ST1-YtnP lactonase from extreme environment: A promising antivirulence agent against multidrug-resistant Pseudomonas aeruginosa

Biomedicine & Pharmacotherapy 190 (2025) 118443

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

ST1-YtnP lactonase from extreme environment: A promising antivirulence agent against multidrug-resistant *Pseudomonas aeruginosa*

Jovana Curcic [a,1], Ivano Merendino [b,1], Danka Matijasevic [a], Branko Jovcic [c], Silvia Spriano [b], Milka Malesevic [a,*]

[a] Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Vojvode Stepe 444a, Belgrade 11042, Serbia
[b] Department of Applied Science and Technology, Politecnico di Torino, Corso Duca degli Abruzzi 24, Turin 10129, Italy
[c] Faculty of Biology, University of Belgrade, Studentski trg 16, Belgrade 11000, Serbia

ARTICLE INFO

Keywords:
Lactonase
Extreme environments
Pseudomonas aeruginosa
Virulence
Antivirulence therapy
Antibiofilm
Caenorhabditis elegans

ABSTRACT

The emergence of multidrug-resistant (MDR) pathogens, particularly *Pseudomonas aeruginosa*, requires innovative therapeutic strategies. This study investigates the potential of ST1-YtnP lactonase, an enzyme isolated from the thermophilic bacterium *Bacillus licheniformis*, which is found in the extreme environment of the Vranjska Banja hot springs. The extreme conditions in this habitat offer untapped potential for the discovery of biotechnologically valuable, resilient enzymes. ST1-YtnP lactonase was shown to effectively degrade acyl-homoserine lactones (AHLs), thereby disrupting the quorum sensing (QS) system of *P. aeruginosa* and reducing its virulence. ST1-YtnP significantly reduced biofilm formation without inhibiting bacterial growth Furthermore, *in vitro* analysis revealed that ST1-YtnP lactonase exhibited a synergistic effect with gentamicin and an additive effect with meropenem, enhancing the efficacy of these antibiotics against *P. aeruginosa* MMA83. *In vivo*, the combination of meropenem and ST1-YtnP lactonase completely rescued *Caenorhabditis elegans* from infection, surpassing the protective effect of meropenem alone. ST1-YtnP lactonase showed no adverse effects on the survival of uninfected nematodes, while it significantly enhanced the survival of *P. aeruginosa*-infected nematodes treated with the enzyme. These findings emphasize the potential of ST1-YtnP lactonase as a novel antivirulence agent with promising biotechnological applications to combat antibiotic-resistant infections.

1. Introduction

The rapid rise of antimicrobial resistance (AMR) is one of the top 10 global health threats according to the World Health Organization (WHO). It is predicted that AMR could cause 10 million deaths annually by 2050, surpassing cancer, diabetes, and heart disease as the leading cause of mortality [1]. The AMR crisis threatens not only human health but also the sustainability of healthcare systems, as the unavailability of effective treatments leads to prolonged hospitalization, higher healthcare costs, and economic burdens due to work absenteeism [2]. Despite the urgent need for new antimicrobial agents to combat multidrug-resistant (MDR) pathogens, the current rate of antimicrobial drug discovery is insufficient to meet this need [3].

In response to this challenge, biocatalysts with antivirulence activity have emerged as a promising alternative to traditional antimicrobial approaches. Quorum quenching (QQ) is one of the most promising antivirulence strategies to combat chronic bacterial infections via disrupting quorum sensing (QS) [4]. QS represents bacterial regulatory mechanism that controls gene expression in response to cell density through the production and detection of signaling molecules known as autoinducers (AI). In pathogenic bacteria, such as *Pseudomonas aeruginosa*, QS plays a key role in the regulation of virulence and biofilm formation [5]. Lactonases - QQ enzymes can degrade these signaling molecules, disrupt QS, and inhibit or attenuate the production of virulence factors, including biofilms formed by multidrug-resistant (MDR) bacteria, thus enhancing the efficacy of clinical antibiotics [6]. In contrast to conventional antibiotics, lactonases do not directly inhibit bacterial growth, which reduces the selective pressure for the development of resistance. Additionally, they act extracellularly in catalytic quantities and exhibit substrate specificity, making them a more selective and potentially sustainable therapeutic option [7].

The WHO has classified *P. aeruginosa* as a critical priority pathogen

* Corresponding author.
E-mail address: milka.malesevic@imgge.bg.ac.rs (M. Malesevic).
[1] Authors are equally contributed.

https://doi.org/10.1016/j.biopha.2025.118443
Received 12 April 2025; Received in revised form 4 August 2025; Accepted 7 August 2025
Available online 9 August 2025

due to its high resistance to antibiotic, its virulence and its ability to form biofilms [8]. Infections associated with medical devices and implants are of critical importance in clinical practice as they can lead to serious complications and prolonged hospitalization [9]. The ability of *P. aeruginosa* to form complex and multi-layered biofilms on medical devices and implants exacerbates this problem. Such biofilm formation complicates treatment as it protects *P. aeruginosa* from both host immune responses and antimicrobial agents, often rendering standard therapies ineffective. In addition, the European Center for Disease Prevention and Control (ECDC) reported in 2016 that *P. aeruginosa* causes a variety of infections acquired in intensive care units and hospitals, including pneumonia, urinary tract infections and bloodstream infections [10]. Studies have shown that *P. aeruginosa* bacteremia can cause severe septic shock and multiple organ failure, leading to high mortality rates of 18–61 % [11].

Biotechnology plays a pivotal role in overcoming these challenges. Enzymes are becoming increasingly attractive for the further development of innovative biotechnological processes due to their numerous advantages such as precision and efficiency, driving the shift towards "greener" and more sustainable industrial practices [12]. Enzymes derived from extreme environments, or extremozymes, have gained increasing attention due to their biotechnologically favorable properties that show significant potential for functionalization in industrial and clinical applications [13]. Extremozymes have the potential to revolutionize biotechnological processes in various industries, as they are stable and active even under harsh conditions, e.g. in the food, feed, textile, pharmaceutical and healthcare industries, as well as in the field of renewable energy. However, the number of extremozymes currently available is not sufficient to meet the growing industrial demand [14].

In response to the pressing need for novel therapeutic strategies against MDR bacterial strains and chronic infections, this study investigates the antivirulence potential of the newly identified ST1-YtnP lactonase, originating from the extreme environment of a Vranjska banja hot spring. This research represents the first investigation of the antibiofilm and antivirulence activities of this lactonase *in vitro* and *in vivo*. Additionally, the toxicity of ST1-YtnP lactonase and its combination with antibiotics was investigated *in vivo* in the *Caenorhabditis elegans* model system, providing important insights into its potential biomedical applications.

2. Material and methods

2.1. Strains, media, and chemicals used in this study

A list of the bacterial strains and plasmids used in this study is presented in Table 1. The Luria-Bertani (LB) broth used for the cultivation of bacterial strains was prepared with distilled water (dH_2O), 1 % Bacto Tryptone (Oxoid, Hampshire, UK), 0.5 % yeast extract (Oxoid), and 0.5 % sodium chloride (NaCl), providing a nutrient-rich environment. The Luria agar (LA) medium contained the same components, but included 1.7 % agar (Oxoid). For the CV026 well-diffusion assay, LB soft-agar containing half the agar concentration (0.85 %) was used. Additionally, Muller-Hinton (MH), prepared according to the manufacturer instructions (Oxoid), was used for the antibiotic susceptibility testing. For ST1-YtnP enzyme expression, LB medium supplemented with 100 μg/ml ampicillin (Sigma, St. Louis, MO, USA) and 50 μg/ml kanamycin (Sigma) was used. Nematode Growth Medium (NGM) was prepared by autoclaving a mixture of 0.25 % peptone (Oxoid), 0.3 % NaCl, and 1.7 % agar in 1 liter dH_2O, followed by the addition of 1 ml cholesterol (5 mg/ml) (Sigma), 1 ml $CaCl_2$, 1 ml $MgSO_4$, and 25 ml KPI buffer (containing 6.6 % K_2HPO_4 and 43.4 % KH_2PO_4). Finally, M9W medium was used for *C. elegans* assays. It consists of 0.3 % KH_2PO_4, 0.6 % Na_2HPO_4, and 0.5 % NaCl per liter dH_2O, prepared and autoclaved with subsequent addition of 0.1 % $MgSO_4$ (1 M). Slow Killing (SK) broth (contains) 3.5 g peptone + 3 g NaCl + 1 l dH_2O + 1 ml $MgSO_4$(1 M) + 25 ml KH_2PO_4 (1 M) + 1 ml $CaCl_2$ + 1 ml cholesterol (5 mg/ml). The liquid Killing (LK) medium used for the slow-killing assay was prepared by mixing equal proportions of SK broth and M9W media.

Table 1
A list of bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Source
Strains		
Bacillus licheniformis ST1	QQ lactonase producer	This study
Chromobacterium subtsugae CV026	ATCC 31532 derivative, cviI:: Tn*5 xylE*, Km^r Sm^r	[15]
Pseudomonas aeruginosa MMA83	NDM−1 positive clinical isolate, Amp^r	[16]
Escherichia coli M15	*lacZ*ΔM15	Qiagen
E. coli DH5α	*supE44* Δ*lacU169* (Φ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi*−1 *relA*	[17]
E. coli OP50	uracil requiring mutant of *E. coli*	[18]
Plasmids		
pJET 1.2	Amp^r, PCR cloning vector	ThermoFisher
pQE30	Amp^r, ColE1 replicon, His6 expression vector	Qiagen
pJETST1-YtnP	*ytnP* lactonase gene cloned into pJET 1.2	This study
pQE30ST1-YtnP	*ytnP* lactonase gene subcloned into pQE30	This study
***C. elegans* strain**		
C. elegans AU37	*C. elegans glp−4* (bn2) I; *sek−1* (km4) X Temperature-sensitive sterility. Maintain at 15°C. Enhanced sensitivity to pathogens.	AU37 strain was provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440)

NDM-1-New-Delhi metallo-β-lactamase 1; Km^r-kanamycin-resistant; Sm^r-streptomycin-resistant; Amp^r-ampicillin-resistant

2.2. In silico analysis of ST1-YtnP lactonase amino acid sequence

The amino acid sequence of ST1-YtnP lactonase was aligned with functionally characterized lactonases from different bacterial species using the ClustalW algorithm (Vector NTI software) [19]. The phylogenetic relationships were analyzed using the maximum likelihood method (MEGA 11 software) [20]. The presence of the signal peptide sequence of the ST1-YtnP lactonase was detected using SignalP version 5.0 [21] and the PrediSi [22] program. Secondary and tertiary structures were predicted using Phyre2 [23], PSIPRED [24], I-TASSER [25], and SWISS-MODEL [26].

