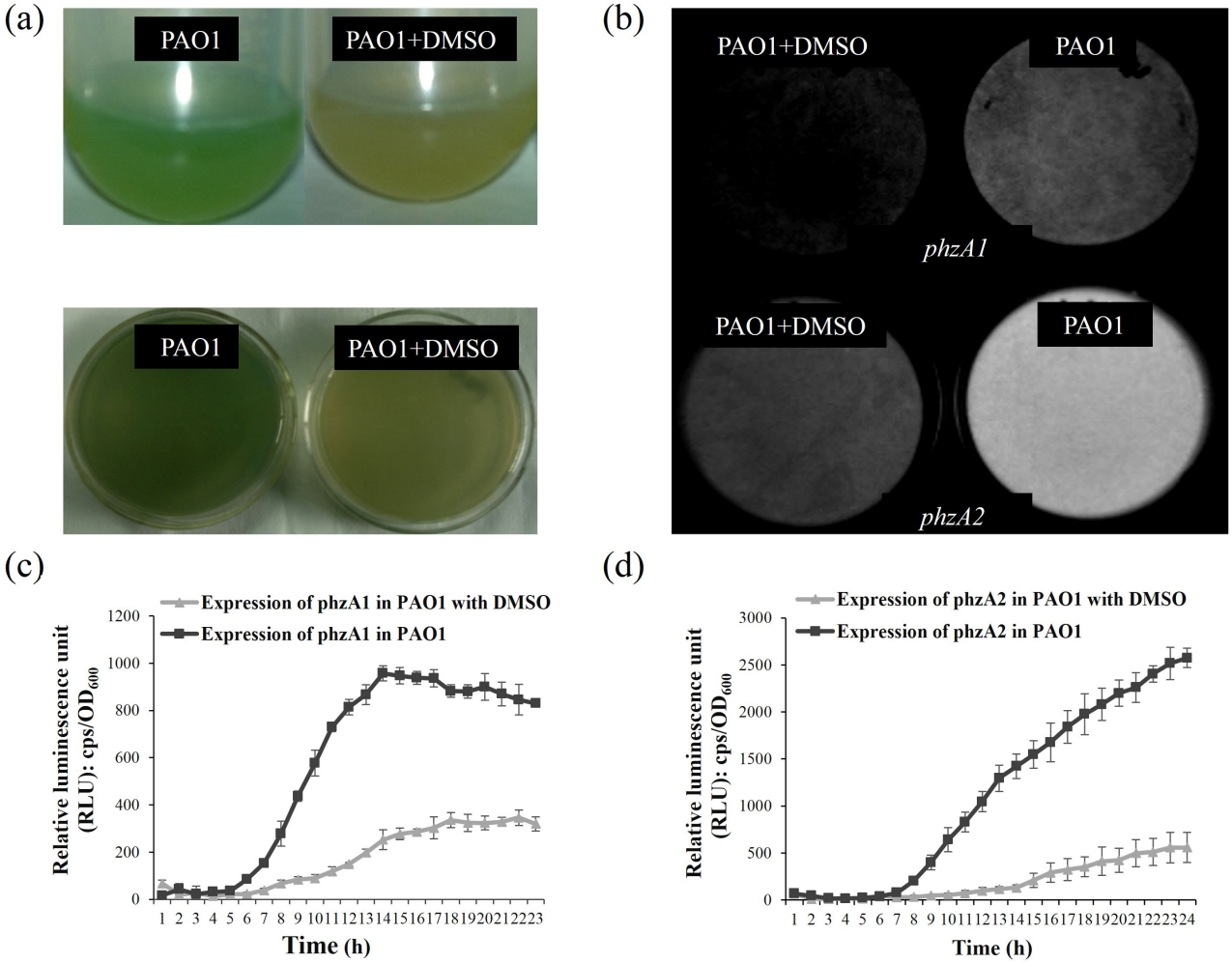

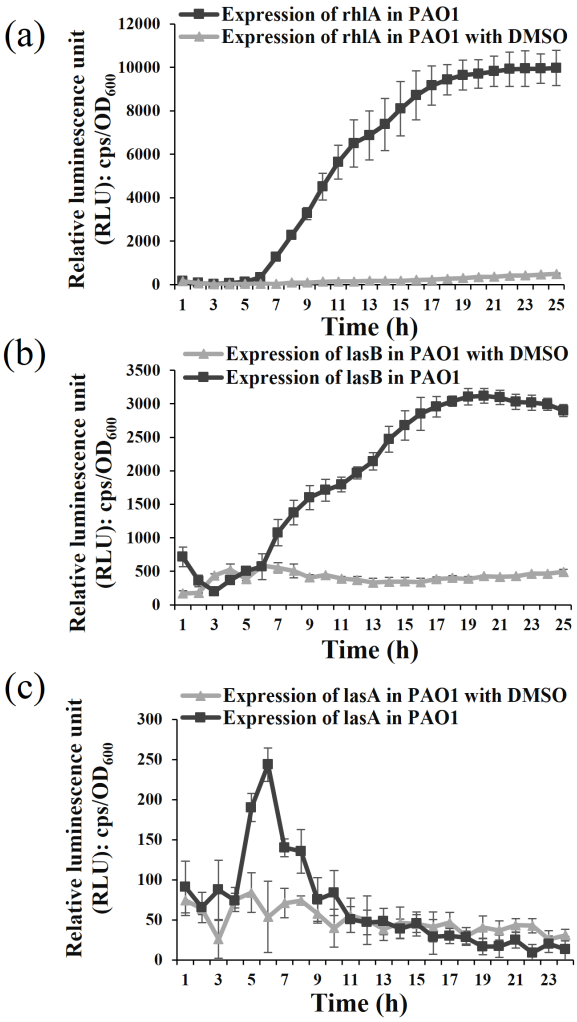

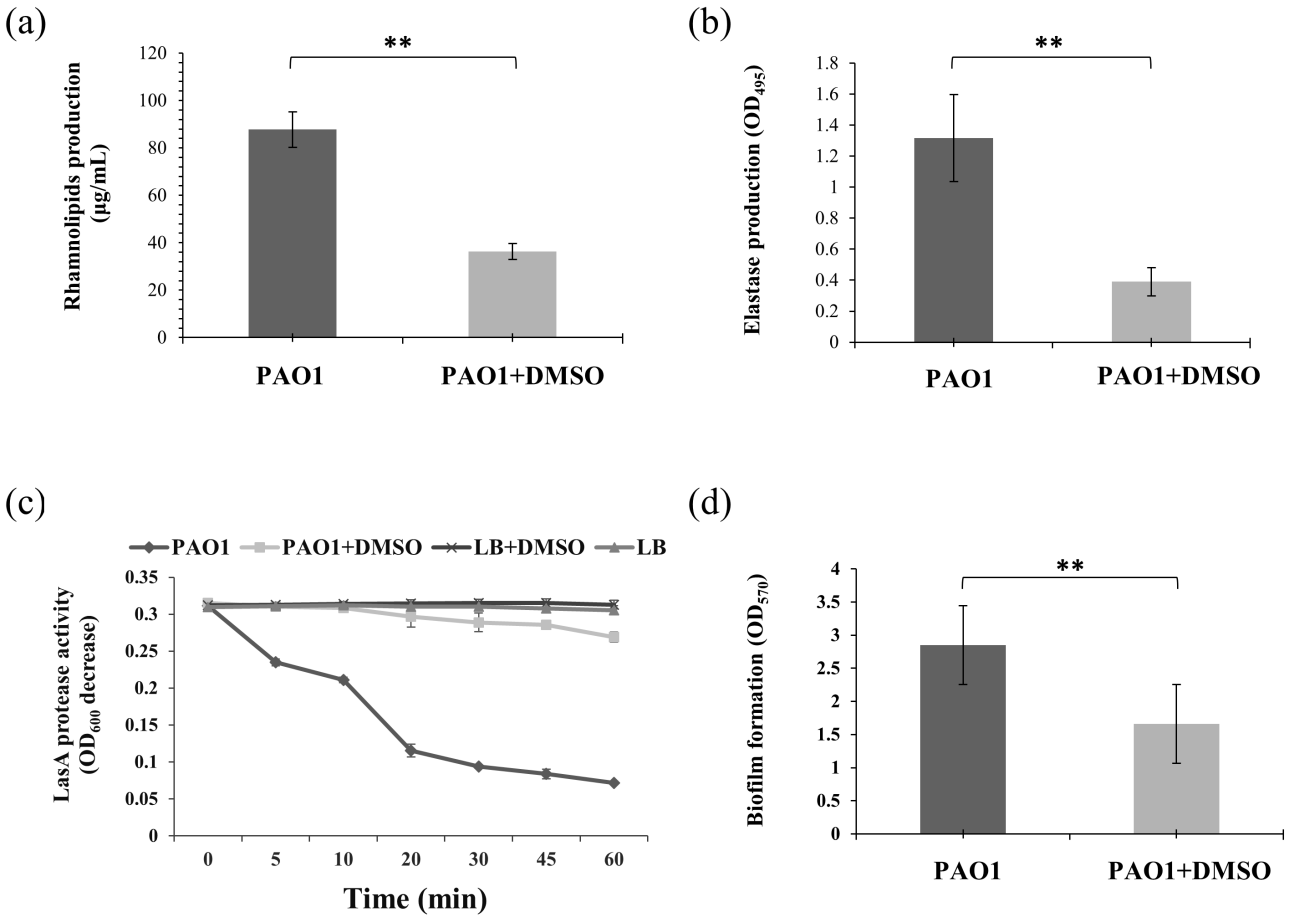

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