Isolation and characterization of HepP: a virulence-related Pseudomonas aeruginosa heparinase

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Abstract

Background: Pseudomonas aeruginosa is an opportunistic pathogen that causes serious infections in immunocompromised hosts including severely burned patients. In burn patients, P. aeruginosa infection often leads to septic shock and death. Despite numerous studies, the influence of severe thermal injuries on the pathogenesis of P. aeruginosa during systemic infection is not known. Through RNA-seq analysis, we recently showed that the growth of P. aeruginosa strain UCBPP-PA14 (PA14) in whole blood obtained from severely burned patients significantly altered the expression of the PA14 transcriptome when compared with its growth in blood from healthy volunteers. The expression of PA14_23430 and the adjacent gene, PA14_23420, was enhanced by seven- to eightfold under these conditions.

Results: Quantitative real-time PCR analysis confirmed the enhancement of expression of both PA14_23420 and PA14_23430 by growth of PA14 in blood from severely burned patients. Computer analysis revealed that PA14_23430 (hepP) encodes a potential heparinase while PA14_23420 (zbdP) codes for a putative zinc-binding dehydrogenase. This analysis further suggested that the two genes form an operon with zbdP first. Presence of the operon was confirmed by RT-PCR experiments. We characterized hepP and its protein product HepP. hepP was cloned from PA14 by PCR and overexpressed in E. coli. The recombinant protein (rHepP) was purified using nickel column chromatography. Heparinase assays using commercially available heparinase as a positive control, revealed that rHepP exhibits heparinase activity. Mutation of hepP resulted in delay of pellicle formation at the air-liquid interface by PA14 under static growth conditions. Biofilm formation by PA14ΔhepP was also significantly reduced. In the Caenorhabditis elegans model of slow killing, mutation of hepP resulted in a significantly lower rate of killing than that of the parent strain PA14.

Conclusions: Changes within the blood of severely burned patients significantly induced expression of hepP in PA14. The heparinase encoded by hepP is a potential virulence factor for PA14 as HepP influences pellicle formation as well as biofilm development by PA14 and the protein is required for full virulence in the C. elegans model of slow killing.

Keywords: Pseudomonas aeruginosa, Heparinase, Virulence, Pellicle, Biofilm, C. elegans killing model

Background

The gram-negative opportunistic pathogen Pseudomonas aeruginosa causes severe infection in immunocompromised hosts such as HIV-infected patients, individuals with cystic fibrosis, cancer patients, transplant patients, and severely burned patients [1, 2]. Damage caused during different P. aeruginosa infections is due to the production of numerous cell-associated and extracellular virulence factors [2–5]. The extracellular factors include proteases such as elastase, hemolsins such as phospholipase C, siderophores such as pyoverdine, and toxins such as exotoxin A. The cell-associated virulence factors include flagellum, alginate and pili [6, 7].

A severe burn destroys the skin barrier and reduces the expression of both local and systemic immune responses [8–11]. Additionally, substances that are produced by the injured cells within the burned wound impair local host immune responses [8, 11, 12]. Although burn wounds are initially sterile they are quickly colonized by bacterial
enzymes produced by Pedobacter heparinus wound repair [20
tumorous processes including, tissue morphogenesis, regen-
and lymphocytes [16, 18–
important modulators of the extracellular matrices, are
β
inases depolymerize heparin and heparan sulfate by
acid by a hydrolytic mechanism whereas bacterial hepar-
heparanases cleave at the reducing end of glucuronic
are known as heparanase or heparinase [16]. Mammalian
neovascularization during tumor progression through their
ability to alter the action of fibroblast growth factor [16, 20,
21, 27]. Despite the proposed benefical effects of the P. heparinus heparanases, it is not known if heparinases play a
role in the pathogenesis of bacterial pathogens, specifically
the ones that cause systemic infection.

In this study, we report the characterization of
PA14_23430, or hepP, and its protein product HepP. hepP
was cloned from PA14 by PCR and overexpressed in E. coli. The recombinant protein (rHepP) was purified using nickel column chromatography. The heparinase assay using commercially available heparinase revealed that
rHepP exhibits heparinase activity. The deletion of hepP resulted in the delay of pellicle formation at the air-liquid interface by PA14 under static growth conditions. The biofilm formation by PA14ΔhepP was also significantly reduced. In the Caenorhabditis elegans model of slow killing, the deletion of hepP resulted in a significantly lower rate of killing than that of the parent strain PA14.

To our knowledge this is the first time a heparinase, or
heparin and heparan sulfate degrading enzyme, has been
characterized from P. aeruginosa. Due to its important
role in PA14 virulence and as P. aeruginosa antibiotic
resistant mutants emerge at an alarming rate, HepP repre-
sent a potential target for new antimicrobial agents.

Methods
Strains, plasmids and general growth conditions
Bacterial strains and plasmids used in this study are listed
in Table 1. The P. aeruginosa strain UCBPP_PA14 (PA14),
originally isolated from an infected wound, or its specific
transposon mutants, PA14ΔzbdP and PA14ΔhepP, were
used in all the experiments [30, 31]. The strains were rou-
tinely grown at 37 °C in Luria Bertani (LB) broth. Gentamicin
was added to the growth medium at a concentration of 60
μg/mL for the PA14 mutants and carbencillin was added at
100 μg/mL for E. coli strains carrying plasmids.

Real time quantitative PCR (qPCR) and reverse transcription PCR (RT-PCR)
Purified RNA samples obtained during a previous study in
which we examined changes in the transcriptome of
PA14 when grown in whole blood from severely burned
patients compared with its growth in whole blood from
healthy volunteers [15] were used in this study. The
blood samples were obtained under an IRB-approved
protocol in compliance with ethical practices [15].

Additional RNA samples were collected to examine the
effect of the hepP mutation on the expression of specific
virulence factors. We grew the strains in specific media,
LB broth for the expression of quorum sensing genes and
calcium-deficient chelated trypticase soy broth dialysate
medium for the expression of the type three secretion sys-
tem (T3SS) genes [32], that are designed to maximally
express those virulence genes. Briefly, PA14 cultures were
pathogens including P. aeruginosa [13, 14]. P. aeruginosa
grows within the wound and translocates to the blood
stream causing bacteremia which is followed by sepsis,
septic shock and multiorgan failure. Despite numerous
studies, the influence of severe burn injury on the patho-
genesis of P. aeruginosa during systemic infection is not
completely understood. We recently followed a unique
approach to address this issue. Instead of growing P. aeru-
ginosa in a regular laboratory medium, we grew it in
whole blood from either healthy volunteers or severely
burned patients and conducted a comparative transcriptome analysis [15]. RNA-seq analysis showed that the
growth of P. aeruginosa strain UCBPP_PA14 (PA14) in
whole blood obtained from severely burned patients sig-
ificantly altered the expression of the PA14 transcriptome when compared with its growth in blood from
healthy volunteers [15]. Among genes not reported in the
previous study whose expression was enhanced were
PA14_23430, which codes for a putative heparinase, and
the adjacent gene PA14_23420.

