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APPLIED GENETICS AND MOLECULAR BIOTECHNOLOGY

Overexpressed recombinant quorum quenching lactonase reduces the virulence, motility and biofilm formation of multidrug-resistant *Pseudomonas aeruginosa* clinical isolates

Masarra M. Sakr[1] · Khaled M. Aboshanab[1] · Walid F. Elkhatib[1] · Mahmoud A. Yassien[1] · Nadia A. Hassouna[1]

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Abstract

The increasing occurrence of resistance among *Pseudomonas aeruginosa* clinical isolates necessitates finding alternatives to antibiotics for controlling the infection of such pathogenic bacteria. In this study, lactonase gene *ahl*-1 from *Bacillus weihenstephanensis* isolate-P65 was successfully cloned and expressed in *Escherichia coli* BL21 (DE3) under the control of *T7* promoter for utilizing its quorum quenching activity against three multidrug-resistant (MDR) *P. aeruginosa* clinical isolates. The biological activity of the overexpressed lactonase enzyme (Ahl-1), tested using a synthetic signal and *Chromobacterium violaceum* CV026 as a biosensor, displayed good catalytic activity using hexanoyl homoserine lactone (HHL) as a substrate and *Chromobacterium violaceum* (CV026) as a biosensor (77.2 and 133 nm min^{-1} for the crude and the purified Ahl-lactonase enzymes, respectively). Upon challenging its ability to inhibit the virulence of three MDR *P. aeruginosa* clinical isolates, recombinant Ahl-1 successfully prevented the accumulation of acylhomoserine lactone signals resulting in a significant reduction in the investigated virulence determinants; protease (from 40 up to 75.5%), pyocyanin (48–75.9%), and rhamnolipids (52.7–63.4%) (P value < 0.05). Ahl-1 also displayed significant inhibitory activities on the swarming motility and biofilm formation of the three tested MDR *P. aeruginosa* clinical isolates (P value < 0.05). Consequently, Ahl-1 lactonase enzyme in this study is considered a promising therapeutic agent to inhibit *P. aeruginosa* pathogenicity with no fear of emergence of resistance.

Keywords Recombinant lactonase Ahl · *P. aeruginosa* · Quorum quenching · *Bacillus weihenstephanensis*

✉ Khaled M. Aboshanab
aboshanab2012@pharma.asu.edu.eg; aboshanab2003@yahoo.com

Masarra M. Sakr
masarra_pharma@yahoo.com

Walid F. Elkhatib
walid2005faisal@yahoo.com

Mahmoud A. Yassien
myassien61@yahoo.com

Nadia A. Hassouna
nadia.hassouna46@gmail.com

[1] Department of Microbiology and Immunology, Faculty of Pharmacy, Ain Shams University, POB: 11566, Organization of African Unity St., Abbassia, Cairo, Egypt

Introduction

Microbial infections have for centuries been classified as major challenges to human progress, side by side with wars and famine. Pandemics and epidemics throughout history had a major impact on mankind (Morens et al. 2004). The biggest challenge in combating deadly infectious diseases is the continuous emergence of resistance among the various strains of microorganisms. Continuous deployment of antimicrobial drugs in treating infections has led to a dramatic increase both in the proportion and absolute number of bacterial pathogens presenting multidrug resistance to antibacterial agents (Loeffl and Stevens 2003). Infections caused by resistant strains have been a matter of concern for years, since they are associated with a threefold higher rate of mortality, a ninefold higher rate of secondary bacteremia, a twofold increase in the length of hospital stay, and a

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considerable increase in healthcare costs (Giamarellou 2002). To the extent that in February 2017, the WHO published its first ever list of antibiotic-resistant "priority pathogens"—a list of bacteria for which new antibiotics are urgently needed—on top of which came the MDR *Pseudomonas* (WHO 2017). *P. aeruginosa* is intrinsically resistant to several antibiotics because of the low permeability of its outer membrane, the constitutive expression of various efflux pumps, and the production of antibiotic-inactivating enzymes (e.g., cephalosporinases) (Hancock 1998). Furthermore, it also has a remarkable capacity to develop or acquire new mechanisms of resistance to antibiotics. This may be related to the large size and the versatility of its genome, and to its distribution in aquatic habitats, which could constitute a reservoir for bacteria carrying other resistance genes (Vaisvila et al. 2001). *P. aeruginosa* is responsible for a variety of pathologies, particularly in neutropenic patients (30–50%) (Maschmeyer and Braveny 2000; Sligl et al. 2006; Mesaros et al. 2007), nosocomial pneumonia (Chastre and Fagon 2002), and cystic fibrosis patients (Stefani et al. 2017). *P. aeruginosa* is also the third leading cause (12%) of hospital-acquired urinary tract infections (Obritsch et al. 2005).

It is clear therefore that MDR *P. aeruginosa* represents one of the most challenging pathogenic bacteria and a threat to the public health for which new therapeutic strategies, including drugs acting on new targets, are urgently needed. Luckily, several functions displayed by *P. aeruginosa* were found to be regulated by quorum sensing, a mechanism by which a population of bacteria can control gene expression and modulate several functions (Viretta and Fussenegger 2004; Schuster and Greenberg 2006; Lee and Zhang 2015). *P. aeruginosa* has aluxI-luxR system, in which luxI encodes the biosynthesis of an acylhomoserine lactone (AHL) signal, and luxR encodes an AHL-dependent transcription factor. Based on this fact, one alternative to antibiotics is finding therapeutic agents that inhibit or interfere with quorum sensing. Some bacteria have been reported to produce enzymes with the ability to degrade AHLs, among which are lactonases (Dong et al. 2001; Leadbetter 2001). Some studies tested the ability of lactonase to inhibit the virulence of some *P. aeruginosa* isolates (Wang et al. 2007; Sakr et al. 2014; Guendouze et al. 2017). However, none of these studies were conducted using MDR isolates.