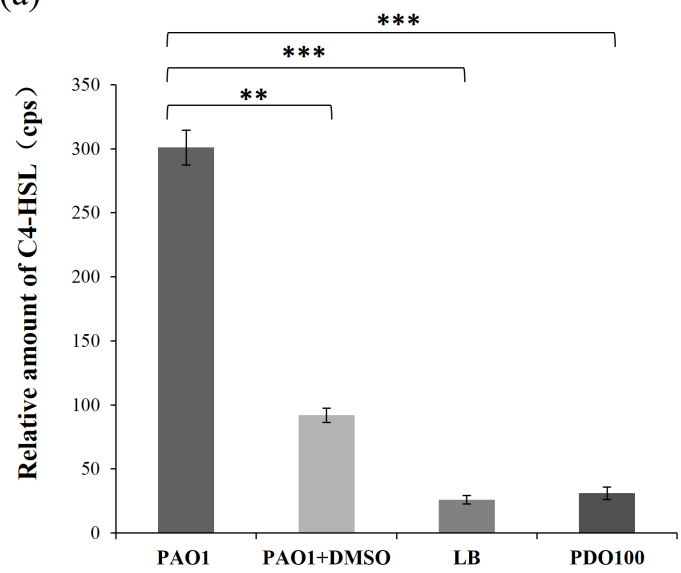

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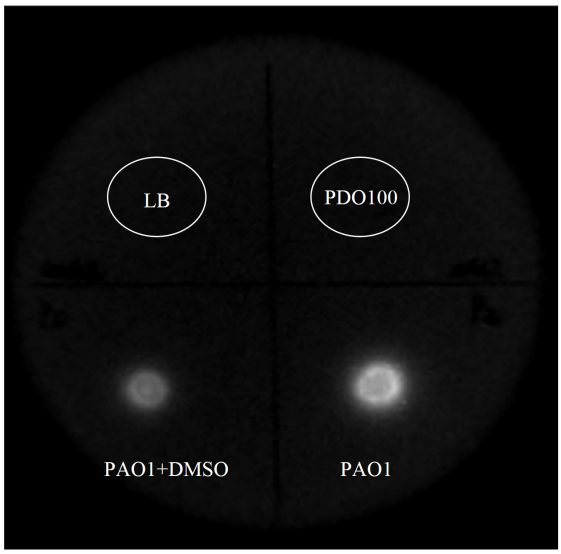

