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A novel thermostable YtnP lactonase from *Stenotrophomonas maltophilia* inhibits *Pseudomonas aeruginosa* virulence in vitro and in vivo

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ABSTRACT

Infections caused by multidrug-resistant pathogens are one of the biggest challenges facing the healthcare system today. Quorum quenching (QQ) enzymes have the potential to be used as innovative enzyme-based antivirulence therapeutics to combat infections caused by multidrug-resistant pathogens. The main objective of this research was to describe the novel YtnP lactonase derived from the clinical isolate *Stenotrophomonas maltophilia* and to investigate its antivirulence potential against multidrug-resistant *Pseudomonas aeruginosa* MMA83. YtnP lactonase, the QQ enzyme, belongs to the family of metallo-β-lactamases. The recombinant enzyme has several advantageous biotechnological properties, such as high thermostability, activity in a wide pH range, and no cytotoxic effect. High-performance liquid chromatography analysis revealed the activity of recombinant YtnP lactonase toward a wide range of *N*-acyl-homoserine lactones (AHLs), quorum sensing signaling molecules, with a higher preference for long-chain AHLs. Recombinant YtnP lactonase was shown to inhibit *P. aeruginosa* MMA83 biofilm formation, induce biofilm decomposition, and reduce extracellular virulence factors production. Moreover, the lifespan of MMA83-infected *Caenorhabditis elegans* was prolonged with YtnP lactonase treatment. YtnP lactonase showed synergistic inhibitory activity in combination with gentamicin and acted additively with meropenem against MMA83. The described properties make YtnP lactonase a promising therapeutic candidate for the development of next-generation antivirulence agents.

1. Introduction

Solving the problem of the antimicrobial resistance crisis is one of the primary challenges currently confronting the healthcare system [1]. Intensive studies on bacteria and their pathobiology over the past two decades have resulted in the development of new strategies to combat antimicrobial resistance (AMR). These strategies include silencing bacterial cell-to-cell communication, utilization of bacteriophages to eradicate bacteria, and manipulating the microbiome to reduce infections caused by pathogens [2].

Quorum sensing (QS) is a cell-to-cell communication system that enables synchronized behavior of bacterial populations. The most prevalent QS signaling molecules are *N*-acyl-homoserine lactones (AHLs), which are predominantly found in Gram-negative bacteria. QS plays a key role in regulating bacterial virulence, biofilm formation, antibiotics and secondary metabolite production, making it an attractive target for developing new therapies to combat bacterial infections [3].

It has been discovered that bacteria naturally produce cell-to-cell communication inhibitors, which can be classified into two categories: quorum sensing inhibitors (QSI) and quorum quenching (QQ) enzymes [4]. Based on their mechanism of action, QQ enzymes are divided into three classes: lactonases, acylases, and oxidoreductases. Lactonases represent a diverse group of enzymes capable of inactivating both short and long-chain AHLs by hydrolyzing the ester bond of the lactone ring, resulting in alterations in QS-related functions. AHL lactonases, such as AidC originating from *Bosea* sp., LrsL from *Labrenzia* sp., and PPH lactonase from *Mycobacterium tuberculosis*, exhibit high catalytic efficiency, but vary in their substrate preferences [5–7]. The diversity observed among lactonases indicates that their different properties could be exploited during the research and development of anti-QS therapeutics

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to find the most suitable ones to combat specific pathogens. QQ enzymes, such as lactonases, show particular promise for combating surface attachment, antimicrobial drug-resistance, their toxicity and pathogenicity. Although QQ enzymes can affect bacterial fitness, they act extracellularly and degrade secreted QS signaling factors. At the optimal concentrations for their antivirulence activity, they do not impair bacterial survival and consequently cause less selective pressure, making them suitable for long-term use due to these properties [8]. In addition, QQ enzymes are promising agents that could substitute or at least supplement antibiotics. QQ enzymes, due to their characteristics, could be used in several economically important fields such as agriculture, aquaculture, biofouling, and health [9].

Pseudomonas aeruginosa causes acute and chronic infections in immunocompromised individuals with cystic fibrosis (CF), transplants, cancer, and ventilator-associated pneumonia (VAP), including those caused by COVID-19 [10]. It is considered a major cause of pulmonary exacerbations and decline in lung function in CF patients, responsible for up to 85 % of deaths [11]. The numerous virulence factors and resistance to most conventional antibiotics have led to *P. aeruginosa* being listed as one of the top-priority pathogens on the ESCAPE pathogen list, highlighting the urgent need for the development of new therapies to combat this pathogen [12]. To achieve its high virulence potential, *P. aeruginosa* possesses one of the most complex signaling networks. The QS network of *P. aeruginosa* consists of four interconnected signaling pathways described so far: las, rhl, pqs, and the still insufficiently explored iqs QS system [13].

P. aeruginosa often coexists with *Stenotrophomonas maltophilia* in various environments, including the rhizosphere of plants and the lungs of CF patients [14]. *S. maltophilia* is a Gram-negative ubiquitous opportunistic pathogen commonly found in water, soil, plant rhizospheres, animals, and the human body [15]. In recent years, it is observed an increasing virulence and spreading of *S. maltophilia* strains [16]. Therefore, it is assumed that *S. maltophilia* has developed a system to disrupt the intercellular communication of its competitors, such as *P. aeruginosa*, in order to survive in polymicrobial communities alongside this successful pathogen.

There is limited literature data available regarding the QQ potential of *S. maltophilia*. In this study, a new lactonase, marked YtnP, originating from a clinical isolate of *S. maltophilia* 6960, was identified and characterized. Our main objective was to investigate the virulence potential of *S. maltophilia* 6960 strain and to evaluate potential of its YtnP lactonase as an antivirulence agent in the therapies designed to combat *P. aeruginosa* infections.

2. Material and methods

2.1. Bacterial strains, cultivation conditions, and chemicals

A list of bacterial strains and plasmids used in this study was presented in Table 1. All bacterial strains were cultivated aerobically in Luria-Bertani (LB) broth medium or Mueller-Hinton medium (MH) at 37 °C except *Chromobacterium subtsugae* CV026 which was grown at 30 °C. Human keratinocyte HaCaT cell line was cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM). Nematode Growth Medium (NGM) was used for the maintenance of *Caenorhabditis elegans*. N-butyryl-DL-homoserine lactone (C4-HSL), N-hexanoyl-L-homoserine lactone (C6-HSL), N-octanoyl-DL-homoserine lactone (C8-HSL), N-decanoyl-DL-homoserine lactone (C10-HSL), N-3-oxododecanoyl-L-homoserine lactone (3-oxo-C12-HSL), and N-tetradecanoyl-DL-homoserine lactone (C14-HSL) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All of the AHL stock solutions (0.5 mM) were prepared in dimethyl sulfoxide (DMSO) and stored at −20 °C for biological assays. AHL stock solutions (100 mM) for high-performance liquid chromatography (HPLC) analysis were prepared in methanol. Isopropyl-β-D-1-thiogalactopyranoside (IPTG; stock solution 1 M) purchased from Serva (Heidelberg, Germany) was applied for the induction of protein expression.

Table 1
Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Source
Strains		
Stenotrophomonas sp. 6960	QQ lactonases producer	[17]
Chromobacterium violaceum CV026 reclassified as *Chromobacterium subtsugae* CV026	ATCC 31532 derivative, cvil:: Tn*5 xylE*, Km^r Sm^r	[18]
Pseudomonas aeruginosa MMA83	NDM-1 positive clinical isolate, Amp^r	[19]
Escherichia coli M15	*lacZ*ΔM15	Qiagen
E. coli DH5α	*supE44* Δ*lacU169* (Φ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi*-1 *relA*	[20]
E. coli OP50	uracil requiring mutant of *E. coli*	[21]
Plasmids		
pJET 1.2	Amp^r, PCR cloning vector	ThermoFisher
pQE30	Amp^r, ColE1 replicon, His6 expression vector	Qiagen
pJETYtnP	*ytnP* lactonase gene cloned into pJET 1.2	This study
pQE30YtnP	*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. Maintained 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)
C. elegans N2 (Bristol)	Wild-type strain. Temperature-sensitive sterility. Maintained at 20 °C.	N2 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. Selection and whole-genome sequencing of AHL degrading strain *Stenotrophomonas sp. 6960*

The strain *Stenotrophomonas* sp. 6960 isolated from the sputum of a CF patient belongs to the collection of clinical isolates of the Laboratory of Molecular Microbiology (LMM), Institute of Molecular Genetics and Genetic Engineering (IMGGE), University of Belgrade. *Stenotrophomonas* sp. 6960 was selected as an AHL-degrading strain among 633 clinical isolates [17].

To investigate the complete genomic potential of the *Stenotrophomonas* sp. 6960, the total DNA from the selected strain was isolated [22] and sequenced using the Illumina HiSeq 2500 platform (MicrobesNG, University of Birmingham, UK). The complete genomic sequence of clinical isolate *S. maltophilia* 6960 was deposited in the NCBI GenBank database under accession number JAIOGM0000000. To elucidate the clinical relevance of *S. maltophilia* 6960, the annotated genome of this strain was searched for genes belonging to resistome and virulome. Tools used for in silico analysis were provided in the Supplementary S.1.1.