Accordingly, the present study focused on AHL quorum quenching as a new approach for the development of an antipathogenic drug effective against MDR *P. aeruginosa* isolates. Therefore, the gene coded for a lactonase enzyme from a *Bacillus* soil isolate was heterologously expressed in standard *Escherichia coli* strain and the expressed lactonase was tested for its ability to reduce some virulence traits, biofilm formation, and motility of some MDR *P. aeruginosa* clinical isolates.

Materials and methods

Bacterial strains, vectors, and growth conditions

Bacillus weihenstephanensis-P65 isolate from Culture Collection Ain Shams university (CCASU) (strain number, CCASU-P65; NCBI accession code KC899665) was previously isolated from soil and studied for its quorum quenching lactonase activity (Sakr et al. 2013). Three multidrug-resistant *P. aeruginosa* isolates were isolated from wound specimens and their resistance profiles were investigated. *P. aeruginosa* isolates coded P1 (strain number CCASU-P1) and *P. aeruginosa* coded P2 (strain number CCASU-P2) were resistant to penicillin/clavulanate combination, cephalosporins, aminoglycosides, flouroquinolones, carbapenems, and lincomycins as well as Co-triamoxazole. They were sensitive to macrolides and one of them, coded P2, displayed sensitivity to tetracycline. Isolate *P. aeruginosa* coded P3 (strain number CCASU-P3) displayed resistance to penicillin/clavulanate combinations, cephalosporins, and Co-triamoxazole. They were all routinely grown in Luria Bertani broth at 37 °Cat 160 rpm. *E. coli* DH5α (Hanahan 1983) and *E. coli* BL21 (DE3) (Studier et al. 1990) were used for cloning and expression of lactonase, respectively. *Chromobacterium violaceum* (CVO26) was used as an acyl homoserine lactone-dependent biosensor purchased from NCTC (NC 13278.T). It was grown in Luria Bertani (LB) broth containing 20 μg/ml kanamycin for purification at 28 °C (Ravn et al. 2001). It can also be grown in LB without kanamycin for maintenance and stored in slant medium or lyophilized for long-term preservation. Plasmid pUCPU21 (U. Wehmeier, Wuppertal Germany) was used as a cloning vector while pET22b (Novagen, Merck, CA, USA) was used as an expression vector. Restriction enzymes, *T4*-DNA ligase, and DNA gel extraction kit were purchased from New England Biolabs, Co. DNA and plasmid extraction kits were purchased from Zymo research (USA). Ni-NTA spin column kit was purchased from Qiagen (Germany).

DNA isolation and manipulation

Genomic DNA extraction of *B. weihenstephanensis* was carried out using Zymo DNA extraction kit according to the manufacturer recommendations. Cloning, preparation, and transformation of competent *E. coli* cells and in vitro DNA manipulation were carried out according to standard protocols (Sambrook and Russell 2001).

PCR amplification

The *ahl*-1 gene coded for acyl homoserine lactonase (accession code KC823046) was amplified by PCR using the extracted genomic DNA as a template and the primers Ahl-F

(*Nde*I) 5′-AAAGGTGGATACCATATGACAG TAAAGAAG-3′ and Ahl-R (*Bam*HI) 5′- ACGTAGGAT CCTGACTTTTTGCACTATATATATTC-3′. The forward primer was designed to introduce a *Nde*I site (underlined) in place of the natural start codon and for the ability to create a start codon fusion of *ahl*-1—downstream the ribosomal binding site (RBS) of the cloning pUCPU21 and expression pET22b vectors. On the other hand, the reverse primer was designed to introduce a *Bam*HI restriction site located immediately downstream of the natural stop codon in order to permit oriented insertion in the respective vectors. The PCR was performed in the thermal cycler (Nyx Technik) using 25 pmole of each primer, 200 ng of genomic DNA, and the PCR mixture (50 μl) was formulated according to the protocol supplied with the Dream Taq master mix kit (Thermo Fisher Scientific, USA). The following conditions were used: initial denaturation took place at 95 °C for 5 min followed by 30 cycles of denaturation (at 95 °C for 30 s), annealing (at 55 °C for 50 s), and extension (at 72 °C for 1 min). Final extension took place at 72 °C for 5 min. The obtained amplicon was analyzed using agarose gel electrophoresis (Sambrook and Russell 2001) and verified by DNA sequencing.

Construction of the recombinant plasmid

The PCR-amplified product was double digested using fast digest R *Nde*I and *Bam*HI (New England Biolabs) and cloned into the appropriate site of pUCPU21 resulting into pUCPU21-*ahl*-1 recombinant plasmids thereafter, transformed into *E. coli* DH5α according to the modified Hanahan method (Hanahan 1983). After transformation, pUCPU21-*ahl*-1 plasmid was extracted and double digested with *Nde*I and *Bam*HI. The resulting passenger DNA (*ahl*-1) was isolated from agarose using DNA gel extraction Kit (MonarchR, NEB, USA), thereafter ligated into *Nde*I-*Bam*HI sites of pET22b generating the recombinant plasmid pET22b-*ahl*-1 vector, transformed into *E. coli* BL21 (DE3).

