Heparinase Is Essential for *Pseudomonas aeruginosa* Virulence during Thermal Injury and Infection

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ABSTRACT The opportunistic pathogen *Pseudomonas aeruginosa* is a major cause of sepsis in severely burned patients. If it is not eradicated from the wound, it translocates to the bloodstream, causing sepsis, multiorgan failure, and death. We recently described the *P. aeruginosa* heparinase-encoding gene, *hepP*, whose expression was significantly enhanced when *P. aeruginosa* strain UCBPP_PA14 (PA14) was grown in whole blood from severely burned patients. Further analysis demonstrated that *hepP* contributed to the *in vivo* virulence of PA14 in the *Caenorhabditis elegans* model. In this study, we utilized the murine model of thermal injury to examine the contribution of *hepP* to the pathogenesis of *P. aeruginosa* during burn wound infection. Mutation of *hepP* reduced the rate of mortality from 100% for mice infected with PA14 to 7% for mice infected with PA14::*hepP*. While comparable numbers of PA14 and PA14::*hepP* bacteria were recovered from infected skin, only PA14 was recovered from the livers and spleens of infected mice. Despite its inability to spread systemically, PA14::*hepP* formed perivascular cuffs around the blood vessels within the skin of the thermally injured/infected mice. Intraperitoneal inoculation of the thermally injured mice, bypassing the need for translocation, produced similar results. The rate of mortality for mice infected with PA14::*hepP* was 0%, whereas it was 66% for mice infected with PA14. As before, only PA14 was recovered from the livers and spleens of infected mice. These results suggest that *hepP* plays a crucial role in the pathogenesis of PA14 during burn wound infection, most likely by contributing to PA14 survival in the bloodstream of the thermally injured mouse during sepsis.

KEYWORDS heparinase, *Pseudomonas aeruginosa*, sepsis, thermal injury, virulence, virulence factor, serum resistance

Following traumatic injury, sepsis is one of the leading causes of death in the days and weeks after the initial injury (1). Besides the direct violation of skin integrity in an invariably contaminated setting that allows inoculation of the wounds with bacteria, other risk factors for infection are also present. These are the immunosuppressive response to injury; the need for invasive ventilation and monitoring, which increase the risk for nosocomial infections; the requirement for blood transfusions; and the relative insulin resistance associated with the response to injury, which leads to hyperglycemia (2, 3). Severe sepsis is a common and frequently fatal condition. Previous epidemiological studies revealed that in the United States 1 million individuals with severe sepsis are hospitalized annually, with the number of deaths being about 200,000 annually (4–6). Despite advances in modern medicine that include vaccines, antibiotics, and more effective acute care practices, if it is not recognized early and treated promptly, sepsis remains the primary cause of death from infection (7).

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Among the many different microorganisms that cause sepsis is *Pseudomonas aeruginosa*. This opportunistic pathogen most commonly infects immunocompromised patients, such as those with cancer, AIDS, cystic fibrosis, and burn injuries. The damage caused during *P. aeruginosa* infection is due to the production of numerous cell-associated and extracellular virulence factors (8–11). Due to the rise in the numbers of multidrug-resistant strains, *P. aeruginosa* is one of the leading organisms associated with nosocomial infections and trauma-related sepsis (12). Despite numerous studies, the pathogenesis of *P. aeruginosa* infection during trauma-induced sepsis has not been defined. In an attempt to understand bacterial pathogenesis during sepsis following burn injury, we recently initiated a new approach, in which we grew *P. aeruginosa* in whole blood and examined the changes in expression of the transcriptome (13). The growth of *P. aeruginosa* in whole blood from severely burned patients significantly enhanced the expression of specific sets of virulence genes, such as iron-regulated genes, quorum sensing-related genes, and type III secretion genes, as well as numerous previously uncharacterized genes, compared with their expression when *P. aeruginosa* was grown in whole blood from healthy volunteers (13). One of the genes whose expression was significantly enhanced upon the growth of *P. aeruginosa* strain UCBBP_PA14 (PA14) in whole blood from severely burned patients was *hepP*, a previously uncharacterized gene encoding a heparinase enzyme. We recently characterized *hepP* by cloning the gene, overexpressing and purifying the protein that it encodes, and confirming its enzymatic activity using a heparin degradation assay (N. Dzvova, J. A. Colmer-Hamood, J. A. Griwsold, and A. N. Hamood, submitted for publication). Further analysis demonstrated that *hepP* contributes to biofilm development, pellicle formation, and the *in vivo* virulence of *P. aeruginosa* in the *Caenorhabditis elegans* model (Dzvova et al., submitted).

