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Human and murine paraoxonase 1 are host modulators of *Pseudomonas aeruginosa* quorum-sensing

Egon A. Ozer [a,b], Alejandro Pezzulo [c], Diana M. Shih [d], Carlene Chun [e], Clement Furlong [f], Aldons J. Lusis [d], Everett P. Greenberg [g], Joseph Zabner [a,*]

[a] *Department of Internal Medicine Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, IA 52242, United States*
[b] *Molecular Biology Graduate Program, Iowa City, IA 52242, United States*
[c] *Universidad Central de Venezuela, Caracas, Venezuela*
[d] *Department of Medicine, University of California at Los Angeles, Los Angeles, CA 90095, United States*
[e] *Department of Medical Microbiology, University of Wisconsin-Madison, Madison, WI 53706, United States*
[f] *Department of Medical Genetics, University of Washington, Seattle, WA 98195, United States*
[g] *Department of Microbiology, University of Iowa, Iowa City, IA 52242, United States*

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Abstract

The pathogenic bacterium *Pseudomonas aeruginosa* uses acyl-HSL quorum-sensing signals to regulate genes controlling virulence and biofilm formation. We found that paraoxonase 1 (PON1), a mammalian lactonase with an unknown natural substrate, hydrolyzed the *P. aeruginosa* acyl-HSL 3OC12-HSL. In in vitro assays, mouse serum-PON1 was required and sufficient to degrade 3OC12-HSL. Furthermore, PON2 and PON3 also degraded 3OC12-HSL effectively. Serum-PON1 prevented *P. aeruginosa* quorum-sensing and biofilm formation in vitro by inactivating the quorum-sensing signal. Although 3OC12-HSL production by *P. aeruginosa* was important for virulence in a mouse sepsis model, *Pon1*-knock-out mice were paradoxically protected. These mice showed increased levels of PON2 and PON3 mRNA in epithelial tissues suggesting a possible compensatory mechanism. Thus, paraoxonase interruption of bacterial communication represents a novel mechanism to modulate quorum-sensing by bacteria. The consequences for host immunity are yet to be determined.

Keywords: *Pseudomonas aeruginosa*; Quorum-sensing; Paraoxonase; Innate immunity

1. Introduction

The pathogenic bacterium *Pseudomonas aeruginosa* uses acyl-homoserine lactone (acyl-HSL) quorum-sensing signals to regulate the expression of virulence genes implicated in infectivity, tissue damage, and the generation of a host inflammatory response [1–5]. Two hierarchically regulated acyl-HSL quorum-sensing molecules are produced and recognized: *N*-(3-oxododecanoyl)-L-homoserine lactone (3OC12-HSL), and *N*-butanoyl-L-homoserine lactone (C4-HSL) [6–8]. These same quorum-sensing signals also coordinate the establishment of biofilms [1–3,9]. One well-described example of the importance of quorum-sensing in establishing *P. aeruginosa* infection is found in the controlled expression

* Corresponding author. Tel.: +319 335 7608; fax: +319 335 7623.
E-mail address: joseph-zabner@uiowa.edu (J. Zabner).

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of the rhamnolipid group of surfactants. Without coordinated expression of these surfactants by quorum-sensing, *P. aeruginosa* does not form structured, well-differentiated biofilms [10,11].

Other organisms have evolved to disrupt quorum-sensing signaling to compete with bacteria that use quorum-sensing to organize their behavior; some environmental strains of bacteria secrete lactonases or acylases to degrade acyl-HSL signals [12–16]. Bacteria expressing these anti-acyl-HSL factors have a competitive edge over their quorum-sensing neighbors [16,17]. Broad protection by anti-acyl-HSL factors has been demonstrated in transgenic experiments. AiiA (an acyl-HSL lactonase from a soil-dwelling species of *Bacillus*) was transgenically expressed in plants and protected them from soft-rot infection by *Erwinia carotovora*, a quorum-sensing plant pathogen [18]. Previously we reported that some acyl-HSL quorum-sensing signals lose biological activity when exposed to mammalian epithelial cells [19]. Therefore, we hypothesized that mammals, like bacteria, also degrade acyl-HSL quorum-sensing signals. Furthermore, we speculated that this innate activity would confer on the host a competitive advantage over pathogenic bacteria by reducing infection and biofilm formation.

The paraoxonases are a family of mammalian lactone hydrolases with high sequence similarity but distinct substrate specificities and expression patterns [20–22]. PON1 is expressed primarily in the liver and secreted into the serum where it associates with high density lipoprotein (HDL) particles [22–24]. PON3 is also expressed in liver and serum but at a lower level than PON1 [22,25], and PON2 is cell-associated and expressed in several tissues [26]. PON1 protects against organophosphate poisoning [27] and has anti-oxidative properties [28]; it is also associated with protection from atherosclerosis, presumably through its ability to hydrolyze oxidized phospholipids and protect serum lipids from oxidative modification [23,28]. Acyl-HSL molecules are substrates for the human paraoxonases (PON), PON1, PON2, and PON3 [29]. Here we report that human and mouse PON1 degrades 3OC12-HSL by hydrolyzing its lactone ring, thereby blocking *P. aeruginosa* quorum-sensing signaling. (A preliminary report of these findings has been presented [30]).

2. Materials and methods

2.1. Molecular analysis

Synthetic 3OC12-HSL was reconstituted in phosphate buffered saline containing magnesium and calcium (PBS) to 10 μM and incubated for 1 h on monolayers of A549 airway epithelial cells or in the absence of cells. 3OC12-HSL was extracted from the medium in acidified ethyl acetate, dried under sterile nitrogen gas, and reconstituted in deuterated chloroform. Proton-nuclear magnetic resonance (NMR) [6], high-pressure liquid chromatography (HPLC) column, and electrospray mass spectrometry (MS) methods are described in supplemental information.

