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D-Methionine-induced DNases disperse established *Burkholderia pseudomallei* biofilms and promotes ceftazidime susceptibility

Rattiyaphorn Pakkulnan [a], Auttawit Sirichoat [a,b], Sorujsiri Chareonsudjai [a,b,*]

[a] Department of Microbiology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand
[b] Research and Diagnostic Center for Emerging Infectious Diseases (RCEID), Khon Kaen University, Khon Kaen, Thailand

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ABSTRACT

Burkholderia pseudomallei biofilm is correlated with pathogenesis, antibiotic resistance, and relapsing cases of melioidosis, leading to challenges in clinical management. There is increasing interest in employing biofilm dispersal agents as adjunctive treatments for biofilm-associated infections. Methionine (Met) has shown promise as an anti-biofilm agent by inducing bacterial DNase production, resulting in the degradation of extracellular DNA (eDNA) and dispersion of bacterial biofilm. In this study, we investigated the impact of 0.05–50 μM D-Met and L-Met on the 24-h established biofilm of a clinical isolate, *B. pseudomallei* H777. Our findings revealed the ability of D-Met and L-Met to disperse the established biofilm in a non-dose-dependent manner accompanied by eDNA depletion. Real-time PCR analysis further identified an up-regulation of bacterial nuclease genes, including *recJ*, *eddB*, *nth*, *xth*, and *recD,* in the presence of 0.05 μM D-Met. Similarly, *recJ* and *eddB* in *B. pseudomallei* were up-regulated in response to the presence of 0.05 μM L-Met. Notably, D-Met enhanced the susceptibility of *B. pseudomallei* H777 biofilm cells to ceftazidime. Our findings indicate a correlation between methionine supplementation and the up-regulation of nuclease genes, leading to eDNA depletion and the dispersal of preformed *B. pseudomallei* H777 biofilm. This enhances the susceptibility of biofilm cells to ceftazidime, showing promise in combating biofilm-associated *B. pseudomallei* infections.

1. Introduction

Melioidosis, caused by the Gram-negative bacterium *Burkholderia pseudomallei*, is endemic in Southeast Asia, Northern Australia, and occurs worldwide [1,2]. The disease has a high mortality rate, reaching 40 % in Thailand [3] and 61.5 % in Cambodia [4]. Globally, an estimated 165,000 human melioidosis cases occur annually, leading to 89,000 deaths [5]. The prevalence of diabetes mellitus significantly heightens the risk of melioidosis, potentially contributing to a rise in melioidosis-related fatalities due to the ever-increasing numbers of diabetes cases globally [6].

Biofilm formation by *B. pseudomallei* is associated with cellular pathogenesis by promoting cellular adhesion and internalization and inducing pro-inflammatory cytokine production that may exacerbate disease [7]. Furthermore, *B. pseudomallei* biofilm is correlated with antibiotic resistance because of the relative inability of antibiotics to penetrate biofilm [8], the exopolysaccharide production [9] and the up-regulation of efflux-pump genes [10]. The biofilm form of *B. pseudomallei* displays a dramatically higher tolerance than planktonic cells to ceftazidime (CAZ) at the minimum biofilm eradication concentration of 2048 μg/ml or more [11]. Notably, relapsing melioidosis is associated with inadequate treatment and biofilm formation by *B. pseudomallei*, posing a threat to life [12,13]. Considerable research attention has been directed towards discovering agents that can effectively improve antibiotic susceptibility of *B. pseudomallei* biofilm-associated infections.

Extracellular DNA (eDNA) serves as a key component in most bacterial biofilms and is a potential target for biofilm dispersal to enhance access by antimicrobial agents [14]. DNase I administration efficiently interrupts *B. pseudomallei* biofilm formation and enhances efficacy of CAZ to eradicate the biofilm cells [15]. This approach also increases the susceptibility of nontypeable *Haemophilus influenzae* biofilms to ampicillin and ciprofloxacin [16]. The combination of dextranase and DNase I increased susceptibility of *Enterococcus faecalis* biofilms to sodium hypochlorite [17]. Furthermore, DNase I exhibited potent anti-biofilm activity and improved antimicrobial susceptibility in *Staphylococcus aureus* and *S. epidermidis* [17]. Therefore, enzymatic breakdown of eDNA has the potential to prevent or disperse biofilm, or to enhance biofilm

* Corresponding author.
E-mail address: sorujsr@kku.ac.th (S. Chareonsudjai).

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susceptibility to antimicrobial agents.

Rather than relying on the requirement for exogenous DNase addition, the supplementation of D and L enantiomers of amino acids offers an effective strategy for eDNA disruption and an anti-bacterial biofilm effect. The studies of D-amino acids on anti-biofilm actions revealed possible mechanisms including biofilm formation interference through metabolic and transport processes, peptidoglycan synthesis and cell division [18,19]. Furthermore, D-amino acids act analogously to prevent biofilm development in *Bacillus subtilis* and *S. aureus* by blocking the subsequent growth of the foci into larger assemblies of cells [20]. Previous studies have documented the ability of D-Leu, D-Met, D-Tyr, and D-Trp to both inhibit the formation of biofilms and disrupt pre-existing biofilms in *B. subtilis* by interfering with protein synthesis [21,22].

Yan and colleagues demonstrated the effectiveness of D-Leu in enhancing the anti-biofilm effect of chlorhexidine in disassembling *Streptococcus mutans* biofilms to release antibiotic-susceptible planktonic cells, offering a potential strategy to minimize dosage required for efficient caries prevention [23]. Furthermore, the combination of D-aspartic acid and D-glutamic acid with ciprofloxacin effectively disrupted the honeycomb-like eDNA meshwork and biofilm matrix leading to the improvement of drug effectiveness to eradicate *S. aureus* biofilm cells [24]. The dispersive activity of D-methionine (D-Met), D-phenylalanine, and D-tryptophan (D-Trp) was effective against preformed biofilms of *S. aureus* and *Pseudomonas aeruginosa* and enhanced activity of antimicrobial agents [25]. The ability of D-alanine, D-serine, D-Met, and D-Trp to promote the dispersal of preformed *Campylobacter jejuni* biofilm and to enhance antimicrobial efficacy of D-cycloserine was established [26]. Furthermore, a mixture of D-Leu, D-Met, D-tyrosine (D-Tyr), and D-Trp not only inhibited biofilm formation but also disrupted established biofilms of *E. faecalis* better than a mixture of the L-forms, and improved the activity of antimicrobial agents [27]. However, L-Met disrupts *P. aeruginosa* biofilm through up-regulation of bacterial DNase genes (*sbcB*, *endA*, *eddB* and *recJ*) leading to increased DNase activity, eDNA eradication and enhancing ciprofloxacin susceptibility. The combining of L-Met with antibiotics was suggested as an effective therapeutic approach against chronic *P. aeruginosa* biofilm infection [28].

The role of D-form and L-form amino acids in the context of anti-biofilm activity is rather controversial. Remarkably, the impact of amino acids on *B. pseudomallei* biofilm has received insufficient attention to date. Therefore, we aimed to test the hypothesis that D-Met and L-Met have potential to disperse *B. pseudomallei* biofilm, and that this was the mechanism through the up-regulation of nuclease genes. Furthermore, the combination of D-Met and L-Met with CAZ was explored to demonstrate their potential as a dual strategy to enhance CAZ susceptibility of *B. pseudomallei* biofilms through amino acid-drug synergy.

2. Methods

2.1. Ethics statement

The clinical isolates *B. pseudomallei* H777, L1, and P1 were not specifically isolated for this study. They were previously collected as part of an epidemiology of *B. pseudomallei* in Khon Kaen Province, conducted by the Melioidosis Research Center, Khon Kaen University. Patient information associated with the isolate was anonymized and de-identified upon receipt of the sample. Both the previous and the current studies received approval from the Khon Kaen University Ethics Committee for Human Research (HE490324) and (HE651568), respectively.