2.3. ST1-YtnP lactonase expression and purification

The DNA sequence encoding ST1-YtnP lactonase from *Bacillus licheniformis* was amplified by PCR using the Phusion™ High-Fidelity DNA Polymerase Kit (ThermoFisher Scientific) using appropriate primers (Forward: AATGGAAACTTTAACAATCGG, Reverse: TGTTCTGCAGGGGAGTCGCAAGG). The ST1-YtnP amplicons were digested with *BsaB*I and *Pst*I and cloned into the pQE30 expression vector (Qiagen) and linearized with the same enzymes. Expression of the recombinant protein was induced with 0.1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) (Qiagen) in *E. coli* M15 pREP4 cells according to the manufacturer's manual. The pQE30ST1-YtnP plasmid construct has been sequenced to confirm the accuracy of the sequences and in-frame incorporation into the pQE30 vector (Macrogen, The Netherlands). The sequence was shown in the Supplementary material Appendix 1. ST1-YtnP protein was purified under denaturing conditions

(8 M urea, 20 mM Tris, 0.5 M NaCl, 10 mM imidazole) using the Ni-NTA Fast Start Kit (Qiagen). During purification, the protein was gradually refolded by stepwise reduction of urea concentration to allow proper renaturation. The His6-ST1-YtnP protein was eluted with 250 mM imidazole in 20 mM Tris, 0.5 M NaCl, pH 8, and desalted with 10 kDa columns (Merck-Millipore) in 20 mM Tris-HCl, pH 7 buffer as previously described [6,27]. The recombinant ST1-YtnP lactonase was stored at −20°C in 50 % (w/v) glycerol and analyzed by 12.5 % SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis). The protein concentration was determined using the Bradford method with bovine serum albumin as standard [28].

2.4. CV026 agar-well diffusion assay

Enzyme activity was assessed using the *Chromobacterium subtsugae* CV026 biosensor assay, as previously described [29]. Purified ST1-YtnP enzyme (100 μg/ml) was preincubated with 10 μM C8-HSL in 20 mM Tris pH 7 for 24 h at 30°C. The reaction mixtures were then added to wells on LB agar (1.7 % w/v) surrounded by LB soft-agar (0.8 % w/v) and inoculated with *C. subtsugae* CV026 (0.6 % v/v). After 24 h of incubation at 30°C, the reduction in diameter of purple pigment violacein around the wells with ST1-YtnP lactonase compared to the positive control (10 μM C8-HSL in 20 mM Tris pH 7) indicates the AHL-degrading activity of ST1-YtnP lactonase.

2.5. pH and thermal activity

The pH and thermal activity of ST1-YtnP lactonase (final concentration of 100 μg/ml) were evaluated. The enzyme was incubated with buffers ranging from pH 4–9 at 30°C for 1 h. To assess activity during exposure to on different temperatures, the enzyme was preincubated in 20 mM Tris buffer (pH 7) at different temperatures (4°C to 90°C) for 1 h. The remaining enzyme activity was measured using the CV026 assay.

2.6. Validation of lactone hydrolysis

Lactone hydrolysis by ST1-YtnP lactonase was confirmed by LC-MS/MS. Purified ST1-YtnP enzyme (100 μg/ml) was incubated with 1 mM C8-HSL and C10-HSL for 2 h at 30°C. The reaction mixture was diluted with LC-MS methanol and then filtered through 0.2 μm PTFE syringe filters and analyzed using a Vanquish™ Core HPLC system coupled to a TSQ FortisTM Plus triple quadrupole mass spectrometer (Thermo Fisher Scientific). The mobile phase consisted of solvent A, water with 0.1 % formic acid, and solvent B, acetonitrile with 0.1 % formic acid. The gradient chromatography procedure began with an isocratic hold at 10 % solvent B for 3 min, followed by a linear increase to 90 % over 10 min. This was followed by an isocratic hold at 90 % solvent B for 5 min and a 3-minute equilibration at 10 % solvent B. AHLs and their degradation products were identified using a modified method by Patel and colleagues [30]. Detection of MS/MS spectra for HSL and its degradation products was conducted in product ion scan mode with a collision energy of 12 V, using the following ionization parameters: 3700 V positive spray voltage, 40 arbitrary units (arb) sheath gas flow rate, 10 arb aux gas flow rate, 300°C ion transfer tube temperature and 350°C vaporizer temperature. The observed molecular ions and their respective masses are listed in Table 2. HSLs were distinguished by the presence of the characteristic 102.06 *m/z* lactone ring fragment, while degradation products were recognized by the presence of the 120 *m/z* fragment indicating a hydrolyzed lactone ring.

Table 2
Homoserine lactones used for LC-MS/MS, their molecular ions, and masses.

Compound name	Molecular ion [M+H] (*m/z*)	Identifying fragment (*m/z*)	Collision energy (V)
C8-HSL standard	228.16	102.06	12
C10-HSL standard	256.19	102.06	12
C8-HSL hydrolysis product	246.16	120.06	12
C10-HSL+hydrolysis product	274.19	120.06	12

2.7. Fluorescence microscopy

The effect of the recombinant ST1-YtnP lactonase on MDR *P. aeruginosa* MMA83 biofilm formation was analyzed using fluorescence microscopy (ZEISS Axioscope 5, Germany, 200 × magnification). ST1-YtnP lactonase (final concentration 100 μg/ml) resuspended in MH medium containing the culture of *P. aeruginosa* MMA83 (adjusted to 10^5 CFU/ml) was added to the 24-well plate with sterile glass slides (Sarstedt, Germany) and incubated for 24 h at 37°C. The control contains the same amount of 20 mM Tris pH7 with 50 % glycerol instead of ST1-YtnP lactonase. After incubation, the planktonic cells were carefully rinsed three times with sterile phosphate-buffered saline 1xPBS. Biofilms were stained with 2.5 μM propidium iodide and 2.5 μM SYTO9 (Thermo Fisher Scientific) and visualized under a fluorescence microscope [31].

2.8. Anti-QS and antivirulence potential of ST1-YtnP lactonase

RT-qPCR was used to measure the impact of ST1-YtnP lactonase on the mRNA levels of QS-related genes (*lasR, lasI, rhlR, rhlI, mvfR, pqsA, pqsH*) and virulence factors (*lasB, phzM, rhlC, algK, pvdS*) in *P. aeruginosa* MMA83. The primers of the selected genes used in this work are listed in Table 3. RT-qPCR analysis was performed using a LineGene 9600 Plus Real Time Thermocycler (Bioer, China). The *rpsL* gene was used as an endogenous control to normalize obtained data using the $2^{-\Delta\Delta CT}$ method [32]. The analysis was performed in duplicate and repeated twice.

2.9. Toxicity and infection model in Caenorhabditis elegans

The *C. elegans* mutant strain AU37 (*glp-4* (bn2) I; *sek-1* (km4)X) with temperature-sensitive sterility and increased sensitivity to pathogens obtained from the Caenorhabditis Genetics Center (CGC) was used to evaluate the toxicity of ST1-YtnP lactonase and the effect on

Table 3
List of the primers used in this study.

Gene	Primer name	Sequence (5′-3′)	Length (bp)	Source
lasI	lasI_Fw	GCGTGCTCAAGTGTTCAAGG	125	[29]
	lasI_Rev	GGGCTTCAGGAGTATCTTCCTGG		
lasR	lasR_Fw	CTGTGGATGCTCAAGGACTAC	133	[29]
	lasR_Rev	AACTGGTCTTGCCGATGG		
rhlI	rhlI_Fw	CCATCCGCAAACCCGCTACATC	151	[29]
	rhlI_Rev	CTCCCAGACCGACGGATCGCTCGGC		
rhlR	rhlR_Fw	GGGCGTGTTCGCCGTCCTGG	143	[29]
	rhlR_Rev	GGTATCGCTCCAGCCAGGCCTTG		
pqsA	pqs_Fw	GACCGGCTGTATTCGATTC	74	[29]
	pqs_Rev	GCTGAACCAGGGAAAGAAC		
mvfR	mvfR_Fw	GTCGGGACGGCTACAAGGTCG	121	[29]
	mvfR_Rev	GATTGCGCGGACCCTTGTTGAG		
pqsH	pqsH_Fw	AGGCGAACGAGGGTATTCCT	149	[6]
	pqsH_Rev	TCAGTGGGAATCGCCCTG		
lasB	lasB_Fw	CGCAGCGTGGAGAACGCCTA	183	[6]
	lasB_Rev	GTCGGAGAACGCTTCGTTCA		
algK	algK_Fw	GGCAAGTGGCTGGCGGCCAA	178	[6]
	algK_Rev	ACTGGTCGATGCGCTGCTGCG		
phzM	phzM_Fw	CCGCGACATGGTGCTGTTCTA	170	[6]
	phzM_Rev	TTCATCGCCAGCAGGAAGCG		
rhlC	rhlC_Fw	TTCCTGCCGGCCATCCATCTCG	139	[6]
	rhlC_Rev	AAGTGGCCGAGGCGCTGGTAG		
pvdS	pvdS_Fw	TACCTGTTCCAGATCGTCCGC	136	[6]
	pvdS_Rev	TTGATGTGCGAGGTTTCCGGC		
rpsL	rpsL_Fw	GCAACTATCAACCAGCTGGTG	231	[29]
	rpsL_Rev	GCTGTGCTCTTGCAGGTTGTG		

P. aeruginosa MMA83 virulence as previously described [6].

To study toxicity, purified recombinant ST1-YtnP lactonase (ranging from 50 μg/ml to 1000 μg/ml) was added to the well containing 10–20 synchronized L4 larval stage of AU37 worms resuspended in M9W medium. The same amount of 20 mM Tris pH 7 was used as a control. The 96 wells were incubated at 25°C with aeration (100 rpm). Worm survival was monitored for 36 h.

To investigate the ability of ST1-YtnP lactonase to reduce the virulence of MMA83 the liquid killing assay was employed [33]. MMA83 in the early exponential growth phase (standardized to 10^6 CFU/ml) was treated with ST1-YtnP lactonase (100 μg/ml). In the control, OP50 was added instead of MMA83. The 10–20 synchronized L4 larval stage of worms were added to the 96 wells in the same amount as a mixture of bacteria and ST1-YtnP lactonase and incubated at 25°C with aeration (100 rpm). Worm survival was monitored for 36 h. The survival rate was determined based on movement; needle sharp and immobile worms are considered dead.

Ethical permit is not required for invertebrate animal models such as *C. elegans*.

2.10. Checkerboard method

2.10.1. In vitro

The combinatorial effect of ST1-YtnP lactonase with meropenem or gentamicin was analyzed using the checkerboard method. The minimal inhibitory concentrations (MICs) of ST1-YtnP against *P. aeruginosa* MMA83 were determined using the 96-well microdilution method. The checkboard method was performed with serially diluted non-MIC concentrations of gentamicin (8, 4, 2, 1, 0.5 mg/ml) or meropenem (0.25, 0.125, 0.0625, 0.0312 mg/ml) in Muller Hinton broth (MH) in combination with different non-MIC concentrations of ST1-YtnP lactonase (0.2, 0.1, 0.05, 0.025 mg/ml). The bacterial suspension of MMA83 (adjusted to 10^6 CFU/ml) was added, and the 96-well plate was incubated for 24 h at 37°C. The resazurin-based colorimetric assessment was used to determine the results. Fractional inhibitory concentrations (FICs) on MMA83 were determined using the checkerboard method as previously described [29]. Experiments were performed in triplicate.