2.2. Serum studies

Blood was collected from euthanized mice through cardiac puncture and serum was separated from red blood cells by centrifugation. Synthetic 3OC12-HSL (10 μM) was prepared in reaction buffer (100 mM HEPES, pH 7.4, 1 mM $CaCl_2$) and exposed to PBS or whole serum. EDTA (25 mM), oxindole (1 mM), isatin (1 mM), or normal saline was added to serum to evaluate inhibition of serum activity against 3OC12-HSL. 1% solutions of human serum, wild-type mouse serum, or PON1 knock-out (KO) serum in reaction buffer were compared for 3OC12-HSL degradation activity. All reactions were performed for 30 min at 37 °C.

2.3. Purified PON1 studies

Human PON1 was purified from serum as previously described [27,31]. Synthetic 3OC12-HSL (10 μM) was prepared in reaction buffer. Purified PON1 was added to the solution and incubated at 37 °C. Samples of the reaction solution were taken at 15 and 30 min, and 3OC12-HSL concentration determined by bioassay.

2.4. Transgene expression

Chinese hamster ovary (CHO) cells were cultured as monolayers in plastic dishes. Recombinant adenovirus serotype 5 (Ad5) expressing hPON1, hPON2, hPON3, or GFP was produced by the University of Iowa Vector and Gene Targeting core. CHO cells were infected with recombinant Ad5 by calcium phosphate transfection [32] and allowed to express for 48 h. Transfection efficiency was evaluated by fluorescence measurement in Ad5-GFP infected cells. Synthetic 3OC12-HSL (10 μM) in PBS was added to transgene expressing cells and incubated at 37 °C, 5.5% CO_2 for 30 min. Remaining 3OC12-HSL in the medium was measured by bioassay.

2.5. P. aeruginosa *quorum-sensing and biofilm formation*

The effect of PON1 in serum on *P. aeruginosa* biofilm formation was studied in vitro by means of a modified Calgary Biofilm Device assay [33]. Initially, a plastic peg was immersed in each well of a 96-well culture plate containing wild-type or *lasI* mutant PAO1 in 1% tryptic soy broth containing 1 mM $CaCl_2$. After 1 h, the pegs were removed from the PAO1 culture and transferred to wells containing 1% wild-type mouse serum, *Pon1*-KO mouse serum with or without purified human

PON1 (20 μg/mL), or control serum (5% bovine serum albumin in lactated Ringer's solution) with or without purified human PON1. The pegs were incubated for 48 h, followed by a wash in water to remove non-adherent bacteria and staining with 0.5% crystal violet solution. After washing non-bound crystal violet, the stain was eluted in ethanol and quantified spectrophotometrically at A_{560} to measure biofilm biomass.

2.6. Infection studies

All experiments were reviewed and approved by the Animal Care and Use Committee of the University of Iowa. Mice were backcrossed at least 10 generations into C57BL/6 background. Controls were weight, gender, and age matched. Mice were allowed access to food and water ad libitum. Male C57BL/6 mice (8–10 weeks old, weighing 20–25 g) were obtained from Harlan Industries, Inc. (Indianapolis, IN). Overnight cultures of PAO1 or PAO1-*lasI*$^-$ were subcultured to OD600 = 0.2–0.4. Cultures were washed 3× and concentrated to the desired OD600 in cold PBS. Bacteria were kept on ice until injection. Colony forming units (CFUs) were quantified by standard plate counting assay. Mice were injected intraperitoneally with 0.1 mL of *Pseudomonas* suspension. Animals were carefully monitored and euthanized when end-point conditions were met (moribund, distressed, and unable to eat or drink).

2.7. Quantitative PCR of epithelia

For RT-PCR analysis, first strand cDNAs were synthesized using total RNA isolated from the airway epithelia and the ThermoScript RT-PCR system (Invitrogen). Real time quantitative PCR was then performed using the QuantiTest SYBR Green PCR kit (Qiagen) in a PerkinElmer Applied Biosystems PRISM 7700 Sequence Detector (Foster City, CA). Known copy numbers of cloned cDNA plasmids for mouse PON1, PON2, PON3, and GAPDH genes were included in the assay to serve as copy number standards. The ratio of PON cDNA copy number relative to GAPDH cDNA copy number of each sample was determined. Primers are included in the supplemental information.

2.8. Acyl-HSL and quantitative acyl-HSL assay

Chemically synthesized 3OC12-HSL was obtained from Vertex Pharmaceuticals (Coralville, IA). 3OC12-HSL was measured by quantitative bioassay as described [34] using *Escherichia coli* MG-4 (pKDT17) [6,35].

2.9. Statistical analysis

P-values of bioassay data were calculated using Student's *t* test in Microsoft Excel software. Survival data was analyzed by log rank test using online software (http://bioinf.wehi.edu.au/software/russell/logrank/).

3. Results

3.1. 3OC12-HSL is degraded through lactone ring hydrolysis

We previously found that the signaling activity of the 3OC12-HSL quorum-sensing molecule was lost when exposed to cultured human airway and other epithelial cells. To characterize the structural changes associated with this loss of activity, three independent methods were used: NMR, HPLC, and MS.

First, analysis of the NMR spectrum of 3OC12-HSL showed that peaks shifted after exposure to A549 airway epithelial cells (Fig. 1A). These shifts reflect structural changes previously reported to correspond to lactone ring hydrogens in 3OC12-HSL [6]. Second, HPLC on a C18 column was used to determine whether exposure to A549-cells did in fact hydrolyze the 3OC12-HSL lactone ring. Since it is known that high pH hydrolyzes acyl-HSL lactone rings [36], we compared the elution