2.2. Bacterial strains and growth condition

Burkholderia pseudomallei H777, L1 and P1, isolated from blood, lung and pus of melioidosis patients, have been previously demonstrated as having the ability to form biofilms [29,30]. These strains, stored in glycerol stock at −80 °C, were grown on Ashdown's agar and incubated at 37 °C for 48 h. The inoculum culture were prepared from a single colony of *B. pseudomallei* in 3 μL of Luria-Bertani (LB) broth and incubated at 37 °C with shaking (200 rpm) for 18–20 h, thereafter, 2 % inoculum was grown in fresh LB. The bacterial starter culture was adjusted to 10^7 or 10^8 CFU/mL before use [29].

2.3. Ceftazidime (CAZ) preparation

Ceftazidime hydrate (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in sterile injected water before being filter-sterilized. The working solution was added into Mueller Hinton broth (MHB) at 512 μg/mL (sub-minimum biofilm elimination concentration (Sub-MBEC)) [11] final concentration.

2.4. Effect of methionine on B. pseudomallei biofilm

Burkholderia pseudomallei biofilm was quantified using 2 % crystal violet staining as previously described, with slight modification [29]. Briefly, 200 μL of bacterial suspension (10^8 CFU/mL) in LB medium was added into a 96-well plate (Nunclon™, Roskilde, Denmark) then statically incubated at 37 °C for 24 h to allow development of a biofilm. This 24-h preformed biofilm was treated with LB contained D-Met or L-Met (Sigma Aldrich, Saint Louis, Missouri, USA) at final concentrations of 0.05, 0.5, 5 and 50 μM in parallel with an untreated control. To assess the extent of any biofilm inhibition, the treated samples were incubated at 37 °C for another 24 h, then washed three times with 200 μL of sterile water. Then, these 48 h biofilms were fixed with 99 % methanol for 15 min and air dried at room temperature. Thereafter, the biofilms were stained with 2 % w/v crystal violet for 5 min before being solubilized with 200 μL of 33 % (v/v) glacial acetic acid. The absorbance of the solution was measured at 620 nm (A_{620}) using a microplate reader (Sunrise™, TECAN, Port Melbourne, Australia). This experiment was performed in eight replicates on each of three independent occasions.

2.5. Confocal laser scanning microscope observation

To visualize the *B. pseudomallei* H777 biofilm structure and eDNA after treatment with D-Met and L-Met, or with these combined with CAZ, the biofilms were grown on glass coverslips using an Amsterdam Active Attachment (AAA) model in 24-well plates (Costar® #3524, Corning, NY, USA). The method was slightly modified from previous studies [7,14,31]. Briefly, 1 mL of bacterial culture (10^8 CFU/mL) was added into 24-well plates to allow biofilm development on a sterile 12 mm-diameter round glass coverslip held on the AAA model lid at 37 °C for 24 h. The biofilms on glass coverslip were washed with sterile phosphate-buffered saline (PBS), pH 7.4 before incubated in LB or MHB broth containing D-Met or L-Met at 0.05 μM, or 512 μg/m CAZ, or the combination of D-Met or L-Met with CAZ, then incubated at 37 °C for an additional 24 h. The 2-day biofilms on the glass coverslips were rinsed three times with sterile PBS prior to staining with 50 μg/mL fluorescein isothiocyanate-concanavalin A (FITC-Con A) ($495_{Ex}/519_{Em}$ nm) (Sigma-Aldrich, Saint Louis, Missouri, USA), which binds to extracellular polysaccharide (biofilms), and 2 μM TOTO-3 ($261_{Ex}/661_{Em}$) (Thermo fisher Scientific, Oregon, USA), which binds to extracellular DNA, then stained in the dark for 20 min. For live/dead staining, the viable cells were visualized using 3.34 μM/mL SYTO 9 ($483_{Ex}/500_{Em}$ nm) and the dead cells were examined using 5 μg/mL propidium iodide (PI) ($305_{Ex}/617_{Em}$) (Invitrogen, Thermo Fisher Scientific, Oregon, USA) stained for 15 min. The stained biofilms were consequently fixed with 2.5 % glutaraldehyde in PBS for 3 h. The excess dye was removed by washing with sterile PBS 3 times and the coverslips air-dried for 24 h at room temperature. The biofilms on glass coverslips were observed under a confocal laser scanning microscope (CLSM, LSM 800, Carl Zeiss, Jena, Germany). The staining intensity was analyzed from z-stack processing using Zen blue software [7,32]. The biofilm biomass and eDNA amount were calculated from 6 CLSM images from each of 3 independent

occasions (n = 18) using COMSTAT computer program [33].

2.6. DNase gene expression using real-time PCR

We selected 5 DNase genes as previous reported from *P. aeruginosa* [28] and known nuclease genes of *B. pseudomallei* by comparing the similarity of each gene between *B. pseudomallei* and *P. aeruginosa* using Nucleotide BLAST NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Each gene sequence was accessed from *Burkholderia* Genome database (https://www.burkholderia.com/) [34]. The *bpsl2279 (recJ)* (single--stranded-DNA specific exonuclease), *bpss1845* (*eddB*) (ExeM/NucH family extracellular endonuclease), *bpsl1103* (*nth*) (endonuclease III), *bpsl2315* (*xth*) (exonuclease III) and *bpsl1284* (*recD*) (exonuclease V) were used for further investigation.

The protein sequence of each gene was accessed from NCBI database (FASTA) for protein prediction. The web-based software, SOSUI (version 1.11), SignalP (version 6.0) and PSORTb (version 3.0.3) were used to determine the protein localization [35–37] (Table 1). The gene-specific primers were designed using Primer-Blast web-based tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 2).

Up-regulation of the DNase genes after treatment with D-Met and L-Met was assessed using real-time PCR. Briefly, RNA was isolated from the 2-day *B. pseudomallei* H777 biofilm grown statically at 37 °C, with or without 0.05 μM D-Met or L-Met, for 24 h using TRIzol® reagent (Invitrogen, Massachusetts, USA) as per manufacturer's procedure for first-strand cDNA synthesis. The isolated RNA was cleaned, and reverse transcribed using a FastKing RT kit with genomic DNase as per the manufacturer's protocol (Tiangen, Beijing, China). The concentration and purity of isolated RNA and cDNA were assessed by Nanodrop spectrophotometry (Thermo Fisher scientific, Massachusetts, USA). A ratio of the absorbance at 260 and 280 nm of around 1.8–2.0 was regarded as acceptable for further investigations. The cDNA was diluted and analyzed for the presence of different DNase genes using specific primers and PowerUp™ SYBR® Green Master Mix (Applied Biosystems, Life technologies, Massachusetts, USA) in the QuantStudio™ 6 Flex Real-Time PCR System instrument (Applied Biosystems, Life Technologies, Massachusetts, USA). The amplification and detection were performed with the following protocol: (i) 1 cycle of 95 °C for 10 min, (ii) 40 cycles of 95 °C for 15 s, (iii) 55 °C for 15 s, (iv) 72 °C for 1 min. The relative gene expression ratio of each target gene was calculated using the comparative $\Delta\Delta C_T$ method by QuanStudio™ real-time PCR software version 10.2 (Applied Biosystem). The quantities of target gene were normalized to the housekeeping gene 16s rRNA of *B. pseudomallei* K96243. These experiments were performed in triplicate on three independent occasions.