Heterologous expression of ahl-1 gene in *E. coli* BL21 (DE3)

Expression of Ahl-1 was carried out as described by Studier et al. (1990). Expression was performed under the control of *T7*-promotor using *E. coli* BL21(DE3) as a host strain. A single colony harboring the plasmid pET22b-*ahl*-1 and a single colony harboring the empty vector (pET22b) were grown overnight in 5 ml LB medium containing ampicillin (100 μg/ml) at 37 °C, 200 rpm in a shaking incubator (preculture). About 200 μl from each of the pre-culture (test) and control was inoculated into 20 ml fresh LB broth containing ampicillin. The cultures were grown at 37 °C/250 rpm until OD600 reached 0.5. *T7*-RNA polymerase production was induced by the addition of 1 mM IPTG. Samples (1 ml) were taken prior to IPTG induction and at the following time intervals after the addition of IPTG: 1, 2, and 4 h. The cells were centrifuged at 13,000 rpm for 3 min, washed twice with ice-cold 25 mM Tris-HCl, pH 7.5 and kept at −20 until use or resuspended in cracking buffer (25 mM Tris-HCl, pH 7.5, 1 mM dithiotheritol) for sonication and appropriate amounts were analyzed using SDS-PAGE according to the protocol described by Sambrook (2000).

Preparation of cell-free lysate containing recombinant Ahl enzyme

Five-milliliter cultures of the 24-h induced *E. coli* BL21 (DE3) pET22b-*ahl*-1 were harvested by centrifugation at 12,000 rpm for 5 min in the cooling centrifuge and washed twice with ice-cold 25 mM Tris-HCl buffer, pH 7.5. The pellet was resuspended in 1.5 ml cracking buffer (25 mM Tris-HCl, pH 7.5, 1 mM dithiotheritol). The cells were then disrupted by intermittent sonication in ice bath for a total time of 2.5 min (15 s of sonication followed by 30 s interval at 60 W). Finally, the cell debris was sedimented by centrifugation (12,000 rpm for 10 min at 4 °C). Protein concentration in the cell-free lysate was then determined using Lowry's method (Lowry et al. 1951) and adjusted to 3 mg/ml.

Purification of the expressed His-tagged Ahl-1

The expressed His-tagged native Ahl-1 was undertaken using Ni-NTA column kit. Purification took place according to the supplied protocol with sonication added as a modification. Cells harvested by centrifugation of 5-ml induced cultures were resuspended as described in NPI-10 supplemented with lysozyme and incubated as described for 30 min. Intermittent sonication was then applied for a total time of 30 s. Cell-free supernatant was then transferred to pre-equilibrated Ni-NTA columns and the rest of the protocol was carried out as described. The eluted His-tagged Ahl-1 protein was analyzed using SDS-PAGE followed by protein determination (Pierce TM protein assay kit, Thermo Fisher, USA). Protein concentration was adjusted to 3 mg/ml.

Assay of the expressed Ahl-1 activity

Determination of the AHL-degrading activity of either crude or purified His-tagged Ahl-1 was done using hexanoyl homoserine lactone (HHL) as a substrate and CV026 as a biosensor. Catalytic activity rate was determined according to Cao and his coworkers (Cao et al. 2012) with some modifications as follows: a concentration of 10 μM HHL in 0.5 ml of the purified enzyme or crude enzyme extract was incubated at 28 °C, then, the reaction was terminated after 30 min by adding 2% SDS. A control was done using plain buffer containing 10 μM HHL. The residual amount of HHL was measured by well diffusion assay as described in

Sakr et al. (2013). One unit of AHL-degrading enzyme activity was defined as the amount of enzyme that hydrolyzed 1 nM HHL per minute.

Extraction of AHL signals and determining quorum sensing productivities of *Pseudomonas* isolates

Extraction of AHL signal was done as previously described by Ravn and his coworkers (Ravn et al. 2001). The concentration of the produced signal was determined by well diffusion assay where the purple zone diameters were measured and compared to a constructed standard curve showing the diameters of the purple zones formed as a function of different HHL concentrations. The quorum sensing (QS) productivities were then calculated relative to the bacterial culture.

In vitro test of the effect of Ahl-1 on some virulence determinants of MDR *P. aeruginosa* clinical isolates

Overnight culture of each *P. aeruginosa* isolate was prepared in 5 ml LB broth incubated at 37 °C at 200 rpm in shaking water bath. About 250 μl of the grown cultures were used to inoculate 25 ml LB broth contained in a 250-ml flask. Flasks were then incubated at 37 °C for 2 h. Then, the 25 ml was divided into five aliquots (5 ml each). Of these, two were used as test experiments supplemented with a final concentration of 0.5 mg/ml of either a crude (T1) or a purified (T2) Ahl-1 enzyme. The remaining three aliquots were used as control experiments as follows: control C1 was prepared by adding 500 μl saline; control C2 was prepared by adding the cell-free lysate of *E. coli* BL21-pET22b and control C3 was prepared by adding NPI-500 buffer (elution buffer used in Ahl-1 purification). The *P. aeruginosa* cultures were then grown overnight, centrifuged, and supernatants collected and the following virulence determinants were measured.