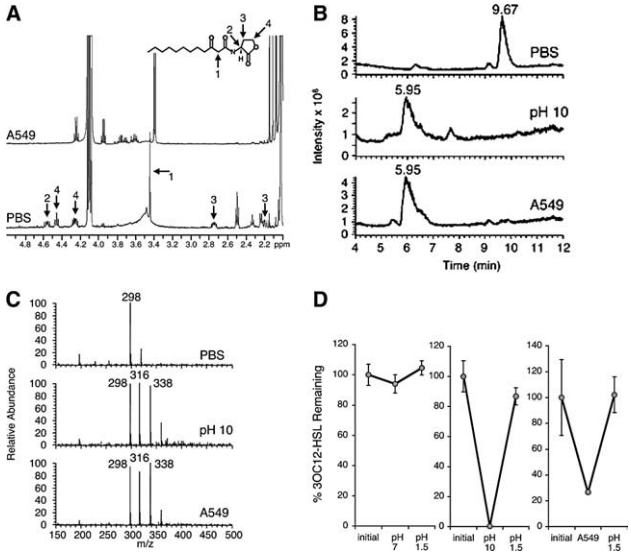

Fig. 1. Lactone ring hydrolysis of 3OC12-HSL exposed to mammalian cells and serum. (A) Synthetic 3OC12-HSL [6] was incubated for 1 h on monolayers of A549 airway epithelial cells (A549) or in the absence of cells (PBS). Ethyl acetate extracts of the medium were analyzed by proton-NMR. Labeled peaks correspond to lactone ring and acyl side chain protons indicated on the 3OC12-HSL structure. (B and C) 3OC12-HSL was incubated in PBS alone (PBS), PBS at pH 10 (pH 10), or PBS on A549 cell monolayers (A549). Extracts were analyzed by C18 reverse phase HPLC–MS. MS of fractions eluting at 9.67 min in PBS, and 5.95 min in pH 10 and A549 samples were examined. (D) 3OC12-HSL was incubated alone, on A549-cells, or in pH 10 PBS. After 1 h the pH was adjusted to 1.5 with HCl. Following a further incubation at room temperature for 72 h, 3OC12-HSL was measured with a bioassay. Data are means ± SEM; $n = 3$.

times of untreated 3OC12-HSL to 3OC12-HSL exposed to high pH or A549-cells. Fig. 1B shows that while untreated 3OC12-HSL eluted at about 10 min, 3OC12-HSL exposed to either high pH or A549-cells eluted from the column identically – at about 6 min. These data show that both treatments make 3O12-HSL less hydrophobic, consistent with hydrolysis of the lactone ring. Finally, MS showed a mass ion at 298 that corresponded to the molecular weight of the unhydrolyzed form (Fig. 1C). After incubation in alkaline conditions, hydrolysis of the lactone ring was confirmed by the appearance of a mass ion of *m*/*z* 316 (Fig. 1C); this represents the molecular weight of 3OC12-HSL plus one water molecule. The MS of 3OC12-HSL inactivated by A549-cells also contained the prominent peak at *m*/*z* 316. Taken together, these data indicate that A549 human epithelial airway cells degrade the *P. aeruginosa* 3OC12-HSL quorum-sensing signal by hydrolyzing its lactone ring.

Previous studies have shown that homoserine lactone rings hydrolyzed at high pH can be recircularized in acidic solutions [36]. Fig. 1D shows that pH 1.5 had no effect on active 3OC12-HSL, but after hydrolysis at pH 10, exposure to pH 1.5 completely restored biological activity. Likewise, pH 1.5 treatment fully restored the signaling activity of 3OC12-HSL degraded by A549-cells. These data suggest that A549-cells degrade 3OC12-HSL by hydrolysis.

3.2. Serum lactonase PON1 degrades 3OC12-HSL

Since the mammalian *Pon* genes encode paraoxonases with lactonase activity, we hypothesized that they may be responsible for degrading the 3OC12-HSL signal. Both PON1 and PON3 are found in serum so we tested whether serum degrades 3OC12-HSL. As with exposure to A549-cells, 3OC12-HSL incubated with wild-type serum is rapidly inactivated (Fig. 2A); this inactivation is completely reversed by incubation at low pH (Fig. 2A). HPLC and MS (Fig. 2B) further confirmed that, similar to A549-cells, serum also contains an activity that hydrolyzes the lactone ring.

To characterize the activity that degrades 3OC12-HSL in serum, we treated human serum with PON inhibitors: EDTA [37], oxindole, and isatin [38]; all blocked degradation of 3OC12-HSL (Fig. 2C). Since PON1 is the most abundant paraoxonase found in serum, and mice with targeted disruptions of the *Pon1* gene (*Pon1*-KO mice) have been previously generated, we assayed serum from *Pon1*-KO mice to more directly test whether PON1 is the enzyme that degrades 3OC12-HSL [39]. Unlike serum from wild-type mice, serum from mice lacking *Pon1* exhibited impaired degradation of 3OC12-HSL (Fig. 2D). Furthermore adding back PON1, purified from human serum, was sufficient to degrade 3OC12-HSL in vitro. Fig. 2E shows that the activity of 3OC12-HSL was inversely related to the amount of purified PON1 added to the reaction mixture. Thus, in vitro PON1 is both required and sufficient to degrade 3OC12-HSL in mouse serum.