2.7. Effect of methionine on ceftazidime susceptibility of B. pseudomallei biofilm

Burkholderia pseudomallei H777 biofilm was established using the Calgary biofilm device (CBD) (Nunc, Thermo Fisher Scientific, Denmark). Two hundred microliters of *B. pseudomallei* (10^7 CFU/mL) in LB broth was added into 96-well plates (Nunclon™, Roskilde, Denmark). The peg lid was immersed into the bacterial suspension and the plates incubated at 37 °C for 24 h to allow biofilm development. The 24-h biofilms on pegs were rinsed with sterile PBS (pH 7.4) for 1 min and incubated with Mueller Hilton broth (MHB) for untreated controls. For experimental treatments, MHB containing test reagents was used. These were as follows: 0.05 μM D-Met or L-Met; 512 μg/mL CAZ; 0.01 U/mL DNase I; the combination of DNase with CAZ; either D-Met or L-Met combined with CAZ. The plates were incubated at 37 °C for another 24 h to achieve 2-day biofilms. After incubation, the biofilms on pegs were rinsed with sterile PBS for 1 min before being transferred to new 96-well plates containing 200 μL MHB and sonicated for 5 min to liberate biofilm cells, followed by serial dilution for bacterial enumeration using the drop plate technique on NA agar which was incubated at 37 °C for 24 h. Results are reported as CFU/mL. The experiments were performed in duplicate in each of 3 independent experiments.

Table 2
Nuclease genes specific primers for real-time PCR.

Nuclease genes		Primers (5′→ 3′)
***bpsl2279* (*recJ*)**	Fwd	TTCGGCGCGAAGATCGATTA
Single-stranded-DNA specific exonuclease	Rv	ATTCCGTTGTCGACCGTGAT
***bpss1845* (*eddB*)**	Fwd	TGATCTACGACAGCCGCAAA
ExeM/NucH family extracellular endonuclease	Rv	TCAGATGATTGACGGCGACC
***bpsl1103* (*nth*)**	Fwd	GACGTATCGGTCAACAAGGC
Endonuclease III	Rv	GACGTTCTTTGCCTTCGTCC
***bpsl2315* (*xth*)**	Fwd	CTGGAACGTCAACTCCCTGA
Exonuclease III	Rv	GGAAACTTCTCGTCGGGGAT
***bpsl1284* (*recD*)**	Fwd	TACTCCTGCGCCTTGATGAC
Exonuclease V	Rv	CGATTCATCTGCGCTTCGAC
16S rRNA	Fwd	TTTTGGACAATGGGCGCAAG
	Rv	TCCACTCCGGGTATTAGCCA

Abbreviations: Fwd = Forward primer, Rv = Reverse primer.

2.8. Statistical analysis

Statistical analyses were performed using SPSS software, version 23 (SPSS Inc., Chicago, IL, USA). One-way ANOVA was followed by Tukey post-hoc tests, or Games-Howell post-hoc tests to correct for variance heterogeneity. The levels required for statistical significance were ** $p < 0.001$ and * $p < 0.05$.

3. Results

3.1. D-Met and L-Met dispersed the preformed B. pseudomallei biofilms

Low concentrations (0.05 and 0.5 μM) of L-Met significantly reduced preformed *B. pseudomallei* H777, L1 and P1 biofilms, while only 0.5 μM of D-Met significantly reduced preformed *B. pseudomallei* H777, L1 and P1 biofilms compared to the untreated control. Dose dependency was not observed (Fig. 1a and b). Conversely, the elevated concentration of 50 μM exhibited a minimal impact on reducing the biofilm. The lowest concentration (0.05 μM) of D-Met and L-Met were chosen for further investigations on *B. pseudomallei* H777.

The 24-h preformed *B. pseudomallei* H777, L1 and P1 biofilms in LB

Table 1
Nuclease of *B. pseudomallei* in this study.

Nuclease	Size (aa)	Name	Encoded gene	Protein topology using SOSUI and Signal 6.0	Predicted cellular localization using PSORTb 3.0.3
RecJ	564	Single-stranded-DNA specific exonuclease	*bpsl2279*	Transmembrane	Cytoplasmic
EddB	624	ExeM/NucH family extracellular endonuclease	*bpss1845*	Soluble	Secreted
Nth	214	Endonuclease III	*bpsl1103*	Soluble	Cytoplasmic
Xth	258	Exodeoxyribonuclease III	*bpsl2315*	Soluble	Cytoplasmic
RecD	890	Exonuclease V	*bpsl1284*	Soluble	Cytoplasmic

Abbreviations: aa = amino acid.

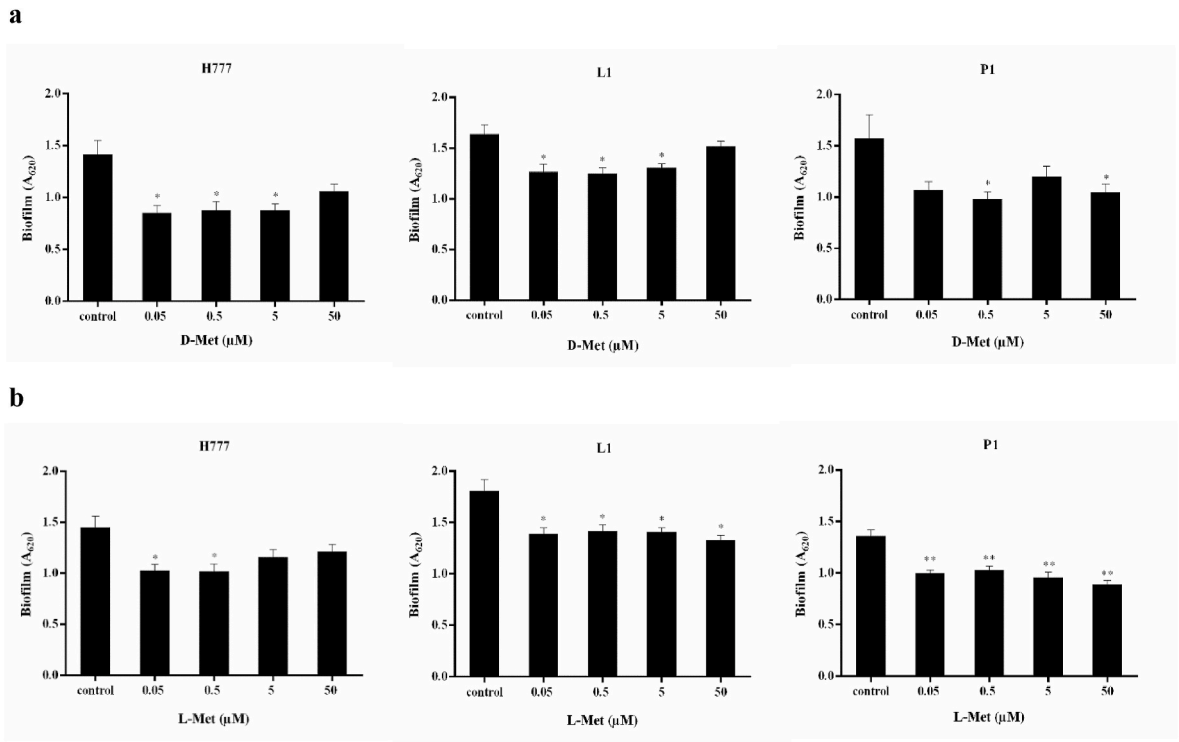

Fig. 1. Effects of D-Met and L-Met on the 24-h preformed *B. pseudomallei* H777, L1 and P1 biofilms.

medium were treated with 0.05, 0.5, 5, and 50 μM D-Met (**a**) and L-Met (**b**) for additional 24 h. The 2-day biofilms were stained with crystal violet and quantified compared to the untreated control. Data were reported as mean and standard deviation from eight replicates in three independent experiments (n = 24). Asterisks indicate statistical significance as ** $p < 0.001$ and * $p < 0.05$.