Protease assay

This assay was carried out as described previously by Nicodème et al. (2005). In an Eppendorf tube, a 125 μl aliquot of 2% *w*/*v* azocasein solution was incubated with 75 μl of the tested supernatants at 37 °C for 45 min. The reaction was stopped by adding 600 μl of 10% trichloroacetic acid for 10 min at room temperature. After that, the mixture was centrifuged for 5 min at 12,500 rpm and 600 μl of the supernatant obtained was transferred to a tube containing 500 μL of 1 M NaOH. The absorbance of the developed orange color was measured spectrophotometrically at 440 nm. Blank was prepared by replacing that the supernatant with plain culture medium. The proteolytic activity was determined using the equation: $Y = 0.2221X + 0.4613$ (Osama et al. 2017) where Y represents the absorbance at 440 nm and X represents the log protease concentration in units per milliliter. The assay was done in triplicates and the mean and standard deviation were calculated.

Determination of rhamnolipids with oil displacement assay

This was done according to Morikawa et al. (2000). In brief, 20 ml of distilled water was added to a Petri dish followed by addition of 20 μl of crude oil to the surface of the water. Ten microliters of the collected supernatants were then added to the oil surface. The oil displaced with an oil-free clearing zone formed and the diameters of these clearing zones were measured. The assay was done in triplicate: each time, the displacement zone was measured five times, and the mean and standard deviation were calculated.

Pyocyanin assay

This was done as described by Essar and Eberly (1990). In this assay, 5 ml supernatants were extracted with 3 ml chloroform and then re-extracted into 1 ml of 0.2 N HCl to give a pink color. The absorbance was then measured at 520 nm. Concentrations (expressed as micrograms of pyocyanin produced per milliliter of culture supernatant) were determined by multiplying the optical density at 520 nm by 17.072. The assay was done in triplicate with the mean and standard deviation calculated. Percentage reduction in the pyocyanin level was determined.

Effect on swarming

As previously reported (Rashid and Kornberg 2000), a volume of 2 μl of diluted *P. aeruginosa* cultures (106 cfu/ml) was inoculated onto the surface of dried swarming plates (1% *w*/*v* tryptone, 0.5% *w*/*v* NaCl, and 0.7% *w*/*v* agar) supplemented with 0.5 mg/ml crude (T1) or purified (T2) Ahl-1. Plates were incubated for 24 h at 30 °C. The diameters of the zones formed were measured and compared. Control plates were prepared as follows: a control plate with no additives (C1), a control to which cell-free lysate obtained from *E. coli* BL21-pET22b was added (C2) and a plate to which NPI-500 was added (C3). The assay was done in triplicate and the mean and standard deviation were calculated.

Effect on biofilm formation

Assay for biofilm formation was carried out as described by Stepanovic et al. (2007). Overnight *P. aeruginosa* cultures were diluted in LB broth to 1×10^6 cfu/ml. Aliquots of 100 μl of the prepared suspension were delivered to the wells of 96-well sterile microtiter plates. For each isolate, aliquots were divided into five groups each of six replicates, crude and purified Ahl-1 were added to the wells of the first (T1) and second (T2) groups, respectively, at a final concentration of 0.3 mg/ml. A volume of 10 μl sterile water was added to group C1. Cell-free lysate obtained from *E. coli* BL21-pET22b was added to group C2 and NPI-500 buffer

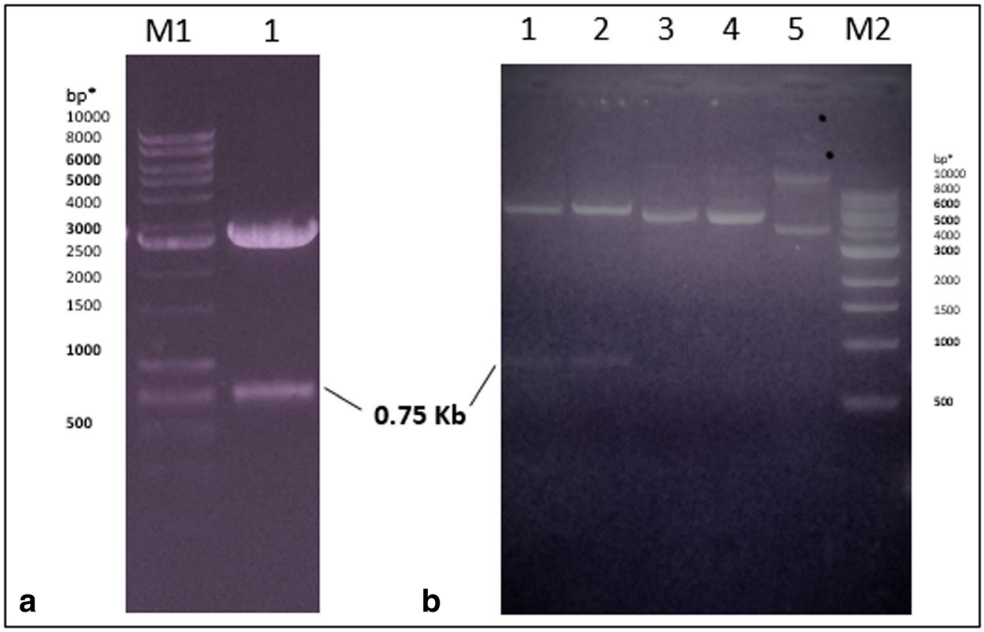

Fig. 1 **a** Gel electrophoresis showing the recombinant plasmid pUCPU21-*ahl*-1 transformed into *E.coli* DH5α. Lane M1, 1 kb DNA ladder (Sigma, USA); lane 1, pUCPU21-*ahl*-1 double digested with *Nde*I and *Bam*HI. **b** Gel electrophoresis showing the recombinant plasmid pET22b-ahl-1 transformed into *E. coli* BL21 (DE3). Lanes 1 and 2, clone 1 and 2; pET22b-ahl-1 double digested with *Nde*I and *Bam*HI; lanes 3 and 4, undigested pET22b-ahl-1 of clones 1 and 2; lane 5, empty pET22b vector; M2, 1 kb DNA ladder (New England Biolabs, USA)

was added to group C3. After incubation for 24 h at 37 °C, the contents of the microtiter plate were gently removed and wells were washed three times with sterile phosphate-buffered saline to remove planktonic cells. The adherent bacterial cells were fixed by adding 100 μl of 99% methanol for 20 min. Methanol was removed, the wells were then stained with aliquots of 100 μl crystal violet (1% *w*/*v*) for 20 min, and any excess dye was removed by washing with distilled water. The plates were air dried and the bound dye was dissolved by adding 80 μl of 33% glacial acetic acid and absorbance at 570 nm was measured.