2.10.2. In vivo

C. elegans mutant strain AU37 was used to study the combinatorial effect of ST1-YtnP lactonase with meropenem and gentamicin *in vivo*. For this purpose, *C. elegans* AU37 was synchronized to the L4 stage, and 10–20 synchronized L4 larvae, resuspended in M9W medium, were added to a 96-well plate in the same volume as SK medium containing a mixture of *P. aeruginosa* MMA83 (standardized to 10^6 CFU/ml), antibiotics separately at their MIC concentrations, and in combination with ST1-YtnP lactonase. The positive control consisted of *C. elegans* AU37 incubated with MMA83 without any antimicrobials, while the negative control consisted of *C. elegans* incubated with OP50. Immediately after setting up the experiment, the 96-well plate was incubated at 25°C with aeration (100 rpm), and the worms were monitored for 36 h.

2.11. Statistics

Statistical analysis and data visualization were performed using GraphPad Prism 9 software. All results are expressed as means ± standard deviations. The differences between the control and experimental groups were assessed using Student's *t*-test. Survival curve differences in *C. elegans* assays were analyzed using the Kaplan-Meier method followed by the log-rank (Mantel-Cox) test. A p-value of < 0.05 was considered statistically significant.

3. Results

3.1. In silico analysis, cloning, expression and purification of ST1-YtnP lactonase

The ST1-YtnP lactonase originates from the species *Bacillus licheniformis*, which was isolated from the Vranjska Banja hot springs in Southern Serbia. The draft genome sequence has been deposited in the NCBI GenBank database under the accession number *Bacillus licheniformis* ST1 JAHZOI000000000 [34].

The ST1-YtnP lactonase is composed of 281 amino acid residues. Using the ProtParam tool, the molecular weight of ST1-YtnP lactonase was calculated to be 31.767 kDa, with an instability index (II) of 35.07, and a Grand Average of Hydropathicity (GRAVY) score of 0.461. The general structural characteristics of ST1-YtnP lactonase predicted by PSIPRED are presented in Table 4. Additional structural features of ST1-YtnP lactonase were predicted by PSIPRED, as shown in Fig. 1. The signal peptide in the amino acid sequence of ST1-YtnP lactonase was not predicted by the SignalP or PrediSi software. PSIPRED predicted that ST1-YtnP is a transmembrane protein with an N-terminal extracellular domain and a C-terminal cytoplasmic domain, with the majority of amino acid residues located extracellularly. These findings are in line with the absence of a signal peptide in the amino acid sequence of ST1-YtnP lactonase, suggesting that the enzyme is not secreted into the extracellular environment (Fig. 1A). PSIPRED was also used for the prediction of the secondary structure of ST1-YtnP lactonase (Fig. 1B) with results consistent with the prediction of Phyre2 software, which detected the presence of alpha-helices (14 %), beta-sheets (28 %), and a transmembrane helix (6 %) (Fig. S1). Tertiary structure analysis using the SWISS-MODEL algorithm indicated a monomeric configuration (Fig. 1C). The 3D structure of the ST1-YtnP lactonase from *Bacillus licheniformis* was also predicted, using I-TASSER (Fig. S2). The identified HXHXDH motif, with highly conserved histidine and aspartic acid residues in the active site of the metallo-β-lactamase superfamily (MBL), suggests that ST1-YtnP lactonase from *Bacillus licheniformis* belongs to this protein family.

The phylogenetic relatedness analysis shows that ST1-YtnP lactonase clusters with YtnP-ZP1 lactonase from *Bacillus paralicheniformis* (98 % similarity) [35], YtnP from *Bacillus licheniformis* (95 % similarity) [36], and YtnP lactonase from *Stenotrophomonas maltophilia* 6960 (43 % similarity) [6] (Fig. 2). Multiple sequence alignment analysis of ST1-YtnP lactonase from *Bacillus licheniformis*, YtnP-ZP1 from *B. paralicheniformis* [UTJ93925.1], YtnP from *S. maltophilia*, and YtnP from *B. licheniformis* [A0A516FJX9] revealed a notable pattern regarding the N-terminal region. Specifically, lactonases exhibiting higher thermostability (*S. maltophilia* and *B. licheniformis*

Table 4
General characteristics of ST1-YtnP lactonase as predicted by PSIPRED.

Feature name	Value
Aliphatic index	82.31
AtomC	0.32
AtomH	0.49
AtomN	0.09
AtomO	0.1
AtomS	0.0
Charge	−3.39
Fraction negative residues	0.14
Fraction positive residues	0.1
Hydrophobicity	−0.46
Isolelectric point	6.04
Length	281.0
Molar extinction coefficient	65290.0
Molecular weight	31767.6
Number of atoms	4427.0
Surface area	54124.0
Volume	38033.7

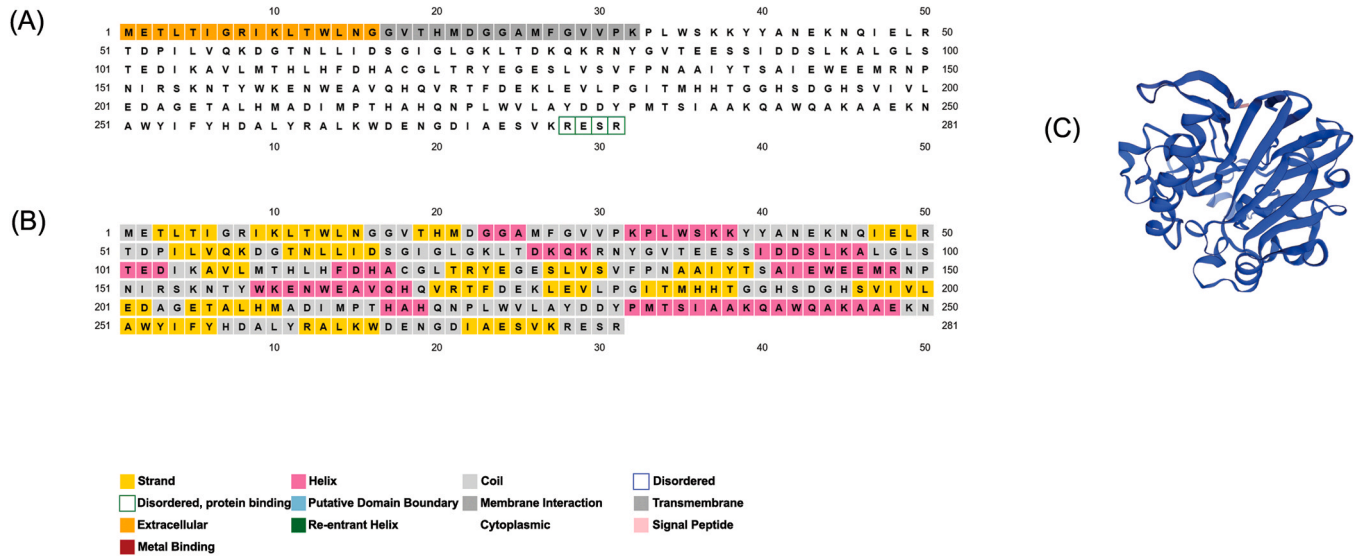

Fig. 1. In silico analysis of amino acid ST1-YtnP sequence. Results obtained by PSIPRED. (A) Prediction of ST1-YtnP amino acid residues position; (B) Secondary structures of ST1-YtnP lactonase; (C) Tertiary structure predicted by SWISS-MODEL algorithm.

[A0A516FJX9]) shared a common absence of several amino acid residues in their N-terminal domain (Fig. S3). In contrast, lactonases with moderate thermostability (ST1-YtnP and YtnP-ZP1) retained the complete N-terminal sequence. This structural variation suggests that the truncation or absence of the N-terminal region may contribute to increased thermal stability of these enzymes.

Cloning and expression of ST1-YtnP was performed using pQE30 expression system. The resulting construct in the pQE30 expression vector pQE30ST1-YtnP was produced as an inclusion body, thus the protein was purified under denaturing conditions using urea gradient. SDS-PAGE analysis (Fig. S4) showed that ST1-YtnP lactonase, purified and refolded using a urea gradient, migrated with an apparent molecular mass of approximately 31 kDa. AHL-degrading activity of ST1-YtnP lactonase was checked using *C. subtsugae* CV026 agar well-diffusion assay. The purified recombinant ST1-YtnP enzyme was found to effectively degrade C8-HSL, as evidenced by the reduction in the diameter of the purple pigment violacein around the well compared to the control (Fig. S5).

3.2. Confirmation of ST1-YtnP lactonase activity

Confirmation of ST1-YtnP lactonase activity is crucial for understanding the biological function of the enzyme and its potential practical applications in inhibiting cell-to-cell communication. The activity of ST1-YtnP lactonase was confirmed by LC-MS/MS with two AHLs (C8-HSL and C10-HSL). The peak at 102.12 *m/z* in the LC-MS/MS chromatogram corresponds to the intact lactone ring, while the peak at 120.06 *m/z*, which represents the hydrolyzed lactone ring, indicates ST1-YtnP lactonase activity upon opening of the lactone ring. This peak was consistent for both lactones, suggesting that the enzyme is capable of degrading AHLs, particularly C8-HSL and C10-HSL (Fig. S6).

3.3. pH and thermal activity of ST1-YtnP lactonase

The optimal pH for ST1-YtnP activity was in the range of 6.5–7.5. The enzyme retained 100 % of its activity at pH values of 6.5, 7, and 7.5, while a 50 % reduction in activity was observed at pH 6. No enzymatic activity was detected at pH values below 6 or above 7.5. ST1-YtnP also showed a broad range of thermal activity, and retained its full activity (100 %) after exposure to temperatures ranging from 25 to 42°C. ST1-YtnP remained stable when preincubated at temperatures up to 50°C and retained 25 % of its hydrolysis rate at this temperature (Fig. S7).

3.4. Inhibition of biofilm formation of Pseudomonas aeruginosa MMA83

Fluorescence microscopy analysis shows a strong effect of ST1-YtnP lactonase on the prevention of biofilm formation (Fig. 3). Compared to the untreated control, biofilm formation of *P. aeruginosa* MMA83 was significantly inhibited in the presence of ST1-YtnP lactonase, resulting in a very loose biofilm and a decrease in confluence and density with more dispersed biofilm aggregates. Based on fluorescence images, no negative effect of ST1-YtnP lactonase on the growth of planktonic cells of *P. aeruginosa* MMA83 was detected.

3.5. Effect of ST1-YtnP lactonase on QS and virulence factors gene expression in Pseudomonas aeruginosa MMA83

The RT-qPCR results indicate that the recombinant ST1-YtnP lactonase significantly affects the relative mRNA level of genes involved in quorum sensing (QS) and virulence factors production in *P. aeruginosa* MMA83. Overall, the expression of all tested genes was significantly reduced in *P. aeruginosa* treated with ST1-YtnP lactonase.