Heparin and heparan sulfate glycosaminoglycans
(HSGAGs), which consist of linear chains of disacchar-ide units of N-acetylated D-glucosamine α (1→4) linked
to glucuronic acids, are present on the surface of different
mammalian cells and the extracellular matrices [16].
Enzymes that degrade HSGAGs by specifically cleaving the 1→4 glycosidic link in heparin and heparan sulfate
are known as heparanase or heparinase [16]. Mammalian
heparanases cleave at the reducing end of glucuronic
acid by a hydrolytic mechanism whereas bacterial hepar-
inases depolymerize heparin and heparan sulfate by
β-elimination cleavage [16, 17]. Heparanases, which are
important modulators of the extracellular matrices, are
present in normal cells including endothelial cells, cyto-
trophoblasts, platelets, mast cells, neutrophils, macrophages,
and lymphocytes [16, 18–21]. Heparanase expression is
increased in certain human carcinomas and hematological
malignancies [20, 21]. Heparanases also function in non-
tumorous processes including, tissue morphogenesis, regen-
eration and repair during embryonic development and
wound repair [20–23].

The best-characterized bacterial heparinases are the three
enzymes produced by Pedobacter heparinus, (previously
known as Flavobacterium heparinum) heparinase I, II, and
III [16, 17, 22, 24–27]. No significant homology exists
between either the DNA sequences of the genes that code
for the three heparinases or the amino acid sequences of
the proteins [28]. The three heparinases proved to be valu-
able tools in analyzing different structural, biochemical and
physiological properties of heparin and heparan sulfate-like
glycosaminoglycans [18, 22, 24, 26]. Additional uses of P. heparinus heparinases include heparinase I as a heparin
antagonist [16, 29] and heparinases I, II, and III to reduce
neovascularization during tumor progression through their
grown and subcultured as described above. At the 8 h time point, cultures grown in the specific medium were mixed with twice the volume of the culture of RNAprotect Bacteria Reagent (Qiagen). After a 5 min incubation at 80 °C, RNA was extracted using the RNeasy Mini Kit (Qiagen), and quantified using a NanoDrop Spectrophotometer (NanoDrop Technologies).

Samples with an A260/280 ratio of 1.8–2.2 were used to synthesize cDNA by means of the QuantiTect Reverse Transcription kit (Qiagen). Two hundred ng of cDNA was mixed with PowerSYBR Green Master mix (Applied Biosystems) and 250 nM of specific primer (Table 2). The StepOnePlus Real-Time PCR System (Applied Biosystems) was then used to detect and quantify amplification of the PCR product. Three independent replicates for RNA samples were used for each experiment; additionally, each qPCR reaction was set up in triplicate. Normalization of the cDNA quantity in the different experiments was done using the 30S ribosomal subunit RNA (rpsL) as an internal standard. The software StepOnePlus version 2.2.2 was used to analyze gene expression. Negative control samples containing RNA as a template and positive control samples containing genomic DNA as a template were included for each experiment. RT-PCR was done as above but the cDNA products were analyzed on agarose gels. Primers used for all experiments involving PCR, RT-PCR or qPCR were synthesized by Integrated DNA Technologies and are listed in Table 2 and (Additional file 1: Table S1).

### Cloning of hepP

The DNA sequence of hepP was amplified with GoTaQ Green Master Mix (Promega) using primers described in Table 2 and genomic DNA from strain PA14 as a template. The 1650-bp amplicon was cloned into pBAD/Thio-TOPO (Invitrogen) to create plasmid pND1. The construction of pND1 was confirmed by restriction enzyme digestion and sequencing analysis. Recombinant HepP (rHepP) expressed from pND1 has the HP-thioredoxin fusion for efficient translation at the N-terminus and a 6xHis fusion for purification at the C-terminus plus the V5 epitope for localization of the protein by Western blotting.

### Expression and purification of recombinant HepP (rHepP)

Plasmid pND1 was transformed into chemically competent OneShot TOP10 Escherichia coli (Invitrogen). TOP10/ pND1 was grown in 50 mL LB broth at 37 °C with shaking, expression of hepP was induced with 0.02% L-arabinose,

### Table 1 Strains and plasmids used in this study

| Strains or plasmids | Characteristics | Reference |
|---------------------|-----------------|-----------|
| Pseudomonas aeruginosa | UCBPP-PA14; prototrophic strain isolated from infected wound | [30, 31] |
| PA14ΔhepP | PA14/Mt7/P:PA14_23430–480; GmR | [30] |
| PA14ΔzbdP | PA14/Mt7/P:PA14_23420–118; GmR | [30] |
| Escherichia coli | TOP10 F- mcrA Δ(mrr-hsrRMS-mcrBC) O8loxZΔA15 ΔacX74 recA1 araD139 Δ(arA-lev)7697 galU galK rpsL (StrR) endA1 nupG | Invitrogen |
| OP50 | Urayl auxotroph; feeder strain for Caenorhabditis elegans | [49] |
| Caenorhabditis elegans | BWS4 Temperature sensitive embryonic lethal strain; proliferates at 15 °C; sterile at 25 °C | Caenorhabditis Genetics Center |
| Plasmids | pBAD/Thio-TOPO pBR322-derived expression vector; cloned genes expressed from PBAD; amino terminus HP-thioredoxin; carboxy terminus V5 epitope and 6xHis; CbR | Invitrogen |
| pND1 | pBAD/Thio-TOPO/hepP, CbR | This study |

**CbR** carbenicillin resistant, **GmR** gentamicin resistant, **StrR** streptomycin resistant

### Table 2 Primers used in this study

| Name | Sequence | Use |
|------|----------|-----|
| hepP-For1 | GGACTGGCAAGCTGTAGGA | Analysis of hepP transcription |
| hepP-Rev1 | CGATACGCAAGGAAGAGGA | Analysis of hepP transcription |
| zbdP-For1 | CGGGTGTTTTGATTTTATTGTTT | Analysis of zbdP transcription |
| zbdP-Rev1 | AACATCCTCGTACGCTTAC | Analysis of zbdP transcription |
| hepP-For2 | CTCTGGGAGCAAGGCTTGC | Analysis of operon |
| hepP-Rev2 | GATAAAGGCTCCAACCTTC | Analysis of operon |
| zbdP-For3 | AGGCGACTATCGCTTGGAA | Confirmation of hepP mutation |
| zbdP-Rev3 | GTGTCTTTCAGCAATGGAG | Confirmation of hepP mutation |
| hepP-ATG | ATGGCCACTTCAAAACTGTCGCG | Cloning hepP |
| hepP-[T-GA] | ACTGCTATTTGCGGGGCTGAA | Cloning hepP |
| Trx Forward | TTCTCAGCACTAACCCTG | Confirmation of cloning |
| pBAD Reverse | GATTGAATCTGTACAGG | Confirmation of cloning |
| hepP-For2 | CGCTGGTGCAACAAAGTAGAA | Analysis of hepP transcription |
| hepP-Rev4 | GCCGTGATACATCGGAGACAA | Analysis of hepP transcription |