2.3. Identification and an in silico analysis of the amino acid sequence of YtnP lactonase

To find the putative AHL lactonase gene, an in silico analysis of the annotated genome of strain *S. maltophilia* 6960 was performed. The global features of YtnP lactonase are predicted using PSIPRED online software [23]. The amino acid sequences of YtnP were searched for the presence of signal peptide sequence using SignalP version 5.0 (http://www.cbs.dtu.dk/services/SignalP/) [24]. In addition, the presence of a signal peptide sequence was also checked using the PrediSi program (http://www.predisi.de) [25]. PSIPRED online program was used for transmembrane topology prediction of YtnP lactonase. The secondary structure of YtnP lactonase from *S. maltophilia* 6960 was predicted using Phyre2, PSIPRED and I-TASSER software [24,26,27]. The tertiary structures of the YtnP lactonase from *S. maltophilia* were predicted using the SWISS-MODEL algorithm (http://swissmodel.expasy.org/) [28,29]. The artificial intelligence (AI) program AlphaFold2 [30] and I-TASSER were used for building the model of protein fold prediction of YtnP lactonase. The sequence of YtnP lactonase was aligned with the selected functionally characterized lactonases from various bacterial species using the ClustalW algorithm of the AlignX program of the Vector NTI software [31]. Phylogenetic trees of the selected amino acid sequences were constructed with MEGA software version 11 [32] using the maximum likelihood (ML) method. Phylogeny analysis results were obtained by setting the default bootstrap parameter to 1000 replicates. SMART (Simple Modular Architecture Research Tool) program (https://smart.embl.de) version 9 [33] was used for the identification and annotation of protein domains in the amino acid sequence of the YtnP lactonase.

To investigate whether the QQ activity of strain 6960 was derived from YtnP lactonase, the growth phase-dependent expression level of the *ytnP* lactonase gene was correlated with the observed QQ activity of this strain (Supplementary S.1.2, S.1.3). The quantitative real-time polymerase chain reaction (RT-qPCR) was used to measure the relative mRNA level of the *ytnP* gene during different growth phases of *S. maltophilia* 6960. A detailed protocol is provided in Supplementary S.1.4. The *Chromobacterium subtsugae* CV026 agar-well diffusion assay [34] was used to visualize the growth phase-dependent QQ activity level of strain 6960 (a detailed protocol is provided in Supplementary S.1.3). To confirm the localization of YtnP lactonase, the preparation of cell-free supernatant and crude extract from *S. maltophilia* 6960 culture is given in detail in Supplementary Material and Methods section S.1.5).

2.4. Cloning, expression, and biochemical characterization of YtnP lactonase

The DNA sequence of YtnP lactonase was amplified by polymerase chain reaction (PCR) with appropriate primers (Supplementary Table S1) using Phusion™ HighFidelity DNA Polymerase Kit (Thermo Fisher Scientific) with a primer annealing temperature of 78 °C. The amplified *ytnP* gene was cloned into the pJET 1.2 cloning vector (Thermo Fisher Scientific), resulting in the pJETYtnP construct, which was transformed into *E. coli* DH5α cells [20]. Subsequently, the pJETYtnP plasmid isolated from *E. coli* DH5α cells was predigested with the restriction enzymes *Bam*HI/*Sal*I and the obtained DNA fragment was cloned into the *BamHI*/*Sal*I predigested pQE30 vector (Qiagen), resulting in the pQE30YtnP construct, which was subsequently transformed into *E. coli* M15 pREP4 expression cells (Qiagen). The obtained pQE30YtnP plasmid was sequenced at the Macrogen Sequencing Service (Macrogen Europe Service, Amsterdam, The Netherlands) to confirm the correctness of the YtnP gene sequence and the in-frame tag incorporation into the pQE30 vector. Expression of recombinant YtnP lactonase in pQE30YtnP transformed *E. coli* M15 pREP4 cells was induced by the addition of 0.1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) according to the protocol (Qiagen). The recombinant His6-YtnP protein was purified using the Ni-NTA Fast Start Kit (Qiagen) under denaturing conditions (8 M, 20 mM Tris, 0.5 M NaCl, 10 mM imidazole) according to the manufacturer's instructions. Ellution of the recombinant His-tagged protein was performed using a buffer containing 0.5 M imidazole [35]. Removal of imidazole and NaCl from the concentrated purified protein was performed using 10 kDa columns (Merck-Millipore) against 50 mM Tris-HCl, pH 7.4 buffer. The recombinant lactonases were stored at −20 °C with 50 % (*w/v*) glycerol. Protein purity was analyzed using 12 % (w/v) SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis). The concentration of total soluble proteins was determined by the Bradford method using bovine serum albumin as a standard [36]. The following analysis was performed using 50 μg/ml of recombinant YtnP lactonase as a final concentration.

The CV026 agar-well diffusion assay [34] using *C. subtsugae* CV026 as a biosensor strain with 10 μM C8-HSL as a substrate was applied to evaluate the residual AHL-degrading activity of the purified recombinant YtnP lactonase in the following biochemical assays. The relative YtnP activity is approximated based on the percentage decrease in the diameter of violacein production around the wells containing YtnP lactonase compared to the untreated control.

2.4.1. pH stability

The pH stability of recombinant YtnP lactonase was analyzed in a pH range of 4 to 9 with a pH interval of 0.5. YtnP lactonase was pre-incubated for 30 min at 25 °C with 50 mM acetate buffer (CH_3COONa, CH_3COOH) pH 4–5.5; 50 mM sodium phosphate buffer ($NaH_2PO_4xH_2O$) pH 6–7; 50 mM Tris-HCl pH 7.5–9 following the previously described method [37]. After the addition of C8-HSL, the reaction mixtures were incubated at 30 °C for the next 20 h.

2.4.2. Thermostability

Recombinant YtnP lactonase was preincubated for 1 h at temperatures ranging from 30 to 100 °C with an interval of 10 °C. The residual activity of lactonase was measured after 20 h of incubation with C8-HSL at 30 °C.

2.5. Substrate specificity of recombinant YtnP lactonase toward AHLs

High-performance liquid chromatography (HPLC) analysis and Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) were used to determine the substrate specificity of YtnP lactonase. YtnP lactonase (50 μg/ml) was incubated with various AHLs, including AHLs with acyl chain lengths of C4, C6, C8, C10, 3-oxo-C12, and C14 HSL, at a final concentration of 1 mM for 2 h at 30 °C. 1 M NaOH was used as a control for lactone hydrolysis. After incubation, the samples were run on the UltiMate™ 3000 UHPLC system (Thermo Fisher Scientific) with an UV/VIS detector at 205 nm using a Hypersil GOLD column over a C18 silica matrix, dimensions: 150 × 4.6 (Thermo Fisher Scientific). Acetonitrile was used as the eluent at a flow rate of 0.5 ml/min according to the method described previously [38].

For LC-MS/MS analysis 100 μl of reaction mixtures were diluted with 900 μl of LC-MS grade methanol and filtered through 0.2 μm PTFE syringe filters. The chromatographic separation was performed using a Vanquish™ Core HPLC system (Thermo Fisher Scientific) equipped with a Acclaim™ PolarAdvantage II C18 (PA2) 3 μm, 2.1 × 50 mm (Thermo Fisher Scientific) column. The mobile phase consisted of A: water with 0.1 % (*w/v*) formic acid and B: acetonitrile with 0.1 % (w/v) formic acid the gradient chromatography profile started with an isocratic hold for 3 min at 10 % of solvent B followed by a linear increase to 90 % over 10 min, isocratic 90 % solvent B for 5 min and a 3 min equilibration at 10 % of solvent B.

AHLs and their degradation product were detected using a modified protocol by Patel et al. [39] via heated electrospray ionization coupled with a TSQ FortisTM Plus triple quadrupole mass spectrometer (Thermo Fisher Scientific). The ionization parameters were: 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. To obtain MS/MS spectra of HSL and the corresponding degradation products detection was carried out in product ion scan mode using 12 V collision energy. A list of molecular ions monitored and their masses is presented in Supplementary Table S2. HLS were identified by the presence of the characteristic 102.06 m/z lactone ring fragment [40] and degradation products were identified by the presence of the 120 m/z fragment of the hydrolyzed lactone ring.

2.6. The examination of antivirulence activity of recombinant YtnP lactonase

A MDR clinical isolate *Pseudomonas aeruginosa* MMA83 [19] from the laboratory collection (LMM, IMGGE) was used as a model system for the determination of lactonase antivirulence potential. To perform experiments, the test strain MMA83 was cultivated in Mueller Hinton (MH) medium (Sigma Aldrich) at 37 °C with aeration (180 rpm).

2.6.1. Biofilm assay

The effect of recombinant YtnP lactonase on *P. aeruginosa* MMA83 biofilm formation and decomposition was monitored in 24-well microtiter plates with sterile glass slides (Sarstedt, Germany) using fluorescence microscopy (ZEISS Axioscope 5, Germany). *P. aeruginosa* MMA83 (previously adjusted to 10^5 CFU/ml) was incubated 24 h with recombinant YtnP lactonase (50 μg/ml) at 37 °C to monitor the prevention of biofilm formation. To evaluate the effect of recombinant YtnP lactonase on biofilm decomposition, the preformed biofilm of MMA83 (24 h) was incubated with the YtnP lactonase under the same conditions (next 24 h). After incubation, the planktonic cells were carefully rinsed three times with sterile phosphate-buffered saline 1xPBS (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl; pH 7.4). Biofilms were stained at room temperature for 30 min with 2.5 μM propidium iodide and 2.5 μM SYTO9 (Thermo Fisher Scientific) resuspended in 1xPBS. The MMA83 culture with 50 mM Tris-HCl (pH 7.4) and 50 % (*w/v*) glycerol was incubated under the same conditions as a control, to exclude any potential effect of these components on the biofilm [34].

2.6.2. Elastase, pyocyanin, and rhamnolipid assay

The effect of recombinant YtnP lactonase (50 μg/ml) on MMA83 virulence factors production (elastase, pyocyanin, and rhamnolipid) was analyzed. The elastase, pyocyanin, and rhamnolipid production in the 24 h MMA83 culture supernatants preincubated with recombinant YtnP lactonase was determined using the previously described methods [41–43]. The effect of YtnP on virulence factors production was determined compared to the untreated control. The experiments were done in triplicate and repeated three times independently.