Fig. 4A illustrates the effects of ST1-YtnP lactonase on genes involved in the QS system of *P. aeruginosa*. In detail, the three QS circuits -las, rhl, and pqs- were analyzed. The expression of the *lasI* gene, which is involved in AI synthesis within the las circuit, and the *lasR* gene, a transcriptional regulator, was reduced by around 2 and 4-fold, respectively, compared to the control. ST1-YtnP lactonase also caused a 2.5-fold reduction in the expression of the *rhlI* gene, which is responsible for C4-HSL production, and the *rhlR*, a response regulator.

A decrease in mRNA levels was observed for the *pqsA* gene, which is involved in PQS synthesis, and for *pqsH*, which is responsible for the conversion of HHQ (a precursor) into PQS, with reductions of 3-fold and 2-fold, respectively, compared to the control. Additionally, the presence of ST1-YtnP lactonase led to an approximately 6-fold decrease in the expression of *mvfR*, a transcriptional regulator.

The results show that the most substantial reduction (6-fold) of mRNA level occurred in the *lasB* gene, which encodes for elastase production. Additionally, in the presence of ST1-YtnP lactonase, the expression of the *rhlC* gene, involved in rhamnolipid production, and the *phzM* gene, associated with pyocyanin production, was reduced 3-fold compared to the control. The mRNA levels of the genes involved in alginate (*algK*) and pyoverdine (*pvdS*) production were also reduced by about 2-fold (Fig. 4B). The detailed statistical analysis was done in Supplementary Table S1.

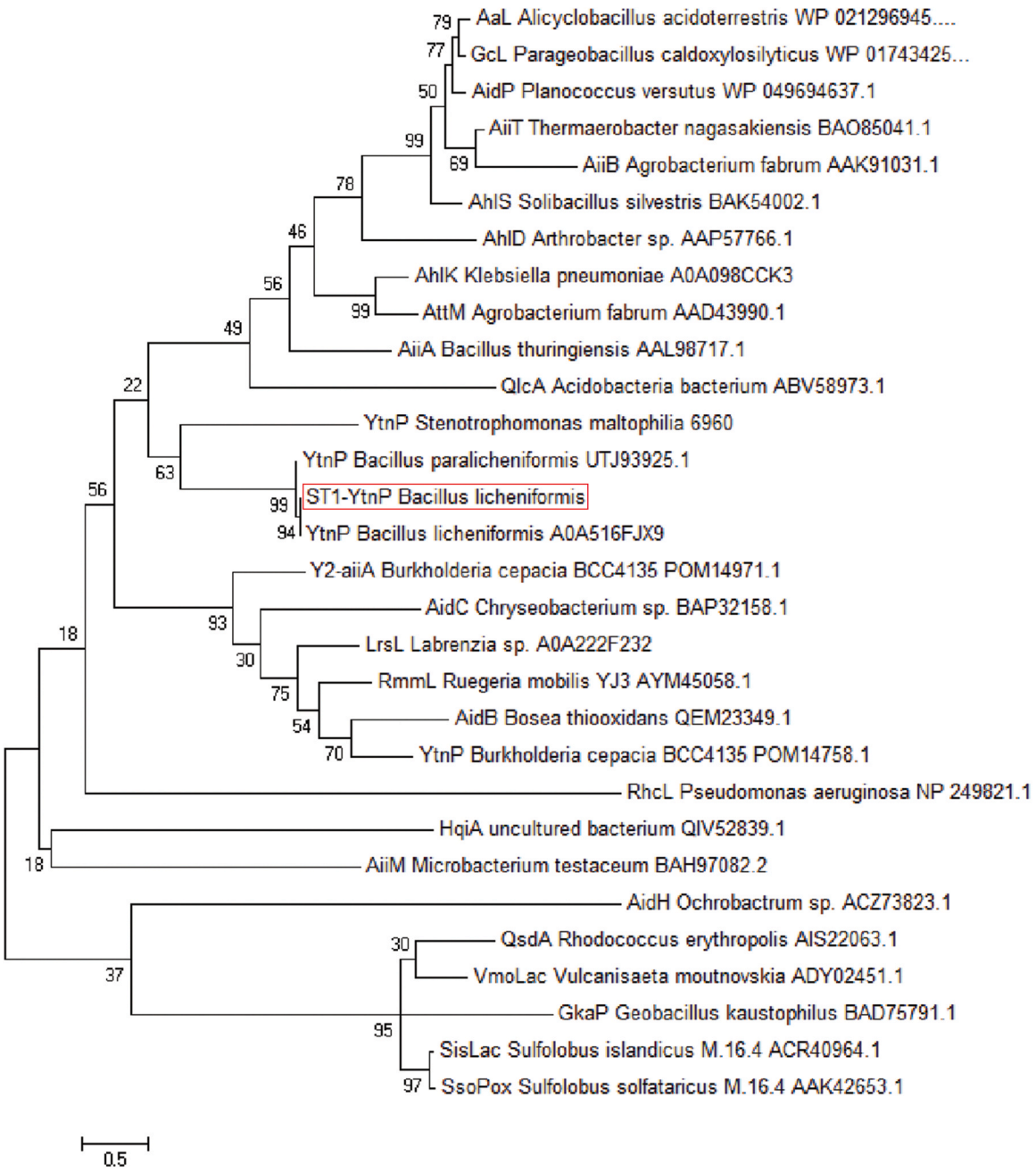

Fig. 2. Phylogenetic tree was obtained by Mega 11. The dendrogram was constructed by the Maximum-likelihood method.

3.6. Toxicity and in vivo examination of antivirulence activity of ST1-YtnP lactonase

The toxicity of ST1-YtnP lactonase was evaluated using the *C. elegans* AU37 model system. The results indicate that ST1-YtnP lactonase has no toxic effects on *C. elegans* (*glp-4; sek-1*) at concentrations from 50 to 1000 μg/ml (Fig. 5A). The addition of ST1-YtnP lactonase had no significant effect on *C. elegans* survival, indicating that the recombinant ST1-YtnP lactonase is not toxic, as shown by the lack of harmful effects on this *C. elegans* strain.

To validate the antivirulence potential of ST1-YtnP lactonase against *P. aeruginosa* MMA83, slow killing assays were performed on *C. elegans*. For this purpose, *C. elegans* AU37 (*glp-4; sek-1*) was used as a model, and the results regarding their survival are shown in Fig. 5B. In a toxin-mediated liquid killing assay, the survival rate of *C. elegans* AU37 mutant strain infected with the clinical isolate *P. aeruginosa* MMA83 increased significantly when treated with ST1-YtnP lactonase compared to untreated controls. Interestingly, there is a 100 % survival rate of *C. elegans* infected with *P. aeruginosa* and treated with ST1-YtnP, while survival rate of worms infected with *P. aeruginosa* alone decreases significantly, dropping to 40 % after 12 h and reaching 0 % after 24 h. The detailed statistical analysis was done in Supplementary Table S1.

3.7. The combined effect of ST1-YtnP lactonase and antibiotics on MIC values of P. aeruginosa MMA83

3.7.1. In vitro

To assess the therapeutic potential of purified recombinant ST1-YtnP lactonase, the efficacy of clinically significant antibiotics in combination with ST1-YtnP lactonase was investigated using the checkerboard method. The effect of ST1-YtnP lactonase was classified as synergistic if the sum of the FICs of antibiotic and ST1-YtnP lactonase (ΣFIC) was

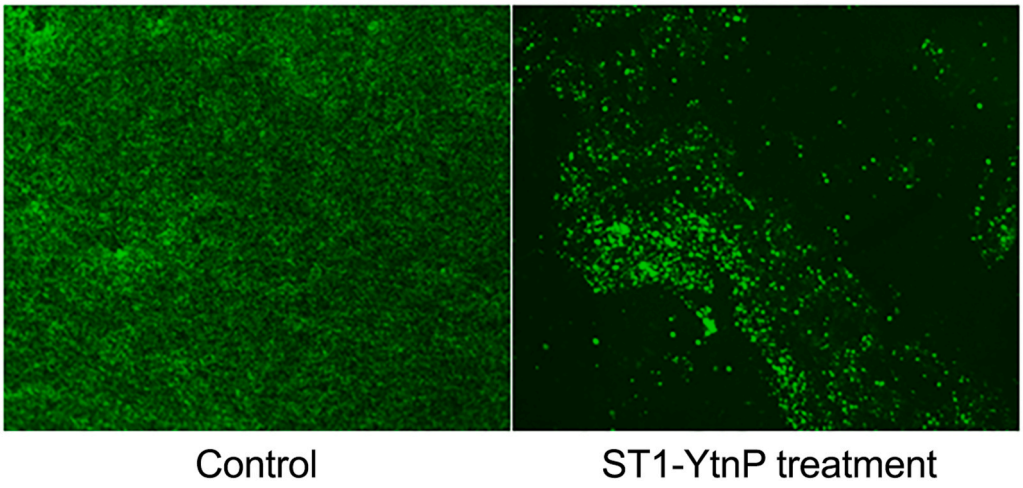

Fig. 3. The effect of YtnP lactonase on biofilm formation of *P. aeruginosa* MMA83. Fluorescence micrographs (200x magnification) show the inhibitory effect of recombinant ST1-YtnP lactonase on biofilm formation. Results were compared with the untreated *P. aeruginosa* MMA83 control.