Primers were purchased from Integrated DNA Technologies.
and the cultures incubated for an additional 4 h. To avoid denaturation of the protein during purification, rHepP was purified under native conditions using a nickel-nitrilotriacetic acid affinity column (Ni-NTA, Qiagen) according to the manufacturer’s protocol. Briefly, following induction, the cells were centrifuged and the pellet was suspended in lysis buffer (50 mM NaH₂PO₄ 300 mM NaCl, 10 mM imidazole at pH 8). The cell suspension was sonicated for 10 cycles of a 10 s burst with a 10 s cooling between bursts. Lysed cells were centrifuged at 10,000 x g for 20 min. The supernatant was applied to a column containing 50% Ni-NTA agarose slurry. The column was capped and the mixture was rotated for 60 min at 4 °C. The column was then uncapped and the flow-through was collected. The column was washed twice with wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole at pH 8) and rHepP was eluted with four applications of elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole at pH 8). The concentration of the purified protein was estimated using the Bradford assay as previously described [33]. The purified protein was analyzed using 10% SDS-PAGE [34, 35]. In addition, we confirmed the production of the recombinant protein by immunoblotting experiments using anti-V5 antibody (Invitrogen).

**Heparinase activity plate assay**

The method is based on the ability of heparinase to cleave heparin and heparan sulfate [36]. Different concentrations of the samples to be tested were spotted on plates containing porcine intestinal heparin (1-mg/mL) (Sigma-Aldrich), 0.25 M sodium acetate, and 0.0025 M calcium acetate in 1.5% agarose at pH 7. The plates were incubated for 1 h at 37 °C and then 2% protamine sulfate solution (Sigma-Aldrich) was poured over the plates. The plates were incubated for 1–2 h at room temperature until a white precipitate formed on the unincoculated portions of the plate. The presence of clear zones indicate heparinase activity, with zones of increasing intensity at the areas where higher amounts of heparinase were added. Commercially available *P. heparinus* heparinase III (0.5–1-U) (Sigma-Aldrich) was used as a positive control. The protein elution buffer was used as a negative control.

**Growth curve analysis**

The strains were grown overnight in LB broth and subcultured into fresh LB broth to a starting OD₆₀₀ of 0.02. Ten-mL aliquots of the inoculum were dispensed into 250-mL flasks, the cultures were grown with vigorous shaking at 37 °C for 48 h, and samples were obtained every 4 h. To determine the number of viable bacterial cells at each time point, we serially diluted each sample (1:10) and 10-μL aliquots were plated on LB agar plates. The plates were incubated at 37 °C overnight and the numbers of colonies produced from each dilution was counted (colony forming units [CFU]). The final CFU/mL is obtained by the formula, CFU/mL = (number of colonies x dilution factor)/sample volume. Each experiment was performed in three independent replicates.

**Analysis of virulence factors**

Different experiments were done to determine the effects of the mutation of *hepP* and *zbdP* on the expression of virulence factors as previously described. For the analysis of elastase (LasB) activity, PA14ΔhepP and PA14ΔzbdP were grown in LB broth at 37 °C; supernatants were collected at 14 h and used in the elastin Congo red tube assay [34, 37]. For pyoverdine production, the strains were grown in chelated trypticase soy broth dialysate (TSB-DC) at 32 °C for 14 h, the supernatants were collected, and pyoverdine levels were determined spectrophotometrically at A₄₀₅ [38]. The strains were grown in glycerol alanine medium at 37 °C for 24 h for evaluation of pyocyanin production [39]. Pyocyanin was extracted from the supernatant fractions with chloroform and 0.2 M HCl [37, 40]. Assays for swimming (flagellar) and twitching (pilus-associated) motilities were done as previously described [41, 42]. Strains grown overnight on LB agar plates at 37 °C were stabbed onto the surface of 1% LB agar plates (twitching motility) and incubated for 16 or 24 h, respectively; zones of motility were measured as described [37, 41, 42].

The analysis of the effect *hepP* and *zbdP* mutations on the expression of quorum-sensing (QS) genes and T3SS genes was done by RT-qPCR. PA14ΔhepP and PA14ΔzbdP were grown for 16 h in LB broth for QS genes and for 16 h in TSB-DC containing nitrilotriacetic acid for T3SS genes [37] and the cells were pelleted. The RNA was extracted, purified, reverse transcribed, and subjected to qPCR as described above in Methods using primers described in Additional file 1: Table S1.

**Formation of pellicle and biofilm**

The air-liquid interface (ALI) method of biofilm development was used to observe both pellicle formation and biofilm development [43–45]. The strains were grown overnight in LB broth and subcultured into fresh LB broth to a starting OD₆₀₀ of 0.02 [41, 46, 47]. Three-mL aliquots of the inoculum were dispensed into 17 mm × 100 mm polystyrene tubes and incubated at 37 °C for 24 to 48 h. For pellicle formation, the tubes were incubated under static conditions and observed at 24 and 48 h post inoculation for pellicle formation and the relative turbidity of the broth below the ALI (planktonic growth). For the analysis of biofilm formation, the tubes were grown with gentle shaking. At 24 or 48 h post inoculation, planktonic growth was removed, the adherent biofilms were washed with distilled water to remove any nonadherent cells, and stained with 1% crystal violet. The biofilms were then washed twice with distilled water, the
crystal violet eluted from the biofilms with 95% ethanol, and the color was measured spectrophotometrically at $A_{590}$ [41, 46, 47].