The confocal laser scanning microscope (CLSM) images demonstrated the dispersed biofilm architecture after being treated with 0.05 μM D-Met or L-Met (Fig. 2a). The quantitative image analysis using COMSTAT revealed a significant reduction in biofilm biomass and eDNA within biofilm compared to the untreated control (Fig. 2b and c). Therefore, both D-Met and L-Met at 0.05 μM exhibited similar ability of disperse the established *B. pseudomallei* H777 biofilm and lowered concentration of eDNA in the biofilm matrix.

3.2. Methionine induced up-regulation of B. pseudomallei H777 nuclease genes

To elucidate the factors contributing to the reduction in both biofilm biomass and eDNA quantity following methionine treatment, real-time PCR analysis was conducted to assess the expression levels of five nuclease gene in *B. pseudomallei* H777. The relative expression ratios demonstrated the up-regulation of *recJ* (single-stranded-DNA specific exonuclease), *eddB* (ExeM/NucH family extracellular endonuclease), *nth* (endonuclease III), *xth* (exonuclease III) and *recD* (exonuclease V) genes in D-Met treated biofilm while L-Met impacted only *recJ* and *eddB* genes compared to the untreated control (Table 3). However, we observed significant up-regulation of *recJ* when treated with both D-Met ($p = 0.013$) and L-Met ($p=0.027$). This indicates that both D-Met and L-Met impact transcriptional up-regulation of DNase genes of *B. pseudomallei.* Furthermore, these DNases were subjected to protein localization prediction through an online tool, PSORTb (version 3.0.3). Most of the predicted DNases were identified as cytoplasmic proteins, while EddB is categorized as a secreted DNase (Table 1).

3.3. Methionine enhanced CAZ susceptibility of B. pseudomallei H777 biofilm

To examine the ability of methionine to increase susceptibility of the pre-existing *B. pseudomallei* H777 biofilm cells to CAZ, cells were liberated and counted from 2-day biofilm treated with a combination of D-Met (or L-Met) and CAZ. The results demonstrated that D-Met combined with CAZ significantly lowered the number of biofilm cells compared to CAZ treatment alone (1 log reduction) and lowered up to 3 log reduction compared to untreated controls, similar to the effect observed with 0.01 U/mL DNase combined with CAZ (Fig. 3).

Visual assessment of confocal images further confirmed that D-Met and L-Met, whether used individually or in combination with CAZ, effectively dispersed the *B. pseudomallei* H777 biofilm in contrast to the untreated control (Fig. 4a). Moreover, the presence of CAZ induced a morphological alteration of *B. pseudomallei* cells from short rods to filamentous forms (red arrow). COMSTAT analysis revealed a significant decrease in both biofilm biomass and eDNA when exposed to the combination of D-Met or L-Met with CAZ in comparison to the untreated control, D-Met or L-Met or CAZ alone (Fig. 4b). Furthermore, live/dead CLSM images (Fig. 5a) and COMSTAT analysis (Fig. 5b) elucidated the combined activity of D-Met and L-Met and CAZ against the biofilm cells. The live/dead ratio indicated that D-Met facilitated CAZ activity, resulting in a lower live/dead ratio compared to that observed with CAZ alone. Taken together, these results suggest that D-Met enhanced the CAZ susceptibility of *B. pseudomallei* H777 biofilm cells.

4. Discussion

Bacterial biofilm can lead to life-threatening conditions where antibiotic treatment fails to affect the bacterial cells. Previous studies have noted the importance of *B. pseudomallei* biofilm in cellular pathogenesis [7], relapsing melioidosis [13] and antibiotic resistance including to CAZ, imipenem, and trimethoprim-sulfamethoxazole [10]. Recently, D- and L-amino acids have been shown to prevent and disrupt biofilm in several Gram-positive and Gram-negative bacteria. In this study, we