Bacterial heparinases degrade heparin and heparan sulfate (HS). Heparan sulfate proteoglycans (HSPGs) are types of glycoproteins that contain one or more covalently attached HS moieties. HSPGs are found at the cell surface and in the extracellular matrix. There are three main types of HSPGs, grouped according to location: membrane HSPGs, such as syndecans and glypicans; the secreted extracellular matrix HSPGs, which include agrin, perlecan, and type XVIII collagen; and the secretory vesicle proteoglycan serglycin (14). Due to the diversity of HSPGs, they can interact specifically with a wide variety of ligands and proteins. These diverse interactions contribute immensely to the biological activities within the body, such as basement membrane integrity and cytokine, chemokine, and growth factor binding. Membrane HSPGs also cooperate with integrins and cell adhesion receptors to facilitate cell attachment to the extracellular matrix, cell-cell interactions, and cell motility (14). Although mammalian heparinases have been linked to inflammatory and autoimmune disorders, such as inflammatory lung syndrome, inflammatory bowel disease, rheumatoid arthritis, and chronic colitis, it is unclear how bacterial heparinases are involved in infection and pathogenesis (14). In this study, we examined the role of the HepP heparinase in *P. aeruginosa*-induced sepsis using the murine model of thermal injury. We found that HepP contributes to *P. aeruginosa* sepsis by aiding the survival of *P. aeruginosa* within the bodies of the thermally injured/infected mice.

RESULTS

Mutation of *hepP* enhances the survival of thermally injured, *P. aeruginosa*-infected mice. Having previously ruled out the possibility that the mutation in *hepP* compromised the growth of PA14 (Dzvova et al., submitted), we tested the *in vivo* virulence of the PA14 transposon mutant PA14/MrT7::PA14_23430–480 (PA14::hepP), in which the Mariner transposon MAR2Tx7 is inserted at nucleotide 480 in the PA14_23430 (*hepP*) gene (Table 1) (15), in the murine model of thermal injury (16). We thermally injured and inoculated groups of mice (five per group) with approximately 200 CFU of PA14::hepP or PA14. The percent survival of mice infected with PA14::hepP was significantly higher than that of mice infected with PA14, which was 0% by day 2 after injury and infection (Fig. 1). By day 7 after injury and infection, 87% of mice infected with

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Characteristics ^a	Reference(s) or source
<i>Pseudomonas aeruginosa</i> strains		
PA14	UCBPP-PA14; prototrophic strain isolated from an infected wound	15, 25
PA14::hepP	PA14/MrT7::PA14_23430-480 Gm ^r	15
PA14::66100	PA14/MrT7::PA14_66100-136 Gm ^r	15
PA14::wzz	PA14/MrT7::PA14_23360-113 Gm ^r	15
Plasmids		
pBAD/Thio-TOPO	pBR322-derived expression vector; cloned genes expressed from P _{BAD} ; amino terminus, HP-thioredoxin; carboxy terminus, V5 epitope and 6× His; Cb ^r	Invitrogen
pND1	pBAD/Thio-TOPO containing the 1,650-bp <i>hepP</i> gene under the control of the arabinose-inducible promoter P _{BAD} ; Cb ^r	Dzvova et al., submitted
pND2	pBAD/Thio-TOPO carrying the 1,213-bp NdeI oriV_pRO1600 fragment for stable replication in <i>P. aeruginosa</i> ; Cb ^r	This study
pND3	pND1 carrying the 1,213-bp NdeI oriV_pRO1600 stability fragment; Cb ^r	This study

^aGm^r, gentamycin resistant; Cb^r, carbenicillin resistant; HP, His patch.

PA14::hepP survived (Fig. 1). These results suggest that in the murine model of thermal injury, the *in vivo* virulence of PA14 is significantly reduced upon the loss of the *hepP* gene and the heparinase enzyme which it encodes.

To confirm these results, we conducted complementation experiments using plasmid pND3 in which *hepP* is expressed from the P_{BAD} promoter (Table 1). The plasmid replicates stably in PA14 (Table 1). To overproduce recombinant HepP (rHepP) in the culture prior to the inoculation of the animals, strain PA14::hepP/pND3 was grown overnight in the presence of 0.02% arabinose. At this level of arabinose induction, a considerable amount of rHepP was produced in *Escherichia coli* TOP10 cells carrying the original *hepP* expression clone pND1 (Table 1) (Dzvova et al., submitted). Initial experiments confirmed that, under similar conditions, PA14::hepP/pND3 produces high levels of rHepP (data not shown). PA14::hepP carrying pND2 (a cloning vector modified for stable replication in PA14) was used as a negative control (Table 1). Compared with the 100% survival of mice inoculated with PA14::hepP/pND2, the presence of pND3 in PA14::hepP reduced the survival rate among thermally injured/infected mice by 50% (Fig. 2). This significant ($P = 0.0247$) but partial complementation with pND3 is possibly due to the limited expression of *hepP* in mice. Although the strain was grown in arabinose prior to the inoculation, the level of rHepP synthesis would likely be reduced during growth within the infected wound in the absence of induction.