**Caenorhabditis elegans slow-killing assay**

This assay was performed as previously described [48]. The temperature-sensitive embryonic lethal *C. elegans* strain BW54 was obtained from the *Caenorhabditis* Genetics Center (https://cgc.umn.edu; accessed 12/12/2017) (Table 1) and maintained at 15 °C for proliferation or 25 °C for storage without proliferation. For the survival studies, 5–15 μL of overnight cultures of the test strains, PA14 as a positive control, or *E. coli* OP50, a traditional feeder source, as the negative control, were spread on 3.5-cm plates containing nematode growth agar and incubated at 37 °C for 24 h [49]. Each plate was then seeded with 10 nematodes and the plates were incubated at 25 °C to prevent proliferation of BW54. Plates were examined every day for 5 days under a dissecting microscope and the numbers of surviving worms recorded. A worm was considered dead when it stopped responding to touch. Each experiment was replicated three to six times.

**Statistical analysis**

Statistical analyses of the results were done using GraphPad Prism 7.0 (GraphPad Software).

**Results**

The expression of *PA14_23420* and *PA14_23430* is influenced by thermal injury-induced changes in blood

We recently examined the effect of thermal injury-induced changes in blood on the expression of *P. aeruginosa* genes [15]. *P. aeruginosa* strain PA14 was grown in whole blood from either healthy volunteers (BHV) or severely burned (thermally-injured) patients (BTI) and the level of gene expression was examined by RNA-seq analysis [15]. Among the genes whose expression was significantly enhanced were the adjacent genes *PA14_23420* and *PA14_23430*. *PA14_23420* (*zbdP*) encodes a hypothetical zinc binding dehydrogenase, while *PA14_23430* (*hepP*) codes for a hypothetical heparinase. Compared with BHV, the growth of PA14 in BTI enhanced the expression of *zbdP* and *hepP* by approximately seven- to eight-fold in each of three male patients (Table 3). Despite differences in age (28-62 years), percentage of total body surface involved in the burn injury (25–50%), and source of burn (fire or explosion) [15], the increase in gene expression was comparable among the three patients (Table 3). We confirmed these results by real time quantitative PCR analysis (qPCR) of the RNA samples obtained during the RNA-seq analysis. Compared with the growth in BHV, the growth of PA14 in BTI increased the expression of *hepP* and *zbdP* by approximately five- and sixfold, respectively (Fig. 1).

| Gene | Protein Function a | Fold change compared to healthy volunteer b |
|------|-------------------|-----------------------------------------|
| zbdP | Zinc-binding dehydrogenase | 6.8 7.5 7.9 |
| hepP | Heparinase | 7.1 7.6 8.1 |

aProtein functions were obtained from the *Pseudomonas* Genome Database (http://pseudomonas.com/; accessed 12/12/2017); [54] bExpression of *zbdP* (*PA14_23420*) and *hepP* (*PA14_23430*) in PA14 that was grown in blood from three burn patients was compared with expression when PA14 was grown in blood from a healthy volunteer. Genes were identified as differentially expressed with $\geq$ two-fold change if they had the false discovery rate correction $p$-value of $\leq 0.01$ [15].

Fig. 1 Growth of PA14 in whole blood from a severely burned patient enhanced expression of *hepP* and *zbdP*. RNA samples obtained from PA14 that was grown for 8 h at 37 °C in LB broth (LBB), whole blood from a healthy volunteer (BHV), or whole blood from a patient with severe thermal injury (BTI) were used in RT-qPCR reactions as described in Methods using primers listed in Table 2. a Expression of *hepP*, b expression of *zbdP*. Values in a and b represent the means of triplicate experiments conducted on three independently obtained samples ± SEM. ****, $P < 0.0001$
In addition to the burn-induced effect, the growth of PA14 in BHV, compared with the growth in a regular laboratory medium such as LB broth, may influence the expression of either or both genes. To examine this possibility, we grew PA14 either in LB broth (LB) or in BHV and analyzed the expression of hepP and zbdP by qPCR. The growth of PA14 in BHV enhanced expression of these genes by seven- and fourfold, respectively (Fig. 1). These results suggest that, with respect to the growth in whole blood, the increase in the expression of hepP and zbdP occurs at two levels. First, there is a blood-related increase (BHV vs LB) that is likely to be triggered by certain components of whole blood; then there is a further increase (BHV vs BTI) that is likely to be triggered by thermal injury-induced changes in blood.

**hepP encodes a potential heparinase while zbdP encodes a potential zinc-binding dehydrogenase**

Computer analysis of the 61.9-kDa predicted protein encoded by hepP using the Conserved Domains database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi; accessed 12/12/2017) revealed the presence of two specific domains within the amino and carboxy terminus regions [50]. The amino-terminus domain (amino acid [aa] 116 to aa 288) has the characteristics of the chondroitin AC/alginate lyase superfamily/heparinase II-III_N domain (Additional file 1: Figure S1a). A number of bacterial species including *P. heparinus* synthesize glycosaminoglycans (GAGs) lyases, such as these chondroitin AC/alginate lyases, to degrade and utilize GAGs as a carbon source [24]. The carboxy terminus domain (aa 324 to aa 547) has the characteristics of the heparinase II/III superfamily, specifically the *P. heparinus* heparinase II/III domain (Additional file 1: Figure S1a). A homology search using Protein BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi; accessed 12/12/2017) and the nonredundant protein sequences database revealed that this domain in HepP is homologous to domains found in predicted proteins encoded by several other bacteria including *Pseudomonas fluorescens*, multiple *Acinetobacter* spp., *Shigella boydii*, *Edwardsiella tarda*, and *Vibrio vulnificus* (Additional file 1: Figure S1b) [51]. The strongest overall homology exists between the PA14 HepP and *P. fluorescens* F113 heparinase (Additional file 1: Figure S1b), and used in RT-PCR reactions with primers corresponding to specific sequences within hepP alone or within both genes (Fig. 2a). A reaction without template was included as a negative control (Fig. 2b). The expected 200-bp product was obtained with the primers for hepP alone (Fig. 2b). Additionally, we detected a 750-bp product using the primers that overlap both genes (Fig. 2b), indicating that zbdP-hepP are transcribed as an operon.

