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Inhibition of quorum sensing-controlled virulence factors in *Pseudomonas aeruginosa* by human serum paraoxonase

Aynur Aybey and Elif Demirkan

Department of Biology, Faculty of Arts and Science, Uludag University, 16059, Bursa, Turkey

Correspondence
Aynur Aybey
aybeyaynur@gmail.com

The role of quorum sensing (QS) in the regulation of virulence factor production in *Pseudomonas aeruginosa* is well established. Increased antibiotic resistance in this bacterium has led to the search for new treatment options, and inhibition of the QS system has been explored for potential therapeutic benefits. If the use of QS inhibitory agents were to lead to a reduction in bacterial virulence, new approaches in the treatment of *P. aeruginosa* infections could be further developed. Accordingly, we examined whether human serum paraoxonase 1 (hPON1), which uses lactonase activity to hydrolyse *N*-acyl homoserine lactones, could cleave *P. aeruginosa*-derived signalling molecules. hPON1 was purified using ammonium sulfate precipitation and hydrophobic interaction chromatography (Sepharose 4B–L-tyrosine-1-naphthylamine). Different concentrations of hPON1 were found to reduce various virulence factors including pyocyanin, rhamnolipid, elastase, staphylolytic LasA protease and alkaline protease. Although treatment with 0.1–10 mg hPON1 ml^{-1} did not show a highly inhibitory effect on elastase and staphylolytic LasA protease production, it resulted in good inhibitory effects on alkaline protease production at concentrations as low as 0.1 mg ml^{-1}. hPON1 also reduced the production of pyocyanin and rhamnolipid at a concentration of 1.25 mg ml^{-1} (within a range of 0.312–5 mg ml^{-1}). In addition, rhamnolipid, an effective biosurfactant reported to stimulate the biodegradation of hydrocarbons, was able to degrade oil only in the absence of hPON1.

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INTRODUCTION

Quorum sensing (QS) is a process of bacterial communication used to collectively control group behaviours (Ng & Bassler, 2009; Rutherford & Bassler, 2012). *Pseudomonas aeruginosa* perceives its local population density using a QS system: as the concentration of the signalling molecules passes a given threshold, the bacteria regulate production of certain virulence factors. To orchestrate the synchronous production of virulence factors, *P. aeruginosa* relies on two major LuxI/R QS systems: the Las and Rhl systems (Pesci *et al.*, 1997; Schuster *et al.*, 2003). The LuxI homologues LasI and RhlI are responsible for synthesis of the Las and Rhl signals, *N*-(3-oxododecanoyl)-L-homoserine lactone (PAI1) and *N*-butanoyl-L-homoserine lactone (PAI2), respectively. PAI1 activates the LuxR-type transcription factor LasR, and, in turn, LasR–PAI1 induces the production of LasB elastase, LasA protease, alkaline protease and LasRI (Gambello *et al.*, 1991, 1993; Seed *et al.*, 1995). PAI2 cooperates with the cognate regulator RhlR to enhance the production of rhamnolipid, pyocyanin and LasB elastase (Latifi *et al.*, 1995; Pearson *et al.*, 1995). Expression of many of the virulence factors in *P. aeruginosa* is controlled by the QS system (Venturi, 2006).

Different strains of *P. aeruginosa* secrete several extracellular proteolytic enzymes that have been implicated as virulence factors. These enzymes include protease IV, alkaline protease, and LasA and LasB elastases (Caballero *et al.*, 2001). These proteases promote replication of the bacteria within an infected host by interfering with the host immune system (Miyoshi *et al.*, 2002; Hung *et al.*, 2005; Hoge *et al.*, 2010). Elastase B is involved in pathogenesis by degradation of human immunologically competent molecules. For example, it has been shown that LasB destroys components of the complement cascade (Schultz & Miller, 1974), cytokines (Parmely *et al.*, 1990), immunoglobulins IgA and IgG (Maeda & Yamamoto, 1996), human airway lysozyme (Jacquot *et al.*, 1985), proteinase-activated receptors (Dulon *et al.*, 2005) and surfactant proteins A and D (Mariencheck *et al.*, 2003). The LasA metalloprotease possesses high staphylolytic activity, lysing *Staphylococcus aureus* cells by cleaving the pentaglycine bridges within the peptidoglycan-rich cell wall, and may also enhance elastolysis by cleaving Gly-Gly peptide bonds abundant in elastin (Toder *et al.*, 1991;

Abbreviations: AHL, acylated homoserine lactone; ECR, elastin–Congo red; hPON1, human serum paraoxonase 1; QS, quorum sensing.