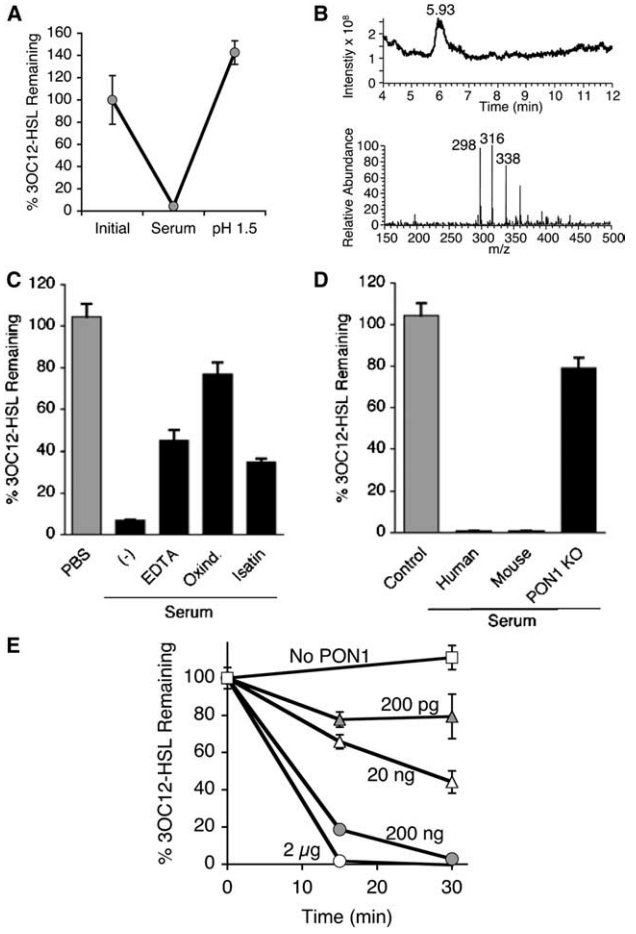

Fig. 2. Serum containing active PON1 degrades 3OC12-HSL. (A) 3OC12-HSL was exposed to 1% mouse serum, followed by adjustment of the reaction buffer pH to 1.5. Data are means ± SEM; $n = 3$. (B) HPLC and MS after incubation of 3OC12-HSL in serum. (C) 10 μM 3OC12-HSL was incubated for 30 min in 1% mouse serum containing normal saline (−), EDTA (25 mM), oxindole (1 mM), or isatin (1 mM). Data are means ± SEM; $n = 3$. (D) 10 μM 3OC12-HSL was incubated 30 min in reaction buffer containing 1% human serum (Human), wild-type mouse serum (Mouse), or PON1-KO serum (PON1-KO). Data are means ± SEM; $n = 3$. (E) Purified human PON1 was added to a solution of 10 μM 3OC12-HSL. 3OC12-HSL was measured in samples taken pre- and post-incubation by bioassay. Data are means ± SEM; $n = 3$.

As stated above, in addition to PON1 mammals express two other lactone ring hydrolases – PON2 and PON3 [40,41]. To test whether these proteins similarly degrade 3OC12-HSL we constructed recombinant Adenoviruses expressing human *Pon* genes. CHO cells were transfected with adenoviruses expressing human PON1, PON2, or PON3 (or GFP as a control) and after 48 h 3OC12-HSL was added to the culture medium and assayed for degradation. After 60 min of incubation the supernatant from cells transfected with both hPON1

and hPON3 had degraded nearly 80% of the added 3OC12-HSL; in media from cells transfected with PON2 nearly 100% of the 3OC12-HSL was degraded (Fig. 3). In comparison, little change was effected by cells expressing GFP. This indicates that all three mammalian paraoxonases degrade 3OC12-HSL with PON2 being the most active.

3.3. *PON1 blocks* P. aeruginosa *biofilm formation*

Previous reports show that biofilm formation is reduced or altered in mutant *P. aeruginosa* that cannot synthesize 3OC12-HSL [9,42]. Based on these findings, we predicted that since serum degrades 3OC12-HSL it would also impair *P. aeruginosa* biofilm formation. We used a previously described in vitro system in which *P. aeruginosa* biofilms grow on plastic pegs immersed in medium [33]. Fig. 4B shows that 0.25% wild-type mouse serum reduced biofilm formation by 75% while a 0.25% solution of BSA (artificial serum) only reduced bilofilm formation by 10%. In contrast, serum from *Pon1*-KO mice showed minimal effects, only reducing *P. aeruginosa* biofilm formation by 15%. Furthermore, adding purified human PON1 to artificial serum, or to *Pon1*-KO mouse serum, inhibited biofilm formation similar to wild-type mouse serum (Fig. 4A). These data show that adding PON1-containing serum to culture media interferes with the ability of *P. aeruginosa* to form biofilm.

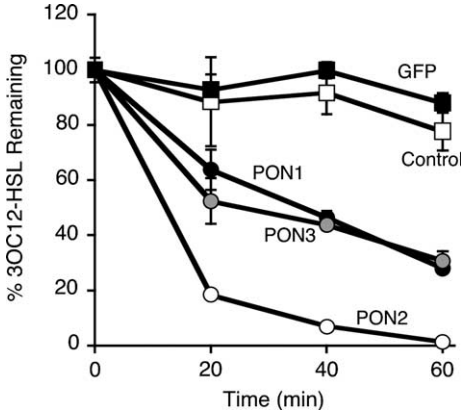

Fig. 3. Human PONs 1, 2, and 3 degrade 3OC12-HSL. CHO cells were transfected with adenovirus expressing hPON1, hPON2, hPON3, or GFP. After 48 h, 10 μM 3OC12-HSL was added to the medium and remaining 3OC12-HSL was measured at 20, 40, and 60 min. Data are means ± SEM; $n = 5$.

To confirm that PON1 inhibits biofilm formation by disrupting *P. aeruginosa* quorum-sensing signaling, we examined biofilm formation by the *lasI* mutant strain of *P. aeruginosa* that does not produce 3OC12-HSL. Fig. 4B shows that, as expected the *lasI* mutant formed less biofilm than wild type *P. aeruginosa* in artificial serum. However, unlike wild-type *P. aeruginosa*, *lasI* mutant bacteria responded the same to both wild-type and *Pon*-KO mouse serum. These results suggest that serum reduces the ability of *P. aeruginosa* to form biofilms in vitro because PON1 degrades the 3OC12-HSL quorum-sensing signal.

3.4. *Quorum-sensing by* P. aeruginosa *in the murine peritonitis/sepsis model*

P. aeruginosa quorum-sensing modulates virulence in several in vivo models of infection. In mice, these include acute and chronic lung infection, and burn infection models [43–45]. To address the role of paraoxonases in host defense mechanisms, we used a mouse model of *P. aeruginosa* peritonitis/sepsis. To test the effect of 3OC12-HSL quorum-sensing on *P. aeruginosa* virulence in this model, wild-type mice were infected