**Characterization of the putative heparinase encoded by hepP**

At this time, we decided to focus our efforts on hepP and its protein product HepP. Therefore, we obtained the 1650-bp fragment that contains the entire structural gene of hepP from PA14 by PCR. The gene was obtained minus the stop codon to allow synthesis of recombinant HepP (rHepP) with a 6xHis tag at the carboxy terminus. The fragment was cloned into the protein expression plasmid pBAD/Thio-TOPO, which carries the arabinose-inducible araBAD promoter P_{BAD} HP-thioredoxin to maximize translation of the recombinant protein, and the 6xHis tag for purification of the protein plus the V5 epitope for recognition of the fusion protein. The resulting plasmid, pND1, was transformed into the *E. coli* expression host TOP10. Preliminary expression experiments determined that 0.02% arabinose produced optimal synthesis of rHepP (data not shown). TOP10/pND1 was then grown in 50 mL of LB broth, induced with 0.02% arabinose, and cells were harvested 4 h post induction and processed as described in Methods. The rHepP was purified using nickel affinity column chromatography. Purification of rHepP was established by SDS-PAGE (Fig. 3a), and confirmed by Western blot analysis using anti-V5 antibody (Invitrogen) to detect the carboxy terminus V5 epitope within rHepP (Fig. 3b).
Once the successful purification of rHepP was confirmed, the next step was to examine its activity. To determine if the protein exhibits heparinase activity, we used the previously described heparinase plate assay [36]. An aliquot containing 28 μg of rHepP in 20 μL of protein elution buffer were spotted on the surface of the heparin-agarose plates along with the buffer as a negative control and heparinase III from P. heparinus as a positive control. Following incubation for 1 h at 37 °C, the plates were flooded with protamine sulfate, which reacts with intact heparin to form a white precipitate, and observed for clear zones indicative of heparinase activity. We detected clear zones with P. heparinus heparinase and with rHepP (Fig. 3c). These results suggest that PA14_23430 or hepP does encode a heparinase enzyme.

**Examination of PA14 mutants defective in hepP or zbdP**

To examine the role of HepP in the virulence of PA14, we utilized PA14 mutant strains defective in the hepP and zbdP genes.
genes. We obtained these mutants from the commercially-available non-redundant whole genome scale PA14 mutant library (PA14NR Set) [30]. These two mutants carry the Mariner transposon MAR2xT7 [30] inserted within the structural genes; in PA14/MrT7::PA14_23430–480 (PA14ΔhepP), the transposon is inserted at nucleotide (nt) 480; while PA14/MrT7::PA14_23420–118 (PA14ΔzbdP), the transposon insertion is at nt 118. To verify the mutation in PA14ΔhepP, we mapped the transposon insertion using PCR and restriction enzyme analyses. PCR analysis was conducted using primers matching sequences 94-bp upstream and 179-bp downstream of the hepP sequence (zbdP-For3/hepP-Rev3, respectively) (Table 2, Additional file 1: Figure S3) and chromosomal DNA from PA14 and PA14ΔhepP as templates. We obtained the expected 1926-bp product from the chromosome of PA14 (the 1653-bp hepP gene plus the additional 273 nt) and the expected 2920-bp product, representing the addition of the 994-bp MAR2xT7 transposon, from the chromosome of PA14ΔhepP (Fig. 4a). We confirmed the presence of MAR2xT7 within PA14ΔzbdP in a similar manner (data not shown).

Computer analysis revealed the presence of an EcoRV restriction enzyme site within MAR2xT7 and the lack of such a site within hepP (http://www.restrictionmapper.org/; accessed 12/12/2017). Therefore, to further confirm the mutation of hepP in PA14ΔhepP, we performed restriction enzyme digestion on the PCR products with EcoRV (New England Biolabs). As expected, there was no reduction in the size of the 1926-bp from PA14 after enzyme digestion (Fig. 4b). In contrast, digestion of the PCR product from PA14ΔhepP produced two fragments of 800 bp and 2120 bp (Fig. 4b). The size of the two bands is consistent with the transposon insertion at 480 bp within hepP.

We then performed a growth curve analysis to determine if the presence of the transposon within either PA14ΔhepP or PA14ΔzbdP affected its growth. PA14, PA14ΔhepP and PA14ΔzbdP were grown in LB broth for 48 h at 37 °C. Samples were obtained every 4 h and serially diluted to determine the CFU/mL. The growth of the mutants paralleled that of their parent strain throughout the time frame of the analysis (Additional file 1: Figure S4).

Loss of hepP or zbdP affects pellicle and biofilm formation by PA14

During static growth of PA14 and PA14ΔhepP or PA14ΔzbdP in LB broth in 17 mm × 100 mm polystyrene tubes, we observed a difference between the three strains in pellicle formation. When grown under static conditions, some bacteria, including P. aeruginosa migrate to the air-liquid interface (ALI) and form biofilm-like structures [43]. These structures consist of bacterial cells and a pellicle, which is a matrix of extracellular polymer [45]. The ALI provides the bacteria with access to a high concentration of oxygen from the air and sufficient nutrients from the liquid. At 24 h of growth, PA14 and PA14ΔzbdP formed a typical pellicle while PA14ΔhepP did not (Fig. 5, red arrowheads). Additionally, there appeared to be more planktonic growth within the LB broth within the tube containing PA14ΔhepP compared to PA14 and PA14ΔzbdP (Fig. 5, yellow arrowhead). At 48 h, PA14ΔhepP appeared to form a thin pellicle, but there was still more growth within the tube compared to PA14 and PA14ΔzbdP (Fig. 5).

Previous studies showed that P. aeruginosa flagella motility contributes to pellicle formation. P. aeruginosa flagellum-deficient mutants either produced pellicles of unusual structure or were delayed in pellicle formation [43, 45, 55]. To assess whether the delay in pellicle formation by PA14ΔhepP was due to a defect in flagellum-mediated motility, we compared all three strains using the swimming and twitching assays [42]. No difference in flagellar motility or pilus-related motility was detected (data not shown).

Since PA14ΔhepP was defective in forming the biofilm-like pellicle structure at the ALI, it may also be defective in forming a biofilm on a solid surface. To examine this possibility, we compared the biofilms formed by PA14, PA14ΔzbdP, and PA14ΔhepP using the ALI method of
biofilm development on the walls of polystyrene tubes [42], similar to the way in which we had observed the difference in pellicle formation. All strains were grown with gentle shaking in LB broth for 24 or 48 h and the amount of biofilm formed was measured using the crystal violet assay as described in Methods [37, 46]. As shown in Fig. 6, PA14ΔhepP formed less biofilm than PA14 or PA14ΔzbdP at both 24 and 48 h. Interestingly, the amount of biofilm formed by PA14ΔzbdP was significantly higher than that formed by PA14 at 24 h ($P < 0.05$) and visibly higher (but not significantly higher) at 48 h (Fig. 6). These results suggest that the mutation in PA14ΔhepP affected the ability of PA14 to form biofilms on solid surfaces as well as the pellicle at the ALI, while the mutation of PA14ΔzbdP enhanced the ability of PA14 to form biofilms on solid surfaces.