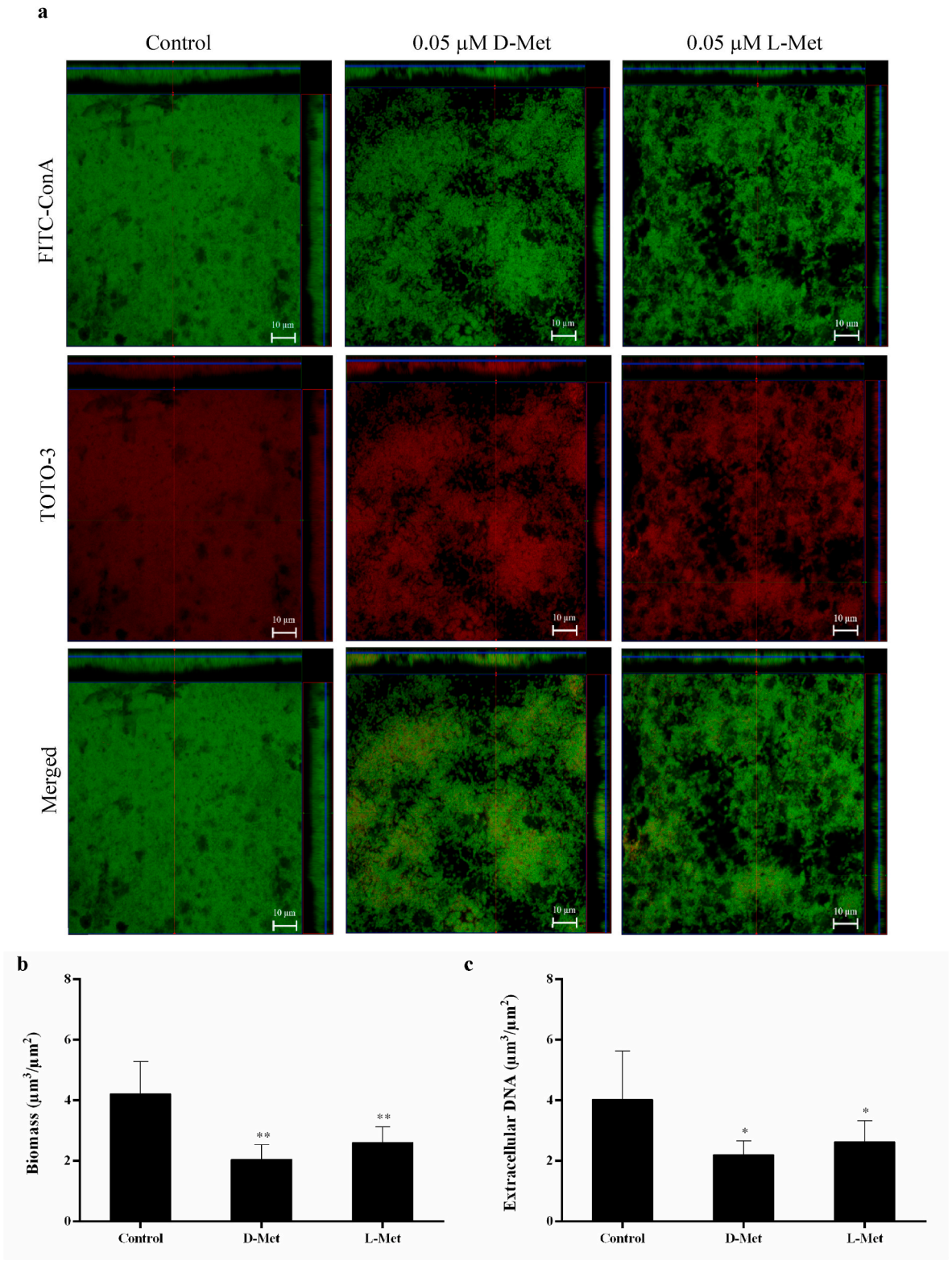

Fig. 2. D-Met and L-Met dispersed the 24 h preformed *B. pseudomallei* H777 biofilm.
(a) The CLSM images of 24 h preformed *B. pseudomallei* H777 biofilms in LB broth treated with 0.05 μM D-Met and L-Met for an additional 24 h. Subsequently, the biofilms were stained with 50 μg/mL FITC-ConA (biofilm, green) and 2 μM TOTO-3 (eDNA, red) in parallel with the untreated controls. These CLSM images of the 2-day biofilm are representatives of three independent experiments. The scale bars represent 10 μm. The images were taken at 630 × magnification under a Zeiss 800 CLSM microscope. The biofilm biomass (b) and eDNA (c) were obtained from 18 CLSM images from three independent experiments using COMSTAT analysis. Data were reported as mean and standard deviation (n = 18). Asterisks indicate statistical significance as follows: ** $p < 0.001$ and * $p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Nuclease gene expression after methionine treatment.

Gene	Relative gene expression	
	0.05 μM D-Met	0.05 μM L-Met
***bpsl2279* (*recJ*)**	1.44 ± 0.13*	1.37 ± 0.16*
***bpss1845* (*eddB*)**	1.13 ± 0.37	1.03 ± 0.23
***bpsl1103* (*nth*)**	1.09 ± 0.05	0.96 ± 0.07
***bpsl2315* (*xth*)**	1.26 ± 0.07	0.94 ± 0.03
***bpsl1284* (*recD*)**	1.15 ± 0.14	0.99 ± 0.10

Expression ratio relative to control if more than 1 is up-regulated, less than 1 is down-regulated.
Data represent as mean ± SD, Asterisks denote statistical significance, with * indicating $p < 0.05$ compared to untreated control.

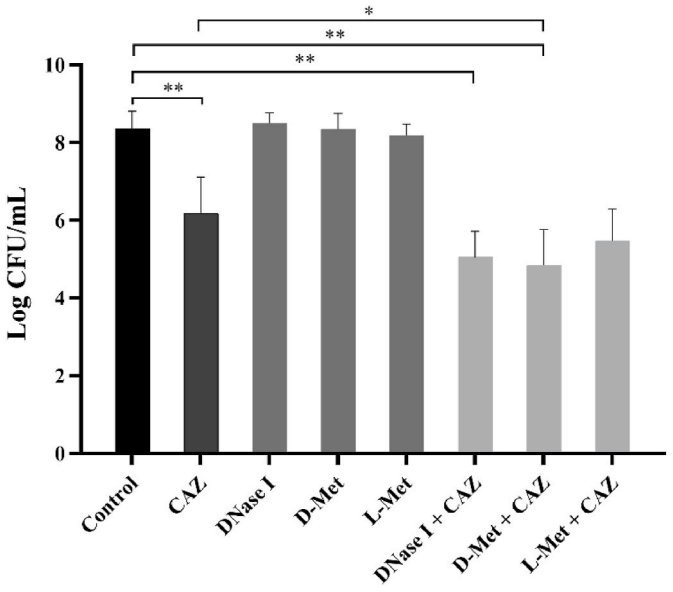

Fig. 3. Effect of D-Met and L-Met combined with CAZ on viable biofilm cells of *B. pseudomallei* H777. The 24 h preformed *B. pseudomallei* H777 biofilms in MHB medium on the pegs in 96 well plates were challenged with 512 μg/mL CAZ, 0.01 U/mL DNase I, 0.05 μM D-Met and L-Met, DNase I combined with CAZ, and D-Met and L-Met combined with CAZ for an additional 24 h. Subsequently, viable biofilm cells of the treated 2-day biofilms were liberated by sonication for bacterial counting. The experiment was performed in duplicates in three independent experiments. Data were reported as mean and standard deviation (n = 6). Asterisks indicate statistical significance as follows: $**p < 0.001$ and $* p < 0.05$.

demonstrated the *in vitro* effect of D-Met and L-Met to disperse a pre-existing *B. pseudomallei* biofilm. One interesting finding was that D-Met and L-Met could induce DNase gene expression that correlated inversely with eDNA levels during biofilm matrix degradation. Importantly, the killing effect of CAZ on the biofilm cells increased when it was combined with D-Met.

The interruption of *B. pseudomallei* biofilm formation using DNase from the initial biofilm culture, followed by the addition of CAZ, significantly eradicated viable biofilm cells, as previously reported [15]. The most exciting finding in this study is the impact of D-Met and L-Met on preformed biofilms, which dispersed biofilm cells and enhance CAZ susceptibility at 512 μg/mL, while a previous report demonstrated the hallmark characteristic antibiotic resistance of biofilm-forming *B. pseudomallei* up to 1024 or 2048 μg/mL of CAZ [11].

The effectiveness of D-Met and L-Met as dispersal agents against the pre-existing *B. pseudomallei* biofilm and their ability to enhance antibiotic susceptibility in this study corroborates other studies in this area. D-Met at a concentration of 20 mM was demonstrated to inhibit both the formation and growth of non-typeable *Haemophilus influenzae* (NTHi) biofilms via peptidoglycan synthesis interference. In addition, 20 mM of D-Met enhances the efficacy of azithromycin treatment in NTHi [18], D-Met at a concentration of 50 mM effectively inhibited *Staphylococcus epidermidis* biofilm formation, particularly in the context of ocular infection [38]. Moreover, D-Met treatment ranging from 1 to 100 mM treatment has the potential to reduce mature biofilms of *Campylobacter jejuni*, while L-Met does not [26]. However, it is noteworthy that some publications suggest that D-Met does not exhibit inhibitory effects on *Pseudomonas aeruginosa* [39]. However, Gnanadhas et al., demonstrated that 0.5 μM L-Met inhibited *P. aeruginosa* biofilm and enhanced ciprofloxacin susceptibility in an *in vivo* study [28]. Furthermore, Cho and colleagues found that L-Met at 0.5 μM inhibited *P. aeruginosa* biofilm and enhanced susceptibility to ivacaftor. However, a high concentration of L-Met at 10 mM did not exhibit an inhibitory effect on *P. aeruginosa* biofilm [40].

The considerably up-regulated DNase genes following L-Met treatment and enhanced antibiotic susceptibility of *P. aeruginosa* biofilm, as reported by Gnanadhas et al. [28], initiated our interest to investigate *B. pseudomallei.* Our study confirms the association between D-Met and L-Met and up-regulation of DNase genes, biofilm dispersal and improved antibiotic susceptibility. Contrary to expectations, our study demonstrated not much increase in relative expression of the DNase genes. The observed up-regulation in response to both D-Met and L-Met treatments suggests a complex interplay between these amino acids and the regulation of DNase genes. The presence of L-Met was suggested in transcriptional up-regulation of DNase genes in *P. aeruginosa* followed by protein secretion through the bacterial secretory systems, lysis mechanism, or increase DNase activity [28]. Treating non-typeable *Haemophilus influenzae* with high concentration of D-Met (20 mM) during biofilm formation reduced biofilm viability and increased its biomass. Quantitative proteomic analyses illustrated the association with proteins involved in metabolic and transport processes, as well as peptidoglycan synthesis [18]. The process of biofilm dispersal regulated by D-amino acids through the dissociation of matrix-associated amyloid fibers of *B. subtilis* cells, was illustrated by Kolodkin-Gal et al. [21]. Various bacterial species produced and release D-amino acids such as D-Met, which play roles in regulating bacterial processes, including cell wall remodeling during the stationary phase, and causing biofilm dispersal in aging bacterial communities [21,41]. Biofilm dispersion could be a potential strategy to make the bacteria easier to kill with antibiotics and widely used to combat biofilm-related infections [42]. Nevertheless, the process through which D-Met and L-Met stimulate DNase production in *B. pseudomallei* has yet to be fully understood.