Kessler *et al.*, 1997). LasA is also responsible for the shedding of the host cell surface proteoglycan syndecan-1 (Preston *et al.*, 1997; Park *et al.*, 2000). Another protease involved in the virulence of *P. aeruginosa* is alkaline protease, a metalloprotease that hydrolyses many biologically important proteins including the cytokines (Parmely *et al.*, 1990), complement factors (Hong & Ghebrehiwet, 1992), matrix metalloproteinases (Twining *et al.*, 1993), human IFN-γ and TNF-α (Parmely *et al.*, 1990).

P. aeruginosa synthesizes a characteristic blue-green, chloroform-soluble compound called pyocyanin (1-hydroxy-5-methylphenazine) (Hassett *et al.*, 1992), an important virulence factor. Pyocyanin is able to interfere with critical host defence mechanisms and contributes to the symptoms associated with *Pseudomonas*-associated lung disease (Denning *et al.*, 1998).

Pseudomonas spp. are well known for their ability to produce rhamnolipid biosurfactants with potential surface-active properties when grown on different carbon substrates (Patel & Desai, 1997). It has been reported that rhamnolipids can mediate the initiation of seeding dispersal in non-mucoid *P. aeruginosa* biofilms (Boles *et al.*, 2005).

Interruption of QS, also known as quorum quenching, can be achieved by targeting the signalling molecule itself (Chan *et al.*, 2010, 2011) the transcriptional activator (Manefield *et al.*, 1999) or the autoinducer synthase (Parsek *et al.*, 1999). Thus, anti-QS treatment may be a plausible way to attenuate bacterial virulence without killing the pathogens, thus potentially preventing the acquisition of drug resistance by the bacteria (Adonizio *et al.*, 2006). The discovery that QS plays a critical role in bacterial virulence and survival makes this signalling pathway a novel and potential target for antimicrobial drugs that can act as QS inhibitors, interfering with and attenuating QS-dependent bacterial pathogenicity (Hentzer *et al.*, 2003; Rasmussen & Givskov, 2006). Inhibitors disrupt QS in various ways: by acting as enzymes [e.g. *N*-acyl homoserine lactone (AHL)-lactonase or AHL-acylase] that destroy signalling molecules, as enzymes that degrade the LuxR protein or as AHL mimics that block signalling molecules (Zhang & Dong, 2004).

Evidence showing that the paraoxonase (PON) enzyme family may play an important role in activity against virulence factors in *P. aeruginosa* has been reported. This bacterium uses AHLs in the form of QS signals to regulate several virulence factors (Smith & Iglewski, 2003; Veesenmeyer *et al.*, 2009). The lactonase activity of PON enzymes led to the hypothesis that they may also play a role in the attenuation of bacterial virulence by interfering with QS (Camps *et al.*, 2011). In line with this hypothesis, it has been shown that overexpression of PON2 increases AHL inactivation in human airway epithelial cell lysates (Stoltz *et al.*, 2008); additionally, of the three PON enzymes, PON2 exhibits the highest activity towards the important AHL, *N*-3-oxododecanoyl homoserine lactone (3OC12HSL). It has also been demonstrated that both PON1 and PON2 are important for the control of *P. aeruginosa* QS (Ozer *et al.*, 2005; Teiber *et al.*, 2008).

Therefore, the aim of the present study was to investigate the effect of lactonase activity of hPON1 on pyocyanin, rhamnolipid, elastase, staphylolytic LasA protease and alkaline protease production as a means for modulating the QS-regulated behaviour of *P. aeruginosa* ATCC 35032.

METHODS

Chemicals, bacterial strains and culture conditions. Sepharose 4B, L-tyrosine, 1-naphthylamine, paraoxon and protein assay reagents were obtained from Sigma-Aldrich. All other chemicals were purchased from Sigma-Aldrich or Merck. All of the experiments were conducted at 37 °C and included at least three independent cultures. The bacterial strain used in this study was *P. aeruginosa* ATCC 35032. *P. aeruginosa* ATCC 35032 was maintained in 2 % Luria–Bertani (LB) broth (1 % tryptone, 0.5 % yeast extract, 0.5 %, w/v, NaCl) with shaking at 200 ***g*** for 24 h and on LB agar (supplemented with 1 %, w/v, agar) at 37 °C and allowed to reach to stationary phase prior to inoculation into the virulence factor assays (data not shown). Purified hPON1 was prepared at different concentrations for each QS-regulated behaviour following filter sterilization using 0.2 μm pore-sized filters.

Purification of hPON1 by hydrophobic interaction chromatography. Human serum was isolated from 50 ml fresh human blood. The blood samples were centrifuged at 1500 ***g*** for 15 min and 10 ml serum was collected. hPON1 was isolated by ammonium sulfate precipitation and hydrophobic interaction chromatography. The precipitation intervals for hPON1 were 60–80 % (Sinan *et al.*, 2006). An incremental amount of gradient of ammonium sulfate from 0 to 1 M was used, and the precipitate was collected by centrifugation at 15 000 ***g*** for 30 min. Precipitated fractions were redissolved in 100 mM Tris–HCl (pH 8.0) and subjected to hydrophobic interaction chromatography using a column with Sepharose 4B–L-tyrosine-1-naphthylamine (Sinan *et al.*, 2006). The column was equilibrated in buffer containing 0.1 M Na_2HPO_4 (pH 8.0) and 1 M ammonium sulfate. The hPON1 was eluted with ammonium sulfate gradient using 0.1 M Na_2HPO_4 with and without 1 M ammonium sulfate (pH 8.0).