As the development of biofilms is controlled by quorum sensing (QS), we also examined the effect of the mutations in hepP or zbdP on QS-controlled virulence factors of P. aeruginosa. We examined the production of pyocyanin, pyo- veridine and elastase (LasB) by PA14ΔhepP or PA14ΔzbdP using the assays previously described [34, 38, 40, 56]. The level of each factor produced by the two mutants was comparable to that produced by PA14 (data not shown). We utilized RT-qPCR to examine the effect of the mutation on the expression of PA14 QS genes rhlA and lasI. We detected no difference in the expression of either gene (data not shown). Using the same approach, we ruled out any potential effect on the expression of the T3SS genes exoU and exsD. Again, the mutation of hepP did not alter the expression of either exoU or exsD (data not shown).

**Loss of hepP reduces PA14 virulence while loss of zbdP does not**

The above results showed that neither hepP nor zbdP mutation affect the production or expression of several specific virulence factors. Therefore, rather than testing additional individual virulence factors, we decided to assess the effect of loss of either gene on the virulence of PA14 using the Caenorhabditis elegans pathogenesis model. This model, which has been used in numerous previous studies, involves the killing of the soil nematode C. elegans by P. aeruginosa [48, 57–59]. Tan et al. [48] previously demonstrated that, depending on the medium in which it is grown, PA14 produced either fast killing or slow killing of C. elegans. Fast killing, which occurs within a few hours, is partially mediated by bacterial secreted factors rather than live bacteria; it is produced when PA14 is grown on a high osmolarity medium. In contrast, slow killing, which occurs over the course of a few days and involves an infection process, is produced when PA14 is grown on a minimal medium (modified nematode growth media) [48].

We examined the slow killing process using C. elegans BW54, a temperature sensitive embryonic lethal mutant, fed on PA14, PA14ΔhepP, PA14ΔzbdP or the E. coli strain OP50 (the common feeder host for maintenance of C. elegans) as a negative control. We monitored the killing/survival of BW54 fed on the different strains for 5 d post infection. The feeding of BW54 on a lawn of OP50 produced the least lethality (90% survival) (Fig. 7). In contrast, on day 5, the survival rate of BW54 that fed on a lawn of PA14 was 0% while the survival rate of worms fed on PA14ΔzbdP was 20% (Fig. 7). However, BW54 that fed on a lawn of PA14ΔhepP had a 70% survival rate on day 5 (Fig. 7). These results suggest that PA14ΔhepP is defective in its in vivo virulence while PA14ΔzbdP is still virulent.

We were puzzled by the phenotypic differences between PA14ΔhepP and PA14ΔzbdP. While PA14ΔhepP produced a significantly reduced biofilm, PA14ΔzbdP formed a well-developed one (Fig. 6). Additionally, while PA14ΔhepP caused only 30% lethality among C. elegans, PA14ΔzbdP caused 80% lethality (Fig. 7). The Pseudomonas Genomic database indicated, and our PCR analysis confirmed, that zbdP and hepP constitute an operon in which zbdP is the first gene and the two genes are separated by eight bp (Fig. 2 and Additional file 1: Figure S3) [54]. Our PCR
analysis confirmed the presence of the operon (Fig. 2b). Therefore, we conducted RT-PCR experiments to determine if hepP is transcribed in PA14ΔzbdP. RNA obtained from PA14ΔzbdP and PA14ΔhepP grown in LB broth was reverse-transcribed and the cDNA used as templates in experiments using primer sets designed to detect internal fragments downstream of the transposon insertion in zbdP (zbdP-For1/zbdP-Rev1) or hepP (hepP-For2/hepP-Rev4) (Additional file 1: Figure S3). As expected, analysis of RNA from PA14ΔzbdP showed no transcription of zbdP (Fig. 8, lane 3). However, we did detect the 201-bp internal hepP fragment from PA14ΔzbdP (Fig. 8, lane 2). Analysis of RNA from PA14ΔhepP showed the presence of the 219-bp internal zbdP fragment (Fig. 8, lane 5), but we detected no transcript from the region of hepP below the transposon insertion (Fig. 8, lane 6). These results show that although zbdP-hepP form an operon, loss of zbdP does not prevent transcription of hepP, which likely explains the observed phenotypic differences between the two mutants. A more detailed description of these experiments is provided in Additional file 1: Figure S5.
Discussion

Our results showed that, compared with its growth in LB broth, the growth of PA14 in whole blood from healthy volunteers (BHV) significantly enhanced the expression of PA14_23430, which encodes a putative heparinase (to be called hepP), and PA14_23420 that codes for a zinc-binding dehydrogenase (to be called zbdP) (Fig. 1). Moreover, compared with its growth in BHV, the growth of PA14 in whole blood from thermally injured patients (BTI) produced an additional increase in the expression of both genes (Fig. 1). These results suggest that these genes are related to the in vivo virulence of PA14. Previous studies have also shown that the growth of different pathogenic bacteria in blood enhanced the expression of numerous genes compared with growth in laboratory media [3, 15, 60]. For example, Graham et al. [60] showed that, compared with its growth in Todd Hewitt broth containing 0.2% yeast extract, the growth of S. pyogenes in blood increased the expression of 62.7% of transcripts including those of virulence factors such as covR-covS (cov, control of virulence); while Kruczek et al. [15] showed that the growth of PA14 in BTI altered (enhanced or reduced) the expression of 2596 genes compared with its growth in BHV.

Interestingly, the hepP and zbdP genes were also recovered in an earlier search for PA14 virulence-related genes done by a screening of the nonredundant PA14 transposon mutant library of 5850 clones that represent 75% of the total genome and 80% of the nonessential ORFs in this P. aeruginosa strain [30, 61]. Using C. elegans infection models and a two-step screening process, Feinbaum et al. in 2012 [61] identified 399 mutants as potentially attenuated in their virulence in the primary screening in which the virulence was measured by the reduction of the C. elegans brood. Of these 399 genes, 180 were identified as potentially attenuated in virulence from the secondary screening based on the slow killing of C. elegans [61]. Both hepP and zbdP, referred to as ORF_11 and ORF_10, respectively, were identified in the primary screening; however, zbdP was not identified in the secondary screening suggesting that only hepP contributes moderately to the in vivo virulence of PA14 [61]. However, in 2006, Feinbaum and colleagues had identified both hepP and zbdP as virulence-related genes in a different study [57]. Using the same C. elegans slow killing assay and the same transposon mutants described (which we call PA14ΔhepP and (PA14ΔzbdP), the results of our current study support the 2012 findings, but contradict the earlier report [57, 61]. Additionally, our results suggest that hepP significantly contributes to PA14 virulence, rather than moderately [61], as feeding C. elegans PA14ΔhepP increased the survival of the nematodes to 70% compared to the 0% survival rate among those fed PA14 (Fig. 7).