2.6.3. The effect of recombinant YtnP lactonase on QS and virulence at the mRNA level

RT-qPCR was used to analyze the effect of YtnP lactonase on the relative mRNA levels of the *P. aeruginosa* MMA83 QS genes (*lasI, lasR, rhlI. rhlR, pqsA, pqsH, mvfR*) as well as virulence factor genes (*lasB*, *algK*, *phzM*, *pvdS*, *rhlC, hcnA*) (Supplementary Table S1) as previously described [34]. The *rpsL* gene was used as an endogenous control to normalize obtained data [44]. The changes in relative mRNA level of analyzing genes were determined compared to untreated control. Analysis was performed in triplicate and repeated three times.

2.7. The effect of recombinant YtnP lactonase on the antibiotics MIC values

The effect of YtnP lactonase on the minimal inhibitory concentration (MIC) values of selected clinically relevant antibiotics (gentamicin and meropenem) against *P. aeruginosa* MMA83 was investigated using the 96-well microdilution method. The bacterial suspension of MMA83 (adjusted to 10^5 CFU/ml) was incubated with serially diluted gentamicin (8, 4, 2, 1, 0.5 mg/ml) and meropenem (0.25, 0.125, 0.0625, 0.0312 mg/ml) in combination with different non-MIC concentrations of YtnP lactonase (0.4, 0.2, 0.1, 0.05 mg/ml) [17]. The effect of this combination on bacterial growth was evaluated after 24 h incubation at 37 °C. The experiments were performed in triplicate and repeated twice. Fractional inhibitory concentrations (FICs) were determined according to the previously described checkerboard method [45]. The effect of YtnP lactonase was defined as synergistic if the sum of the two FICs (FIC of the antibiotic and FIC of the YtnP lactonase) was ≤ 0.5; additive if $0.5 < \sum\text{FIC} \leq 1$; indifferent if $1 < \sum\text{FIC} < 4$; antagonistic if $\sum\text{FIC} > 4$.

2.8. The antivirulence potential of the YtnP lactonase in the Caenorhabditis elegans in vivo model system

The wild-type N2 (Bristol) strain and AU37 (glp-4(bn2) I; sek-1(km4) X) strain, with temperature sensitive sterility and enhanced sensitivity to pathogens obtained from the Caenorhabditis Genetics Center (CGC) were used in the study. *C. elegans* was used as a model system to investigate whether recombinant YtnP lactonase is able to reduce the virulence of MMA83 and consequently prolong the survival of infected *C. elegans*. Liquid assay was performed in the 96-well plate as previously described with slight modifications [46]. The AU37 worms were maintained at 15 °C, while N2 worms were grown at 20 °C on Nematode Growth Medium (NGM) plates seeded with *E. coli* OP50 strain and synchronized by using standard protocols [47]. Briefly, 15–30 worms in the L4 stage were resuspended in a medium containing 95 % (*w/v*) M9 buffer, 5 % (w/v) LB Broth and 10 μg/ml of cholesterol (Sigma Aldrich) and aliquoted in each well. The culture of MMA83 in the early exponential growth phase (standardized to 10^6 CFU/ml) was preincubated with YtnP lactonase (50 μg/ml) at 30 °C for 3 h. The bacteria growth medium was incubated with the 50 mM Tris-HCl and 50 % (w/v) glycerol in the same amount and was used as a control to exclude any potential effect of these compounds on *C. elegans* survival. Treatments were added to the wells and the survival rate of the worms was determined during 24 h. The 96-well plate was incubated at 25 °C with aeration (150 rpm) to maintain worms' number and sterility of AU37 strain. Assay with N2 strain were carried on at 20 °C with aeration (150 rpm) with addition of 20 μM 5-Fluorodeoxyuridine (FudR, Sigma-Aldrich) to avoid hatching of the eggs. The opaque needle shape worms that showed no movement were classified as dead. The experiments were performed in triplicates and repeated independently three times.

2.9. Cytotoxicity assay

The cytotoxicity of recombinant YtnP lactonase was investigated in vitro using a LDH (eng. Lactate dehydrogenase) assay kit (Thermo Fisher Scientific) with immortalized human keratinocytes HaCaT cell line as a model system. The detailed protocol was provided in Supplementary S.1.6. Additionally, the cytotoxicity level of recombinant YtnP lactonase (final concentration of 50 μg/ml) was assessed in vivo using *C. elegans* AU37 strain. The effect of recombinant YtnP lactonase (50 μg/ml) on the survival of *C. elegans* was conducted simultaneously with the fast-killing liquid assay under the same conditions as previously described.

2.10. Statistical analysis

The statistical analysis and visualization were done using GraphPad Prism 9 software. All data are shown as means ± standard deviations. The differences between control and experimental groups were compared using Student's *t*-test. The differences between survival curves in *C. elegans* measurement were analyzed by the Kaplan-Meier method following the log-rank (Mantel-Cox) test. A p value cutoff of <0.05 was considered to be statistically significant.

3. Results and discussion

3.1. Genomic analysis of clinical isolate S. maltophilia 6960 revealed the presence of diverse virulome and resistome

To investigate the basis of the observed AHL-degrading activity and the pathogenic potential of this strain, the whole-genome sequencing of the *Stenotrophomonas* sp. 6960 was performed. According to the genomic sequence analysis, *Stenotrophomonas* sp. 6960 was classified as a member of the species *Stenotrophomonas maltophilia*. *S. maltophilia* was listed as one of the top 10 globally most important resistant bacteria (TOp TEn resistant Microorganisms-TOTEM) isolated from hospital environments [48]. MLST analysis revealed that *S. maltophilia* 6960 belongs to the already-known sequence type ST137. The MLST results were provided in Supplementary Table S3. *S. maltophilia* ST137 was previously isolated in Germany from the human oropharynx in 2012 [49]. The presence of this strain in the sputum of cystic fibrosis patients in the territory of Serbia indicates increasing spreading and the necessity for intensive monitoring of this strain in order to timely control its dissemination. The basic characteristics of the genome of the strain *S. maltophilia* 6960 are provided in Supplementary Table S4.

The annotated genome of *S. maltophilia* 6960 revealed the presence of a putative AHL lactonase gene, designated as *ytnP*. Additionally, in the genome of the clinical isolate *S. maltophilia* 6960, numerous virulence factors (proteases, endonucleases, pigments, esterases, hemolysins, toxins, iron absorption proteins, secretion system type II and IV) genes were identified. The detailed list is provided in Supplementary Table S5. It is assumed that these genes represent an adaptation to the stressful conditions of the hospital environment [50]. CARD and ResFinder have predicted chromosomally encoded genes associated with resistance to fluoroquinolones, aminoglycosides, tetracyclines, and β-lactam antibiotics (Supplementary Table S6), which is consistent with literature data on the presence of genes for resistance to these classes of antibiotics in strains of *S. maltophilia* [51]. An in silico search using the Plasmid Finder program did not reveal the existence of a plasmid in the genome of *S. maltophilia* 6960, which is unusual for clinical isolates of *S. maltophilia* [52]. The plasmid absence in the genome of clinical isolate *S. maltophilia* 6960 indicates the reduced ability of this strain to rapidly spread antibiotic resistance. Additionally, in the genome of the *S. maltophilia* 6960 genes encoding type IV secretion system VirB/D4 T4SS were found, which suggests the possible capability of this strain to modulate apoptosis of human cells and promote competition against heterologous bacteria [53].

3.2. Quorum quenching and quorum sensing of S. maltophilia 6960

The annotated genome of *S. maltophilia* 6960 revealed the presence of a putative AHL lactonase gene, designated as *ytnP*. The correlation between the growth phase-dependent level of QQ activity of strain 6960 (the highest QQ activity was recorded at 10 h of cultivation) (Supplementary Fig. S1, Supplementary Fig. S2) and the relative mRNA level of the *ytnP* lactonase gene (the highest mRNA level of the *ytnP* gene is observed at 10 h of cultivation) (Supplementary Fig. S3) indicates that the QQ activity of this strain was derived from YtnP lactonase activity. The *ytnP* gene expression corresponds to the production trend of YtnP lactonase with maximum expression during the exponential growth phase [34,54].

Quorum sensing (QS) of *S. maltophilia* is based on DSF (Diffusible Signal Factor cis-11-methyl-2-dodecenoic acid) [55], and to date, AHL production by *S. maltophilia* species has not been described. To confirm that strain 6960 does not synthesize AHLs, the genome of strain 6960 was searched for the presence of genes involved in AHL-based QS. A potential AHL synthase and receptor genes were not detected in the genome of strain *S. maltophilia* 6960, but a gene for an AHL efflux protein (*B*) was found.

To get an insight into the cell-to-cell communication of the strain 6960, the annotated genome was searched for genes involved in DSF-based QS. Yero et al. have made a significant contribution by demonstrating that *S. maltophilia* strains possess distinct variants within the *rpf* cluster, namely *rpf-1* and *rpf-2*, which can be used to categorize these strains into two groups. It's noteworthy that only the *rpf-1* strains have been shown to produce detectable DSF, while the *rpf-2* variant strains are suggested to employ a strategy referred to as "social cheating" [56]. The genes for sensory kinase (*rpfC*) and a response regulator (*rpfG*) were detected, but no DSF synthase gene (*rpfF*) was found in the annotated genome of this strain. The absence of the DSF synthase gene makes *S. maltophilia* 6960 a "social cheater". The cheating group refers to the loss of ability to produce signaling molecules, but can still benefit from extracellular (shared) QS-dependent products synthetized by co-operators [57]. At the same time, it has become dependent on DSF-producing strains from the environment. The rapid emergence of cheaters in QS communities has recently been documented [58], exemplified by the identification of *P. aeruginosa* cheats in the lungs of cystic fibrosis patients with chronic infections [59]. Given the increasing incidence of *S. maltophilia* as a significant pathogen in CF patients [60], it is plausible that its QS signal cheating could be sustained by detecting QS signaling molecules from other bacteria in the open environment. This strategy may contribute to competition for both the host and the surrounding environment. Considering that QS mutants/cheaters represent an unstable population that cannot spread and therefore remains localized, with the risk of being excluded from the consortium either by competition or by the collapse of the local population [59], the assumption is that the acquisition of the AHL lactonase gene represents an adaptation and a survival strategy that occurs by suppressing competitors in the environment. The loss of the DSF synthase gene might have facilitated the acquisition of new and beneficial genes, such as the quorum quenching *ytnP* gene. This phenomenon highlights an intriguing aspect of bacterial communities, indicating a tendency toward increased structure and specialization within a given niche. As bacterial populations evolve, there appears to be a distribution of roles and a greater degree of specialization among their members.