L-amino acids are predominantly found in nature, whereas D-enantiomers of amino acids have traditionally been considered to play minor roles in biological processes [41]. Research on non-typeable *Haemophilus influenzae* demonstrated that D-Met had a more effect on biofilm viability, bacterial growth involved in peptidoglycan synthesis and cell division compared to L-Met [18]. Furthermore, unusual D-amino acids observed in *Vibrio cholerae* and *Bacillus subtilis* have been suggested to downregulate peptidoglycan synthesis [19]. Our current study showed that D-Met had a slightly greater impact on *B. pseudomallei* biofilm biomass, eDNA, relative nuclease gene expression, log CFU, and live/-dead ratio of treated samples compared to L-Met. This could be attributed to the irregular form of the D-enantiomer, which may affect bacterial cell integrity and thereby increase antibiotic susceptibility.

The findings in this study should be considered with potential limitations. Using only a single bacterial strain, *B. pseudomallei* H777, may not fully represent other strains. Future research with diverse biofilm-forming capabilities is necessary to determine the generalizability of amino acids' effects on biofilm formation. Furthermore, while the 16S rRNA gene was used as a reference gene for normalization of qPCR, its expression stability under the tested conditions should be considered. Future studies should include a validation of reference gene to ensure their consistent expression across different experimental conditions.

These findings revealed for the first time that D-Met and L-Met

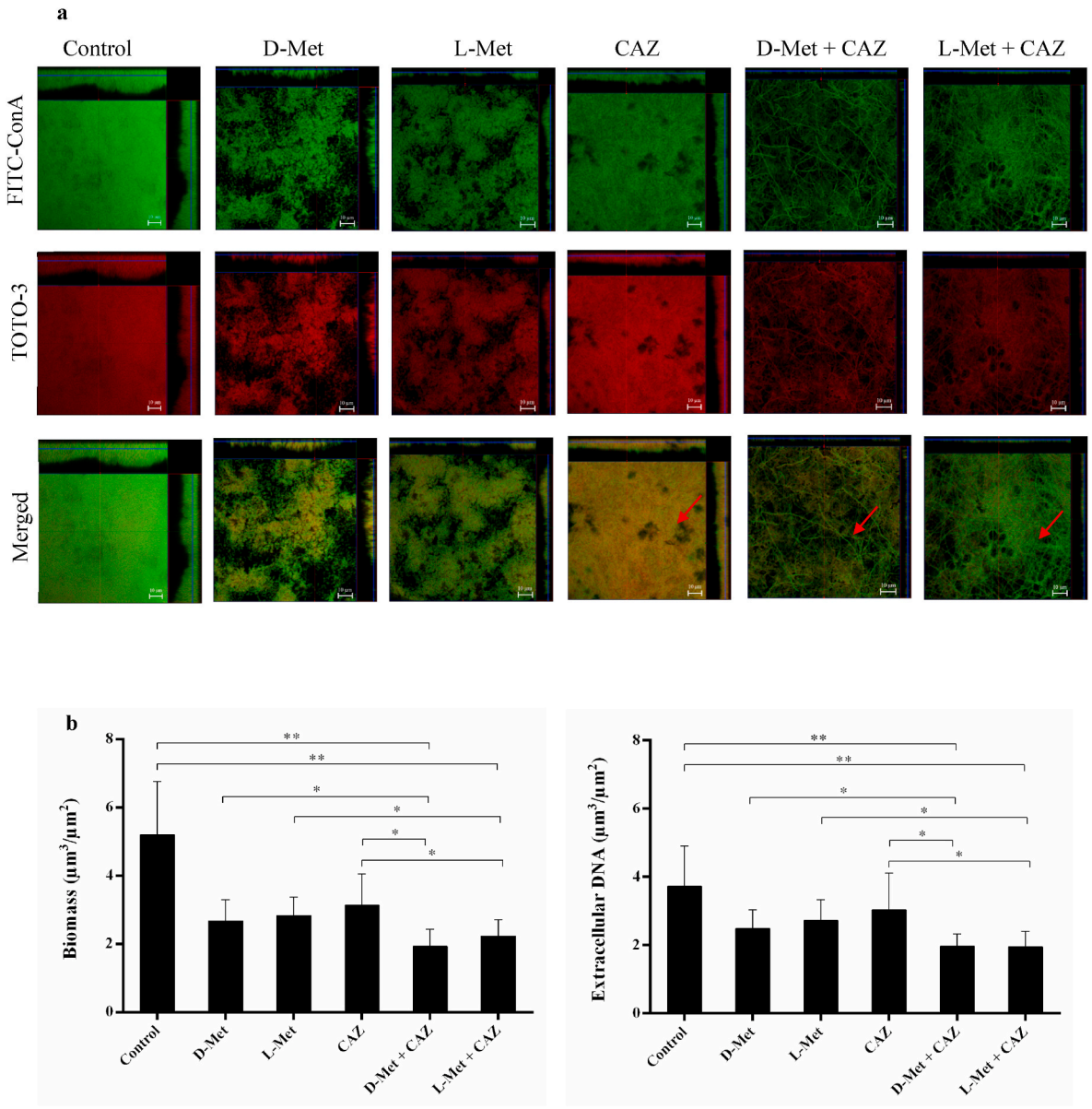

Fig. 4. The combination of methionine and CAZ effectively dispersed and minimized eDNA within the preformed *B. pseudomallei* H777 biofilms.
(a) CLSM images of *B. pseudomallei* H777 biofilms in MHB medium after exposed to 0.05 µM D-Met and L-Met, 512 µg/mL CAZ, and D-Met or L-Met combined with CAZ before staining with FITC-ConA (biofilm, green) and TOTO-3 (eDNA, red). The red arrow in merged image indicates filamentous cells. (b) Quantification of biomass and eDNA was conducted through COMSTAT analysis of 18 CLSM images derived from three independent experiments. Data were reported as mean and standard deviation (n = 18). The scale bar indicates a length of 10 µm. The images were captured at 630 × magnification using a Zeiss 800 CLSM microscope. Statistical significance is denoted by asterisks, with ** representing $p < 0.001$ and * $p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

destabilized the pre-existing *B. pseudomallei* biofilm through DNase gene up-regulation and eDNA degradation. Furthermore, the combination of D-Met with CAZ improved the antibiotic susceptibility of *B. pseudomallei* biofilm. Methionine may be a potential component of a therapeutic strategy involving ceftazidime treatment to combat *B. pseudomallei* biofilm-associated infection.

5. Conclusion

In the present study, we demonstrated *in vitro* the effect of D-Met and L-Met as biofilm dispersal agents through DNase gene up-regulation and breakdown of eDNA in biofilm matrix. This, in turn, enhanced the susceptibility of *B. pseudomallei* cells to antibiotics. These findings indicate the potential for a combination of methionine and antibiotic to prevent treatment failure against *B. pseudomallei* biofilm-associated infections.