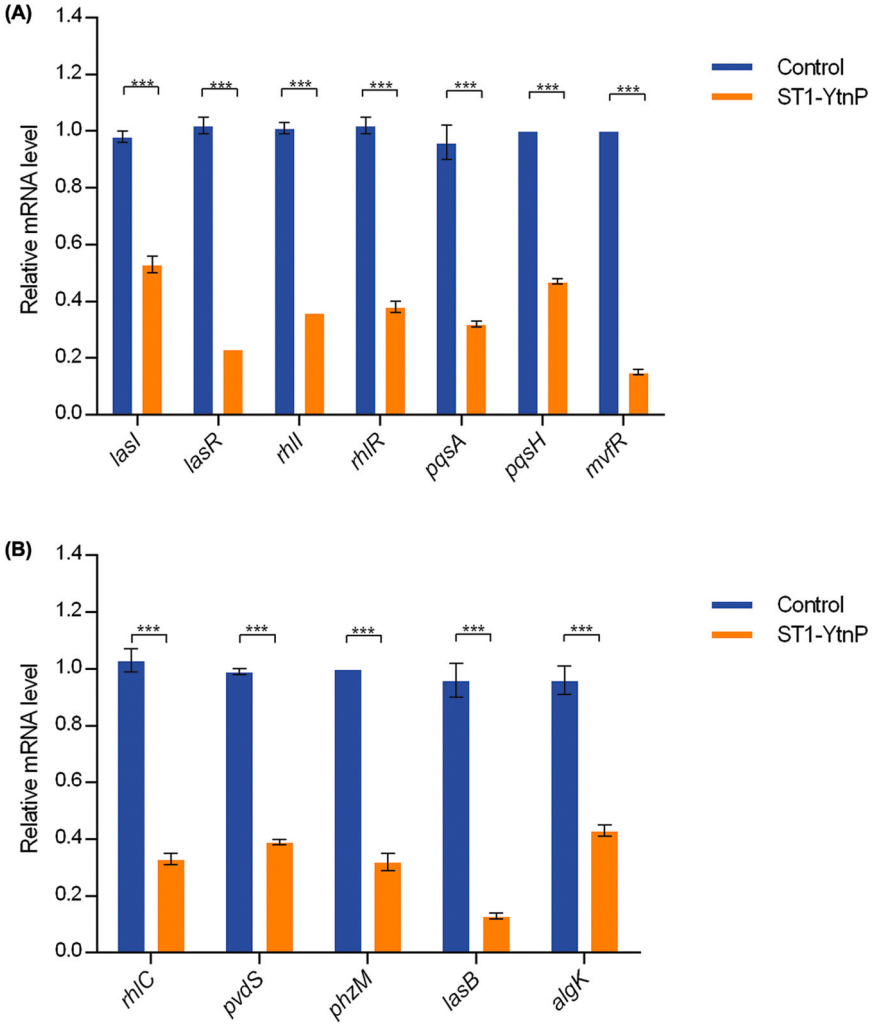

Fig. 4. The effect of ST1-YtnP lactonase on transcription of QS and virulence factors genes of *P. aeruginosa* MMA83. (A) Relative mRNA expression levels of key genes involved in the QS regulatory network (*lasI*, *lasR*, *rhlI*, *rhlR*, *pqsA*, *pqsH*, and *mvfR*) in *P. aeruginosa* MMA83 treated with ST1-YtnP lactonase, compared to untreated control. (B) Relative mRNA levels of representative virulence factor genes (*rhlC*, *pvdS*, *phzM*, *lasB*, and *algK*) under the same treatment conditions. The data represent the mean ± standard deviation (SD) of duplicates repeated twice (N = 4). Statistical significance between control and treated groups was assessed using an unpaired two-tailed Student's *t*-test with Welch's correction. A significant reduction in transcript levels was observed in all tested genes upon ST1-YtnP treatment (***$p < 0.001$), indicating strong inhibition of QS signaling and virulence factors gene expression.

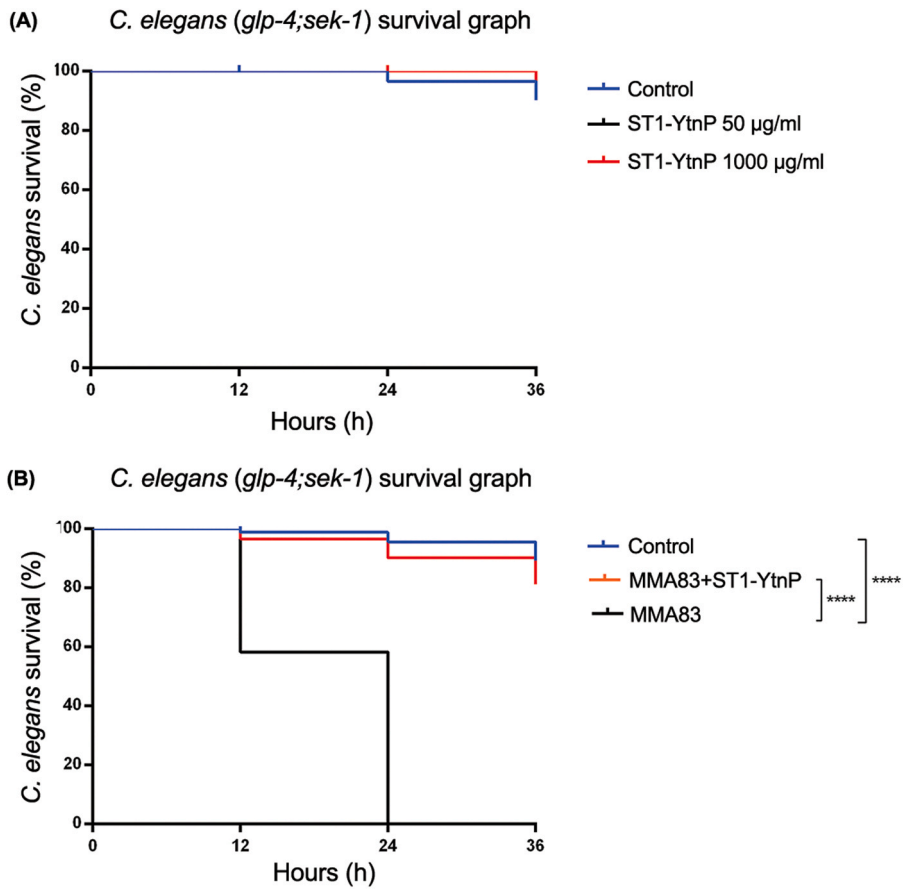

Fig. 5. Kaplan–Meier survival curves of *C. elegans* AU37 (glp-4; sek-1) in toxicity and infection assays with ST1-YtnP lactonase. (A) Toxicity assessment of ST1-YtnP lactonase. No statistically significant reduction in survival was observed in ST1-YtnP-treated nematodes compared to the untreated control group, indicating that ST1-YtnP lactonase is not toxic in the concentrations ranging from 50 to 1000 µg/ml. (B) Antivirulence effect of ST1-YtnP lactonase in *C. elegans* AU37 infected with *P. aeruginosa* MMA83. A statistically significant increase in survival was observed in the group treated with ST1-YtnP compared to the untreated infected group (****$p < 0.0001$) indicating the protective effect of the lactonase. Survival assays were performed in triplicate and repeated independently twice.

≤ 0.5, additive if $0.5 < \Sigma FIC \leq 1$, indifferent if $1 < \Sigma FIC < 4$, and antagonistic if $\Sigma FIC > 4$. The combination showed a synergistic effect with gentamicin ($\Sigma FIC = 0.312$) and an additive effect with meropenem ($\Sigma FIC = 0.75$) (Table 5). These findings suggest that ST1-YtnP lactonase could enhance the efficacy of antibiotics and thus possibly contribute to the elimination of infections caused by MMA83.

3.7.2. *In vivo*

The *C. elegans* infection model was used to evaluate the toxicity of gentamicin and meropenem and their combinatorial effect with ST1-YtnP lactonase against *P. aeruginosa* MMA83. The results showed that meropenem alone and in combination with lactonase did not significantly affect the survival of *C. elegans* after 12 and 24 h. As shown in Fig. 6A, meropenem exhibited no toxicity to *C. elegans* after 12 and 24 h, while meropenem alone resulted in approximately 50 % mortality at its MIC concentration after 36 h. In contrast, the combination of meropenem and ST1-YtnP lactonase was not toxic even after 36 h.

As shown in Fig. 6B, *C. elegans* infected with *P. aeruginosa* MMA83 exhibited approximately 40 % mortality after 12 h, while worms treated with meropenem alone or in combination with ST1-YtnP lactonase survived almost completely. After 24 h, untreated *C. elegans* infected with MMA83 had a mortality rate of 100 %. At the same timepoint (after 24 h), approximately 70 % of *C. elegans* infected with MMA83 survived when treated with meropenem alone, while the combination of meropenem and ST1-YtnP lactonase resulted in a survival rate of almost 100 %. After 36 h, the survival rate of worms treated with meropenem alone declined to approximately 20 %, while worms treated with the combination of meropenem and ST1-YtnP lactonase survived at nearly 100 %. These results indicate that the combination of meropenem and ST1-YtnP lactonase significantly improves survival of *C. elegans* and provides protection against mortality caused by MMA83. The detailed statistical analysis was done in Supplementary Table S1.

In contrast, gentamicin exhibited significant toxicity to *C. elegans* at its MIC concentration, and reduced survival by approximately 50 % after 12 h, with complete lethality observed after 24 h. Furthermore, the combination of gentamicin with ST1-YtnP lactonase resulted in mortality of more than 50 % of *C. elegans* after 24 h, with complete lethality occurring by 36 h (Fig. S8). The detailed statistical analysis was done in Supplementary Table S1.

Table 5
Checkerboard method showing the effect of ST1-YtnP, meropenem, and gentamicin against *P. aeruginosa* MMA83 *in vitro*.

	P. aeruginosa MMA83				
	MIC of each antimicrobial (mg/ml)				
Antimicrobials	Alone	Combination	FIC	∑FIC	Outcome
ST1-YtnP	0.4	0.100	0.25	0.75	additive
Meropenem	0.5	0.250	0.50		
ST1-YtnP	0.4	0.025	0.06	0.31	synergistic
Gentamicin	16.0	4.000	0.25		

FIC – fractional inhibitory concentration, FIC = MIC combination / MIC alone
∑FIC – sum of two FICs, ∑FIC = FIC of antibiotic + FIC of ST1-YtnP lactonase

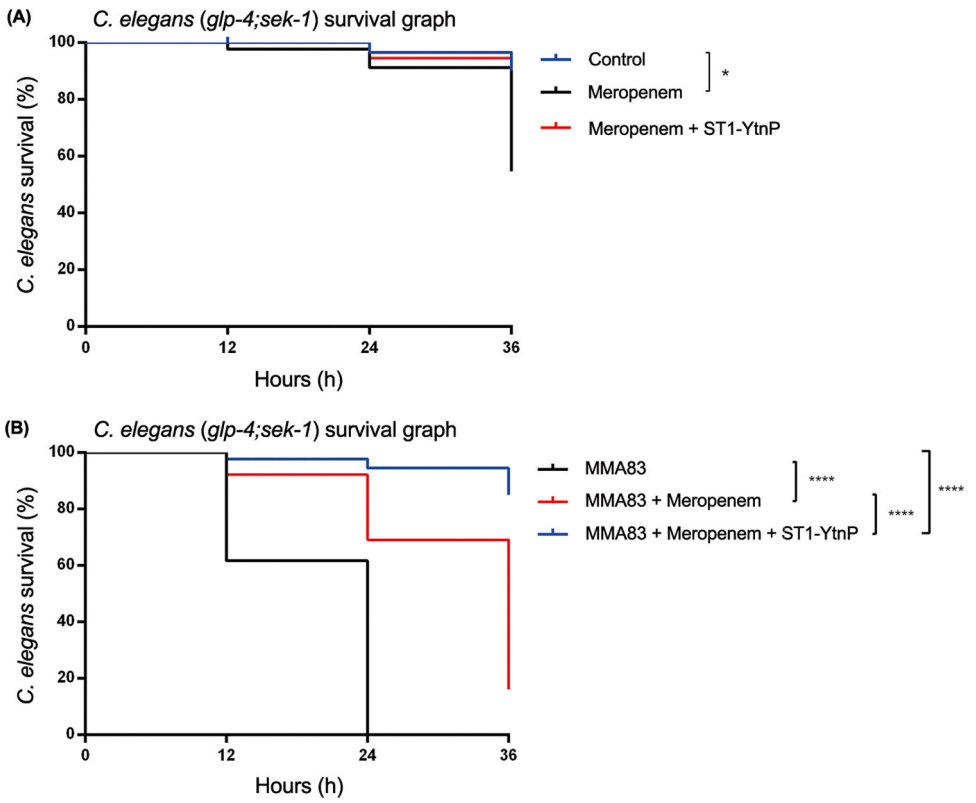

Fig. 6. Kaplan–Meier survival analysis of *C. elegans* AU37 (glp-4; sek-1) in response to meropenem and ST1-YtnP lactonase treatment. (A)Toxicity assessment of meropenem and its combination with ST1-YtnP lactonase in *C. elegans* AU37. A slight, but statistically significant reduction in worm survival was observed in the meropenem-treated group compared to the untreated control (*$p < 0.05$). The combination of meropenem with ST1-YtnP lactonase resulted in improved *C. elegans* survival compared to meropenem alone. (B) Survival analysis of *C. elegans* AU37 infected with *P. aeruginosa* MMA83 and treated with meropenem alone, or meropenem in combination with ST1-YtnP lactonase. Infection with MMA83 alone led to a significant reduction in worm survival. Treatment with meropenem significantly improved survival (****$p < 0.0001$), and in combination with ST1-YtnP lactonase further enhanced survival rates, showing a highly significant difference compared to both the untreated and meropenem-only treated groups (****$p < 0.0001$).