Paraoxonase assay. Paraoxonase activity towards paraoxon was quantified spectrophotometrically by the method described by Gan *et al.* (1991). The reaction was followed for 2 min at 37 °C by monitoring the appearance of *p*-nitrophenol at 412 nm in a Biotek automated recording spectrophotometer. A final substrate concentration of 2 mM was used during each enzyme assay and all measurements were taken in duplicate and corrected for the non-enzymic hydrolysis. hPON1 activity (1 U l^{-1}) was defined as 1 μmol *p*-nitrophenol formed min^{-1}. In order to maintain activity, purified hPON1 samples were kept in the presence of 2 mM $CaCl_2$ at 4 °C, which controlled activity prior to inoculation into each of the virulence factor assays or each cultivation. In previous studies, it was found that PON1 is stable for 2 years at −80 °C (Huen *et al.*, 2009), 6 months at −20 °C (Beekhof *et al.*, 2012), more than 1 year at 4 °C (Demir *et al.*, 2011) and 24–96 h at 37 °C (Leviev *et al.*, 2001; Golmanesh *et al.*, 2008; Ceron *et al.*, 2014).

Determination of protein concentration. For each purification, protein concentrations of purified hPON1 samples were determined as within a range of 5–10 mg ml^{-1} with similar activity (data not shown) using the Lowry method (Lowry *et al.*, 1951) by measuring absorbance at 600 nm, using BSA as a standard. Differences in protein concentrations of enzyme samples depended on the different human serums used.

Pyocyanin assay. Pyocyanin was produced in modified pyocyanin medium (10 g whey powder, 1.4 g $MgCl_2$, 10 g KH_2PO_4, 10 g $C_6H_5Na_3O_7$) at 37 °C, which was inoculated with *P. aeruginosa* ATCC 35032 that had been grown previously in LB medium for 24 h. Bacterial biomass was monitored spectrophotometrically at A_{600} for 120 h. Pyocyanin production was examined in the presence of 0.312–5 mg hPON1 ml^{-1}. Pyocyanin was extracted from the supernatants of cultures grown in the presence or absence of hPON1 and measured by the method described previously by Essar *et al.* (1990). A volume of 5 ml supernatant (to maximize pyocyanin production) was extracted with 3 ml chloroform. The chloroform layer was transferred to a fresh tube and re-extracted with 1 ml 0.2 M HCl to give a pink to deep red solution. After centrifugation, concentrations of pyocyanin were determined based on measurement of A_{520} and expressed as μg pyocyanin produced (ml culture supernatant)$^{-1}$, calculated using the following formula: $A_{520} \times 17.072/A_{600}$.

Rhamnolipid assay. Rhamnolipid agar plates were prepared and used to detect extracellular glycolipid production by modification of a previously published protocol (Siegmund & Wagner, 1991; Köhler *et al.*, 2000). The CTAB (cetyltrimethylammonium bromide) medium composition was based on M9 minimal salts medium supplemented with 2 mM $MgSO_4$, 0.0005 % methylene blue and 0.02 % CTAB and solidified with 1.8 % agar. A final concentration of 0.05 % glutamate was then added as the sole nitrogen source. After solidification, the plates were inoculated from the strain grown in fresh LB broth without and with 0.312–5 mg hPON1 ml^{-1}. The plates were incubated at 30 °C for 24 h and then kept for at least 48–72 h at room temperature until a blue halo appeared around the colonies, indicating the production of rhamnolipid (Gunther *et al.*, 2005; Pinzon & Ju, 2009). A quantitative method based on the interaction of methylene blue, CTAB and rhamnolipids, as illustrated in the CTAB agar test, was used. This assay involves extraction of the rhamnolipids in chloroform, followed by the addition of methylene blue and CTAB. The resulting formation of a complex is detected by measuring A_{638} (Pinzon & Ju, 2009). The rhamnolipid was produced in agar-free CTAB medium and inoculated with *P. aeruginosa* ATCC 35032 grown in LB medium for 24–48 h. Rhamnolipid production was investigated in the presence of 0.312–5 mg hPON1 ml^{-1}. The amount of methylene blue, CTAB and rhamnolipid complex biomass in the presence of hPON1 was monitored by meaduring A_{638}.