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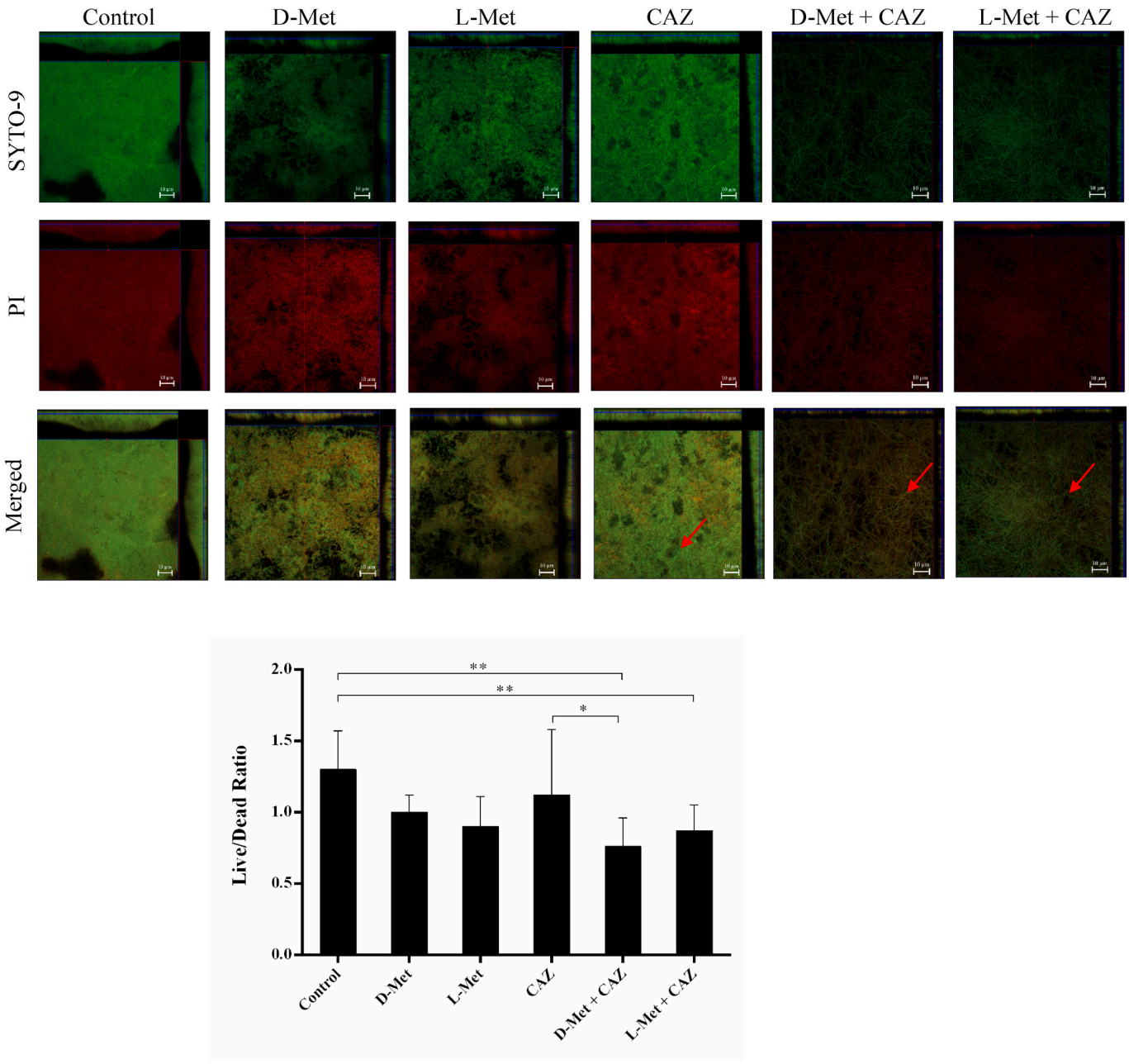

Fig. 5. The combination of D-Met and CAZ effectively reduced the viability of preformed *B. pseudomallei* H777 biofilm cells. (a) Confocal images of the 24-h preformed *B. pseudomallei* H777 biofilms in MHB medium were treated with 0.05 μM D-Met, 0.05 μM L-Met, 512 μg/ml CAZ, and D-Met or L-Met combined with 512 μg/ml CAZ for an additional 24 h, subsequently stained with 3.34 μM SYTO-9 (live cell, green) and 5 μg/ml PI (dead, red). The red arrows indicate filamentous cells. (b) Live/Dead ratios were calculated from 18 CLSM images using COMSTAT analysis. These data are representatives of three independent experiments and reported as mean and standard deviation (n = 18). The scale bar represents 10 μm. The images were taken at 630 × magnification under a Zeiss 800 CLSM microscope. Statistical significance is denoted by asterisks, with ** representing $p < 0.001$ and * $p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Approval of the submitted version of the manuscript

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CRediT authorship contribution statement

Rattiyaphorn Pakkulnan: Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Conceptualization. **Auttawit Sirichoat:** Writing – review & editing, Supervision, Methodology, Formal analysis. **Sorujsiri Chareonsudjai:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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References