4. Discussion

In the last few decades, the demand for new effective biocatalysts has increased, particularly those with greater stability and resistance to support a more sustainable, economically profitable, and bio-based economy [37]. Fortunately, there are many extreme environments in nature with as yet unexplored microorganisms that represent an excellent source of extremozymes. Thanks to their exceptional properties, extremozymes could have a large impact from a commercial and biotechnological perspective. However, despite their potential, extremozymes are still relatively rare, emphasizing the need for further research to fully exploit their capabilities [38]. Thermophilic microorganisms inhabiting hot areas can be classified into three categories: moderate thermophiles, extreme thermophiles, and hyperthermophiles with optimal growth temperatures of 50–60°C, 60–80°C, and 80–110°C, respectively. They are a great source of biocatalysts with unusual properties, such as increased stability and efficiency [39].

B. licheniformis is a thermophilic bacterium isolated from the Vranjska Banja hot spring. The Vranjska Banja hot springs are located in the Southern Serbia and with a water temperature of 63–95°C, are considered the hottest thermal springs in Europe, besides the Icelandic springs [34]. Given the global challenge of MDR infections and the limitations of conventional antibiotics, this study aimed to identify new antivirulence enzymes from extremophilic bacteria *B. licheniformis*. The novel ST1-YtnP lactonase was identified, characterized, and evaluated for its antivirulence potential to provide a basis for innovative therapeutic strategies against pathogenic bacteria.

In silico analysis revealed that ST1-YtnP lactonase belongs to the MBL superfamily, which is characterized by the conserved HXHXDH motif, common to most described lactonases [40]. The ability of ST1-YtnP to degrade AHLs, the QS signaling molecules in *P. aeruginosa*, underlines its potential as an antivirulence agent.

Recent studies have identified several lactonases from extremophiles with promising QQ activity. The lactonase from *Thermobacter mariaensis* is highly thermostable, providing activity at high temperatures [41]. Similarly, the AHL lactonase from *B. licheniformis* DAHB1 has been noted for its effective inhibition of *Vibrio* biofilm formation and colonization [36]. Additionally, MzmL lactonase from *Mesoflavibacter zeaxanthinifaciens*, another extremophile, exhibits unique properties related to its enzymatic activity and stability [42]. Enzymes originating from extreme environments exhibit some valuable properties, such as stability under harsh conditions including activity at high temperatures, extreme pH, organic solvents, and high ionic strength. Their robustness makes them suitable for various biotechnological applications such as medical biotechnology, biomaterials, bioengineering, agricultural, and industrial biotechnology. In this study, the properties of the novel ST1-YtnP lactonase were thoroughly investigated and described from various perspectives to provide a comprehensive insight into its biotechnological potential. The pH stability of ST1-YtnP lactonase in a range of 6–8 and its moderate thermostability between 25 and 42°C are consistent with the literature on YtnP-ZP1 lactonase which almost completely maintains its activity between 25 and 40°C [35]. Interestingly, sequence alignment revealed that both ST1-YtnP and a non-thermostable YtnP homolog from *B. paralicheniformis* retain a complete N-terminal region, in contrast to the thermostable lactonase sequences from *S. maltophilia* and *B. licheniformis* [A0A516FJX9], which lack this part of the protein [6,36]. The absence of the N-terminal region in these thermostable lactonases could contribute to their increased

thermal stability by enabling a more compact and stable protein structure. Moreover, it is important to emphasize that enzymes originating from thermal environments do not necessarily exhibit high thermostability [43,44]. Conversely, it has been shown that enzymes from mesophilic organisms can exhibit considerable thermostability due to specific structural and functional adaptations [45,46]. Altogether, these data justify the moderate thermostability of ST1-YtnP despite its origin from a thermal source and its phylogenetic relatedness to thermostable YtnP lactonases from *B. licheniformis* [A0A516FJX9] and *S. maltophilia*.

In this study, we used as a model system MDR clinical isolate *P. aeruginosa* MMA83, a New Delhi metallo-β-lactamase-producer, whose phenotype correlates with a severe outcome of infection [16]. Obtained results demonstrated that ST1-YtnP possesses antibiofilm and antivirulence activity, evidenced by reduced relative mRNA levels of QS and virulence-related genes. It was shown that ST1-YtnP lactonase significantly prevents *P. aeruginosa* MMA83 biofilm formation, which is consistent with previous studies demonstrating the ability of lactonases to prevent biofilm formation and reduce the production of virulence factors in MDR strains of *P. aeruginosa* [6,29,35].

Initial assessments of novel compounds often rely on *in vitro* methods, which have their limitations, particularly in terms of toxicity, pharmacokinetics, and *in vivo* efficacy. Early *in vivo* testing is essential for the evaluation of efficacy and toxicity and helps to eliminate toxic compounds and optimize resources. *C. elegans* has become a valuable model in drug discovery, particularly for studying antimicrobial compounds [47]. The use of *C. elegans* aligns with the 3 R principles (Replacement, Reduction, Refinement) in scientific research by reducing reliance on vertebrates, enabling high-throughput studies with simple organisms due to its fast life cycle, and allowing non-invasive observation of biological processes due to its transparency [48]. Its use facilitates ethical and sustainable research. Although traditionally used to evaluate antibiotics, recent studies have highlighted the toxicity of clinical antibiotics in *C. elegans* and emphasized the need for safer treatments. However, its use in evaluating QQ enzymes like novel lactonases as antivirulence agents is still underexplored. Our study contributes to this gap by investigating the therapeutic potential of a newly identified lactonase from *B. licheniformis* in the *C. elegans* infection model. By focusing on both efficacy and toxicity, this research highlights the enzyme's promise as a safer alternative in fighting bacterial infections without the harmful side effects associated with conventional antibiotics. In this study, the toxicity of the novel QQ enzyme ST1-YtnP lactonase was investigated using the liquid killing assay. The purified recombinant ST1-YtnP lactonase showed no toxicity in the *C. elegans* model system. These results are consistent with the findings of previous studies on the lack of toxic effects of certain QQ enzymes on *C. elegans* and eukaryotic cells [6,29,35]. This is also in line with the tolerance of SsoPox lactonase in rat lung [49]. All together, these results indicate that recombinant ST1-YtnP lactonase can be considered safe to use. In addition, ST1-YtnP lactonase significantly prolonged the survival of *C. elegans* infected with *P. aeruginosa* MMA83. These results are supported by previous findings that QQ lactonases can prolong the lifespan of *C. elegans* [6] and rats infected with *P. aeruginosa* [49]. Since mortality in the liquid killing assay is primarily due to toxins secreted by the pathogen [50], it is likely that ST1-YtnP lactonase exerts its protective effect by reducing the production of these virulence factors. The protective effects of ST1-YtnP lactonase against MMA83-induced pathogenicity in *C. elegans*, coupled with its absence of toxicity, make ST1-YtnP lactonase a promising prophylactic agent with antivirulence properties.

In the face of increasing antibiotic resistance, which is a major challenge in human healthcare, QQ has emerged as a promising alternative for combating bacterial infections [51]. By targeting the QS system of pathogenic bacteria without exerting selective pressure, QQ enzymes offer a valuable tool against MDR pathogens. Disrupting cell-to-cell communication in pathogenic bacteria and disabling their virulence not only strengthens the host's defense mechanisms but also enables the immune system and normal microbiota to prevent colonization and eliminate infections. The synergistic and additive effects observed between ST1-YtnP lactonase and gentamicin and meropenem are in line with previous studies that have shown that the QQ enzymes can improve the efficacy of antibiotics [6,29,35], suggesting that these enzymes could be integrated into existing treatment regimens to improve outcomes in infections caused by *P. aeruginosa*.

C. *elegans* is a well-established *in vivo* model for evaluating combinatorial antimicrobial therapies, due to its sensitivity to bacterial virulence, conserved innate immunity, and suitability for high-throughput survival assays [52,53]. This model has been used to investigate the QS inhibition, bacterial biofilm disruption, and survival outcomes in infections caused by *P. aeruginosa*, and other clinically relevant pathogens [54]. To our best knowledge, no previous studies have reported direct toxic effects of antibiotics on *C. elegans*. The observed toxicity of gentamicin on *C. elegans* may result from the high antibiotic concentrations required due to the resistance of the strain. Given the extreme resistance of MMA83 to gentamicin, which exceeds the EUCAST breakpoints by more than 1,000-fold ($\leq$ 4 μg/ml), the observed toxic effects of gentamicin at MIC and MIC 1/4 concentrations disabled its evaluation for *C. elegans* survival against MMA83 infection. Interestingly, a previous study demonstrated that gentamicin combined with phytochemicals improved the survival of *C. elegans* when infected with *P. aeruginosa* PAO1 [55], suggesting that gentamicin, in combination with ST1-YtnP lactonase, may be effective against less-resistant bacterial strains.

Additionally, the MIC of meropenem against *P. aeruginosa* MMA83 was approximately 200-fold higher than the EUCAST breakpoint ($\leq$ 2 μg/ml). While meropenem alone at MIC concentration significantly increased *C. elegans* survival, the combination of meropenem (MIC 1/2) and ST1-YtnP lactonase (100 μg/ml) completely rescued *C. elegans* from MMA83 infection. The prolonged lifespan of *C. elegans* infected with *P. aeruginosa* MMA83 treated with a combination of antibiotics and ST1-YtnP lactonase aligns with previous studies emphasizing the beneficial effects of QQ enzymes on host survival in bacterial infections [55–57]. Notably, supplementing meropenem with ST1-YtnP lactonase reduces the effective antibiotic concentration, and this combination may protect *C. elegans* from MMA83 infection.