3.3. Structural characteristics of YtnP lactonase

YtnP lactonase contains 278 amino acid residues with a molecular weight of 31.02 kDa. The general features of YtnP lactonase predicted by PSIPRED are shown in Table 2. Signal sequence prediction software did not detect a leader peptide in the amino acid sequence of the YtnP lactonase, indicating that YtnP is not secreted into the extracellular environment. According to the results of PSIPRED (Fig. 1A) and Phyre2 (Supplementary Fig. S5), YtnP lactonase is predicted to be a

Table 2
Global features of YtnP lactonase calculated from the sequence data are derived from the sequence. The values were calculated using the PSIPRED software.

Feature name	Value
Aliphatic index	89.86
atomC	0.32
atomH	0.49
atomN	0.09
atomO	0.09
atomS	0.00
Charge	−4.39
fraction negative residues	0.14
Fraction positive residues	0.10
Hydrophobicity	−0.24
Isoelectric point	5.97
Length	278
Molar extinction coefficient	34,850.00
Molecular weight	31,020.80
Number of atoms	4335.00
Surface area	48,510.00
Volume	37,167.40

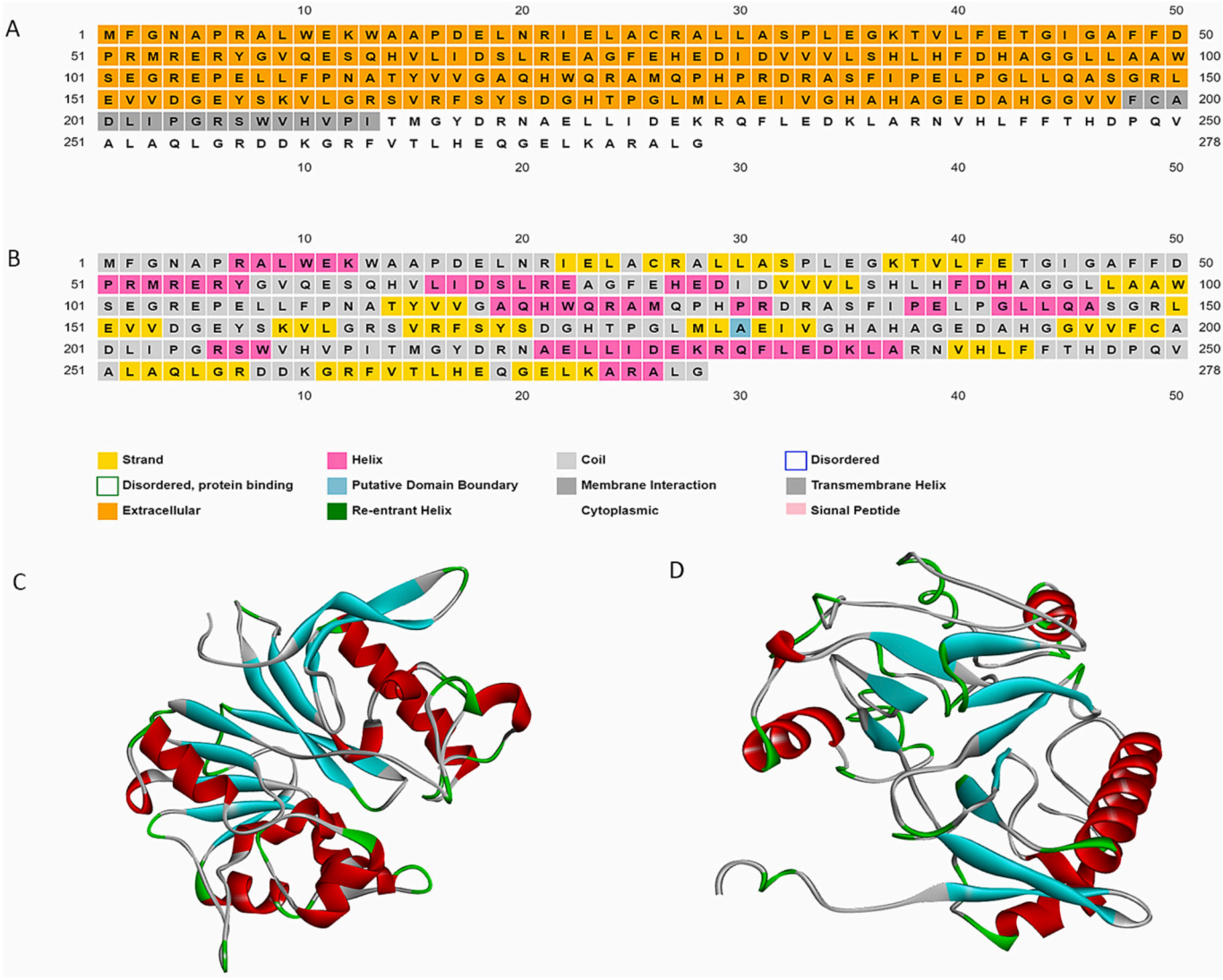

Fig. 1. Computational analysis of YtnP lactonase. (A) PSIPRED prediction of the localization of YtnP amino acid residues; (B) Secondary structure prediction of the YtnP lactonase; (C) Tertiary structure prediction models of YtnP lactonase obtained by AlphaFold2, with the selected model ranked highest (score 0); (D) Tertiary structure prediction models of YtnP lactonase obtained by I-TASSER, based on the highest C score, and validated by the "Qmean" score.

transmembrane protein with N-terminal extracellular domain and C-terminal cytoplasmic domain with mostly extracellular amino acid residues. The QQ activity of *S. maltophilia* 6960 culture toward exogenous AHLs, confirms this prediction and indicate the role of YtnP lactonase in modulating the QS of the surrounding bacteria. The observation of QQ activity in the crude extract and not in the cell-free supernatant of bacterial strain 6960 indicates that the YtnP lactonase is active within the bacterial cells (Supplementary Fig. S6). This result is consistent with the absence of a predicted signal peptide in the amino acid sequence of YtnP lactonase, suggesting that its activity is not destined for extracellular release. In summary, YtnP lactonase is likely to be intracellularly localized and, based on the presence of a predicted transmembrane domain, possibly associated with membranes.

The Phyre2 (Supplementary Fig. S5), PSIPRED (Fig. 1B) and I-TASSER (Fig. 1D) predicted the coils, strands, and helixes in the secondary structure of YtnP lactonase. The results obtained by secondary structure prediction show that the secondary structure of YtnP lactonase has an approximately equal abundance of alpha-helixes and beta-sheets. The prediction of the tertiary structure of YtnP lactonase using the SWISS-MODEL algorithm, showed that it is a homodimer structure with four Zn^{2+} binding sites (see Supplementary Fig. S7) and that its 3D structure is most similar to the 3D structure of AidC lactonase isolated from the bacterium *Chryseobacterium* sp., one of the most efficient wild type lactonases described so far [61]. Fig. 1 shows the predicted tertiary structure of YtnP lactonase modeled with AlphaFold2 (Fig. 1C) and I-TASSER (Fig. 1D).

Domain structure analysis using the SMART program revealed that the domain Lactamase_B is present in the amino acid sequence of YtnP lactonase (Supplementary Fig. S4). Multiple amino acid sequence alignments with close clustered experimentally characterized lactonases revealed the presence of conserved HXHXDH (H140LHFDH145 ~ H245), the dinuclear Zn^{2+} binding motif in the amino acid sequences of YtnP lactonase (Fig. 2A). The HXHXDH Zn^{2+} binding motif with highly conserved vital histidine and aspartic acid residues in the active site is specific for the MBL family [62], confirming that YtnP lactonase belongs to this family of proteins.

To determine the phylogenetic relatedness of YtnP to other functionally characterized lactonases, phylogenetic analysis was performed. YtnP showed close phylogenetic clustering with YtnP from *Bacillus paralicheniformis* [63], *Bacillus licheniformis* [64], and AidC from *Chryseobacterium* sp. [61], sharing nearly 29 % of identity with the first two lactonases, and 28.51 % with the AidC at the amino acid sequence level (Fig. 2B).