Oil-spreading method. Overnight cultures of *P. aeruginosa* ATCC 35032 grown in agar-free CTAB medium with and without hPON1 at different concentrations were centrifuged and 100 μl supernatant was added to crude vegetable oil on Petri dishes as described by Nasr *et al.* (2009). The appearance of a zone of clearing was an indication of rhamnolipid production acting as a biosurfactant, and zone diameters were then determined. The amount of rhamnolipid in cultures with and without hPON1 was confirmed by displacement of oil around the supernatant.

Elastolytic assay. The elastolytic activity of extracellular supernatants was assayed in the following manner. First, 0.1 ml samples of supernatant taken from cultures of *P. aeruginosa* ATCC 35032 grown in LB medium at 37 °C for 24 h were added to Eppendorf tubes containing 20 mg elastin–Congo red (ECR; Sigma) suspended in 0.9 ml ECR buffer [100 mM Tris/HCl (pH 7.5), 1 mM $CaCl_2$] and 0.1–10 mg hPON1 ml^{-1}. After 4 h incubation with agitation, insoluble ECR was removed by centrifugation (20 000 ***g***, 5 min, 4 °C). The absorbance of the supernatants measured at A_{495} was divided by the A_{600} of the culture. LB medium was used as a negative control (Ohman *et al.*, 1980).

Staphylolytic LasA protease assay. Staphylolytic LasA protease activity was determined by the modified method of Kessler *et al.* (1997). The overnight cells of *S. aureus* ATCC 6538P grown in LB medium were harvested by centrifugation at 15 000 ***g*** for 15 min at 4 °C and the OD_{600} was adjusted to 0.8 and suspended in 1 ml supernatant of *P. aeruginosa* ATCC 35032-produced LasA protease that had been grown in LB medium at 37 °C for 24 h. Next, 0.1–10 mg hPON1 ml^{-1} was added and the mixture incubated at 25 °C for 30 min. The activity was measured by monitoring the decrease in OD of a live cell suspension of *S. aureus* ATCC 6538P. One unit of enzyme was defined as the amount resulting in a decrease in the OD of 1 U min^{-1} at OD_{650}. A control without enzyme was also used.

Alkaline protease assay. The production of alkaline protease was measured by spreading 0.1–10 mg hPON1 ml^{-1} onto an alkaline skimmed milk agar plate (1.0 % skimmed milk, 0.1 % peptone, 0.5 % NaCl, 2.0 % agar, pH 10.0); a colony from overnight cultures of *P. aeruginosa* ATCC 35032 was inoculated using a toothpick at the centre of the plates and the plates were then incubated at 37 °C for 24 h. The production of alkaline protease was confirmed by the formation of a clear zone around the colonies and the halo diameters were measured (Déziel *et al.*, 2003). Data on alkaline protease production were compared against a control for zero inhibition exhibited on medium containing no enzyme.

Statistical analysis. Student's *t*-test was used to analyse the significance of the effect of hPON1 on pyocyanin and rhamnolipid production.

RESULTS

Inhibition of pyocyanin production

Before investigation of the inhibition, the production rate of pyocyanin and growth of biomass was monitored as a function of time. The growth of biomass reached a maximum after 120 h, while pyocyanin production was in the beginning of the stationary phase at this time (Fig. 1a). Therefore, measurement of pyocyanin production for longer (120 h) periods is important, instead of the generally monitored 24–48 h (Prithiviraj *et al.*, 2005).

It was found that pyocyanin produced by *P. aeruginosa* ATCC 35032 was significantly lower in the presence of 0.312–5 mg purified hPON1 ml^{-1}. At a concentration of 5 mg ml^{-1}, pyocyanin production began to decrease (A_{520} from 1.9 ± 0.15 to 0.6 ± 0.1 after 120 h; Fig. 1b). However, in the presence of 5 mg hPON1 ml^{-1}, *P. aeruginosa* ATCC 35032 was still able to produce 19.37–8.78 μg pyocyanin ml^{-1} after 120 h (Table 1).

Inhibition of rhamnolipid production

Rhamnolipids consist of a polar head group and a non-polar tail, such that when they combine with different cationic substances, such as CTAB, they form insoluble ion pairs in aqueous solution, which precipitates as dark blue zones against a blue background in methylene blue agar (Siegmund & Wagner, 1991). The size of the zone of precipitation is proportional to the amount of biosurfactant present in the sample. Dark blue zones showed rhamnolipid production (Fig. 2). Additionally, as shown in Fig. 3, the production of rhamnolipid at different hPON1 concentrations (0.312–5 mg ml^{-1}) began to