Very little homology was discovered for hepP or zbdP at the DNA level, with only three strains of Pseudomonas aeruginosa (PA14OR, serotype 010, and H47921) carrying genes matching the hepP and zbdP sequences. Similarly, PCR analysis of several different P. aeruginosa clinical isolates previously obtained from patients at University Medical Center or the Texas Tech Physicians clinics, Lubbock, TX did not detect either hepP or zbdP. However, interrogation of the nonredundant protein database (NCBI Protein BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi; accessed 12/12/2017) revealed that proteins with 96–100% aa identity to HepP can be found in 38 P. aeruginosa strains including PA14, BL04, BL16, BWH054, BWH058, BWHPSA043, BWHPSA044, CI127, HMSC057H01, and HMSC072F09; but are missing from the strains PA01, PA103 and PAK as well as numerous other sequenced P. aeruginosa strains (data not shown). An additional search of the Pseudomonas Ortholog Groups (http://www.pseudomonas.com/orthologs/list?id=1654617; accessed 12/12/2017) found orthologous heparinases in conjunction with zinc-binding dehydrogenases in P. aeruginosa strains AZPAE15025 and PA14_CIA. Similarly, proteins 96–100% homologous to ZbdP were found in 31 P. aeruginosa including all of the strains named above except CI127. Dehydrogenase orthologs (not all were classified as zinc-binding proteins) were found in conjunction with heparinases in a number of different P. aeruginosa strains including 11 different AZPAE strains, WS136, Pae_CF67.11p, Pae_CF67.10q, PADK2_CF510, CF_PA39, H11, BWPSA008, BWPSA010, and BWPSA040 (data not shown).

Strains PA01 and PA14 were originally isolated from infected wounds and both strains have been utilized extensively in in vitro and in vivo virulence studies. However, PA14 was shown to be more virulent than PA01 in various infection models including the murine model of thermal injury and the nematode C. elegans, the plant Arabidopsis thaliana, and the wax moth Galleria mellonella infection models [31, 48, 62]. Using the C. elegans killing model, Lee et al. [57] also found PA14 to be one of the more virulent strains in their comparison of the in vivo virulence of 20 P. aeruginosa laboratory strains, environmental isolates and clinical isolates obtained from urine, blood, burn wounds, and respiratory secretions from cystic fibrosis (CF) patients. The CF lung isolate CF18 ranked first in virulence, PA14 ranked second, and PA01 ranked eleventh [57]. While the PA14 and PA01 genomes are remarkably similar, the PA14 chromosome is larger than that of PA01 (6.5 Mb vs 6.3 Mb) [57]. Many of the PA14 genes that do not exist in PA01 contribute to the pathogenicity of PA14 and are located within two pathogenicity islands, PAP-1 and PAP-2 [57, 63]. PAP-1 is absent from the reference strain of PA01 and only a portion of PAP-2 is present [63]. Neither hepP nor zbdP is found within PAP-1 or PAP-2; instead they are found within a cluster of genes named PA14R38, which is present in PA14 [57]. PA14R38 was found in all 20 strains analyzed by Lee et al. [57], although the sequence of this gene cluster is so
divergent that hepP and zbdP are not present in PA14R38 or any of the other 19 strains. These results suggest that hepP and zbdP genes are unique to PA14 and a number of other P. aeruginosa strains and were not horizontally transferred directly from them to other bacteria. However, similar heparinases can be found in the company of similar dehydrogenases in other Pseudomonas species, such as P. fluorescens F113, where the genes are in the same order and the proteins share a level of homology (66% identity and 78% positive with no gaps for HepP; 74% identity, 85% positives with no gaps for ZbdP). The strains P. bauza-nensis W13Z2, P. chloritidimutans AW-1, P. chlororaphis HT66, P. denitrificans 148-PDEN, P. fluorescens AU6026, AU6308, and PA4C2, Pseudomonas sp. PAMC 26793, and P. stutzeri TS44 also carry sequences encoding proteins homologous to HepP and ZbdP. These findings suggest the functions of these two proteins are conserved among many different Pseudomonas species.

The microorganism from which heparinase was fully characterized is the nonpathogenic soil microorganism P. heparinus, which produces three heparinases: heparinase I, which acts on heparin; heparinase II, which acts on both heparin and heparan sulfate; and heparinase III, which acts on heparin; heparinase II, which acts on both heparan sulfate and heparin; and heparinase III, which produces three heparinases: hepP, hepP, and hepP. Regardless of its exact nature as a heparinase (I, II, or III), HepP contributes to the virulence of P. aeruginosa as shown by the significant decrease in mortality of C. elegans fed PA (Fig. 7). To our knowledge, this is the first characterized virulence-related heparinase in a bacterial pathogen.

Based on the presence of the alginate lyase domain in HepP, the protein may play a role in the cleavage of proteoglycans within the human host. Proteoglycans are composed of core proteins to which chains of anionic polysaccharides and glycosaminoglycans (GAGs) are attached. GAGs consist of repeated disaccharide units [22, 70]. Depending on the composition of these units, GAGs are classified as heparin or heparan sulfate, dermatan sulfate, chondroitin sulfate, keratin sulfate and hyaluronic acid [16, 70]. Among these classes, heparan sulfate proteoglycans (HSPGs) are the most complex and most physiologically relevant. HSPGs play a critical role in cellular processes including cell adhesion, organization of extracellular matrix, cytoskeleton organization, differentiation and morphogenesis, and tissue repair and inflammation [16, 71]. HSPGs also serve as coreceptors for cytokines, chemokines, and growth factors, a role that is associated with signal transduction; the binding of these ligands also protects them from proteolysis [71]. Shedding of the extracellular domain, leaving the membrane-spanning region with its intracellular domain intact, has been shown to play a key role in regulating the host response to tissue injury and inflammation [16, 18, 71, 72].

Many pathogenic bacteria utilize heparan sulfate for initial colonization and adherence [73]. Other pathogens, including Enterococcus faecalis and Listeria monocytogenes, utilize this mechanism for invasion and internalization [74]. The heparin-binding hemagglutinin of Mycobacterium tuberculosis not only facilitates adherence to epithelial cells but is also important in extrapulmonary dissemination of the infection [16, 75, 76]. P. aeruginosa induces shedding of the HSPG syndecan, which appears to augment its invasion of host cells [77, 78]. The shed syndecan binds proline- and arginine-rich antimicrobial peptides leading to a reduced host defense and enhanced bacterial survival and pathogenesis [71]. Despite much evidence for the importance of heparan sulfate in bacterial pathogenesis, with the exception of our current study, no previous studies have connected heparinase with bacterial virulence. One earlier study investigating the production of heparinase by Bacteroides concluded that heparinase produced by anaerobes is not likely to contribute to the regional thrombophlebitis that occurs during anaerobic infections [79].