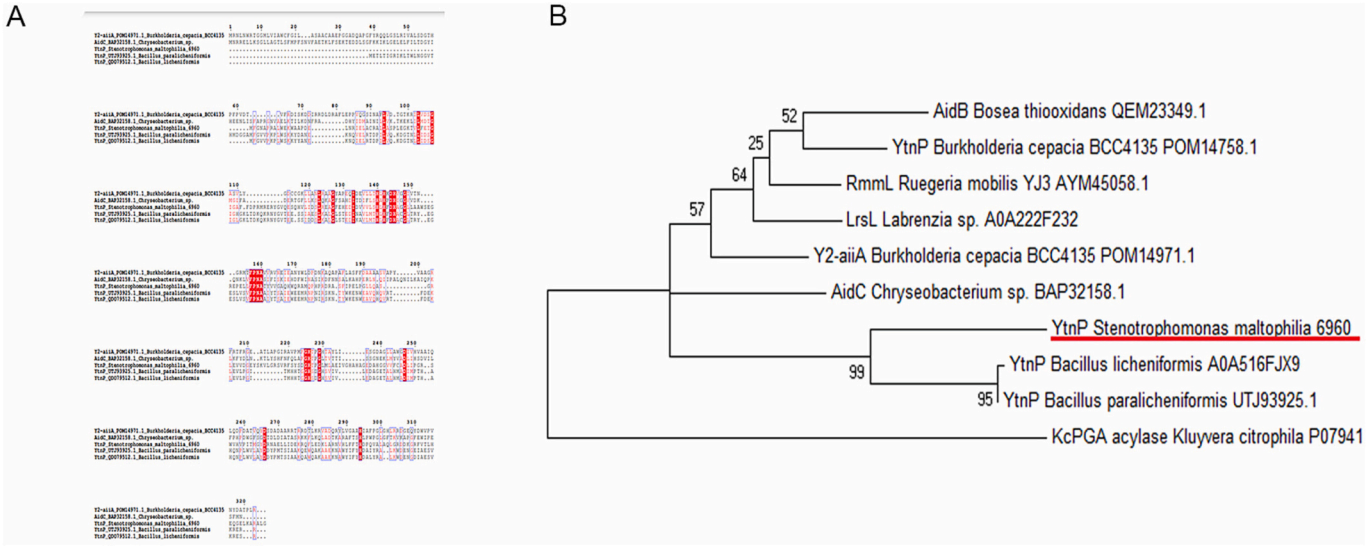

Fig. 2. Phylogenetic analysis of YtnP lactonase and selected functionally characterized lactonases from various bacterial species. (A) Alignment of the amino acid sequence of YtnP lactonase from strain *S. maltophilia* 6960 with amino acid sequences of functionally characterized lactonases from other bacterial species. Multiple alignments of amino acid sequences were performed using ClustalW software. (B) The dendrogram was constructed by the Maximum-Likelihood (ML) method using the ClustalW algorithm of the AlignX program of the Vector NTI software. The end of each branch shows the name of the QQ enzyme, followed by the name of the host bacteria and the GenBank accession number.

3.4. Purification and biochemical characteristics of YtnP lactonase

Given that the strain *S. maltophilia* 6960 possesses numerous resistance genes and virulence factors, it is unsafe for direct application as a quorum quenching strain against competitors. In pharmacological applications, the isolation of the enzyme from the clinical isolate has many advantages. By purifying YtnP enzyme using pQE30 system, we can harness its specific catalytic properties while minimizing the potential risks associated with using the entire clinical isolate. This approach not only ensures a targeted and controlled application of the enzyme but also mitigates concerns related to the clinical isolate's pathogenicity.

3.4.1. YtnP lactonase has a wide range of thermal and pH stability

In addition to catalytic activity, the properties of lactonases that limit their use as therapeutics are insufficient thermal and pH stability [65]. The previously described lactonases such as YtnP-ZP1 and Y2-aiiA retain >50 % activity up to approximately 50 °C [34,63]. The recombinant YtnP lactonase from 6960 retained almost complete activity after exposure to temperatures ranging from 30 to 100 °C (Fig. 3A). Besides YtnP lactonase from *S. maltophilia* 6960, the only characterized thermostable lactonase originating from mesophilic bacteria is AidB lactonase from *Bosea* sp. [66]. The analysis of YtnP lactonase from *S. maltophilia* 6960 revealed a close phylogenetic relatedness with the thermostable YtnP lactonase found in *Bacillus licheniformis* [64]. To unravel the basis of YtnP lactonase high thermostability, multiple sequence alignments of YtnP lactonase with other closely clustered lactonases were performed. This analysis revealed the common absence of the N-terminal 63-amino acid of YtnP lactonase and the previously characterized thermostable lactonase from *B. licheniformis* (Fig. 2A) [64]. This indicates the possibility that N-terminal 63-amino acid residue could disrupt the spherical organization and consequently affect the thermostability of the proteins [67]. A comparison of the amino acid abundances in the primary structure of YtnP and other functionally characterized lactonases has been presented in the form of a heat map (Supplementary Fig. S8). This map shows a relatively tight grouping of the YtnP enzyme with thermostable lactonases. A high proportion of leucine, alanine, glycine, and other hydrophobic amino acids in the amino acid sequence of YtnP lactonase, indicates the importance of hydrophobicity due to its role in tightly folding the proteins thus increasing resistance to high temperatures [68]. Notably, the amino acid sequence of YtnP lactonase is also characterized by a significant abundance of glutamic acid and arginine. This information suggests a potential molecular basis for the thermostability of YtnP lactonase and highlights the importance of specific amino acid residues in maintaining the enzyme's structural integrity at elevated temperatures. High proportion of glutamic acid and arginine, which can form salt bridges indicates that this strong interaction could contribute thermal stability of YtnP lactonase [69]. However, the mechanism contributing to the high thermostability of YtnP lactonase remains to be elucidated and requires further analysis for confirmation of proposed structural features' role in it.

YtnP lactonase showed a wide range of pH stability (Fig. 3B). It was found that the optimal pH for the activity of the YtnP enzyme was in the range of 6 to 8 at 30 °C, while it reached its maximum activity at pH 7. Moreover, the activity of YtnP enzyme is not negligible even at lower pH values.which is in line with the pH optimum determined for other characterized lactonases, YtnP-ZP1 and AidC [61,63]. This work represents an initial step in a comprehensive analysis, for the potential utilization of lactonase as an antivirulence therapeutic and laying the groundwork for further exploration. Exploring the stability of YtnP lactonase for potential use in antivirulence therapy requires further investigation beyond the information presented in the manuscript.

3.4.2. The affinity of the YtnP lactonase for long-chain AHLs

Determining the substrate specificity of the QQ enzyme is important for understanding the biological role of the enzyme and its potential application, as the QQ enzyme has a significant impact on modulating QS-associated behavior in pathogenic bacteria [4]. Previously characterized lactonases exhibit a broad substrate spectrum and hydrolyze short and long-chain AHLs with similar specificity and efficiency, such as GcL lactonase or show a preference for long-chain AHLs, such as SsoPox lactonase [70]. The HPLC analysis shows that the retention times obtained for the standards were 5.9, 10.3, 13.22, 15.1, 16.1, and 21.3 for C4-HSL, C6-HSL, C8-HSL, C10-HSL, 3-oxo-C12-HSL, and C14-HSL, respectively. Analysis of the filtered reactions of homoserine lactones of different acyl chain lengths incubated with YtnP lactonase shows the presence of a major degradation product in each reaction. The peaks of the degradation products were obtained at retention times of 3.3, 3.4,

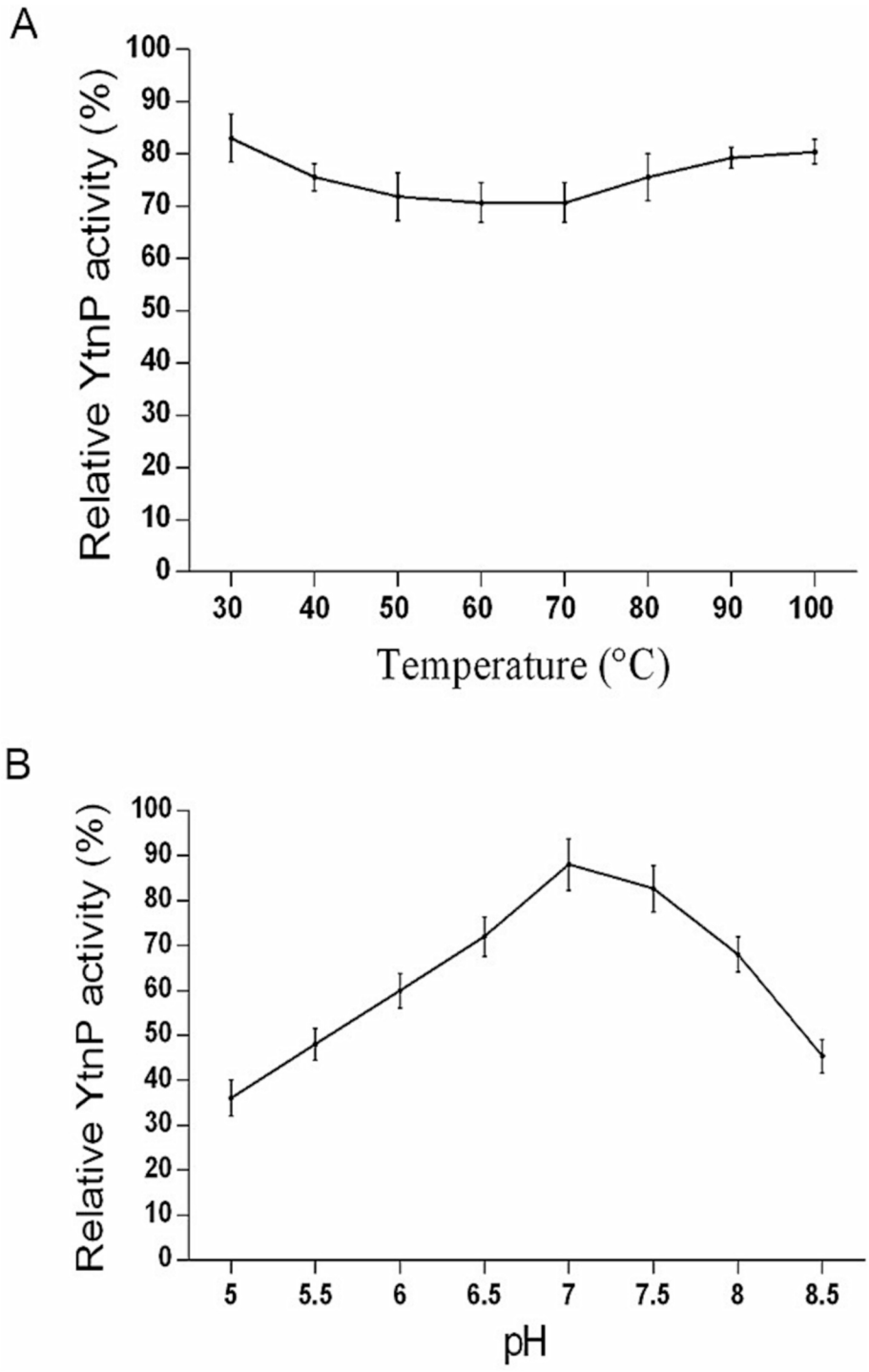

Fig. 3. The biochemical characteristics of the recombinant YtnP enzyme: (A) temperature stability and (B) pH stability. The CV026 agar-well diffusion assay was used to determine the residual YtnP activity. Relative YtnP activity is estimated by the percentage of decreased diameter of violacein production around the wells compared to the positive control. C8-HSL with 50 mM Tris-HCl pH 7.4 was used as a positive control.