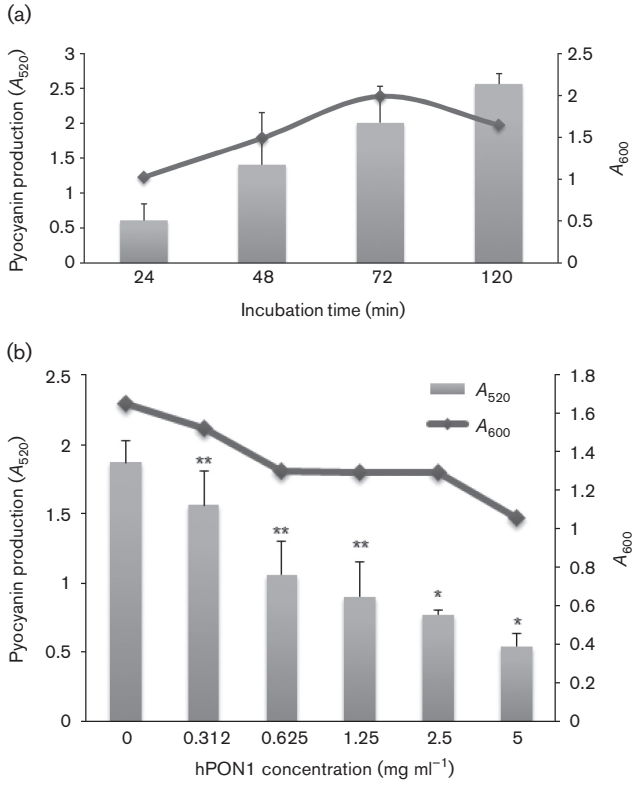

Fig. 1. (a) Growth curve and pyocyanin production of *P. aeruginosa* ATCC 35032 at 24, 48, 72 and 120 h. (b) Decreasing effect of hPON1 on pyocyanin production. Bars, mean pyocyanin concentration (A_{520}); squares, mean A_{600} value. Error bars represent SD of three replicates. The strain was cultivated in LB medium at 37 °C for 24 h in the presence of hPON1 at different concentrations and then cultivated in pyocyanin broth for pyocyanin production under the same conditions. Bars show the mean ± SEM (n=3 independent experiments) with statistical differences at P<0.05 and P<0.01 indicated by * and **, respectively.

Table 1. Pyocyanin concentrations produced by *P. aeruginosa* ATCC 35032 in the presence of hPON1

hPON1 (mg ml^{-1})	Pyocyanin (µg ml^{-1})
0 (control)	19.37
0.312	17.53
0.625	13.93
1.25	11.84
2.5	10.17
5	8.78

The strain was cultivated for 24 h without and with 0.312–5 mg hPON1 ml^{-1} in LB broth. *P. aeruginosa* ATCC 35032 pyocyanin was produced in modified pyocyanin medium for 120 h. The amount of pyocyanin in supernatants was calculated using the formula: $A_{520} \times 17.072/A_{600}$).

decrease. Increased absorbance values showed that higher rhamnolipid–CTAB–methylene blue complexes were present in samples containing lower concentrations of hPON1 (Fig. 3).

Biodegradation of oil

The effect of rhamnolipid-mediated biosurfactant activity on the degradation of crude vegetable oil was measured in the biosurfactant-producing strain *P. aeruginosa*. Supernatants of *P. aeruginosa* grown in the absence of hPON1 in agar-free CTAB medium showed larger zones of clearance than those grown in the presence of hPON1, due to an ability to displace the oil around the colony, which was indicative of biosurfactant production (images not shown) (Table 2).

Effects of hPON1 on *P. aeruginosa* exoprotease production

For examination of staphylolytic LasA protease production by *P. aeruginosa* following treatment with hPON1, cell lysis was measured spectrophotometrically and compared with that of the control (not treated with hPON1). The effect of hPON1-treated *P. aeruginosa* supernatant staphylolytic LasA protease activity on bacterial cell lysis is shown in Fig. 4. The influence of incubation time on cell lysis with staphylolytic LasA protease was investigated over the course of 30 min. We showed that the staphylolytic LasA protease produced by *P. aeruginosa* was not markedly inhibited by the presence of hPON1 at concentrations ranging from 0.1 to 10 mg ml^{-1}. Staphylolytic LasA protease can lyse bacterial cells in the stationary phase of growth (Barequet *et al.*, 2004). However, hPON1 did not have a significant effect on the production of the staphylolytic LasA protease and lower protease levels could not lyse many more *S. aureus* cells.

P. aeruginosa ATCC 35032 supernatants containing different concentrations of hPON1 did not show a significant reduction in proteolysis or staphylolytic LasA protease production (Fig. 4) or in elastolysis or elastase production (Fig. 5), when compared with control supernatants lacking hPON1. Elastolytic activity or elastase production of supernatants treated with hPON1 did not markedly decrease with an increase in hPON1 concentration.

Alkaline protease production or proteolytic activity was observed as a halo around colonies grown on medium in the presence of 0.1–10 mg hPON1 ml^{-1} (Fig. 6). These growth conditions resulted in significant effects on alkaline protease activity or production in the presence of even the lowest concentration of hPON1 (0.1 mg ml^{-1}). As seen in Fig. 6, smaller zones indicated inhibition of alkaline protease activity.