Mutation of *hepP* eliminates the systemic spread of PA14 after infection of thermal injury. We examined whether the defect in PA14::hepP is due to the inability

FIG 1 The loss of *hepP* significantly enhanced the survival of thermally injured mice infected with PA14. Adult Swiss Webster mice (five per group) were thermally injured and inoculated with ~200 CFU of either PA14 or PA14::hepP. The mice were monitored for survival daily for 7 days after burn infection. Values represent the averages for 15 mice used in 3 independent experiments. Comparison of survival curves using the log-rank (Mantel-Cox) test produced a P value of <0.0001 .

FIG 2 Complementation of PA14::hepP with a plasmid expressing *hepP* partially restores the virulence phenotype. Adult Swiss Webster mice (four per group) were thermally injured and inoculated with ~200 CFU of PA14, PA14::hepP/pND2 (negative control), or PA14::hepP/pND3 (expressing *hepP*). The mice were monitored for survival daily for 6 days after burn infection. Values represent the averages for 8 mice used in 2 independent experiments. Comparison of the survival curves of mice infected with PA14::hepP/pND2 and PA14::hepP/pND3 using the log-rank (Mantel-Cox) test produced a *P* value of 0.0247.

of the mutant either to grow and spread within the injured skin or to spread systemically within the thermally injured mouse. Thermally injured mice infected with either PA14 or PA14::hepP were euthanized at 24, 48, 72, or 96 h postinfection, and skin samples and organs were recovered. To examine the horizontal (local) spread within the thermally injured skin, 5-mm by 5-mm skin samples were obtained from the inoculation site and from a section of the skin 15 mm away from the inoculation site (referred to here as the distant site). Systemic spread was assessed by determining the bacterial load within the liver and spleen. Serial 10-fold dilutions of tissues homogenized in 1 ml of phosphate-buffered saline (PBS) were plated to determine the number of CFU per gram of tissue of each bacterial strain. At 24 h after injury and infection, PA14::hepP showed reduced numbers of CFU at the distant site compared to the numbers of CFU of PA14 ($P < 0.05$) (Fig. 3A), but by 48 h after injury and infection, the number of CFU of PA14::hepP was comparable to that of PA14 (Fig. 3B). However, PA14::hepP was unable to spread systemically by 48 h after injury and infection, while PA14 had reached the liver and spleen within 24 h (Fig. 3A and B), and by 48 h, all the mice had succumbed to PA14 infection. Despite continued growth at both the inoculation site and the distant site comparable to that of PA14, PA14::hepP was unable to spread systemically even by 96 h after injury and infection, as no microorganisms were recovered from the livers or spleens of infected mice (Fig. 3C and D). These results suggest that while PA14::hepP was somewhat delayed in its local spread, it was not defective in its growth within the injured skin but was defective in its systemic spread within the bodies of the thermally injured/infected mice. Therefore, PA14::hepP may be defective in either its ability to translocate from the infected skin into the bloodstream or its ability to survive within the blood of thermally injured/infected mice, or both.

Mutation of *hepP* does not affect *P. aeruginosa* PVC. The dermatologic manifestation known as ecthyma gangrenosum (EG) has been considered a classic sign of *P. aeruginosa* septicemia. Although EG can be caused by other microorganisms, including fungi, 70 to 75% of all cases are caused by *P. aeruginosa* (17, 18). In EG, biopsy of the skin lesion shows perivascular cuffing (PVC), which consists of a profusion of bacteria around blood vessels, with the bacteria being present in the media and adventitia of vessels but not usually being present in the intima (17, 19). No inflammatory response is seen in the lesion (17–19). EG often represents the hematogenous dissemination of *P. aeruginosa* from the lung, gastrointestinal tract, or urinary tract (17, 19–21). However, the same phenomenon has been observed in burn wounds, where PVC precedes translocation into the bloodstream (17, 21). We previously observed a strong association between PVC and *P. aeruginosa* sepsis in the murine model of thermal injury (22). Therefore, we assessed the skin of thermally injured/infected mice for PVC as an indication of the ability of PA14 to translocate from the infected wound into the bloodstream. Mice were thermally injured and infected with either PA14 or PA14::hepP

FIG 3 Mutation of *hepP* reduced the local spread and eliminated the systemic spread of *P. aeruginosa* PA14. Mice were thermally injured and infected with either PA14 or PA14::*hepP* by inoculation of ~200 CFU under the burn. At 24 (A), 48 (B), 72 (C), or 96 (D) h postinfection, the mice were euthanized and the skin samples, livers, and spleens were obtained. The tissues were weighed, homogenized in PBS, serially diluted 10-fold, and plated to determine the number of CFU per gram of tissue. Local spread within the burn wound was indicated by recovery of the strain from a site 15 mm away from the inoculation site (the distant site). Systemic spread was determined by recovery of the strain from the liver and spleen. Values represent the means from 3 independent experiments $\pm$ SEMs. *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, no significant difference.