5.3, 8.3, 9.5, and 11.1 for C4-HSL, C6-HSL, C8-HSL, C10-HSL, 3-oxo-C12-HSL, and C14-HSL, respectively. Since almost the same retention times were obtained for the reaction products of the same HSLs treated with NaOH, this is a confirmation of lactone hydrolysis by YtnP lactonase. YtnP lactonase comparison of peak heights of intact HSLs and their degradation products shows that YtnP lactonase completely hydrolyzed C14-HSL under the conditions described above. The HPLC analysis shows that YtnP lactonase is active for a wide range of AHLs, but shows higher efficacy in degrading long-chain AHLs (Fig. 4). The ability of YtnP lactonase to hydrolyze homoserine lactones was confirmed by LC-MS/MS (with C8-HSL and C10-HSL) (Supplementary Fig. S9). Considering that each bacterial species uses a unique AHL or a unique combination of AHLs in cell-to-cell communication [3], substrate specificity of YtnP lactonase makes it suitable for targeted suppression of QS of certain bacterial species or modulating QS outputs. Assuming that strain 6960 acquired lactonase activity as a competitive strategy to suppress the QS system of environmental competitors, the higher prevalence of YtnP lactonase for long acyl chain lengths can be explained by the fact

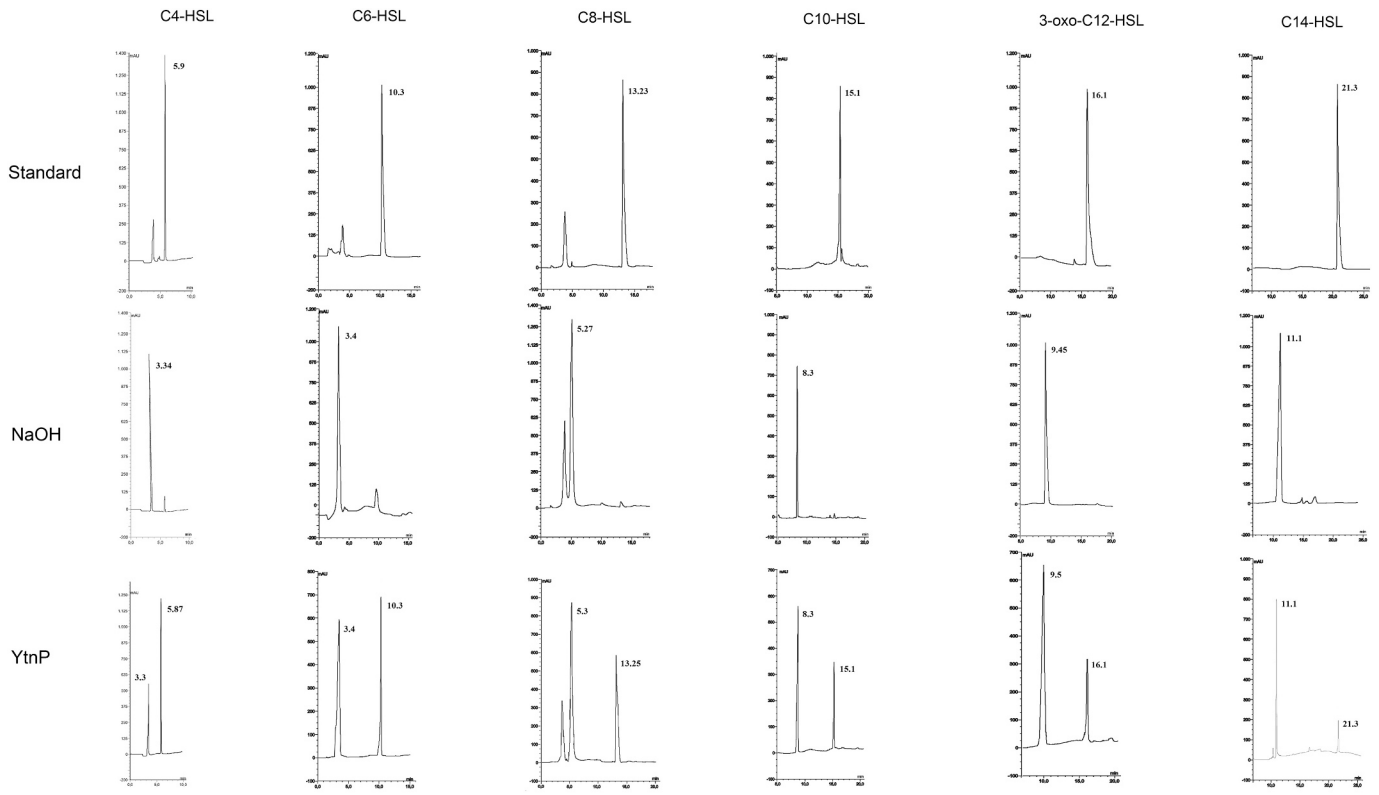

Fig. 4. HPLC substrate specificity profile of recombinant YtnP lactonase on AHLs with different acyl chain lengths (from C4 to C14). 1 M NaOH was used as a lactone hydrolysis control. AHLs standards, YtnP treated AHLs, and AHLs treated with 1 M NaOH were incubated under the same conditions, and used for HPLC analysis. Main ion peaks of AHLs standards, as well as AHLs degradation products were presented.

that *S. maltophilia* is frequently isolated together with pathogens such as *P. aeruginosa* or *Acinetobacter baumanii*, which use long-chain AHLs to induce the expression of virulence factors [71,72].

3.5. Functional characteristics of YtnP lactonase

3.5.1. YtnP lactonase inhibits biofilm formation, induces decomposition of preformed P. aeruginosa MMA83 biofilms, and suppresses virulence factors production

The key properties of QQ enzymes that make them useful for antimicrobial therapy are their ability to disrupt QS and interfere with biofilm and virulence factors production [73]. To evaluate the effect of

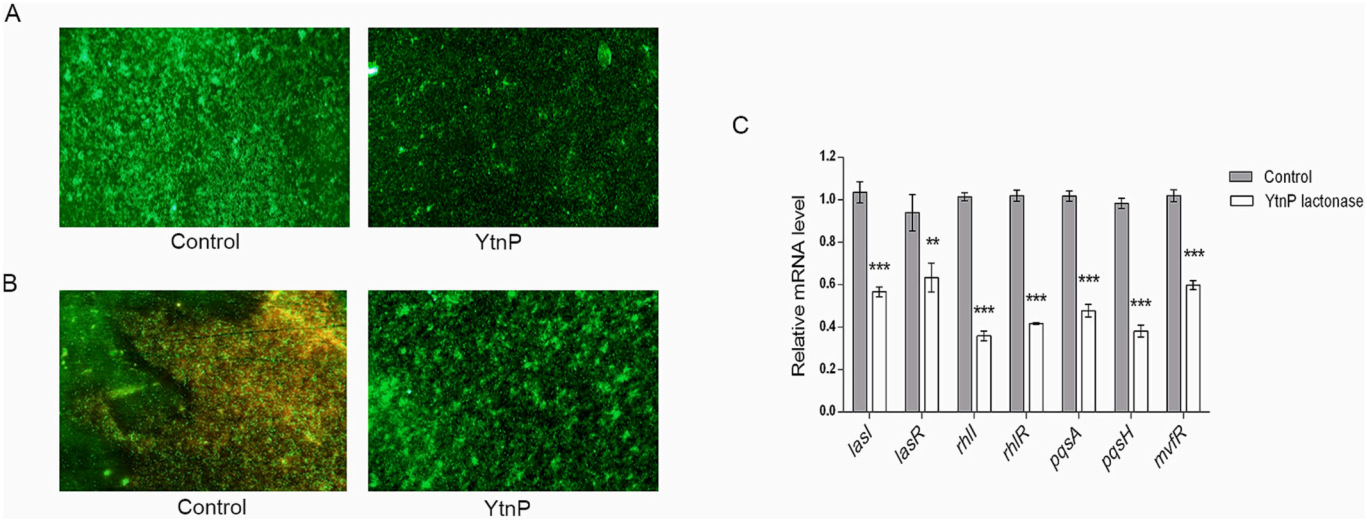

Fig. 5. The effect of YtnP lactonase on biofilm formation, decomposition of preformed biofilm and QS system of *P. aeruginosa* MMA83. Fluorescence micrographs of the MMA83 biofilm (60× magnification) show the effect of recombinant YtnP lactonase in (A) prevention of biofilm formation and (B) induction of biofilm decomposition. Results obtained by RT-qPCR (C) after treatment of *P. aeruginosa* MMA83 strain with YtnP lactonase were compared with the untreated MMA83 control. Student's *t*-test was used to evaluate if there was a significant difference between the control and treated group (**$p < 0.01$, ***$p < 0.001$).