The obtained results suggest that ST1-YtnP lactonase promotes the elimination of pathogenic bacteria and thus possibly restores the effectiveness of antibiotics against resistant strains. This approach could be especially valuable for the treatment of highly resistant strains such as MMA83. Our findings emphasize the importance of optimizing antibiotic dosing in host-pathogen interaction models to minimize unintended physiological effects. Furthermore, they highlight ST1-YtnP lactonase as a potent antivirulence agent with a potential for developing novel therapeutic strategies against *P. aeruginosa* infections when used in combination with antibiotics. Overall, our results support the therapeutic value of QQ-based approaches in combating bacterial infections and underscore the need for further research to optimize their application.

5. Conclusion

In conclusion, ST1-YtnP lactonase effectively disrupts QS and reduces virulence of MDR *P. aeruginosa* MMA83, positioning it as a promising antivirulence agent. Its ability to reduce biofilm formation further highlights its potential in combating biofilm-associated infections. Additionally, the combination of meropenem and ST1-YtnP lactonase significantly improved survival rate of *C. elegans* against MMA83, indicating the enzyme's potential to boost efficacy of antibiotics and combat resistant strains. These findings emphasize the potential of ST1-YtnP lactonase to restore the efficacy of antibiotics against resistant bacteria. While these results support the therapeutic value of QQ strategies, further *in vivo* studies are required to fully explore their clinical applications and integrate them into future antimicrobial therapies.

Author contributions

MM designed the study. JC and IM performed the experiments and did the analysis. JC wrote the initial version and MM revised the manuscript. All authors interpreted the data, critically read, reviewed, and agreed to publish the manuscript.

CRediT authorship contribution statement

Silvia Spriano: Writing – review & editing, Validation, Resources, Project administration, Formal analysis. **Branko Jovcic:** Writing – review & editing, Validation, Project administration, Investigation, Funding acquisition. **Danka Matijasevic:** Writing – review & editing, Visualization, Validation, Formal analysis, Data curation. **Milka Malesevic:** Writing – review & editing, Supervision, Resources, Investigation, Funding acquisition, Conceptualization. **Ivano Merendino:** Visualization, Methodology, Investigation, Formal analysis. **Jovana Curcic:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis.

Funding

This work was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia [Grant No. 451-03-136/2025-03/200042 and Grant No. 451-03-137/2025-03/200178].

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.

Acknowledgements

We are grateful to dr Brana Pantelic and dr Nemanja Stanisavljevic for their help.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2025.118443.

Data availability

Data will be made available on request.

References

[1] M.E. de Kraker, A.J. Marlieke, A.J. Stewardson, S. Harbarth, Will 10 million people die a year due to antimicrobial resistance by 2050? PLoS Med. 13 (11) (2016) e1002184 https://doi.org/10.1371/journal.pmed.1002184.

[2] S.H.O. Zarroug, J.S. Bajaman, F.N. Hamza, R.A. Saleem, H.K. Abdalla, *caenorhabditis elegans* as an *in vivo* model for the discovery and development of natural plant-based antimicrobial compounds, Pharmaceutics 16 (8) (2023) 1070, https://doi.org/10.3390/ph16081070.

[3] S.K. Ahmed, S. Hussein, K. Qurbani, R.H. Ibrahim, A. Fareeq, K.A. Mahmood, M. G. Mohamed, Antimicrobial resistance: impacts, challenges, and future prospects, J. Med. Surg. Public Health 2 (2024) 100081, https://doi.org/10.1016/j.glmedi.2024.100081.

[4] M. Gonzales, B. Kergaravat, P. Jacquet, R. Billot, D. Grizard, E. Chabriere, L. Plener, D. Daude, Disrupting quorum sensing as a strategy to inhibit bacterial virulence in human, animal, and plant pathogens, Pathog. Dis. 82 (2024) ftae009, https://doi.org/10.1093/femspd/ftae009.

[5] B. Rémy, L. Plener, P. Decloquement, N. Armstrong, M. Elias, E. Chabriere, Lactonase specificity is key to quorum quenching in *pseudomonas aeruginosa*, Front. Microbiol 11 (2020) 762, https://doi.org/10.3389/fmicb.2020.00762.

[6] J. Curcic, M. Dinic, K. Novovic, Z. Vasiljevic, M. Kojic, B. Jovcic, M. Malesevic, A novel thermostable YtnP lactonase from *stenotrophomonas maltophilia* inhibits *pseudomonas aeruginosa* virulence *in vitro* and *in vivo*, Int. J. Biol. Macromol. 264 (2024) 130421, https://doi.org/10.1016/j.ijbiomac.2024.130421.

[7] M. Gonzales, L. Plener, J. Armengaud, N. Armstrong, E. Chabriere, D. Daude, Lactonase-mediated inhibition of quorum sensing largely alters phenotypes, proteome, and antimicrobial activities in *burkholderia thailandensis* E264, Front. Cell. Infect. Microbiol 13 (2023) 1190859, https://doi.org/10.3389/fcimb.2023.1190859.

[8] E. Sendra, A.F. Munoz, L. Zamorano, A. Oliver, J.P. Horcajada, C. Juan, S. G. Zorilla, Impact of multidrug resistance on the virulence and fitness of *pseudomonas aeruginosa*: a microbiological and clinical perspective, Infection (2024) 1–34, https://doi.org/10.1007/s15010-024-02313-x.

[9] Z. Khatoon, C.D. McTiernan, E.J. Suuronen, T.F. Mah, Bacterial biofilm formation on implantable devices and approaches to its treatment and prevention, Heliyon 4 (12) (2018) e01067, https://doi.org/10.1016/j.heliyon.2018.e01067.

[10] S. Qin, W. Xiao, C. Zhou, Q. Pu, X. Deng, L. Lan, H. Liang, X. Song, M. Wu, *pseudomonas aeruginosa*: pathogenesis, virulence factors, antibiotic resistance, interaction with host, technology advances and emerging therapeutics, Signal Transduct. Target. Ther. 7 (1) (2022) 199, https://doi.org/10.1038/s41392-022-01056-1.

[11] Y. Zhang, Y. Li, J. Zeng, Y. Chang, S. Han, J. Zhao, Y. Fan, Z. Xiong, X. Zou, C. Wang, B. Li, H. Li, J. Han, X. Liu, Y. Xia, B. Lu, B. Cao, Risk factors for mortality of inpatients with *pseudomonas aeruginosa* bacteremia in China: impact of resistance profile in the mortality, Infect. Drug Resist (2020) 4115–4123, https://doi.org/10.2147/IDR.S268744.

[12] C. Burkhardt, L. Baruth, N.M. Heydecke, B. Klippel, A. Margaryan, A. Paloyan, H. H. Panosyan, G. Antranikian, Mining thermophiles for biotechnologically relevant enzymes: evaluating the potential of european and caucasian hot springs, Extremophiles 28 (1) (2024) 5, https://doi.org/10.1007/s00792-023-01321-3.

[13] K. Dumorne, D.C. Cordova, M.A. Elo, P. Renganathan, Extremozymes: a potential source for industrial applications, J. Microbiol. Biotechnol. 27 (4) (2017) 649–659, https://doi.org/10.4014/jmb.1611.11006.

[14] N.M. Mesbah, Industrial biotechnology based on enzymes from extreme environments, Front. Bioeng. Biotechnol. 10 (2022) 870083, https://doi.org/10.3389/fbioe.2022.870083.

[15] K.H. McClean, M.K. Winson, L. Fish, A. Taylor, S.R. Chhabra, M. Camara, M. Daykin, J.H. Lamb, S. Swift, B.W. Bycroft, G.S.A. Stewart, P. Williams, Quorum sensing and *chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones, Microbiology 143 (12) (1997) 3703–3711, https://doi.org/10.1099/00221287-143-12-3703.

[16] B. Jovcic, Z. Lepsanovic, V. Suljagic, G. Rackov, J. Begovic, Lj Topsirovic, M. Kojic, Emergence of NDM-1 metallo-β-lactamase in *pseudomonas aeruginosa* clinical isolates from Serbia, Antimicrob. Agents Chemother. 55 (8) (2011) 3929–3931, https://doi.org/10.1128/aac.00226-11.

[17] D. Hanahan, Studies on transformation of *escherichia coli* with plasmids, J. Mol. Biol. 166 (4) (1983) 557–580, https://doi.org/10.1016/S0022-2836(83)80284-8.

[18] S. Brenner, The genetics of *caenorhabditis elegans*, Genetics 77 (1) (1974) 71–94, https://doi.org/10.1093/genetics/77.1.71.

[19] M.A. Larkin, G. Blackshields, N.P. Brown, R. Chenna, P.A. McGettigan, H. McWilliam, F. Valentin, I.M. Wallace, A. Wilm, R. Lopez, J.D. Thompson, T. J. Gibson, D.G. Higgins, Clustal w and clustal x version 2.0, Bioinformatics 23 (21) (2007) 2947–2948, https://doi.org/10.1093/bioinformatics/btm404.

[20] K. Tamura, G. Stecher, S. Kumar, MEGA11: molecular evolutionary genetics analysis version 11, Mol. Biol. Evol. 38 (7) (2021) 3022–3027, https://doi.org/10.1093/molbev/msab120.

[21] J.J. Almagro Armenteros, K.D. Tsirigos, C.K. Sonderby, T.N. Petersen, SignalP 5.0 improves signal peptide predictions using deep neural networks, Nat. Biotechnol. 37 (4) (2019) 420–423, https://doi.org/10.1038/s41587-019-0036-z.

[22] K. Hiller, A. Grote, M. Scheer, R. Munch, D. Jahn, PrediSi: prediction of signal peptides and their cleavage positions, Nucleic Acids Res 32 (_2) (2004) W375–W379, https://doi.org/10.1093/nar/gkh378.

[23] L.A. Kelley, S. Mezulis, C.M. Yates, M.N. Wass, M.J.E. Sternberg, The Phyre2 web portal for protein modeling, prediction, and analysis, Nat. Protoc. 10 (6) (2015) 845–858, https://doi.org/10.1038/nprot.2015.053.

[24] D.W.A. Buchan, D.T. Jones, The PSIPRED protein analysis workbench: 20 years on, Nucleic Acids Res 47 (W1) (2019) W402–W407, https://doi.org/10.1093/nar/gkz297.

[25] X. Zhou, W. Zheng, Y. Li, R. Pearce, C. Zhang, E.W. Bell, G. Zhang, Y. Zhang, I-TASSER-MTD: a deep-learning-based platform for multi-domain protein structure and function prediction, Nat. Protoc. 17 (10) (2022) 2326–2353, https://doi.org/10.1038/s41596-022-00728-0.