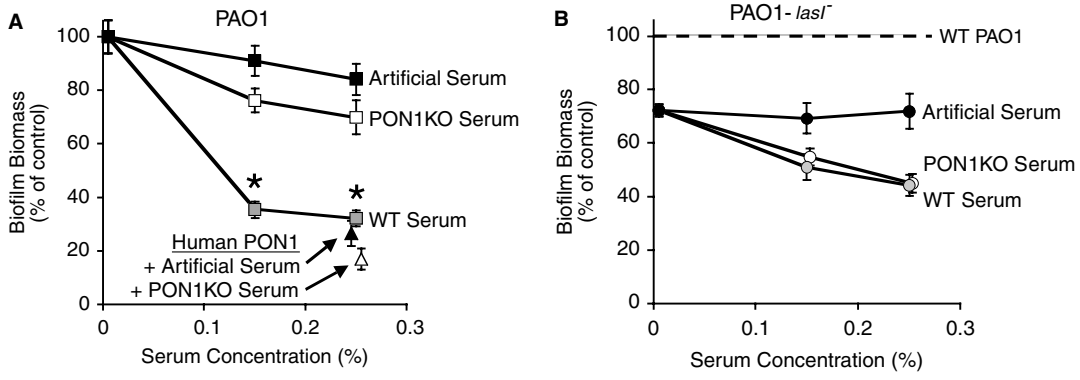

Fig. 4. PON1 blocks *P. aeruginosa* biofilm formation. (A) *P. aeruginosa* biofilms were cultured on plastic pegs for 48 h in the presence of increasing concentrations of artificial serum (5% BSA in lactated Ringer's solution), wild-type mouse serum, or PON1-KO mouse serum. Purified human PON1 was added to the highest concentration of PON1-KO serum (white) or artificial serum (black). Biofilm biomass was measured by spectrophotometric analysis of crystal violet stain bound to the bacteria on the pegs. Data are means ± SEM; $n = 18$ for samples without human PON1, $n = 6$ for samples with added human PON1. (B) Biofilm biomass of LasI$^-$ mutant PAO1 biofilms grown on pegs in the presence of artificial serum, wild type mouse serum, or PON1-KO serum. Data are means ± SEM; $n = 12$. *$p < 0.05$.

with the LD50 (1.9×10^7–3.8×10^7 CFU) of PAO1 (a wild-type strain of *P. aeruginosa*) or an equal dose of the *lasI* mutant strain (Fig. 5A). While 55% of the mice infected with the PAO1 strain died after 100 h, less than 10% those infected with the *lasI* mutant died, suggesting that 3OC12-HSL is important for causing mortality. These findings are consistent with *C. elegans* and other mouse models that show decreased virulence in infections with quorum-sensing deficient strains of *P. aeruginosa* [15,43–45].

3.5. Pon1 *deficient mice are paradoxically protected from quorum-sensing dependent* P. aeruginosa *infection*

Our results and those of others suggest that disrupting *P. aeruginosa* quorum-sensing signaling decreases mortality in the infected host [44,46]. We therefore hypothesized that *Pon1*-KO mice would be more sensitive to PAO1 infection. Contrary to our prediction, at 50 h post-infection 100% of the *Pon1*-KO mice survived as compared to 50% of wild-type mice (Fig. 5B). These results implied that disrupting *Pon1* actually protects the mice from succumbing to infection. We then compared the outcome of infection by the *lasI* mutant, which cannot synthesize quorum-sensing signals. We first determined the LD50 of *lasI* mutant *P. aeruginosa* in wild type mice (6.3×10^7 CFU). When infected with this dose of the *lasI* mutant strain, wild-type and *Pon1*-KO mice survived at the same rate (Fig. 5C). These results demonstrate that the paradoxical difference in mortality between wild-type and *Pon1*-KO mice infected with PAO1 is related to 3OC12-HSL.

These unexpected findings suggest that other protective factors compensated for the loss of PON1 in knockout mice. We hypothesized that the expression of *Pon2* or *Pon3* could be increased in the *Pon1*-KO mouse. Our hypothesis was confirmed by semi-quantitative PCR of airway epithelia from *Pon1*-KO mice: both PON2 and PON3 mRNA levels were doubled in the *Pon1*-KO mice as compared to the wild-type mice (Fig. 5D). Therefore, increased tissue expression of PON2, PON3, or both in *Pon1*-KO mice may degrade 3OC12-HSL more effectively in vivo thereby protecting the KO mice from sepsis caused by *P. aeruginosa.*

4. Discussion

Previously we reported that the *P. aerugionosa* quorum-sensing molecule 3OC12-HSL loses signaling