Both heparan sulfate and dermatan sulfate exist in the region of the terminal web and brush border of C. elegans intestine [80, 81]. Furthermore, mutations in C. elegans genes coding for heparin, heparan sulfate, HSPG core proteins, and their biosynthetic enzymes produced several
phenotypic abnormalities including defects in cytokinesis and vulval morphogenesis, developmental abnormalities at embryonic stages, abnormal axon branching, defects in the formation and maintenance of the muscle myofilament lattice, and embryonic lethality [82, 83]. Considering our current findings, heparinase may enhance PA14 virulence in C. elegans by disrupting numerous physiological processes and pathways that depend on HSPGs. Therefore, the observed significant reduction in the in vivo virulence of PA14ΔhepP may be due to the failure of the strain to interfere with or disrupt one or more of these vital processes.

Another role for hepP in the virulence of PA14 is its contribution to pellicle formation at the air-liquid interface (ALI) and biofilm formation on a solid surface. With respect to pellicle formation, our results showed that when grown in a polystyrene tube under static conditions, thick pellicles are formed at the ALI by PA14 at 24 h post inoculation, but pellicle formation was delayed in PA14ΔhepP until 48 h, at which time a thinner pellicle became visible (Fig. 5). Previous studies showed that flagellum-mediated motility is required for pellicle formation in P. aeruginosa strain PA01 [42]. A recent comparison of pellicle formation by PA14, a PA14 flagellum mutant (PA14Δflgk) and a PA14 pilus mutant (PA14ΔpilB) also showed that loss of flagellum-mediated motility, but not pilus-mediated motility, resulted in delayed pellicle formation [84]. We ruled out the possibility that hepP influences flagellar- or pilus-related motility using swimming and twitching motility assays in which PA14ΔhepP was equal to PA14 in its motility (data not shown). Similar to the observations of Holscher et al. [84], the medium under the pellicle formed by PA14ΔhepP remained turbid compared to PA14 (Fig. 5). The Pel exopolysaccharide is required for the formation of pellicles at the ALI [85]. Using RT-qPCR we investigated the possibility that hepP influences pellicle formation through pelC or pelE (Additional file 1: Table S1). However, amount of pelC and pelE transcripts produced by PA14ΔhepP was comparable to that produced by PA14 (data not shown).

With respect to biofilm development on a solid surface, PA14ΔhepP failed to form a robust biofilm at 24 h post inoculation compared with PA14 (Fig. 6). Besides ruling out any effect of hepP on PA14 motility and pel transcription, both critical factors in biofilm initiation, we have also examined the possible effect of hepP on other potential contributors to biofilm development including the QS system. Assays for factors controlled by QS, such as LasB, pyocyanin, and pyoverdine, and the transcription of the QS genes rhlA and lasI produced the same results with PA14ΔhepP and PA14 (data not shown). Whether hepP affects the production of other biofilm-related factors is yet to be determined.

The genes zbdP and hepP constitute an operon (Fig. 2) whose expression in PA14 is enhanced by thermal injury-induced changes in blood (Fig. 1 and Table 3). However, it is clear that despite transposon insertion in zbdP, hepP is still transcribed in PA14ΔzbdP (Fig. 8). Additionally, PA14 mutants defective in these genes showed phenotypic differences, with both biofilm production and lethality in the C. elegans model of slow killing reduced in PA14ΔhepP but not in PA14ΔzbdP (Figs. 6 and 7). These results suggest that hepP is reinitiated from a region within zbdP below the transposon insertion. We are currently conducting experiments designed to identify the role of the potential zinc-binding protein encoded by zbdP and its relationship to the heparinase HepP.

Our work is significant because, to our knowledge, this is the first time a heparinase or heparin- and heparan sulfate-degrading enzyme has been characterized from P. aeruginosa. Such enzymes have been shown to have value as heparin antagonists and antineoplastic agents because of their ability to reduce neoangiogenesis [16] and heparinases I, II, and III to reduce neovascularization during tumor progression through their ability to alter the action of fibroblast growth factor [16, 20, 21, 27, 29]. In the future, heparinases may gain therapeutic value as targets for antimicrobials. We are currently conducting experiments to elucidate the effect of the HepP heparinase on PA14 virulence in a murine model.

Conclusions
Changes within the blood of severely burned patients significantly induced expression of hepP in PA14. The gene codes for an active heparinase enzyme, HepP, which influences PA14 in vivo virulence. To our knowledge this is the first time a heparinase, or heparin and heparan sulfate degrading enzyme, has been characterized from P. aeruginosa. HepP influences pellicle formation as well as biofilm development by PA14 and the protein is required for full virulence in the C. elegans model of slow killing.

Additional file

Additional file 1: Figure S1. Characteristics of HepP and its homology to other proteins. Figure S2. Amino acid sequence of the 78-kDa predicted protein encoded by zbdP. Figure S3. Nucleotide sequences of zbdP and hepP showing locations of primers used in experiments to analyze these two genes. Figure S4. Mutation of hepP or zbdP does not significantly alter the growth of PA14 in vitro. Figure S5. Detailed experiment to confirm transcription of hepP in PA14ΔzbdP. Table S1. Primers used to compare virulence factor gene transcription in PA14, PA14ΔhepP, and PA14ΔzbdP. (PDF 1972 kb)

Abbreviations
aa: Amino acid; ALI: Air-liquid interface; BHV: Whole blood from healthy volunteers; BTI: Whole blood from severely burned (thermally-injured) patients; Cb: Carbenicillin; CFU: Colony forming units; GAGs: Glycosaminoglycans; Gm: Gentamicin; HSGAGs: Heparin and heparan sulfate...
sulfate glycosaminoglycans; HSPGs: Heparin sulfate proteoglycans; LB: Luria-Bertani; nt: Nucleotide; qPCR: Real time quantitative PCR; QS: Quorum sensing; RT-PCR: Reverse transcriptase PCR; Str: Streptomycin; TSS: Type three secretion system; TSB-DC: Chelated trypticate soy broth dialysate

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Availability of data and materials
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors’ contributions
ND conducted all the experiments and wrote the manuscript. ANH coordinated the study, designed portions of the experiments and participated in the initial draft and the revision of the manuscript. JACH critically analyzed the data and revised the manuscript. JAG participated in the design of several experiments and provided critical clinical evaluation of the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Human samples used in this study were previously obtained in a study approved by the Texas Tech University Health Sciences Center Institutional Review Board. The authors thank Dr. Cassandra Kruczek for her expert assistance with the RNA analyses, Dr. K. Rumbaugh for her kind gift of E. coli strain OP50 and Joanna E. Swickard for critical reading of this manuscript.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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