[26] A. Waterhouse, M. Bertoni, S. Bienert, G. Studer, G. Tauriello, R. Gumienny, F. T. Heer, T.A.P. de Beer, C. Rempfer, L. Bordoli, R. Lepore, T. Schwede, Swiss-MODEL: homology modelling of protein structures and complexes, Nucleic Acids Res. 46 (W1) (2018) W296–W303, https://doi.org/10.1093/nar/gky427.

[27] J. Chen, H. Yang, Y. Feng, Q. Shi, Z. Li, Z. Tao, J. Fan, Y. Jin, S. Li, J. Cheng, X. Lu, A single nucleotide mutation drastically increases the expression of tumor-homing NGR-TNFα in the *e. coli* M15-pQE30 system by improving gene transcription, Appl. Microbiol. Biotechnol. 105 (2021) 1447–1460, https://doi.org/10.1007/s00253-021-11136-x.

[28] N.J. Kruger, The bradford method for protein quantitation. Methods in Molecular Biology, Humana Press, Totowa, NJ, 2009, pp. 17–24, https://doi.org/10.1385/0-89603-268-x:9.

[29] M. Malešević, N. Stanisavljević, K. Novović, N. Polović, Z. Vasiljević, M. Kojić, B. Jovčić, *burkholderia cepacia* YtnP and Y2-aiiA lactonases inhibit virulence of *pseudomonas aeruginosa* via quorum quenching activity, Microb. Pathog. 149 (2020) 104561, https://doi.org/10.1016/j.micpath.2020.104561.

[30] N.M. Patel, J.D. Moore, H.E. Blackwell, D.A. Noguez, Identification of unanticipated and novel N-acyl L-homoserine lactones (AHLs) using a sensitive

non-targeted LC-MS/MS method, PLoS One 11 (10) (2016) e0163469, https://doi.org/10.1371/journal.pone.0163469.
[31] M. Malešević, F.D. Lorenzo, B. Filipić, N. Stanisavljević, K. Novović, L. Senerovic, N. Polović, A. Molinaro, M. Kojić, B. Jovčić, *pseudomonas aeruginosa* quorum sensing inhibition by clinical isolate *delftia tsuruhatensis* 11304: involvement of N-octadecanoylhomoserine lactones, Sci. Rep. 9 (1) (2019) 16465, https://doi.org/10.1038/s41598-019-52955-3.
[32] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method, Methods 25 (4) (2001) 402–408, https://doi.org/10.1006/meth.2001.1262.
[33] V.C. Scoffone, L.R. Chiarelli, V. Makarov, G. Brackman, A. Israyilova, A. Azzalin, F. Forneris, O. Riabova, S. Savina, T. Coenye, G. Riccardi, S. Buroni, Discovery of new diketopiperazines inhibiting *burkholderia cenocepacia* quorum sensing *in vitro* and *in vivo*, Sci. Rep. 6 (1) (2016) 32487, https://doi.org/10.1038/srep32487.
[34] M. Malesevic, N. Stanisavljevic, D. Matijasevic, J. Curcic, V. Tasic, S. Tasic, M. Kojic, Metagenomic analysis of bacterial community and isolation of representative strains from vranjska banja hot spring, Serbia, Microb. Ecol. 86 (4) (2023) 2344–2356, https://doi.org/10.1007/s00248-023-02242-6.
[35] L. Djokic, N. Stankovic, I. Galic, I. Moric, N. Radakovic, S. Segan, A. Pavic, L. Senerovic, Novel quorum quenching YtnP lactonase from *bacillus paralicheniformis* reduces *pseudomonas aeruginosa* virulence and increases antibiotic efficacy *in vivo*, Front. Microbiol. 13 (2022) 906312, https://doi.org/10.3389/fmicb.2022.906312.
[36] G. Vinoj, B. Vaseeharan, S. Thomas, A.J. Spiers, S. Shanthi, Quorum-quenching activity of the AHL-lactonase from *bacillus licheniformis* DAHB1 inhibits *vibrio* biofilm formation in vitro and reduces shrimp intestinal colonization and mortality, Mar. Biotechnol. 16 (2014) 707–715, https://doi.org/10.1007/s10126-014-9585-9.
[37] O.G. Ndochinwa, Q.J. Wang, O.C. Amadi, T.N. Nwagu, C.I. Nnamchi, E.S. Okeke, A.N. Moneke, Current status and emerging frontiers in enzyme engineering: an industrial perspective, Heliyon (2024), https://doi.org/10.1016/j.heliyon.2024.e32673.
[38] J.A. Coker, E.P. Williams, B.K. Gregory, Extremophiles and biotechnology: current uses and prospects, F1000Res 5 (2016), https://doi.org/10.12688/f1000research.7432.1.
[39] R. Mehta, P. Singhal, H. Singh, D. Damle, A.K. Sharma, Insight into thermophiles and their wide-spectrum applications, 3 Biotech 6 (2016) 1–9, https://doi.org/10.1007/s13205-016-0368-z.
[40] S.M. Diene, P. Pontarotti, S. Azza, N. Armstrong, L. Pinault, E. Chabriere, P. Colson, J.M. Rolain, D. Raoult, Origin, diversity, and multiple roles of enzymes with metallo-β-lactamase fold from different organisms, Cells 12 (13) (2023) 1752, https://doi.org/10.3390/cells12131752.
[41] T. Morohoshi, Y. Tominaga, N. Someya, T. Ikeda, Characterization of a novel thermostable N-acylhomoserine lactonase from the thermophilic bacterium *thermaerobacter marianensis*, J. Biosci. Bioeng. 120 (1) (2015) 1–5, https://doi.org/10.1016/j.jbiosc.2014.11.014.
[42] L. Hao, J. Liang, S. Chen, J. Zhang, Y. Zhang, Y. Xu, MzmL, a novel marine-derived N-acyl homoserine lactonase from *mesoflavibacter zeaxanthinifaciens* that attenuates *pectobacterium carotovorum* subsp. *carotovorum* virulence, Front. Microbiol. 15 (2024) 1353711, https://doi.org/10.3389/fmicb.2024.1353711.
[43] P.K. Sharma, K. Singh, R. Singh, N. Capalash, A. Ali, O. Mohammad, J. Kaur, Characterization of a thermostable lipase showing loss of secondary structure at ambient temperature, Mol. Biol. Rep. 39 (3) (2012) 2795–2804, https://doi.org/10.1007/s11033-011-1038-1.
[44] O. López-López, M.E. Cerdán, M.I. González-Siso, Hot spring metagenomics, Life 3 (2) (2013) 308–320, https://doi.org/10.3390/life3020308.
[45] J. Fitter, R. Herrmann, N.A. Dencher, A. Blume, T. Hauss, Activity and stability of a thermostable alpha-amylase compared to its mesophilic homologue: mechanisms of thermal adaptation, Biochemistry 40 (35) (2001) 10723–10731, https://doi.org/10.1021/bi010808b.
[46] P.F. Fox, L. Stepaniak, Isolation and some properties of extracellular heat-stable lipases from *pseudomonas fluorescens* strain AFT 36, J. Dairy Res. 50 (1) (1983) 77–89, https://doi.org/10.1017/s0022029900032544.
[47] B. Ruiz-Díez, P. Sanchez, F. Baquero, J.L. Martinez, A. Navas, Differential interactions within the *caenorhabditis elegans - pseudomonas aeruginosa* pathogenesis model, J. Theor. Biol. 225 (4) (2003) 469–476, https://doi.org/10.1016/s0022-5193(03)00288-1.
[48] A. Wellenberg, L. Weides, J. Kurzke, T. Hennecke, J. Bornhorst, B. Crone, U. Karst, V. Brinkmann, G. Fritz, S. Honnen, Use of *c. elegans* as a 3R-compliant *in vivo* model for the chemoprevention of cisplatin-induced neurotoxicity, Exp. Neurol. 341 (2021) 113705, https://doi.org/10.1016/j.expneurol.2021.113705.
[49] S. Hraiech, J. Hiblot, J. Lafleur, H. Lepidi, L. Papazian, J.M. Rolain, D. Raoult, M. Elias, M.W. Silby, J. Bzdrenga, F. Bregeon, E. Chabriere, Inhaled lactonase reduces *pseudomonas aeruginosa* quorum sensing and mortality in rat pneumonia, PLoS One 9 (10) (2014) e107125, https://doi.org/10.1371/journal.pone.0107125.
[50] A.L. Conery, J. Larinks-Ford, F.M. Ausubel, N.V. Kirienko, High-throughput screening for novel anti-infectives using a *c. elegans* pathogenesis model, Curr. Protoc. Chem. Biol. 6 (1) (2014) 25–37, https://doi.org/10.1002/9780470559277.ch130160.
[51] F. Chen, Y. Gao, X. Chen, Z. Yu, X. Li, Quorum quenching enzymes and their application in degrading signal molecules to block quorum sensing-dependent infection, Int. J. Mol. Sci. 14 (9) (2013) 17477–17500, https://doi.org/10.3390/ijms140917477.
[52] S.H.O. Zarroug, J.S. Bajaman, F.N. Hamza, R.A. Saleem, H.K. Abdalla, *caenorhabditis elegans* as an in vivo model for the discovery and development of natural Plant-Based antimicrobial compounds, Pharmaceuticals 16 (8) (2023) 1070, https://doi.org/10.3390/ph16081070.
[53] E. van Tilburg Bernardes, L. Charron-Mazenod, D.J. Reading, S.L. Reckseidler-Zenteno, S. Lewenza, Exopolysaccharide-Repressing small molecules with antibiofilm and antivirulence activity against *pseudomonas aeruginosa*, Antimicrob. Agents Chemother. 61 (5) (2017) e01997-16, https://doi.org/10.1128/AAC.01997-16.
[54] M. Malešević, B. Jovčić, Targeting Gram-Negative bacterial biofilm with innovative therapies: communication silencing strategies, Future Pharm. 5 (3) (2025) 35, https://doi.org/10.3390/futurepharmacol5030035.
[55] J. Chadha, K. Harjai, S. Chhibber, S. Kaur, Gentamicin augments the quorum quenching potential of cinnamaldehyde *in vitro* and protects *caenorhabditis elegans* from *pseudomonas aeruginosa* infection, Front. Cell. Infect. Microbiol. 12 (2022) 899566, https://doi.org/10.3389/fcimb.2022.899566.
[56] L. Kumar, R.N. Brenner, J. Brice, J. Klein-Seetharaman, S.K. Sarkar, Cephalosporins interfere with quorum sensing and improve the ability of *caenorhabditis elegans* to survive *pseudomonas aeruginosa* infection, Front. Microbiol. 12 (2021) 598498, https://doi.org/10.3389/fmicb.2021.598498.
[57] H. Yao, A. Xu, J. Liu, F. Wang, H. Yao, J. Chen, Evaluation of *in vivo* antibacterial drug efficacy using *caenorhabditis elegans* infected with carbapenem-resistant *klebsiella pneumoniae* as a model host, Front. Pharm. 13 (2022) 973551, https://doi.org/10.3389/fphar.2022.973551.