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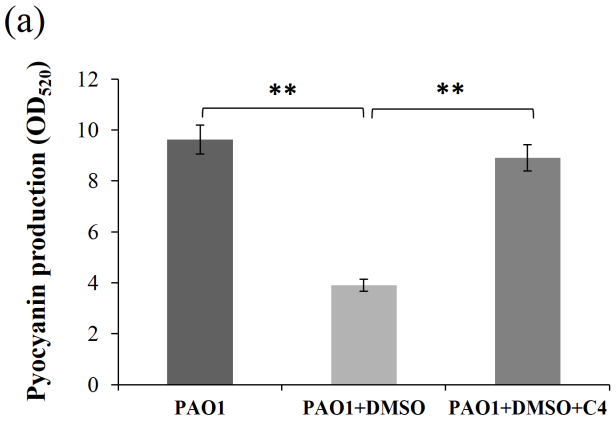

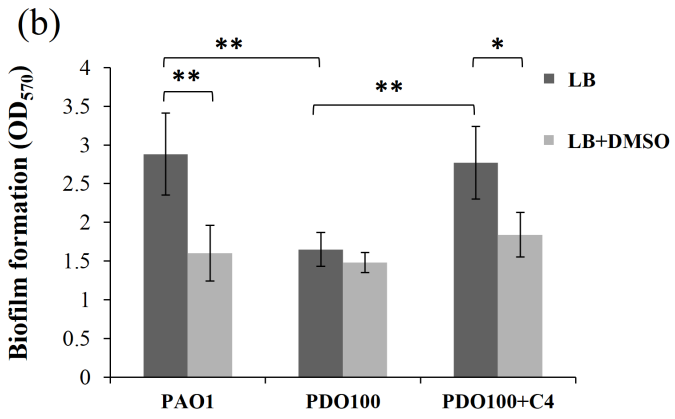

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