as described above. At 24 h postinfection, the mice were euthanized and 15-mm sections of skin that included the margins of the burn, where the burned and healthy tissues merged, were collected. Hematoxylin and eosin (H&E) staining of the tissues revealed the presence of distinct PVC in skin sections obtained from mice infected with both PA14 and PA14::*hepP* (Fig. 4). The presence of PVC was associated with thrombosis within the blood vessels (Fig. 4), as we had previously demonstrated (22). Thus, although we detected no systemic spread in mice infected with PA14::*hepP*, we still detected PVC within the wound. Its ability to grow within the infected wound and surround the blood vessels suggests that PA14::*hepP* may not be defective in its ability to translocate into the bloodstream. Rather, it may be defective in its ability to survive within the bloodstream.

We have already excluded the possibility that the avirulent phenotype of PA14::*hepP* is due to its failure to grow in blood. To determine the influence of *hepP* on the expression of PA14 genes, we grew PA14 and PA14::*hepP* in whole blood and conducted transcriptome analysis (N. Dzvova, J. A. Colmer-Hamood, S. Dissanaik, J. A. Griswold, and A. N. Hamood, unpublished results). Both strains grew to comparable levels (data not shown).

Heparinase HepP is essential for the survival of PA14 within the blood of thermally injured mice. It is possible that PA14::*hepP* translocates into blood vessels but fails to replicate within the bloodstream and cause sepsis. To examine this possibility, we modified our model of thermal injury to bypass the translocation step. In the modified model, the mice are prepared and thermally injured to produce the same immunocompromising conditions described above. However, instead of injecting

FIG 4 PA14::*hepP* is not defective in perivascular cuffing. Sections of the injured/infected skin tissues were stained with hematoxylin and eosin and examined for the blue haze surrounding the blood vessels that denotes perivascular cuffing. The areas of PVC are demarcated by yellow outlines. Thrombosis is visible in the cross sections of the blood vessels. Skin tissue from thermally injured mice infected with PA14 (A) or PA14::*hepP* (B) is shown. Magnifications, $\times 40$.

the bacteria within the injured skin, they are injected intraperitoneally, thereby bypassing the step of bacterial translocation from the infected wound to the bloodstream. In this regard, the modified model resembles the established model of murine sepsis (23). To establish the model, we conducted preliminary experiments in which mice were injured and infected only with PA14. As a control, shaved but uninjured mice received an intraperitoneal injection of PA14. The animals were monitored daily for up to 4 days postinfection, at which time only 33% of the injured/infected mice had survived (Fig. 5A). However, we observed 100% survival among the uninjured but infected mice (Fig. 5A).

FIG 5 *PA14::hepP* failed to disseminate from the peritoneum of compromised mice. Mice in groups of 5 were shaved and thermally injured (compromised) or shaved and left uninjured (noncompromised) and infected with either *PA14* (A) or *PA14::hepP* (B) by inoculation of 200 to 300 CFU into the peritoneal cavity. The mice were monitored daily for 4 days for mortality. Comparison of the survival curves in panel A using the log-rank (Mantel-Cox) test produced a *P* value of 0.0190. (C) At 24 h after thermal injury and infection, the mice were euthanized and the livers and spleens were recovered. The organs were weighed, homogenized in PBS, serially diluted 10-fold, and plated to determine the number of CFU per gram of tissue. Values represent the means from 3 independent experiments $\pm$ SEMs. **, *P* < 0.01; ****, *P* < 0.0001.

Once the modified model was established, we utilized it to assess the ability of *PA14::hepP* to cause sepsis. Uninjured or thermally injured mice were intraperitoneally infected with *PA14::hepP*. By 4 days postinfection, we observed no mortality (100% survival rate) among both the control (uninjured) mice and the thermally injured mice (Fig. 5B). To further investigate the survival of *PA14* and *PA14::hepP* within the thermally injured mice in the modified model, we examined the livers and spleens for microorganisms. We recovered *PA14* from both organs but did not recover *PA14::hepP* from either organ (Fig. 5C). These results strongly suggest that, in contrast to *PA14*, *PA14::hepP* is defective in its ability to systemically spread and survive within the body of the thermally injured mouse.