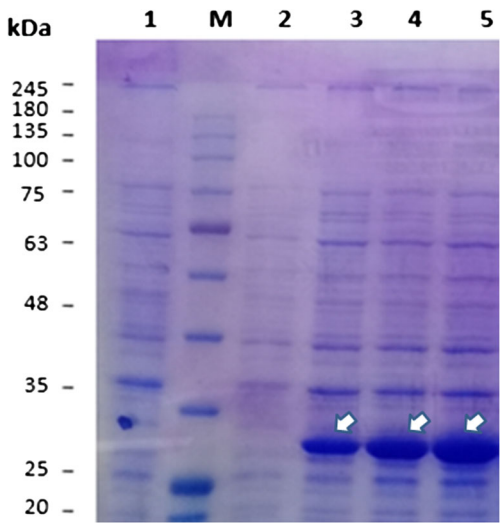

Fig. 2 Detection of overexpressed Ahl-1 lactonase by SDS-PAGE after IPTG induction: 1, control (no band observed at 28 kDa). M: Protein marker 2, Sample at zero time before induction (non-induced BL21-pET22b-ahl-1); 3, sample after 1 h of induction with IPTG showing Ahl-1 lactonase protein at about 28 kDa; 4, sample after 2 h of induction with IPTG; 5, sample after 4 h of induction with IPTG

Statistical analysis

All the experiments were carried out in triplicates and the results were represented as respective average values ± standard deviation. Data were analyzed using GraphPad InStat-3 software (GraphPad Software Inc., USA).

Results

Construction of recombinant plasmids

The amplified *ahl*-1 gene was successfully cloned into the cloning pUCPU21 plasmid (Fig. 1a) and the expression plasmid pET22b (Fig. 1b).

Detection of overexpressed Ahl-1 using SDS-PAGE

As shown in Fig. 2, the expression of recombinant Ahl-1 analyzed using SDS-PAGE showed that Ahl-1 was overproduced as a soluble N-terminal His-tagged protein in *E. coli* BL21 (DE3). An additional band of about 28 kDa present in the soluble fraction of the cell-free lysate corresponding in size to the expected molecular mass (28.14 kDa) of the His-tagged protein was detected. The maximal overexpression was obtained at 35 °C and 4 h after IPTG induction.

Measuring the activity of the crude and purified Ahl-1 enzyme

Catalytic activity rates of crude and purified Ahl-1 were found to be 77.2 nm min^{-1} and 133 nm min^{-1}, respectively. Crude

Table 1 The relative QS signal productivities of the MDR *P. aeruginosa* isolates used in the present study

P. aeruginosa isolate code no.	Amount of signal produced in growth supernatant in terms of HHL (μM)
P1	0.179 ± 0.041
P2	0.85 ± 0.13
P3	0.068 ± 0.02

recombinant enzyme displayed lactonase activity of 77.2 U/mg while purified enzyme displayed activity of 88.7 U/mg protein.

The AHL signal productivities of the three MDR clinical *P. aeruginosa* isolates

The isolates displayed AHL signal productivities ranging between 0.068 and 0.85 μM as shown in Table 1.

In vitro testing of the effect of Ahl-1 on some virulence determinants of the MDR *P. aeruginosa* isolates

Protease

Results (Fig. 3) showed that Ahl-1 successfully decreased protease production in the three tested isolates both in its crude and purified forms. Percentage reduction in protease concentration in the supernatants of isolates P1, P2, and P3 was 57.9%, 60.66%, and 44%, respectively, when crude Ahl-1 was used and 63.9%, 75.3%, and 48.8% when the purified enzyme was used.

Rhamnolipids

Ahl-1 reduced rhamnolipid production in the three tested *P. aeruginosa* isolates expressed in terms of diameter of oil displacement zone. Percentage reduction for isolates P1, P2, and P3 was 52.7%, 63.4%, and 55%, respectively, when crude Ahl-1 was used and 55.4%, 64.9%, and 56.5% when the purified enzyme was used (Fig. 4).

Pyocyanin

Results (Fig. 5) showed that Ahl-1 decreased the production of pyocyanin pigment in the three *P. aeruginosa*-tested isolates. Percentage reduction in pyocyanin concentration in the supernatants of isolates P1, P2, and P3 was 72.3%, 73%, and 48%, respectively, when crude Ahl-1 was used and 74.9%, 75.9%, and 53.1% when purified Ahl-1 was used.

Swarming

Figure 6 showed that the presence of Ahl-1 in the test medium both in its crude and purified forms decreased the swarming motility of the three MDR *P. aeruginosa* isolates. It was also reported that the buffer used in lactonase purification (NPI-500), present in control (C3), displayed an inhibitory activity on *P. aeruginosa* swarming motility. Results for the crude and purified enzymes were calculated relative to the control prepared for each. The percentage reduction in swarming motility of the three tested isolates P1, P2, and P3 was 47.2%, 52.2%, and 50.3%, respectively, when crude Ahl-1 was used and 26.3%, 44.4%, and 39.5% when purified Ahl-1 was used.