DISCUSSION

In a study, AHLs were detected in bacterial supernatants of late exponential- and stationary-phase culture broths

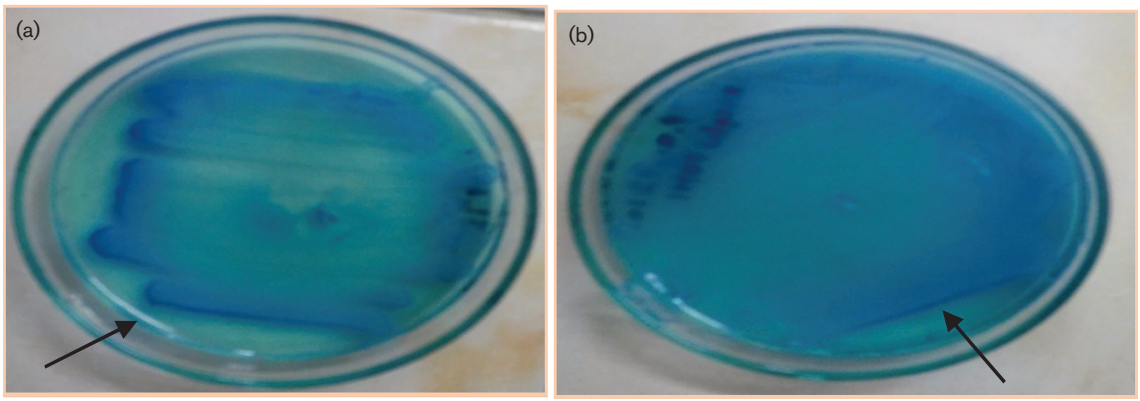

Fig. 2. Representative images of rhamnolipid production in the absence (a) and presence (b) of 5 mg hPON1 ml^{-1} on *P. aeruginosa* ATCC 35032. The strain was cultivated in fresh LB broth without and with hPON1 at 37 °C for 24 h. After cultivation, 100 µl was added to plates contained CTAB medium agar and incubated at 30 °C for 24 h and then kept for at least 48–72 h at room temperature until a blue zone appeared around the colonies.

(Fekete *et al.*, 2010). It has been reported that signal molecules are produced maximally when cultures reach the late stationary phase of growth (Gallagher *et al.*, 2002; Lépine *et al.*, 2003). The stationary-growth-phase time of *P. aeruginosa* ATCC 35032 was determined as 24 h (data not shown) and stationary-phase cells were inoculated into virulence factor medium. In a previous study, the hydrolytic activity of PON1 purified from human serum against the *P. aeruginosa* 3OC12HSL signal was demonstrated (Ozer *et al.*, 2005). PON enzymes seemed to be most active with long-chain AHL signals, such as 3OC12HSL (Chun *et al.*, 2004; Yang *et al.*, 2005). Therefore, we used hPON1 as a lactonase that hydrolyses the homoserine lactone ring of AHL to indirectly inhibit the QS-controlled virulence factors of *P. aeruginosa.*

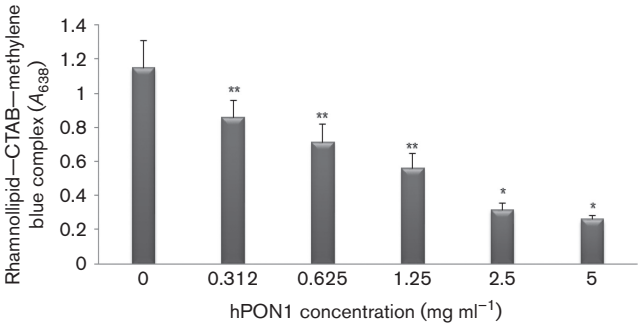

Fig. 3. Effect of different concentrations of hPON1 on rhamnolipid production. *P. aeruginosa* ATCC 35032 was cultivated in fresh LB broth with and without different concentrations of hPON1 at 37 °C for 24 h and added to agar-free CTAB medium and incubated for 48 h. The amount of rhamnolipid–CTAB–methylene blue complex biomass in the presence of the hPON1 was determined. Error bars represent SD of three replicates with statistical differences at $P<0.05$ and $P<0.01$ indicated by * and **, respectively.

Additionally, newly purified hPON1 at different protein concentrations and with similar activities was used in each virulence factor assay: hPON1 was used at concentrations of 0.1–10 and 0.312–5 mg ml^{-1} in the exoprotease assays and other assays, respectively.