Mutation of *hepP* compromises the serum resistance of *PA14*. One possible mechanism for the failure of *PA14::hepP* to survive in the bloodstream (as indicated by the lack of systemic spread within the thermally injured mice) is that the mutant is compromised in its serum resistance. We examined this possibility using a serum sensitivity assay (24). Aliquots of an overnight culture of *PA14* or *PA14::hepP* were incubated in either 50% pooled normal human serum (50% NHS) or medium without serum (0% NHS). After 2 h of incubation at 37°C, the cultures were serially diluted and plated, and the numbers of CFU per milliliter were determined. *PA14* was serum resistant (>100% survival after 2 h of exposure to 50% NHS) (Fig. 6). In contrast, no CFU

FIG 6 Mutation of *hepP* compromised the serum resistance of PA14. Aliquots of overnight cultures of PA14, PA14::*hepP*, PA14::66100, or PA14::wzz diluted 1:10,000 in M9 minimal medium (M9MM) were incubated in equal volumes of 50% pooled normal human serum (50% NHS) or M9MM (0% NHS) for 2 h at 37°C. The cultures were serially diluted and plated to determine the number of CFU per milliliter. The serum resistance of each strain was calculated as the cell survival after exposure to 50% serum for 2 h at 37°C, given as a percentage of the number of CFU of the strain at time zero. PA14::66100 and PA14::wzz, which are defective in O-antigen/LPS biosynthesis, were used as controls, as similar mutants in strain PAO1 are known to be serum sensitive. An aliquot of PA14::*hepP* was incubated with heat-inactivated (56°C for 30 min) normal human serum (HI-NHS) to confirm that the serum sensitivity of PA14::*hepP* was due to complement activity. Values represent the means from 2 independent experiments $\pm$ SEMs. *, $P < 0.05$; ***, $P < 0.001$; nd, not done.

of PA14::*hepP* was recovered (Fig. 6). However, the mutant was resistant to 50% heat-inactivated normal human serum (50% HI-NHS) (Fig. 6). These results suggest that PA14::*hepP* was no longer resistant to the complement-mediated inhibitory effect of serum.

P. aeruginosa strains with mutations in the genes for lipopolysaccharide (LPS) or O-antigen biosynthesis are known to be serum sensitive. Therefore, we examined two PA14 mutants, PA14::66100 and PA14::wzz, which are defective in O-antigen/LPS biosynthesis (Table 1) (15). In PA14::66100, the MAR2xT7 transposon is inserted at nucleotide 136 in PA14_66100, a gene that codes for a hypothetical membrane protein involved in O-antigen biosynthesis/O-antigen ligase activity (15, 25). In PA14::wzz, the transposon is inserted at nucleotide 113 in PA14_23360 (*wzz*), a gene that codes for a hypothetical membrane protein involved in lipopolysaccharide biosynthesis (15, 25).

Compared with the percent survival of their parent strain, PA14, the percent survival of PA14::66100 and PA14::wzz after exposure to 50% NHS was 16% and 25%, respectively (Fig. 6). Analysis of PA14::66100 and PA14::wzz in the murine model of thermal injury suggested that the serum-sensitive phenotype does not necessarily reflect its *in vivo* virulence phenotype during infection of thermally injured wounds. The number of CFU obtained from the livers and spleens of injured mice infected with either mutant paralleled that obtained from the livers and spleens of mice infected with PA14 (Fig. 7). In contrast, no CFU of PA14::*hepP* was obtained from the livers or spleens of infected mice (Fig. 3C and D).

DISCUSSION

Our results clearly show that *hepP* is essential for the PA14-induced sepsis in the murine model of thermal injury. While the loss of the gene had no effect on the growth of PA14 in the wound, it prevented the growth of PA14 within the liver and spleen of the infected mouse (Fig. 3). Additionally, serum sensitivity assays clearly showed that the PA14::*hepP* mutant is serum sensitive (Fig. 6). On the basis of these *in vivo* and *in vitro* study findings, a likely function for the HepP heparinase is that it protects PA14 from complement-mediated lysis, although direct experimental evidence confirming that the defect in the *in vivo* virulence of PA14::*hepP* is due to its serum sensitivity is currently lacking. LPS contributes significantly to the serum resistance phenotype in *P. aeruginosa*. Previous studies demonstrated that *P. aeruginosa* mutants with altered LPS structures were both serum sensitive and defective in their *in vivo* virulence (26–28). Using the murine model of thermal injury, Cryz et al. (29) demonstrated that, compared

FIG 7 Neither PA14::66100 nor PA14::wzz is defective in its systemic spread within the bodies of thermally injured/infected mice. Mice were thermally injured and infected with either strain by inoculation of ~200 CFU under the burn. At 24 h postinfection, the mice were euthanized and the livers and spleens were obtained. The tissues were weighed, homogenized in PBS, serially diluted 10-fold, and plated to determine the number of CFU per gram of tissue. Values represent the means from 3 independent experiments $\pm$ SEMs. No significance differences were observed.