Biofilm formation

Results displayed the ability of Ahl-1 to inhibit biofilm formation in the three tested isolates both in its crude and purified forms. NPI-500 (in C3) also displayed relative reduction in the biofilm formation when compared to the control with no supplements (C1). In Fig. 7, biofilm formation in C1 was considered 100% to which relative biofilm formation of the other test groups was calculated. From the data, it was found that crude Ahl-1 reduced the biofilm formation in P1, P2, and P3 with a percentage of 36.7%, 40.7%, and 29.2%, respectively, when compared to its

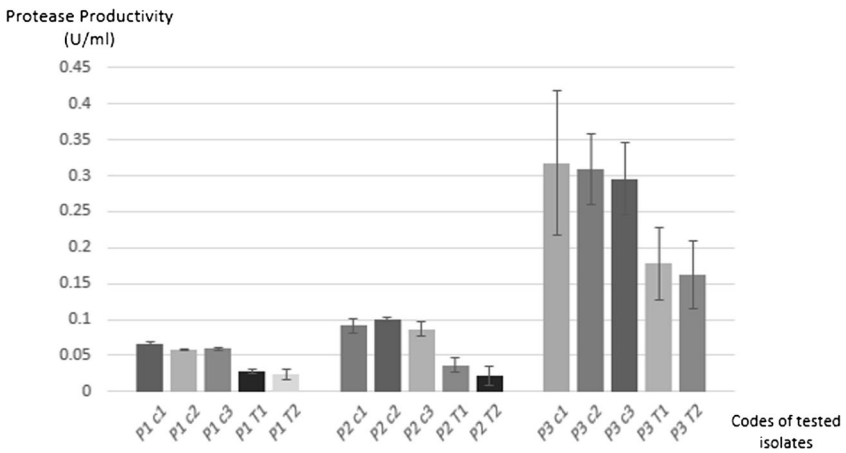

Fig. 3 Protease concentration in the supernatants of *P. aeruginosa* cultures in the presence and absence of recombinant Ahl-1. C1, the control grown with no supplements; C2, control for the crude Ahl-1, prepared by supplementing the medium with *E. coli* BL21-pET22b cell-free lysate. C3, control for purified Ahl-1, prepared by supplementing the medium with the elution buffer NPI-500. T1, test using crude Ahl-1. T2, test using purified Ahl-1

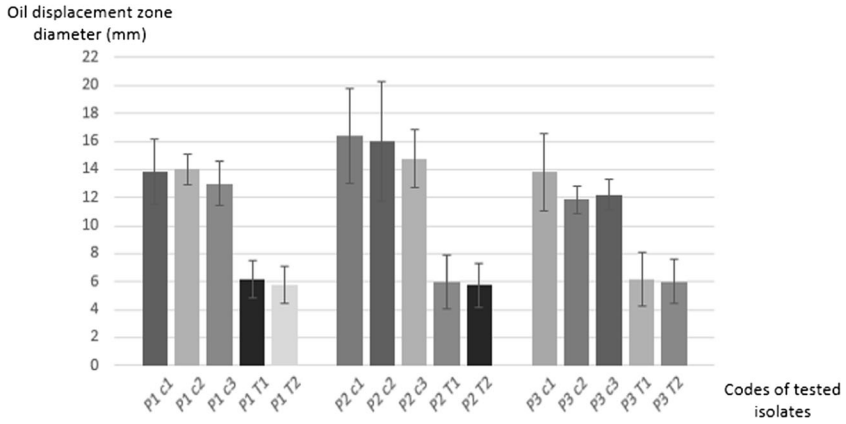

Fig. 4 Rhamnolipid production by the *P. aeruginosa* isolates in terms of oil displacement zone diameter in the presence and absence of Ahl-1. C1, the control grown with no supplements. C2, control for the crude Ahl-1, prepared by supplementing the medium with *E. coli* BL21-pET22b cell-free lysate. C3, control for purified Ahl-1, prepared by supplementing the medium with the elution buffer NPI-500. T1, test using crude Ahl-1. T2, test using purified Ahl-1

control (C2). The purified enzyme reduced the biofilm formation in P1, P2, and P3 with a percentage of 25.7%, 39%, and 19.7%, respectively, when compared to its control (C3) and 47.4%, 55.7%, and 35% when compared to C1.

Discussion

In this study, we used a recombinant lactonase enzyme, Ahl-1 to inhibit the virulence of three MDR-resistant *P. aeruginosa* clinical isolates. The prestudied resistance profiles for the *P. aeruginosa* isolates were as follows: isolates coded P1 and P2 were found to be resistant to penicillin/clavulanate combination, cephalosporins, aminoglycosides, flouroquinolones, carbapenems, and lincomycins as well as Co-triamoxazole. They were sensitive to macrolides and one of them, isolate coded P2, displayed sensitivity to tetracyclins. However, isolate coded P3 displayed resistance to penicillin/clavulanate combinations, cephalosporins, and Co-triamoxazole. It is therefore obvious that finding an alternative for antibiotics capable of suppressing the virulence of such bacteria would be of great help in infection control.