Our studies showed that, in addition to being generally effective, hPON1 may be able to act as a quorum-quenching agent sufficient to inhibit the virulence factors produced by *P. aeruginosa.* Pyocyanin is a virulence factor that can cause death, and research indicates that salicylic acid can inhibit pyocyanin production (Prithiviraj *et al.*, 2005). We concluded that pyocyanin extracted from *P. aeruginosa* can be affected by different concentrations of hPON1. Although pyocyanin has antibacterial activity, hPON1 can effectively decrease the production of pyocyanin and therefore could be used more effectively as an antibacterial agent against pathogenic bacteria. Previous studies have investigated the effects of using low

Table 2. Oil-spreading zone diameters at different concentrations of hPON1

hPON1 (mg ml^{-1})	Zone diameter (mm)
0 (control)	37±6
0.312	36±7
0.625	36±3
1.25	27±4
2.5	18±3
5	15±5

Results are shown as means±SD of three replicates. Displacement of oil clearly showed rhamnolipid as an extracellular surfactant present in the supernatant.

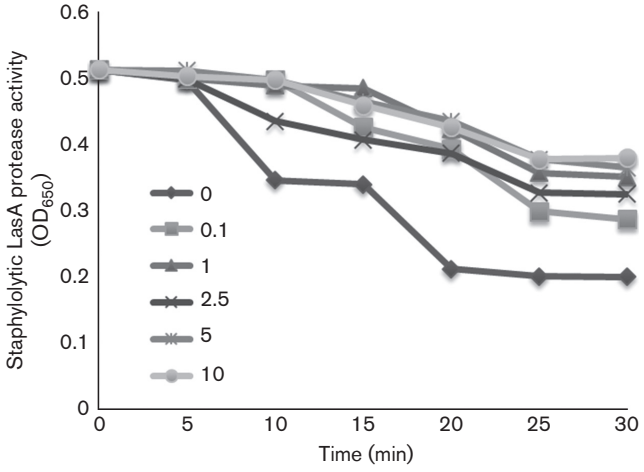

Fig. 4. Staphylolytic assay of *P. aeruginosa* in the presence of hPON1 at different concentrations. The decrease in OD_{650} represents lysis of *S. aureus* cells by *P. aeruginosa* cultures treated with hPON1. Each curve shows the staphylolytic activity of *P. aeruginosa* at different concentrations of hPON1 over time. 0, Control.

concentrations of different compounds administered for short periods of time on the production of pyocyanin in *P. aeruginosa* (Prithiviraj *et al.*, 2005; Bandara *et al.*, 2006). However, our results demonstrated that, under appropriate growth conditions, *P. aeruginosa* survived to a great extent in the presence of hPON1 and that *P. aeruginosa* ATCC 35032 produced pyocyanin for longer periods of time than observed previously. Therefore, to maintain the inhibition for longer periods of time, significantly higher concentrations of hPON1 were necessary; as a result, we used high concentrations of hPON1 to inhibit the production of pyocyanin.

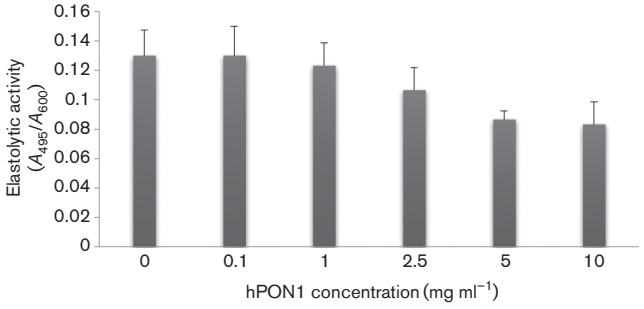

Fig. 5. ECR assay. The strain was cultivated in LB medium at 37 °C for 24 h in the presence of hPON1 and then added to ECR. Elastolytic activity was performed in ECR buffer. Each bar represents the mean ± SEM of three independent assays of a culture supernatant. Experiments were repeated with similar results. 0, Control.

P. aeruginosa is a typical strain for rhamnolipid production (Zhang *et al.*, 2005). Our results indicated that hPON1 has the potential to control *P. aeruginosa* behaviour, as well as the syntheses of rhamnolipids. *P. aeruginosa* ATCC 35032 produced rhamnolipids in CTAB medium as judged by the blue agar plate method. When grown on plates, *P. aeruginosa* secreted detectable levels of rhamnolipid, as visualized by the dark blue haloes in the plate assay. In contrast, *P. aeruginosa* grown with hPON1 did not release detectable levels of rhamnolipids. Similar results were found by a quantitative method based on the interaction of methylene blue, CTAB and rhamnolipids.

A similar approach was used in the oil-spreading test, in which a drop of bacterial supernatant treated or not with hPON1 was added on top of an oil/water interface (Morikawa *et al.*, 2000). The presence of a surface-active molecule such as rhamnolipid will cause the oil to be repelled, forming a clearing zone whose diameter can be correlated with the activity of the tensioactive compounds in the supernatant (Morikawa *et al.*, 2000). We investigated the oil degradation by microbial processes, focusing on the effects of hPON1 on rhamnolipid production.