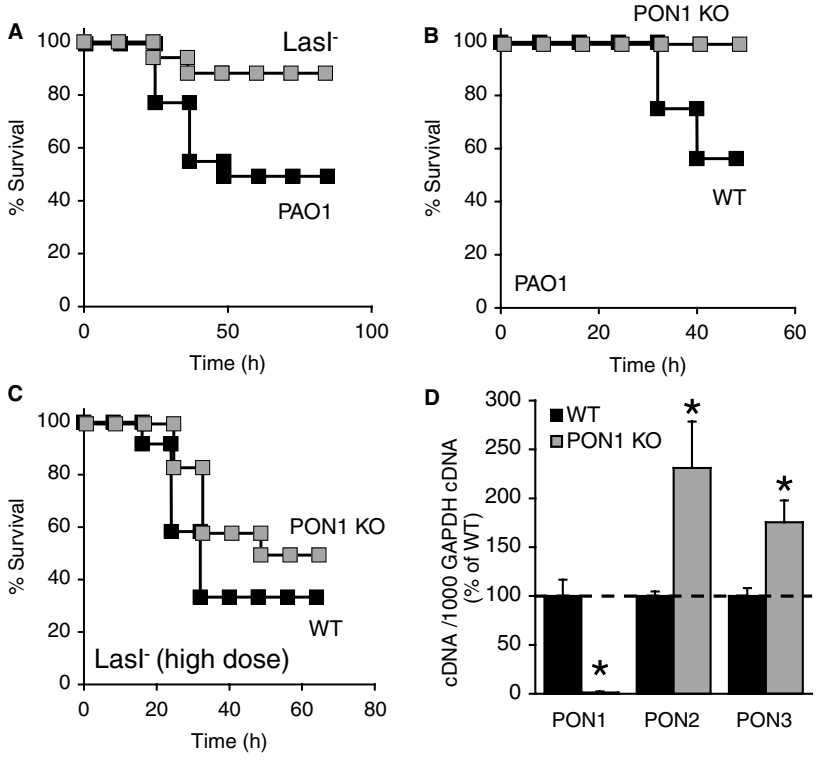

Fig. 5. PON1 knockout protects mice from *P. aeruginosa* sepsis. (A) Survival of mice following intraperitoneal injection of 1.3×10^7 CFU of PAO1 (black squares) or PAO1-*lasI*$^-$ (gray squares). $n = 18$, $p < 0.02$. (B) Survival of wild-type mice (black squares) and PON1-KO mice (gray squares) following intraperitoneal injection of 2.9×10^7 CFU of PAO1. $n = 16$, $p < 0.01$. (C) Survival of wild-type mice (black squares) and PON1-KO mice (gray squares) following intraperitoneal injection of 6.3×10^7 CFU of PAO1- *lasI*$^-$. $n = 12$, N.S. (D) Copies of mouse PON1, PON2, and PON3 cDNA in cultured airway epithelia from wild-type (black) and PON1-KO mice (gray). Values are normalized for copies of GAPDH per epithelium and expressed as percent of copies of PON1, PON2, or PON3 per wild-type epithelium. Data are means ± SEM. $p < 0.01$.

activity after incubation with primary airway epithelia and other cultured cells [19]. In this study we identify the mechanism of acyl-HSL degradation as lactone ring hydrolysis. This is consistent with findings in prokaryotes showing that quorum-sensing inhibitor systems degrade the lactone ring of acyl-HSLs [12–14,16,47–49], such as was found for the quorum-sensing inhibitor AiiA from the bacteria *Bacillus* sp.

We found that, like airway cells, human serum degrades 3OC12-HSL through lactonolysis. We therefore suggested that a likely candidate for this activity is PON1 – a lactonase abundant in serum. Serum from *Pon1*-KO mice was impaired in degrading this quorum-sensing signal. The importance of serum to host defense cannot be understated, as it is a vital medium for transport of immune cells and defense factors to sites of infection. The discovery that both serum-PON1 and purified PON1 reduced *P. aeruginosa* biofilm formation showed that PON1 can significantly impact a complex process that relies heavily on quorum-sensing [9,11,50].

Both pathogen and host contribute to microbial pathogenicity, [reviewed in Casadevall and Pirofski [51]]. In this model, both a weak and an overzealous host response can enhance virulence. Thus, elevated 3OC12-HSL concentrations in the *Pon1*-KO mouse may provoke different responses from both the *P. aeruginosa* and the host defense mechanisms. Similar to findings in other animal models of infection [43–45], causing sepsis in mice using a quorum-sensing deficient strain of *P. aeruginosa* did not kill them as effectively as sepsis caused by wild-type bacteria. These results confirmed the hypothesis that attenuating quorum-sensing signaling reduces bacterial virulence. In apparent discord with these findings, however, *Pon1* deficient mice were resistant to *P. aeruginosa* infection, surviving infection more frequently. Quorum-sensing appears to be involved since a 3OC12-HSL-deficient mutant strain of *P. aeruginosa* killed both *Pon1*-KO and wild-type mice with the same frequency. Both PON2 and PON3, although not present in serum, were increased in the PON1-KO mice epithelia, as compared to wild-type mice. PON2 is expressed ubiquitously, including in endothelial cells and bloodstream monocytes [26,40]. It is possible that increased acyl-HSL lactonase activity in these cells compensates for the absence of PON1 in serum. However, to test this hypothesis, it will be necessary to generate a triple knockout animal that lacks endogenous paraoxonase expression. Thus, based on our data we cannot conclude that modulation of *P. aeruginosa* quorum-sensing by PON1 is a host defense mechanism.

Although paraoxonases are conserved from *C. elegans* to mammals [21], their physiological substrates are not known [20,21]. Our data, in conjunction with previous studies [29,52], propose that PON1 may have evolved to degrade bacterial acyl-HSLs. Hence, a quorum-sensing blockade by PON1 and other members of the family may mediate a number of bacterial biofilm, virulence- and inflammation-dependent processes in host organisms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.femsle.2005.09.023.

References

[1] Smith, R.S. and Iglewski, B.H. (2003) *Pseudomonas aeruginosa* quorum-sensing systems and virulence. Curr. Opin. Microbiol. 6, 56–60.
[2] de Kievit, T.R. and Iglewski, B.H. (2000) Bacterial quorum sensing in pathogenic relationships. Infect. Immun. 68, 4839–4849.
[3] Schuster, M., Lostroh, C.P., Ogi, T. and Greenberg, E.P. (2003) Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. J. Bacteriol. 185, 2066–2079.
[4] Wieland, C.W., Siegmund, B., Senaldi, G., Vasil, M.L., Dinarello, C.A. and Fantuzzi, G. (2002) Pulmonary inflammation induced by *Pseudomonas aeruginosa* lipopolysaccharide, phospholipase C, and exotoxin A: role of interferon regulatory factor 1. Infect. Immun. 70, 1352–1358.
[5] Rumbaugh, K.P., Hamood, A.N. and Griswold, J.A. (2004) Cytokine induction by the *Pseudomonas aeruginosa* quorum sensing system during thermal injury. J. Surg. Res. 116, 137–144.
[6] Pearson, J.P., Gray, K.M., Passador, L., Tucker, K.D., Eberhard, A., Iglewski, B.H. and Greenberg, E.P. (1994) Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. Proc. Natl. Acad. Sci. USA 91, 197–201.
[7] Latifi, A., Foglino, M., Tanaka, K., Williams, P. and Lazdunski, A. (1996) A hierarchical quorum-sensing cascade in *Pseudomonas aeruginosa* links the transcriptional activators LasR and RhIR (VsmR) to expression of the stationary-phase sigma factor RpoS. Mol. Microbiol. 21, 1137–1146.
[8] Pesci, E.C. and Iglewski, B.H. (1997) The chain of command in *Pseudomonas* quorum sensing. Trends Microbiol. 5, 132–134.
[9] Davies, D.G., Parsek, M.R., Pearson, J.P., Iglewski, B.H., Costerton, J.W. and Greenberg, E.P. (1998) The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science 280, 295–298.