The goal of our study was to combat *P. aeruginosa* pathogenicity by degrading AHL quorum sensing signals, thereby preventing the accumulation of the signals and inhibiting the expression of some virulence factors. To achieve this, lactonase-coding gene, *ahl*-1 (accession KC823046), was amplified using primers that were designed with mismatches to create restriction sites for *Nde*I and *Bam*HI enzymes. The gene was successfully cloned into pUCPU21 and transformed into *E. coli* DH5α. Blue and white selection were used to select the right clone harboring *ahl*-1 which was then cloned into pET22b and transformed into *E. coli* BL21 (DE3). Having a *T7*-promoter,

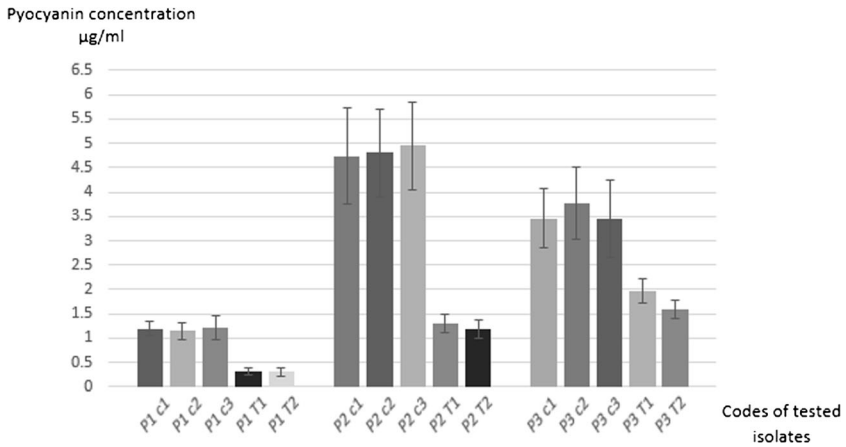

Fig. 5 Pyocyanin concentration in the supernatants of *P. aeruginosa* cultures in the presence and absence of recombinant Ahl-1. C1, the control grown with no supplements. C2, control for the crude Ahl-1, prepared by supplementing the medium with *E. coli* BL21-pET22b cell-free lysate. C3, control for purified Ahl-1, prepared by supplementing the medium with the elution buffer NPI-500. T1, test using crude Ahl-1. T2, test using purified Ahl-1

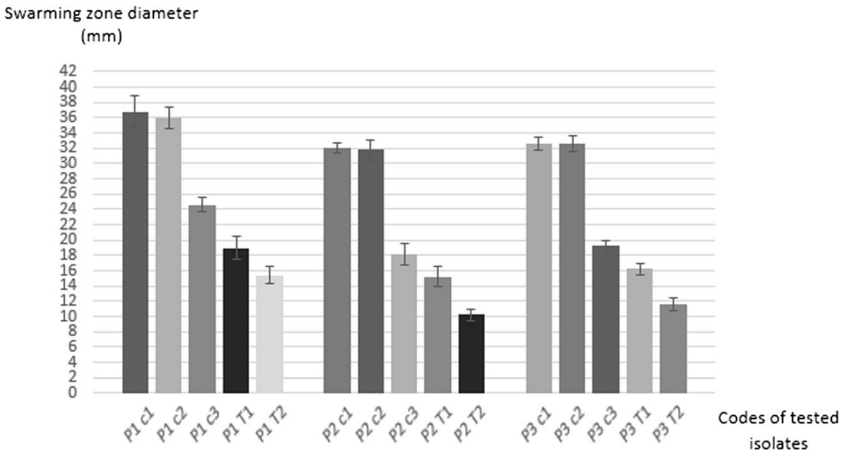

Fig. 6 Swarming of the tested *P. aeruginosa* isolates in the presence and absence of recombinant Ahl-1. C1, the control grown with no supplements. C2, control for the crude Ahl-1, prepared by supplementing the medium with *E. coli* BL21-pET22b cell-free lysate. C3, control for purified Ahl-1, prepared by supplementing the medium with the elution buffer NPI-500. T1, test using crude Ahl-1. T2, test using the purified Ahl-1

pET22b was used as an expression vector where induction of heterologous Ahl-1 was achieved using IPTG. SDS-PAGE showed the production of adequate amounts of the overexpressed Ahl-1 in addition to increase in its production over time. SDS-PAGE was also used to detect the purified enzyme. When tested for its AHL degrading activity using CV026 as a biosensor strain, the AHL lactonase activity of the crude Ahl-1 was found to be 77.2 U/mg protein which increased to 88.7 U/mg upon purification. Prior to testing the ability of the recombinant Ahl-1 to reduce the virulence of some MDR clinical *P. aeruginosa* isolates, the AHL signals produced by these isolates were extracted and quantified in terms of HHL. A great variation in the quantity of signal produced was observed among the three isolates. The isolate coded P3—displaying the least AHL productivity—showed around 8% of the productivity of P2—the highest. This variation might be due to the sensitivity of CV026 used as the biosensor which produces violacein only in response to AHL signals with side chain length between C4 and C8 and can also be due to the complex nature of the quorum sensing circuit in *P. aeruginosa*.