LasA protease is a staphylolytic endopeptidase secreted by *P. aeruginosa*. It is reported to have lytic action on heat-killed cells of *S. aureus* (Kessler *et al.*, 1997; Caballero *et al.*, 2001). It is a 20 kDa endopeptidase that cleaves peptide bonds following Gly-Gly residues, hydrolysing the pentaglycine bridge required for peptidoglycan stabilization in the cell wall of staphylococci. This activity enables the lysis of *S. aureus in vitro* by cleaving the peptidoglycan layer (Barequet *et al.*, 2009) and is the basis for the high level of staphylolytic activity of this protease (Gustin *et al.*, 1996; Kessler & Ohman, 1998). LasA protease activity on *P. aeruginosa* culture medium treated with different concentrations of hPON1 was determined not to be significant. In addition, the results presented here revealed a lower elastase activity of *P. aeruginosa* in the presence of 0.1 mg hPON1 ml^{-1}. These exoproteases use the same signalling pathways and AHL molecules and the same genes related to the QS system. Our results indicated that the staphylolytic LasA protease and elastase contribute to elastolysis, as determined using an ECR assay and a staphylolytic LasA protease assay; each was found to be active in the absence of the other, but by themselves these enzymes had significantly reduced activities in the presence of hPON1. In contrast to the modest role of elastase in the ECR assay, LasA protease was absolutely necessary for activity in the staphylolytic LasA assay.

Alkaline protease can lead to LasA protease maturation, although far less effectively than elastase and the post-lysine-cleaving enzyme. This component may be important in strains lacking these proteases (Kessler *et al.*, 1998). We observed smaller zones of clearance on alkaline protease plates containing between 0.1 and 10 mg hPON1 ml^{-1}, indicative of an inhibition of alkaline protease production.

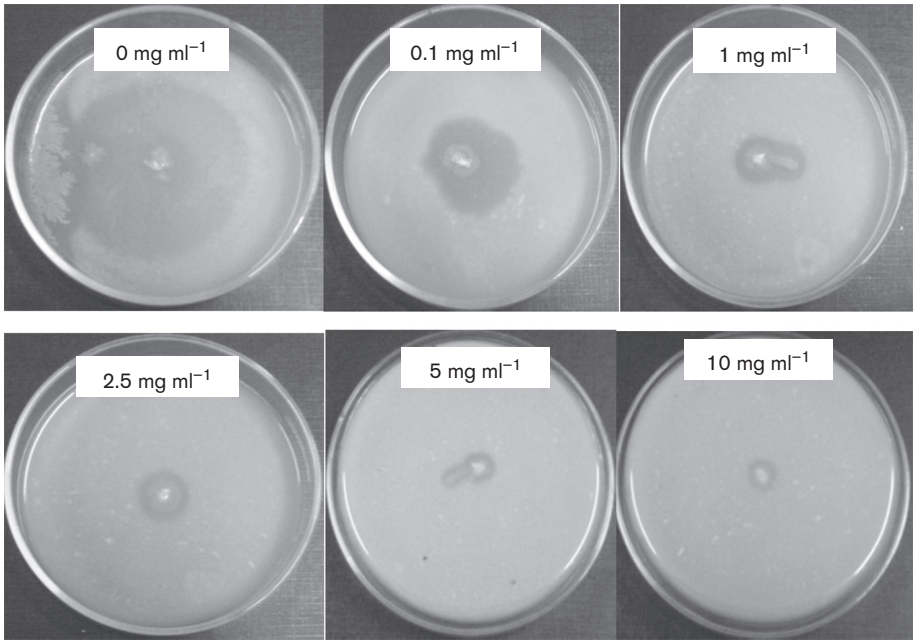

Fig. 6. Dose-dependent inhibition of *P. aeruginosa* alkaline protease production in the presence of hPON1. A colony of the overnight cultures of *P. aeruginosa* ATCC 35032 was added to alkaline skimmed milk agar with 0–10 mg hPON1 ml^{-1} as indicated and incubated at 37 °C for 24 h.

We suggest that hPON1 could potentially be exploited to control QS-regulated behaviours of *P. aeruginosa*. Additionally, it could be used as a lead lactonase enzyme in the screening for antimicrobial agents based on a new antimicrobial target. As a lactonase treatment, hPON1 administration significantly reduced the production of pyocyanin and exoprotease including alkaline protease but not elastase and staphylolytic LasA protease, which are controlled by the *las* QS system, and additionally reduced rhamnolipid production, which is controlled by the *rhl* QS system. Further investigation is required to determine whether hPON1 may be used as an effective therapeutic tool against infectious diseases, contributing to an increase in the efficacy of antibiotic treatments.

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