[1] Cheng AC, Currie BJ. Melioidosis: epidemiology, pathophysiology, and management. Clin Microbiol Rev 2005;18(2):383–416.
[2] Wiersinga WJ, Currie BJ, Peacock SJ. Melioidosis. N Engl J Med 2012;367(11):1035–44.
[3] Limmathurotsakul D, Wongratanacheewin S, Teerawattanasook N, Wongsuvan G, Chaisuksant S, Chetchotisakd P, et al. Increasing incidence of human melioidosis in Northeast Thailand. Am J Trop Med Hyg 2010;82(6):1113–7.
[4] Rammaert B, Beaute J, Borand L, Hem S, Buchy P, Goyet S, et al. Pulmonary melioidosis in Cambodia: a prospective study. BMC Infect Dis 2011;11:126.
[5] Limmathurotsakul D, Golding N, Dance DA, Messina JP, Pigott DM, Moyes CL, et al. Predicted global distribution of *Burkholderia pseudomallei* and burden of melioidosis. Nat Microbiol 2016;1:15008.
[6] Wiersinga WJ, Virk HS, Torres AG, Currie BJ, Peacock SJ, Dance DAB, et al. Melioidosis. Nat Rev Dis Prim 2018;4:17107.
[7] Kunyanee C, Kamjumphol W, Taweechaisupapong S, Kanthawong S, Wongwajana S, Wongratanacheewin S, et al. *Burkholderia pseudomallei* biofilm promotes adhesion, internalization and stimulates proinflammatory cytokines in human epithelial A549 cells. PLoS One 2016;11(8):e0160741.
[8] Pibalpakdee P, Wongratanacheewin S, Taweechaisupapong S, Niumsup PR. Diffusion and activity of antibiotics against *Burkholderia pseudomallei* biofilms. Int J Antimicrob Agents 2012;39(4):356–9.
[9] Mongkolrob R, Taweechaisupapong S, Tungpradabkul S. Correlation between biofilm production, antibiotic susceptibility and exopolysaccharide composition in *Burkholderia pseudomallei bpsI, ppk,* and *rpoS* mutant strains. Microbiol Immunol 2015;59(11):653–63.
[10] Sawasdidoln C, Taweechaisupapong S, Sermswan RW, Tattawasart U, Tungpradabkul S, Wongratanacheewin S. Growing *Burkholderia pseudomallei* in biofilm stimulating conditions significantly induces antimicrobial resistance. PLoS One 2010;5(2):e9196.
[11] Anutrakunchai C, Sermswan RW, Wongratanacheewin S, Puknun A, Taweechaisupapong S. Drug susceptibility and biofilm formation of *Burkholderia pseudomallei* in nutrient-limited condition. Trop Biomed 2015;32(2):300–9.
[12] Limmathurotsakul D, Chaowagul W, Chantratita N, Wuthiekanun V, Biaklang M, Tumapa S, et al. A simple scoring system to differentiate between relapse and Re-infection in patients with recurrent melioidosis. PLoS Neglected Trop Dis 2008;2(10):e327.
[13] Limmathurotsakul D, Paeyao A, Wongratanacheewin S, Saiprom N, Takpho N, Thaipadungpanit J, et al. Role of *Burkholderia pseudomallei* biofilm formation and lipopolysaccharide in relapse of melioidosis. Clin Microbiol Infect 2014;20(11):O854–6.
[14] Okshevsky M, Regina VR, Meyer RL. Extracellular DNA as a target for biofilm control. Curr Opin Biotechnol 2015;33:73–80.
[15] Pakkulnan R, Thonglao N, Chareonsudjai S. DNase I and chitosan enhance efficacy of ceftazidime to eradicate *Burkholderia pseudomallei* biofilm cells. Sci Rep 2023;13(1):1059.
[16] Cavaliere R, Ball JL, Turnbull L, Whitchurch CB. The biofilm matrix destabilizers, EDTA and DNaseI, enhance the susceptibility of nontypeable *Hemophilus influenzae* biofilms to treatment with ampicillin and ciprofloxacin. Microbiologyopen 2014;3(4):557–67.
[17] Kaplan JB, Izano EA, Gopal P, Karwacki MT, Kim S, Bose JL, et al. Low levels of beta-lactam antibiotics induce extracellular DNA release and biofilm formation in *Staphylococcus aureus*. mBio 2012;3(4):e0019812.
[18] Dawe H, Berger E, Sihlbom C, Angus EM, Howlin RP, Laver JR, et al. D-methionine interferes with non-typeable *Haemophilus influenzae* peptidoglycan synthesis during growth and biofilm formation. Microbiology 2017;163(7):1093–104.
[19] Lam H, Oh DC, Cava F, Takacs CN, Clardy J, de Pedro MA, et al. D-amino acids govern stationary phase cell wall remodeling in bacteria. Science 2009;325(5947):1552–5.
[20] Hochbaum AI, Kolodkin-Gal I, Foulston L, Kolter R, Aizenberg J, Losick R. Inhibitory effects of D-amino acids on *Staphylococcus aureus* biofilm development. J Bacteriol 2011;193(20):5616–22.
[21] Kolodkin-Gal I, Romero D, Cao S, Clardy J, Kolter R, Losick R. D-amino acids trigger biofilm disassembly. Science. 2010;328(5978):627–9.
[22] Leiman SA, May JM, Lebar MD, Kahne D, Kolter R, Losick R. D-amino acids indirectly inhibit biofilm formation in *Bacillus subtilis* by interfering with protein synthesis. J Bacteriol 2013;195(23):5391–5.
[23] Yan Y, Zheng B, Lekbach Y, Liu X, Zheng Y, Xu D, et al. d-leucine enhances antibiofilm activity of chlorhexidine against caries-causing *Streptococcus mutans* biofilm. Int Biodeterior Biodegrad 2021;157:105135.
[24] Warraich AA, Mohammed AR, Perrie Y, Hussain M, Gibson H, Rahman A. Evaluation of anti-biofilm activity of acidic amino acids and synergy with ciprofloxacin on *Staphylococcus aureus* biofilms. Sci Rep 2020;10(1):9021.
[25] Sanchez Jr CJ, Akers KS, Romano DR, Woodbury RL, Hardy SK, Murray CK, et al. D-amino acids enhance the activity of antimicrobials against biofilms of clinical wound isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 2014;58(8):4353–61.
[26] Elgamoudi BA, Taha T, Korolik V. Inhibition of *Campylobacter jejuni* biofilm formation by D-amino acids. Antibiotics (Basel) 2020;9(11).
[27] Zilm PS, Butnejski V, Rossi-Fedele G, Kidd SP, Edwards S, Vasilev K. D-amino acids reduce *Enterococcus faecalis* biofilms *in vitro* and in the presence of antimicrobials used for root canal treatment. PLoS One 2017;12(2):e0170670.
[28] Gnanadhas DP, Elango M, Datey A, Chakravortty D. Chronic lung infection by *Pseudomonas aeruginosa* biofilm is cured by L-Methionine in combination with antibiotic therapy. Sci Rep 2015;5:16043.
[29] Pakkulnan R, Anutrakunchai C, Kanthawong S, Taweechaisupapong S, Chareonsudjai P, Chareonsudjai S. Extracellular DNA facilitates bacterial adhesion during *Burkholderia pseudomallei* biofilm formation. PLoS One 2019;14(3):e0213288.
[30] Pakkulnan R, Thonglao N, Chareonsudjai S. DNase I and chitosan enhance efficacy of ceftazidime to eradicate *Burkholderia pseudomallei* biofilm cells. Sci Rep 2023;13(1):1059.
[31] Anutrakunchai C, Bolscher JGM, Krom BP, Kanthawong S, Chareonsudjai S, Taweechaisupapong S. Impact of nutritional stress on drug susceptibility and biofilm structures of *Burkholderia pseudomallei* and *Burkholderia thailandensis* grown in static and microfluidic systems. PLoS One 2018;13(3):e0194946.
[32] Mann EE, Rice KC, Boles BR, Endres JL, Ranjit D, Chandramohan L, et al. Modulation of eDNA release and degradation affects *Staphylococcus aureus* biofilm maturation. PLoS One 2009;4(6):e5822.
[33] Heydorn A, Nielsen AT, Hentzer M, Sternberg C, Givskov M, Ersbøll BK, et al. Quantification of biofilm structures by the novel computer program COMSTAT. Microbiology (Read) 2000;146(Pt 10):2395–407.
[34] Winsor GL, Khaira B, Van Rossum T, Lo R, Whiteside MD, Brinkman FS. The *Burkholderia* Genome Database: facilitating flexible queries and comparative analyses. Bioinformatics 2008;24(23):2803–4.
[35] Mitaku S, Hirokawa T, Tsuji T. Amphiphilicity index of polar amino acids as an aid in the characterization of amino acid preference at membrane-water interfaces. Bioinformatics 2002;18(4):608–16.
[36] Teufel F, Almagro Armenteros JJ, Johansen AR, Gíslason MH, Pihl SI, Tsirigos KD, et al. SignalP 6.0 predicts all five types of signal peptides using protein language models. Nat Biotechnol 2022;40(7):1023–5.
[37] Yu NY, Wagner JR, Laird MR, Melli G, Rey S, Lo R, et al. PSORTb 3.0: improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. Bioinformatics 2010;26(13):1608–15.
[38] Ramon-Perez ML, Diaz-Cedillo F, Ibarra JA, Torales-Cardena A, Rodriguez-Martinez S, Jan-Roblero J, et al. D-Amino acids inhibit biofilm formation in *Staphylococcus epidermidis* strains from ocular infections. J Med Microbiol 2014;63(Pt 10):1369–76.
[39] Kao WTK, Frye M, Gagnon P, Vogel JP, Chole R. D-amino acids do not inhibit *Pseudomonas aeruginosa* biofilm formation. Laryngoscope Investig Otolaryngol 2017;2(1):4–9.
[40] Cho D-Y, Lim D-J, Mackey C, Weeks CG, Peña Garcia JA, Skinner D, et al. l-Methionine anti-biofilm activity against *Pseudomonas aeruginosa* is enhanced by the cystic fibrosis transmembrane conductance regulator potentiator, ivacaftor. International Forum of Allergy & Rhinology 2018;8(5):577–83.
[41] Cava F, Lam H, de Pedro MA, Waldor MK. Emerging knowledge of regulatory roles of D-amino acids in bacteria. Cell Mol Life Sci : CM 2011;68(5):817–31.
[42] Wille J, Coenye T. Biofilm dispersion: the key to biofilm eradication or opening Pandora's box? Biofilms 2020;2:100027.