with its parent strain, a serum-sensitive *P. aeruginosa* mutant was nonvirulent. The mutant failed to spread systemically and establish an infection within the burned skin (the bacteria did not grow within the injured/infected skin) (29). We considered such a possibility in our current study. However, available results suggest that the defect in the *in vivo* virulence of PA14::hepP is not likely to be due to defects in its LPS. In contrast to the phenotype of the LPS mutant described by Cryz et al. (29), PA14::hepP was not defective in its ability to spread within the thermally injured skin. At 24, 48, 72, and 96 h postinfection, the number of CFU per gram of tissue of the infected skin was high (about 10⁷) (Fig. 3). Additionally, we tested two PA14 LPS/O-antigen biosynthesis mutants (PA14::66100 and PA14::wzz) (24) and found their phenotype to be different from that of PA14::hepP. Both mutants were serum sensitive, but they were not as serum sensitive as PA14::hepP (Fig. 6). However, PA14::66100 and PA14::wzz were able to translocate from the infected wound and spread within the bodies of infected mice. At 24 h postinfection, the numbers of CFU per gram within the livers and spleens of mice infected with either mutant paralleled that within the livers and spleens of mice infected with PA14 (Fig. 7). Thus, serum resistance may still contribute to the *in vivo* virulence of PA14 in the murine model but through an LPS-independent mechanism. PA14 may produce a factor(s) that interferes with the complement-mediated antimicrobial effect. Such an effect would resemble the effect of *P. aeruginosa* LasB (elastase), which inactivates and degrades serum complement factors (30–32). Hong and Ghebrehiwet (30) showed that degradation of the key recognition molecules of complement, C1q and C3, by LasB enhanced the virulence of *P. aeruginosa* by abolishing complement-mediated killing.

Heparinase may interfere with the role of heparin sulfate proteoglycans (HSPGs) in host innate immunity. Although many bacterial pathogens utilize HSPGs in their initial colonization and attachment to, invasion of, and internalization by their target cells within the host (33–38), degradation of the HSPGs may contribute to the virulence of certain bacterial pathogens. We clearly demonstrated that hepP plays an essential role in the pathogenesis of PA14 during sepsis in the murine model of thermal injury. One possible scenario is interference with the suggested role that HS from neutrophils and endothelial cells plays in modulating innate immunity. Inactivation of the HS uronyl 2-O-sulfotransferase in neutrophils substantially reduced their bactericidal activity (39). A deficiency in this enzyme enhanced the susceptibility of mice to systemic infection with the pathogenic bacterium group B *Streptococcus* (39). Thus, by cleaving HS from HSPGs and compromising this aspect of innate immunity, HepP may enhance the virulence of PA14 during sepsis.

Alternatively, HepP may manipulate the host innate immune response by interfering either with the interaction of HS with neutrophil bactericidal effectors (such as elastase,

cathepsin G, and defensins) or with the HSPG binding of the cytokines and chemokines that are released during the inflammatory processes. Such interactions are important for the rapid recruitment of neutrophils to the site of infection (40, 41). Besides killing microbes via the production of reactive oxygen species and the release of antimicrobial peptides and mediators, neutrophils may expel their nuclear contents to form neutrophil extracellular traps that help control microbial infections by binding and trapping pathogens (39). Additionally, the DNA from the neutrophils, the neutrophil extracellular traps, contain extracellular fibers, granule proteins, and HSPGs (39, 42). Enzymes such as heparinases may modulate the immune response by regulating the release of HSPG-bound chemokines, growth factors, and cytokines, consequently affecting immune cells indirectly.

Another potential mechanism through which heparinase may compromise the host innate resistance and affect sepsis is via the degradation of the endothelial surface layer (ESL). The ESL is a carbohydrate-rich layer lining the vascular endothelium on the luminal side that contains HSPGs, such as heparin and HS. Previous studies demonstrated that sepsis-induced acute lung injury is initiated by degradation of the pulmonary ESL by activated heparinase degradation of HS in the ESL. The ESL loss exposes neutrophil adhesion sites, resulting in neutrophil influx, leading to inflammatory lung injury (43). Therefore, *P. aeruginosa* can cause ESL loss in two ways. First, the mere presence of *P. aeruginosa* in the host can trigger endotoxemia and the activation of mammalian heparinase. Second, the *P. aeruginosa* heparinase, together with the activated host heparinase, may degrade HS in the ESL with subsequent acute lung injury.