[10] Davey, M.E., Caiazza, N.C. and O'Toole, G.A. (2003) Rhamnolipid surfactant production affects biofilm architecture in *Pseudomonas aeruginosa* PAO1. J. Bacteriol. 185, 1027–1036.
[11] Lequette, Y. and Greenberg, E.P. (2005) Timing and localization of rhamnolipid synthesis gene expression in *Pseudomonas aeruginosa* biofilms. J. Bacteriol. 187, 37–44.
[12] Leadbetter, J.R. and Greenberg, E.P. (2000) Metabolism of acyl-homoserine lactone quorum-sensing signals by *Variovorax paradoxus*. J. Bacteriol. 182, 6921–6926.
[13] Borchardt, S.A., Allain, E.J., Michels, J.J., Stearns, G.W., Kelly, R.F. and McCoy, W.F. (2001) Reaction of acylated homoserine lactone bacterial signaling molecules with oxidized halogen antimicrobials. Appl. Environ. Microbiol. 67, 3174–3179.
[14] Dong, Y.H., Xu, J.L., Li, X.Z. and Zhang, L.H. (2000) AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of *Erwinia carotovora*. Proc. Natl. Acad. Sci. USA 97, 3526–3531.
[15] Lin, Y.H., Xu, J.L., Hu, J., Wang, L.H., Ong, S.L., Leadbetter, J.R. and Zhang, L.H. (2003) Acyl-homoserine lactone acylase from *Ralstonia* strain XJ12B represents a novel and potent class of quorum-quenching enzymes. Mol. Microbiol. 47, 849–860.
[16] Dong, Y.H., Gusti, A.R., Zhang, Q., Xu, J.L. and Zhang, L.H. (2002) Identification of quorum-quenching *N*-acyl homoserine lactonases from *Bacillus* species. Appl. Environ. Microbiol. 68, 1754–1759.
[17] Leadbetter, J.R. (2001) Plant microbiology. Quieting the raucous crowd. Nature 411, 748–749.
[18] Dong, Y.H., Wang, L.H., Xu, J.L., Zhang, H.B., Zhang, X.F. and Zhang, L.H. (2001) Quenching quorum-sensing-dependent bacterial infection by an *N*-acyl homoserine lactonase. Nature 411, 813–817.
[19] Chun, C.K., Ozer, E.A., Welsh, M.J., Zabner, J. and Greenberg, E.P. (2004) Inactivation of a *Pseudomonas aeruginosa* quorum-sensing signal by human airway epithelia. Proc. Natl. Acad. Sci. USA 101, 3587–3590.
[20] Li, H.L., Liu, D.P. and Liang, C.C. (2003) Paraoxonase gene polymorphisms, oxidative stress, and diseases. J. Mol. Med. 81, 766–779.
[21] Draganov, D.I. and La Du, B.N. (2004) Pharmacogenetics of paraoxonase: a brief review. N-S Arch. Pharmacol. 369, 78–88.
[22] Primo-Parmo, S.L., Sorenson, R.C., Teiber, J. and La Du, B.N. (1996) The human serum paraoxonase/arylesterase gene (PON1) is one member of a multigene family. Genomics 33, 498–507.
[23] Mackness, M.I., Arrol, S. and Durrington, P.N. (1991) Paraoxonase prevents accumulation of lipoperoxides in low-density lipoprotein. FEBS Lett. 286, 152–154.
[24] Sorenson, R.C., Bisgaier, C.L., Aviram, M., Hsu, C., Billecke, S. and La Du, B.N. (1999) Human serum Paraoxonase/Arylesterase's retained hydrophobic N-terminal leader sequence associates with HDLs by binding phospholipids: apolipoprotein A-I stabilizes activity. Arterioscler. Thromb. Vasc. Biol. 19, 2214–2225.
[25] Reddy, S.T., Wadleigh, D.J., Grijalva, V., Ng, C., Hama, S., Gangopadhyay, A., Shih, D.M., Lusis, A.J., Navab, M. and Fogelman, A.M. (2001) Human paraoxonase-3 is an HDL-associated enzyme with biological activity similar to paraoxonase-1 protein but is not regulated by oxidized lipids. Arterioscler. Thromb. Vasc. Biol. 21, 542–547.
[26] Ng, C.J., Wadleigh, D.J., Gangopadhyay, A., Hama, S., Grijalva, V.R., Navab, M., Fogelman, A.M. and Reddy, S.T. (2001) Paraoxonase-2 is a ubiquitously expressed protein with antioxidant properties and is capable of preventing cell-mediated oxidative modification of low density lipoprotein. J. Biol. Chem. 276, 44444–44449.
[27] Li, W.F., Costa, L.G., Richter, R.J., Hagen, T., Shih, D.M., Tward, A., Lusis, A.J. and Furlong, C.E. (2000) Catalytic efficiency determines the in-vivo efficacy of PON1 for detoxifying organophosphorus compounds. Pharmacogenetics 10, 767–779.
[28] Watson, A.D., Berliner, J.A., Hama, S.Y., La Du, B.N., Faull, K.F., Fogelman, A.M. and Navab, M. (1995) Protective effect of high density lipoprotein associated paraoxonase. Inhibition of the biological activity of minimally oxidized low density lipoprotein. J. Clin. Invest. 96, 2882–2891.
[29] Draganov, D.I., Teiber, J.F., Speelman, A., Osawa, Y., Sunahara, R. and La Du, B.N. (2005) Human paraoxonases (PON1, PON2, and PON3) are lactonases with overlapping and distinct substrate specificities. J. Lipid Res. 46, 1239–1247.
[30] Greenberg, E.P., Chun, C.K., Ozer, E.A., Welsh, M.J. and Zabner, J. (2004) Enzymatic inactivation of a *Pseudomonas aeruginosa* quorum-sensing signal by human airway epithelia. Abstract S5:1. In: ASM Conference on Cell–Cell Communication in Bacteria. Banff, Alberta, Canada.
[31] Furlong, C.E., Richter, R.J., Chapline, C. and Crabb, J.W. (1991) Purification of rabbit and human serum paraoxonase. Biochemistry 30, 10133–10140.
[32] Fasbender, A., Zabner, J., Chillon, M., Moninger, T.O., Puga, A.P., Davidson, B.L. and Welsh, M.J. (1997) Complexes of adenovirus with polycationic polymers and cationic lipids increase the efficiency of gene transfer in vitro and in vivo. J. Biol. Chem. 272, 6479–6489.
[33] Ceri, H., Olson, M.E., Stremick, C., Read, R.R., Morck, D. and Buret, A. (1999) The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. J. Clin. Microbiol. 37, 1771–1776.
[34] Schaefer, A.L., Hanzelka, B.L., Eberhard, A. and Greenberg, E.P. (1996) Quorum sensing in *Vibrio fischeri*: probing autoinducer-LuxR interactions with autoinducer analogs. J. Bacteriol. 178, 2897–2901.
[35] Passador, L., Tucker, K.D., Guertin, K.R., Journet, M.P., Kende, A.S. and Iglewski, B.H. (1996) Functional analysis of the *Pseudomonas aeruginosa* autoinducer PAI. J. Bacteriol. 178, 5995–6000.
[36] Yates, E.A., Philipp, B., Buckley, C., Atkinson, S., Chhabra, S.R., Sockett, R.E., Goldner, M., Dessaux, Y., Camara, M., Smith, H. and Williams, P. (2002) *N*-Acylhomoserine lactones undergo lactonolysis in a pH-, temperature-, and acyl chain length-dependent manner during growth of *Yersinia pseudotuberculosis* and *Pseudomonas aeruginosa*. Infect. Immun. 70, 5635–5646.
[37] Kuo, C.L. and La Du, B.N. (1995) Comparison of purified human and rabbit serum paraoxonases. Drug Metab. Dispos. 23, 935–944.
[38] Billecke, S., Draganov, D., Counsell, R., Stetson, P., Watson, C., Hsu, C. and La Du, B.N. (2000) Human serum paraoxonase (PON1) isozymes Q and R hydrolyze lactones and cyclic carbonate esters. Drug Metab. Dispos. 28, 1335–1342.
[39] Shih, D.M., Gu, L., Xia, Y.R., Navab, M., Li, W.F., Hama, S., Castellani, L.W., Furlong, C.E., Costa, L.G., Fogelman, A.M. and Lusis, A.J. (1998) Mice lacking serum paraoxonase are susceptible to organophosphate toxicity and atherosclerosis. Nature 394, 284–287.
[40] Rosenblat, M., Draganov, D., Watson, C.E., Bisgaier, C.L., La Du, B.N. and Aviram, M. (2003) Mouse macrophage paraoxonase 2 activity is increased whereas cellular paraoxonase 3 activity is decreased under oxidative stress. Arterioscler. Thromb. Vasc. Biol. 23, 468–474.
[41] Draganov, D.I., Stetson, P.L., Watson, C.E., Billecke, S.S. and La Du, B.N. (2000) Rabbit serum paraoxonase 3 (PON3) is a high density lipoprotein-associated lactonase and protects low density lipoprotein against oxidation. J. Biol. Chem. 275, 33435–33442.
[42] Parsek, M.R. and Greenberg, E.P. (2000) Acyl-homoserine lactone quorum sensing in gram-negative bacteria: a signaling mechanism involved in associations with higher organisms. Proc. Natl. Acad. Sci. USA 97, 8789–8793.