YtnP lactonase on virulence factors production of clinical isolate *P. aeruginosa* MMA83, several assays were performed. Fluorescence microscopy analysis shows a strong effect of YtnP lactonase on preventing biofilm formation (Fig. 5A) and initiating the decomposition of the preformed biofilm of *P. aeruginosa* MMA83 (Fig. 5B). Compared with the control, biofilm formation of *P. aeruginosa* MMA83 was significantly inhibited in the presence of YtnP lactonase, resulting in a very loose biofilm with a considerable number of individual cells (Fig. 5A). After treatment of the preformed biofilm with YtnP lactonase, the MMA83 biofilm was hindered, and a significant decrease in confluence and density with more dispersed biofilm aggregates was observed. Based on fluorescence images, no negative effect of YtnP lactonase on the growth of *P. aeruginosa* MMA83 planktonic cells was detected (Fig. 5B). These results are in line with previously reported data about the ability of QQ enzymes to effectively inhibit *P. aeruginosa* biofilm formation [74]. However, the efficiency of YtnP lactonase to induce the decomposition of preformed biofilms is not a common characteristic of QQ enzymes and could have a high significance in facilitating the eradication of previously established infection. Considering the poor penetration of antibiotics into biofilms and the easy spread of antimicrobial resistance genes between bacteria within the biofilm [75], preventing biofilm formation and disrupting preformed biofilms may increase the susceptibility of *P. aeruginosa* to antibiotics or phages. Considering that QS plays a vital role in biofilm formation by *P. aeruginosa* [76], we further evaluated the expression of the QS genes belonging to three QS networks-las, rhl, and pqs. Results shows that all tested genes of *P. aeruginosa* MMA83 treated with YtnP lactonase were significantly downregulated (Fig. 5C). The most striking effect of YtnP lactonase on QS genes was observed in the reduction of the transcription of the *rhlI*, C4-HSL synthase gene, whose mRNA level was about 2.8-fold lower compared to the control. In the presence of YtnP lactonase, the relative mRNA level of the response regulator gene *rhlR* was reduced by 2.4-fold. The relative mRNA level of the *lasI/lasR* gene was slightly less reduced. The transcription of autoinducer synthase gene *lasI* and the transcriptional regulator *lasR* were found to be reduced 1.9 and 1.5-fold, respectively, after treatment with YtnP lactonase. The relative expression of PQS, the third QS system of *P. aeruginosa*, was also significantly reduced after YtnP lactonase treatment. The relative mRNA levels of *pqsA*, the first gene in the PQS synthesis gene cluster, and *pqsH*, the gene responsible for the conversion of HHQ to PQS, were reduced by 2.1-fold and 2.6-fold, respectively, while the relative expression of *mvfR*, the transcriptional regulator, was 1.7-fold lower after treatment with YtnP lactonase compared with control. The effectiveness of YtnP lactonase in interfering with the QS signaling of *P. aeruginosa* could explain the reduction in biofilm formation of MMA83 treated with YtnP lactonase. However, the mechanism of YtnP lactonase in inducing the decomposition of preformed biofilm in MMA83 remains unclear and requires further investigation. Overall, this property makes YtnP lactonase a potential anti-biofilm agent that could be used for coating medical implants intended for long-term or permanent use. The antibiofilm activity of YtnP lactonase may be specifically useful for combating serious health issues based on biofilm formation, such as in the oral cavity and cystic fibrosis [77].

Treatment with YtnP lactonase also reduced the production of virulence factors of the *P. aeruginosa* MMA83 strain including elastase, pyocyanin, and rhamnolipid by 50 %, 60 %, and 60 %, respectively (Fig. 6A, B, C). The reduction of MMA83 virulence factors production was in accordance with the results of RT-qPCR which show that treatment with YtnP lactonase decreases the relative mRNA level of genes

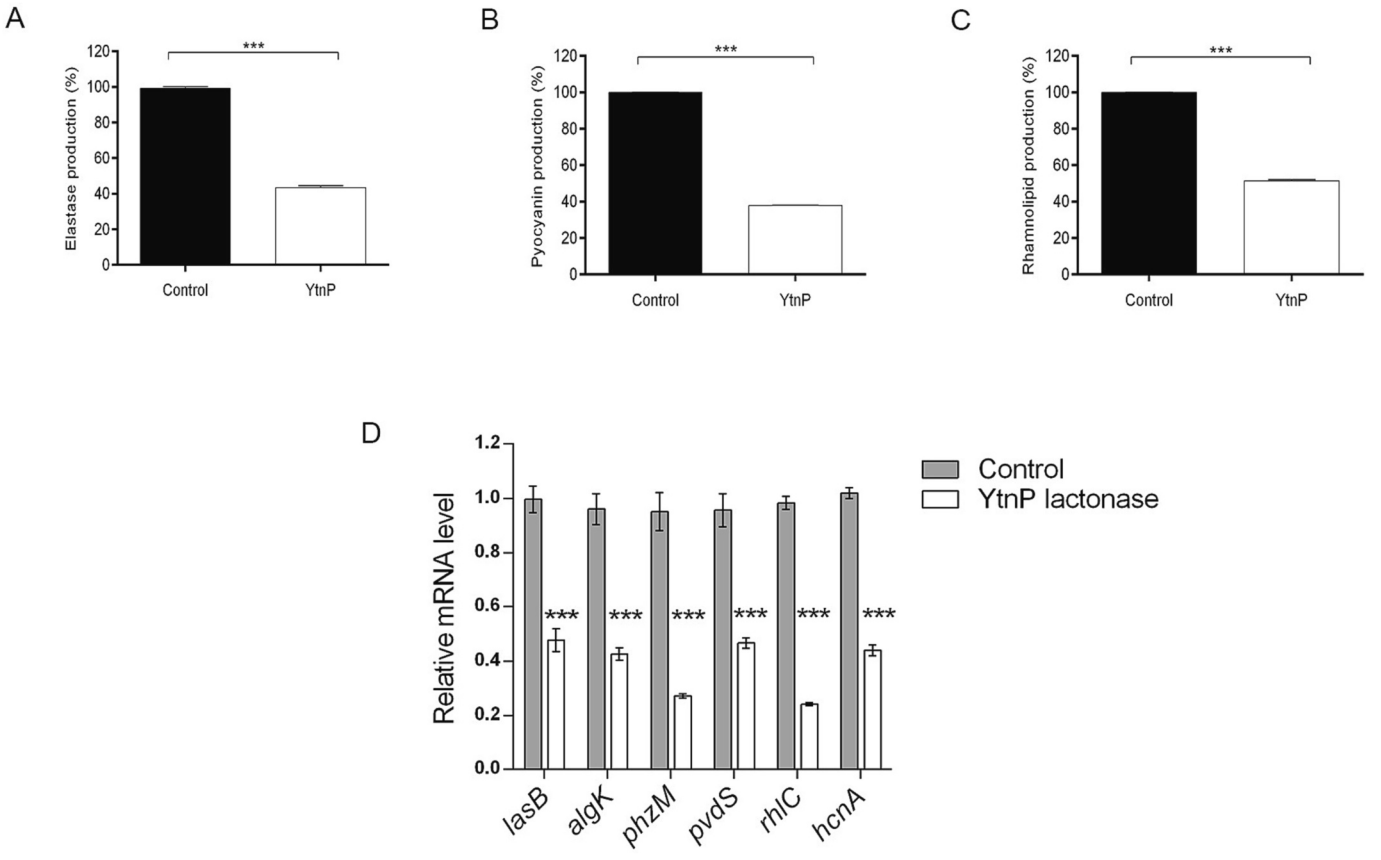

Fig. 6. The effect of YtnP lactonase on virulence factors production and transcription of virulence factors genes of *P. aeruginosa* MMA83. The impact of YtnP lactonase on (A) elastase, (B) pyocyanin, (C) rhamnolipid production and (D) relative mRNA levels of virulence factors genes of *P. aeruginosa* MMA83. *P. aeruginosa* MMA83 untreated with YtnP lactonase was used as control. Student's t-test was used to evaluate if there was a significant difference between the control and treated groups (***$p < 0.001$).

involved in the production of elastase (*lasB* about 2-fold), alginate (*algK* about 2.2) pyocyanin (*phzM* about 3.5-fold), pyoverdine (*pvdS* about 2-fold), rhamnolipid (*rhlC* about 4-fold), and hydrogen cyanide (*hcnA* about 2.2 fold) (Fig. 6D), suggesting that YtnP lactonase exerts an antivirulence effect at the transcription level, and confirms the effectiveness of lactonases in reducing *P. aeruginosa* virulence factor production [4]. These results indicate that the antivirulence activity of YtnP lactonase is the result of the downregulation of QS-related genes, which is consistent with the previously reported data that biofilm formation and virulence factor production is QS dependent [78].

3.5.2. YtnP exhibits a synergistic effect with gentamicin and additive with meropenem against P. aeruginosa MMA83

To further investigate the therapeutic potential of YtnP lactonase, the effectiveness of clinically significant antibiotics combined with recombinant YtnP lactonase was evaluated using the checkerboard method. The MICs of gentamicin and meropenem against MMA83 were 16 mg/ml and 0.5 mg/ml, respectively (Table 3) and MMA83 was susceptible at 0.8 mg/ml YtnP lactonase concentration. YtnP lactonase showed a synergistic effect with gentamicin ($\sum$FIC = 0.375) and an additive effect with meropenem ($\sum$FIC = 0.5625). These results suggest that the potential use of YtnP lactonase in combination with clinically important antibiotics could increase the efficacy of therapy without causing the adverse effects associated with high antibiotic dosing regimens [79]. These results are consistent with previously reported data that QQ enzymes can significantly increase bacterial susceptibility and enhance antibiotic efficacy in vitro and in vivo and facilitate the eradication of infections [63,80].