Upon testing the ability of the overexpressed Ahl-1 to inhibit the virulence of the three *P. aeruginosa* isolates in hand, it was found that the enzyme successfully decreased protease production in the three tested isolates. Purified Ahl-1 reduced protease production in isolate coded P2 to less than 25% that of the control. The least affected was isolate P3 in which protease production decreased to 51.2% which is also a significant reduction. Rhamnolipids produced by isolate coded P2 also decreased to less than 36% of that of the control and to less than 44% and 43% for the other two isolates. Pyocyanin production as well was greatly affected secondary to AHL signal degradation upon incorporation of Ahl-1 in the growth medium. Purified Ahl-1 reduced pyocyanin concentration in the growth supernatant of isolates coded P1 and P2 to less than 24% relative to the control. Findings in our study here support the role of quorum sensing signals in controlling the virulence of *P. aeruginosa* apart from its antibiotic resistance pattern. Efficiency of Ahl-1 to reduce virulence of the tested isolates in this study comes in agreement with previous studies in which quorum sensing manipulation resulted in reducing some virulence determinants in *P. aeruginosa* (Sio et al. 2006; Wang et al. 2007). In the two previous studies, quorum quenching was found to reduce elastase and pyocyanin production (Sio et al. 2006; Wang et al. 2007). A recent study also reported the

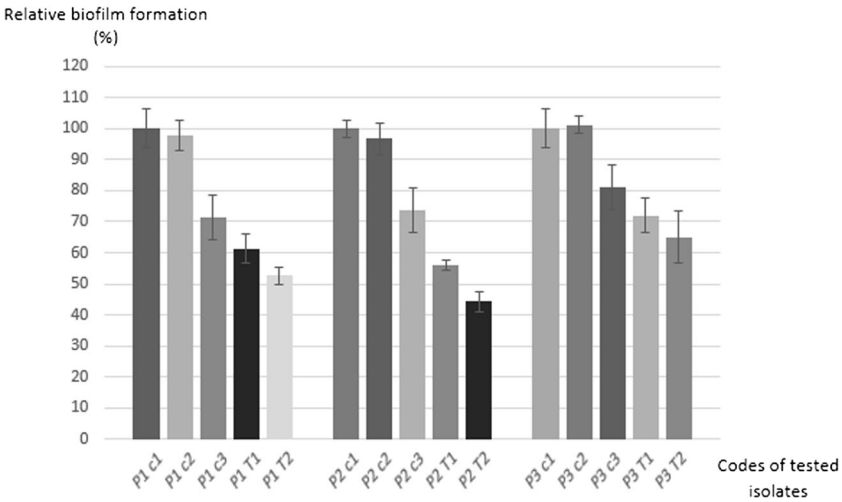

Fig. 7 Relative biofilm formation of *P. aeruginosa* isolates in the presence and absence of recombinant Ahl-1 compared to the biofilm formed in (C1). C1, the control grown with no supplements. C2, control for the crude Ahl-1, prepared by supplementing the medium with *E. coli* BL21-pET22b cell-free lysate. C3, control for purified Ahl-1, prepared by supplementing the medium with the elution buffer NPI-500. T1, test using crude Ahl-1. T2, test using the purified Ahl-1

ability of paraoxonase-1 (hPON1), a human serum enzyme which uses lactonase activity, to hydrolyze AHL, reducing pyocyanin, rhamnolipid, and protease production (Aybey and Demirkan 2016). Another study displayed the ability of lactonase enzyme to decrease the secretion of two virulence factors, proteases and pyocyanin in *P. aeruginosa* isolates, from diabetic foot ulcers (Guendouze et al. 2017).

The results in our study here showed that motility of the tested isolates was also inhibited by recombinant Ahl-1 lactonase. Crude Ahl-1 reduced swarming by around 50% in the three isolates. When the purified enzyme was tested, further reduction in the swarming zone was observed. It was also noted that the buffer NPI-500, used in lactonase purification and as a control for the purified enzyme (C3), reduced swarming of the three isolates. The possible explanation for this is that imidazole present in high concentration in the elution buffer was responsible for this reduction in swarming. This finding itself is quite interesting and can further be investigated. Ability of lactonase to inhibit swarming in bacteria was previously reported in some studies (Wang et al. 2007; Torres et al. 2017). The importance of inhibiting swarming as a means of virulence control lies in the role of swarming as a complex adaptation that has been correlated to an increase in the production of virulence factors and resistance to antibiotics (Overhage et al. 2008). Studying the effect of lactonase on biofilm formation, a reduction in the formed biofilm was observed. A previous study stated that lactonase played significant roles in reducing *P. aeruginosa* biofilm formation (Wang et al. 2007; Christiaen et al. 2014). In this study, Ahl-1 represents a better option as an anti-biofilm agent since it is well known that in the biofilm form, bacteria are more resistant to various antimicrobial treatments and to the host's immune system (Rabin et al. 2015). Having a promising anti-biofilm activity, lactonase is therefore a promising tool for combating *P. aeruginosa* infections. From the previous results, it is clear that the quorum quenching activity of the recombinant Ahl-1 enzyme used in this study can significantly reduce the virulence of the three tested MDR *P. aeruginosa* clinical isolates. It is worth mentioning that reducing the virulence of *P. aeruginosa* through quorum quenching has the advantage of the very limited, or actually the expected no risk, of developing resistance. This is because—in contrast to conventional antibiotics—quorum quenching does not directly affect bacterial survival, instead it depends on degrading the quorum sensing signals and the substantial reduction in the virulence gene expression. In conclusion, a recombinant Ahl-1 lactonase enzyme produced in this study is a promising tool to combat MDR *P. aeruginosa* isolates. It could significantly reduce protease production, rhamnolipids, and pyocyanin production in all the tested isolates. It also displayed an inhibitory activity on the swarming motility and the biofilm formation. To the best of our knowledge, this is the first study in which lactonase quorum quenching activity was evaluated against MDR *P. aeruginosa* clinical isolates. Further studies on the in vivo activity of this enzyme are still conducted in our lab for the aim of developing it into a drug capable of controlling *P. aeruginosa* infections.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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