Finally, HepP heparinase may affect the innate immunity-related mechanism that involves the serine protease inhibitor heparin cofactor II (HCII) (44). Upon its interaction with cell surface glycosaminoglycans, the inert HCII undergoes conformational changes that activate it and induce novel biological functions, including endotoxin binding and antimicrobial properties (45, 46). Peptide epitope mapping localized the antimicrobial properties of HCII to helices A and D (45, 47). *In vivo* analysis revealed that HCII-deficient mice were more susceptible to *P. aeruginosa* infection than their wild-type counterparts, while challenge of wild-type mice with *P. aeruginosa* reduced the level of HCII within their bodies (45). Using a murine model of *P. aeruginosa* sepsis, Papareddy et al. (47) demonstrated that intraperitoneal injection of mice with synthetic NFL20, a peptide that corresponds to the D helix of HCII, significantly enhanced their survival. Additionally, the number of *P. aeruginosa* bacteria recovered from the liver, kidney, and spleen of NFL20-treated mice was significantly reduced compared with the number recovered from those organs of untreated infected mice (47). Thus, it is possible that in our model of *P. aeruginosa* sepsis, the HepP heparinase produced by PA14 during bacteremia cleaves HSPGs and prevents HCII activation. In the absence of HCII antimicrobial activity, PA14 disseminates to the liver and spleen, resulting in a high mortality rate (Fig. 1). Conversely, the lack of heparinase activity in mice infected with PA14::hepP allowed the efficient elimination of the bacteria through the uncompromised HCII activity.

Although the evidence suggests that PA14::hepP is defective in its ability to survive after translocation from the wound, we cannot rule out a possible defect in the translocation from the wound into the bloodstream. One mechanism through which such an effect may be accomplished is through the effect of HepP on syndecan shedding. Syndecans (syndecan-1 through syndecan-4) constitute a family of HSPGs that play different roles in the inflammatory process. They are released into the host cell environment as intact soluble proteoglycan ectodomains through a proteolytic process called syndecan shedding (48). Although the level of syndecan-1 that is shed in the biological fluids of healthy individuals is low, the level is increased in human dermal wound fluid (49). Using the murine model of thermal injury, we previously demonstrated that shedding of syndecan-1 plays a role in *P. aeruginosa* sepsis (50). The mortality rate among syndecan-1 knockout mice infected with *P. aeruginosa* was significantly lower than that among their wild-type counterparts (50). Furthermore,

while PAO1 grew within the injured/infected skin of the syndecan-1 knockout mice, no microorganisms were detected within the livers (50). However, when the syndecan-1 knockout mice were treated with HS, which compensates for the lack of syndecan shedding, we detected bacteria within the livers (50). Typical perivascular cuffing was observed in skin sections of infected wild-type mice but not in skin sections of infected syndecan-1 knockout mice (50). This led us to suggest that syndecan-1 shedding is important in the translocation of *P. aeruginosa* from the infected/injured skin into the bloodstream (50).

Comparison of the results of our current study with those of the syndecan-1 study suggests that, depending on the stage of infection, *P. aeruginosa* utilizes either the host-released HS or its own heparinase. Similar to the syndecan-1 knockout mice infected with PAO1, the wild-type mice infected with PA14::hepP showed significantly reduced mortality (Fig. 1) and failure of the mutant to spread systemically (Fig. 3). In contrast to the syndecan-1 knockout mice, perivascular cuffing occurred in the skin of the wild-type mice infected with PA14::hepP (Fig. 4) (50). Thus, syndecan-1 shedding may play a critical role in the translocation of *P. aeruginosa* from the infected wound into the bloodstream, while HepP heparinase is essential for the survival of *P. aeruginosa*, once the organism reaches the bloodstream. To confirm this possibility, we plan to conduct studies using wild-type mice, syndecan-1 knockout mice, wild-type PA14, and the PA14::hepP heparinase mutant.

MATERIALS AND METHODS

Strains and general growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* strain UCBPP_PA14 (PA14), originally isolated from an infected burn wound, or its specific transposon mutants that carry the Mariner transposon MAR2xT7 within the structural genes of interest were used in all the experiments (15, 51). The strains were obtained from the PA14 transposon insertion mutant library (<http://pa14.mgh.harvard.edu/cgi-bin/pa14/home.cgi>; accessed 16 October 2017). For routine growth, strains were grown at 37°C in Luria-Bertani (LB) broth. Antibiotics were used at the following concentrations: for PA14 mutants, 60 µg gentamicin/ml; to maintain plasmids in the PA14 mutants, 300 µg carbenicillin/ml. For the serum sensitivity experiments, the tested strains were grown in tryptic soy broth (TSB; Remel, Lenexa, KS) and on tryptic soy agar (TSA) (52). The cultures were suspended and diluted in M9 minimal medium (M9MM) (53).

Preparation of bacterial inoculum. A 100-µl aliquot of an overnight culture of either *P. aeruginosa* strain PA14 or *P. aeruginosa* strain PA14::hepP was subcultured into fresh LB broth. The cultures were grown at 37°C for 3 h to an optical density at 600 nm (OD₆₀₀) of 0.5. A 100-µl aliquot of culture was serially diluted 10-fold to yield an infective dose of 200 to 300 CFU in 100 µl. This dose has previously been shown to produce 94 to 100% lethality in Swiss Webster mice by 2 days after thermal injury and infection with *P. aeruginosa* (54).