[43] Pearson, J.P., Feldman, M., Iglewski, B.H. and Prince, A. (2000) *Pseudomonas aeruginosa* cell-to-cell signaling is required for virulence in a model of acute pulmonary infection. Infect. Immun. 68, 4331–4334.

[44] Wu, H., Song, Z., Givskov, M., Doring, G., Worlitzsch, D., Mathee, K., Rygaard, J. and Hoiby, N. (2001) *Pseudomonas aeruginosa* mutations in *lasI* and *rhlI* quorum sensing systems result in milder chronic lung infection. Microbiology 147, 1105–1113.

[45] Rumbaugh, K.P., Griswold, J.A., Iglewski, B.H. and Hamood, A.N. (1999) Contribution of quorum sensing to the virulence of *Pseudomonas aeruginosa* in burn wound infections. Infect. Immun. 67, 5854–5862.

[46] Hentzer, M., Wu, H., Andersen, J.B., Riedel, K., Rasmussen, T.B., Bagge, N., Kumar, N., Schembri, M.A., Song, Z., Kristoffersen, P., Manefield, M., Costerton, J.W., Molin, S., Eberl, L., Steinberg, P., Kjelleberg, S., Hoiby, N. and Givskov, M. (2003) Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. EMBO J. 22, 3803–3815.

[47] Zhang, H.B., Wang, L.H. and Zhang, L.H. (2002) Genetic control of quorum-sensing signal turnover in *Agrobacterium tumefaciens*. Proc. Natl. Acad. Sci. USA 99, 4638–4643.

[48] Park, S.Y., Lee, S.J., Oh, T.K., Oh, J.W., Koo, B.T., Yum, D.Y. and Lee, J.K. (2003) AhlD, an *N*-acylhomoserine lactonase in *Arthrobacter sp*., and predicted homologues in other bacteria. Microbiology 149, 1541–1550.

[49] Dong, Y.H. and Zhang, L.H. (2005) Quorum sensing and quorum-quenching enzymes. J. Microbiol. 43 (Spec No), 101–109.

[50] De Kievit, T.R., Gillis, R., Marx, S., Brown, C. and Iglewski, B.H. (2001) Quorum-sensing genes in *Pseudomonas aeruginosa* biofilms: their role and expression patterns. Appl. Environ. Microbiol. 67, 1865–1873.

[51] Casadevall, A. and Pirofski, L.A. (2003) The damage-response framework of microbial pathogenesis. Nat. Rev. Microbiol. 1, 17–24.

[52] Yang, F., Wang, L.H., Wang, J., Dong, Y.H., Hu, J.Y. and Zhang, L.H. (2005) Quorum quenching enzyme activity is widely conserved in the sera of mammalian species. FEBS Lett. 579, 3713–3717.