3.5.3. YtnP lactonase shows no cytotoxic effect and prolongs the life span of C. elegans infected with YtnP-pretreated P. aeruginosa MMA83

The recombinant YtnP lactonase does not show any cytotoxic effects, neither on the immortalized human HaCaT cell line (data not shown) nor on two strains of *C. elegans* including immunocompromized AU37 and N2 wild type (Fig. 7A, B). Based on the results of the toxicity analysis conducted both in vitro and in vivo, it has been determined that recombinant YtnP lactonase could be considered safe for use. These findings are consistent with previously reported data on the lack of toxic effects of certain QQ enzymes on eukaryotic cells, as stated in the study by Djokic and coauthors [63].

Additionally, *C. elegans* was used as a model system to test the antivirulence potential of recombinant YtnP lactonase against *P. aeruginosa* MMA83 in vivo. The toxin-mediated killing liquid assay showed that the survival rates of immunocompromised *C. elegans* AU37 mutant and N2 wild type strain infected with the clinical isolate *P. aeruginosa* MMA83 pre-treated with YtnP lactonase are significantly higher compared to untreated control (Fig. 7A, B). Beside potent protective response we observed in wild type animals, YtnP lactonase

Table 3
The checkerboard method showing the effect of antimicrobials against *P. aeruginosa* MMA83.

	Pseudomonas aeruginosa MMA83				
	MIC of each antimicrobial (mg/ml)				
Antimicrobials	Alone	Combination	FIC	$\sum$FIC	Outcome
YtnP lactonase	0.8	0.05	0.0625	0.5625	Additive
Meropenem	0.5	0.25	0.5		
YtnP lactonase	0.8	0.2	0.25	0.375	Synergistic
Gentamycin	16	2	0.125		

FIC – fractional inhibitory concentration, FIC = MIC combination / MIC alone. $\sum$FIC – sum of two FICs, $\sum$FIC = FIC of antibiotic + FIC of YtnP lactonase. The outcome was defined as a synergistic if the sum of two FICs (FIC of the antibiotic and FIC of the YtnP lactonase) was ≤ 0.5; additive if $0.5 < \sum FIC \leq 1$; indifferent if $1 < \sum FIC < 4$; antagonistic if $\sum FIC > 4$.

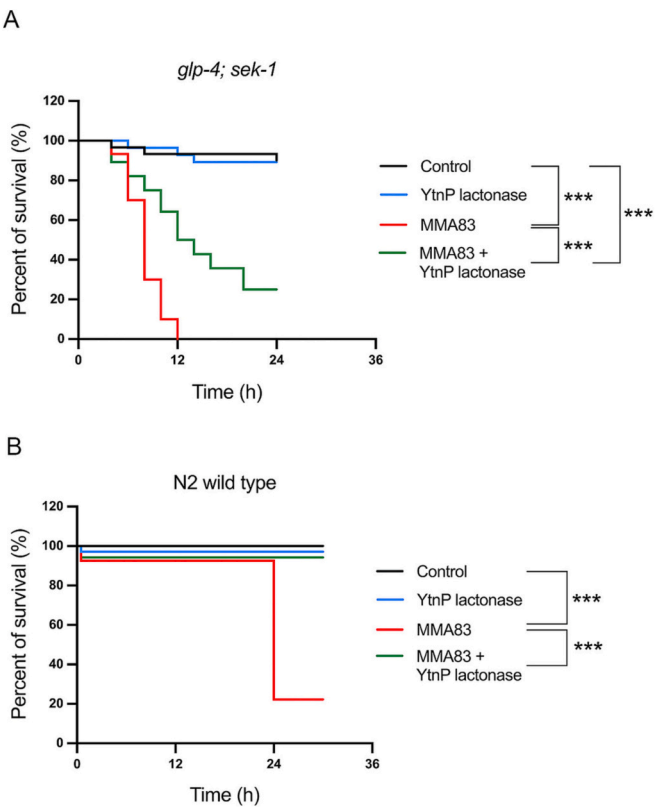

Fig. 7. The examination of cytotoxicity and antivirulence potential of recombinant YtnP lactonase using *C. elegans* in vivo model system. (A) The percentage of survival of pathogen sensitive *C. elegans* AU37 mutant after 24 h of treatment with recombinant YtnP lactonase and *P. aeruginosa* MMA83. (B) The effects of recombinant YtnP lactonase on the survival rate of N2 wild type animals and its activity in worms infected with *P. aeruginosa* MMA83. The survival curves were plotted using the Kaplan-Meier method, and the log-rank (Mantel-Cox) test was used to analyze the difference in survival rates in GraphPad Prism 9.0. A statistically significant difference in survival rate (***$p < 0.001$) was observed in the treatment groups.

significantly prevent mortality of the pathogen sensitive AU37 worms which is of a great importance for the application in patients struggling with infections of different etiology with already weakened immune response. The efficacy of YtnP lactonase in extending the lifespan of both *C. elegans* strains infected with *P. aeruginosa* is consistent with previous findings of QQ enzymes reducing the pathogenicity of *P. aeruginosa* in the *C. elegans* infection model [81]. In liquid infection assays, *C. elegans* death is mediated by the production of virulence factors by pathogens rather than bacterial intestinal accumulation in the form of clamps, which supports our assumption that YtnP lactonase exhibit potent antivirulence activity [82]. Hydrocyanic acid produced by *P. aeruginosa* is reported to exert a major pathogenic effect on *C. elegans* by causing rapid neuromuscular paralysis and subsequently death [83]. The production of hydrogen cyanide is mainly regulated by the las and rhl QS systems [84]. YtnP lactonase downregulates the relative mRNA level of the *hcnA* gene, which is the first gene in the *hcnABC* gene cluster responsible for hydrogen cyanide synthesis in *P. aeruginosa*. As hydrogen cyanide is associated with the virulence of *P. aeruginosa* and its lethality in *C. elegans*, the proposed hypothesis suggests that the reduction in hydrogen cyanide levels through YtnP lactonase activity may contribute to the protection of *C. elegans* from MMA83-induced death [83]. The prolonging of the life span of *C. elegans* infected with *P. aeruginosa* MMA83 pretreated with YtnP lactonase and lack of toxicity, indicate the potential of YtnP lactonase to be used as a prophylactic agent.

However, QQ enzymes face several challenges before becoming a feasible therapeutic option. Notably, the expression of some virulence

factors in bacteria is not controlled only by the QS system [85]. Another potential issue is that interference with QS systems might affect pathogen growth, exerting selective pressure and potentially facilitating the emergence of resistant pathogens [86]. Furthermore, in polymicrobial infections, where interactions between pathogens are mediated by QS-controlled factors, interference with QS systems in one pathogen could potentially enhance the pathogenicity and antibiotic resistance of co-infecting pathogens [87]. Additionally, having diagnostic tools sensitive enough to detect infecting pathogens at low cellular densities is crucial. This sensitivity allows for the implementation of QQ strategies before the pathogen reaches the quorum necessary to trigger its full pathogenic potential [88,89].

4. Conclusion

In this study, the new clinical isolate *S. maltophilia* 6960 was described for the first time in the territory of Serbia. Genomic analysis revealed important virulence and antibiotic resistance determinants of this strain. Considering the rapid emergence and spread of MDR pathogens, there is an urgent need to find new alternative approaches to control infections caused by pathogenic bacteria. In addition to pathogenic potential, *S. maltophilia* 6960 also exhibited QQ activity. The YtnP lactonase gene encoding novel QQ enzyme, was identified in the genome of this strain, and further characterized. YtnP lactonase belongs to the MBL family of proteins and degrades both short- and long-chain AHLs, with a higher preference for long-chain AHLs. This substrate specificity allows recombinant YtnP lactonase to modulate the QS of many pathogens and act as an antivirulence agent. The main focus of this research was to investigate the antivirulence potential of YtnP lactonase against the MDR clinical isolate *P. aeruginosa* MMA83. Recombinant YtnP lactonase was shown to inhibit biofilm formation and disrupts existing biofilms, as well as reduce virulence factors production of the MMA83. The efficacy of YtnP lactonase in reducing virulence of MMA83 was confirmed in vivo using *C. elegans* as a model system. The synergistic effect of YtnP lactonase in combination with gentamicin and the additive effect with meropenem against MMA83 indicate the potential of YtnP lactonase to be used in combination with antibiotics to reduce antibiotic dosage and increase the efficiency of antibiotic therapy. The advantageous biochemical properties, such as high temperature and pH stability, the lack of cytotoxicity, and significant antivirulence potential make YtnP lactonase a promising candidate for the design of innovative therapeutics to combat bacterial infections caused by MDR *P. aeruginosa*. Additionally, further studies need to be performed to confirm the in silico structural prediction of YtnP lactonase and to investigate the antivirulence potential of YtnP lactonase against other clinically important pathogens.

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CRediT authorship contribution statement

Jovana Curcic: Data curation, Investigation, Methodology, Writing – original draft. **Miroslav Dinic:** Methodology, Validation, Writing – review & editing. **Katarina Novovic:** Investigation, Methodology, Visualization, Writing – review & editing. **Zorica Vasiljevic:** Resources, Writing – review & editing. **Milan Kojic:** Funding acquisition, Resources, Writing – original draft. **Branko Jovcic:** Conceptualization, Funding acquisition, Resources, Writing – review & editing. **Milka Malesevic:** Conceptualization, Formal analysis, Investigation, Writing – original draft, Writing – review & editing.

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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