Murine model of thermal injury. Adult female Swiss Webster mice (The Jackson Laboratory, Sacramento, CA) weighing between 22 and 24 g were utilized in the murine model of thermal injury (16). Briefly, mice were anesthetized by intraperitoneal injection of 0.44 to 0.48 ml of 5% sodium pentobarbital (Nembutal; Abbott Laboratories, North Chicago, IL) at 5 mg/ml, and their backs were shaved. The mice were placed in a template that exposes approximately 15% of the total body surface area. Thermal injury was induced by placing the exposed surface in 90°C water for 8 s. Such an injury is nonlethal but results in a third-degree burn. Immediately following the injury, the mice were given 0.5 ml of a 0.9% NaCl solution as fluid replacement therapy, and 100 µl of a bacterial inoculum containing approximately 200 CFU was injected directly under the injured skin. The mice were immediately placed on a warming mat for recovery. Mortality was recorded every day after thermal injury-infection for up to 7 days. Animals were treated humanely and in accordance with the protocol approved by the Animal Care and Use Committee at the Texas Tech University Health Sciences Center (TTUHSC), Lubbock, TX.

To bypass the process of translocation of the bacteria from the infected wound to the bloodstream, we developed a modified intraperitoneal infection model. Mice were thermally injured and received fluid replacement therapy as described above. However, the bacterial inoculum (200 to 300 CFU, which was the same as that described above) was delivered by intraperitoneal injection rather than under the injured skin. Mice were immediately placed on a warming mat for recovery.

Quantification of bacterial load within the skin, livers, and spleens of infected animals. At specified time points after thermal injury and infection (24, 48, 72, or 96 h), the mice were euthanized with CO₂ in accordance with the protocol approved by the Animal Care and Use Committee at TTUHSC. Mice infected with PA14 were carefully watched for signs of morbidity so that the organs could be collected prior to their death. Skin sections (approximately 5 mm by 5 mm) were excised from the inoculation site and from a site 15 mm away from the inoculation site (the distant site) of the infected mice. The livers and spleens of the animals were also recovered. The individual skin samples or organs were weighed, suspended in 2 ml of PBS, and homogenized (Precellys24 tissue homogenizer; Bertin Instruments, Rockville, MD). A 100-µl aliquot of the homogenate was serially diluted (10-fold dilutions) in PBS, and a 10-µl aliquot of each dilution was plated on *Pseudomonas* isolation agar plates (Criterion;

Hardy Diagnostics, Santa Monica, CA) to determine the number of bacteria present. The number of CFU per gram of tissue from each sample was calculated. The bacterial load in the distant site indicated local spread, while the bacterial load in the liver and spleen indicated systemic spread.

Sample preparation and histopathologic analysis. For microscopic analysis, samples were taken from the underlining of the burned mouse tissue using a scalpel. Specimens were sent to the TTUHSC Department of Pathology (Lubbock, TX) for paraffin embedding and sectioning. Tissue sections on glass slides were deparaffinized in three changes of xylene and rehydrated by washing in 100% and 95% ethanol. The tissues were then rinsed with water and then stained with hematoxylin and eosin. Stained tissue sections were dehydrated by dipping them in 95% ethanol, 100% ethanol, and xylene. Coverslips were placed on the slides using Permount mounting medium (Thermo Fisher Scientific, Waltham, MA), and light microscopy was used to visualize perivascular cuffing (Nikon Eclipse 80i; Nikon, Melville, NY).

Serum sensitivity assay. The serum sensitivity assay was done as previously described (24). The tested strains were grown on TSA plates. The massed growth was scraped off the plates and suspended in TSB to an OD₆₅₀ of 0.1. The cultures were grown at 37°C to an OD₆₅₀ of 0.4. The cells were then pelleted, resuspended in an equal volume of M9MM, and diluted 1:10,000 in M9MM. An aliquot (100 µl) of each dilution was mixed with 100 µl of fresh pooled normal human serum (50% NHS; Fisher BioReagents, Thermo Fisher Scientific, Pittsburgh, PA), and the mixture was incubated at 37°C for 2 h. A 100-µl aliquot of each mixture was serially diluted (10-fold), and 10 µl of each dilution was spotted on TSA plates to determine the number of CFU. Serum resistance was calculated as the cell survival after exposure to 50% serum for 2 h at 37°C, given as a percentage of the number of CFU at time zero. To confirm that the serum sensitivity of PA14::hepP was due to complement activity, an aliquot of the strain was incubated with heat-inactivated (56°C for 30 min) normal human serum (HI-NHS).

Statistical analyses. Statistical analyses of the results were done using GraphPad Prism (version 7.0) software (GraphPad Software, La Jolla, CA).

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We report no conflicts of interest.

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