Serine Protease PKF of *Acinetobacter baumannii* Results in Serum Resistance and Suppression of Biofilm Formation

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*Acinetobacter baumannii* is an important nosocomial pathogen. Infections are often preceded by intubation or catheter use, promoting the formation of biofilm, and some strains are able to cause severe cases of bacteremia because of their ability to resist killing by complement. We identified a secreted serine protease, termed “PKF,” that provided resistance to complement killing and suppressed biofilm formation. Serum resistance was abrogated in *A. baumannii* treated with protease inhibitors, as well as in a PKF-negative mutant. Serum resistance could be restored by recombinant PKF, which was shown to reduce the complement activity of normal human serum by almost 50%. PKF was shown to inhibit biofilm formation, because the PKF-negative mutant and wild-type *A. baumannii* treated with protease inhibitors produced biofilm that could be inhibited by addition of recombinant PKF. Our data indicate that PKF is required for serum resistance and that it suppresses biofilm formation in *A. baumannii*.

Keywords. *Acinetobacter baumannii*; complement; serum resistance; biofilm; innate immunity.

*Acinetobacter baumannii* is emerging as an important pathogen within the nosocomial setting. Although *A. baumannii* is often found in environmental samples, it can also be isolated from healthy individuals’ skin and other body sites [1]. This gram-negative, nonfermentative coccobacillus has relatively low virulence, most commonly infecting immunocompromised patients or those already hospitalized with another condition [2]. Common pathologies include urinary tract infections, pneumonia, bacteremia, or skin and soft tissue infections [3]. Epidemics are frequent in the hospital setting, with *A. baumannii* being the fifth most common pathogen in intensive care units, and mortality can be high because of the pathogen’s resistance to most commonly prescribed antibiotics [4, 5]. Some of this resistance is due to an 86-kb genomic resistance island that contains resistance genes imported from *Salmonella, Escherichia coli*, and *Pseudomonas*. This island is the largest of its kind identified to date [6, 7]. Although drug resistance remains one of the most prevalent topics when discussing the pathogenesis of *A. baumannii* [8], the other virulence factors of this pathogen are not well understood. Some isolates are able to evade the innate immune system by resisting the killing action of normal human serum [9]. This is due in part to the presence of a thick polysaccharide capsule that prevents deposition of complement [10]. *A. baumannii* has a polysaccharide capsule that contains the common surface polysaccharide beta-(1→6)-poly-N-acetyl-D-glucosamine (PNAG) seen in other pathogens, such as *E. coli, Staphylococcus aureus*, and *Yersinia pestis* [11]. PNAG could be involved in antibody-mediated immunity to this organism. A different capsular polysaccharide, K1, has also been identified that may be involved in virulence [12]. It has been
shown, however, that the capsule of *A. baumannii* does not significantly contribute to resisting killing by the innate immune system [10], suggesting that the capsule plays more of a role in adherence than it does in immune evasion.

We have previously shown that the complement-evasion strategy of binding factor H is not used by *A. baumannii* [9], and therefore another mechanism must be at work. Secreted bacterial proteins can mediate serum resistance. In particular, secreted proteases are often responsible for degrading essential complement components such as C3, or complement regulators such as properdin [13], downregulating the cascade response. Secreted proteases can also function in virulence by cleaving antibodies or breaking down host defenses and barriers [14, 15].

Some clinical isolates of *A. baumannii* are able to form substantial biofilm. This is especially important in nosocomial infections because the use of ventilators or catheters, ideal surfaces for the adherence of bacteria and production of biofilm, often precede colonization and infection [16, 17]. This complex biofilm matrix composed of polysaccharide polymers, proteins, and nucleic acids can decrease the bacteria’s susceptibility to antibiotics, making it even more formidable and difficult to treat [18].

In this study, we identified a secreted serine protease which we have termed “protease required for resistance to complement killing and suppression of biofilm formation” (hereafter, “PKF”). We show that PKF is important in serum resistance and reduces the formation of biofilm, possibly by preventing steps involved in initial attachment.

**MATERIALS AND METHODS**

**Bacterial Strains**

*A. baumannii* clinical isolates, designated LK10, LK41, and LK88, were obtained from the G. V. Sonny Montgomery Veteran’s hospital clinical laboratory and stored at −80°C in Luria-Bertani (LB) broth supplemented with 20% glycerol. These clinical isolates vary in their susceptibility to antibiotics, making it even more formidable and difficult to treat [18].

In this study, we identified a secreted serine protease which we have termed “protease required for resistance to complement killing and suppression of biofilm formation” (hereafter, “PKF”). We show that PKF is important in serum resistance and reduces the formation of biofilm, possibly by preventing steps involved in initial attachment.

**Cloning**

The gene encoding PKF from *A. baumannii* LK41 was amplified by polymerase chain reaction (PCR) using primers LSM810F (5′CTACGTGATCTTCTCATCAC3′) and LSM810R (5′TTTACGTGATCTAAACCAACAGTTG3′). It was cloned into the pET100D expression vector in which the recombinant protein was expressed with an attached N-terminal 6xHis tag in *E. coli* BL21*" and was purified over a nickel-chelated column.

A mutant of LK41 that failed to express PKF was created using the suicide vector PCR2.1topo. An interior fragment of *pkf* (LSM815F 5′ATGCAGGTTTCTCATCA3′ and LSM815R 5′TACGTGATCTACCCGCAA3′) was cloned into the vector, which was propagated in *E. coli*. The plasmid was then isolated and electroporated into LK41, creating an insertion-inactivation mutant. The mutant was confirmed by PCR and sequencing and was designated LK41.3.

**Serum Survival Assay**

As previously described, LK41 was grown to approximate mid-log phase with shaking at 37°C. The cells were washed in sterile phosphate-buffered saline (PBS) and diluted to 10⁴ cells/mL. These cells were incubated with 100 μL of a protease inhibitor cocktail (Sigma-Aldrich) for 10 minutes at room temperature with shaking. The protease inhibitor cocktail was suspended in dimethyl sulfoxide (DMSO); for a negative control, cells were incubated with 100 μL of 20% DMSO. The cells were then washed 4 times in sterile PBS and used in our previously described serum survival assay [9]. In short, the cells were incubated for 3 hours at 37°C in 40% normal human serum (NHS). At time 0 and at 3 hours, cells were serially diluted and plated to determine colony-forming units (CFU) per milliliter. As a control for lethality of protease inhibitors, heat-inactivated serum controls were treated with the cocktail.

We then determined which individual class of protease was responsible for the inhibition of serum resistance by incubating cells with individual inhibitors prior to incubation in serum. Each inhibitor present in the cocktail, 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), bestatin, pepstatin, E-64, and ethylenediaminetetraacetic acid, was tested in the concentration found in the cocktail.

Repeated efforts were made to genetically complement LK41.3. In a separate experiment, LK41.3 was used in a serum survival assay, and CFU per milliliter were compared to findings for wild-type *A. baumannii*. Recombinant PKF was added to these reactions at a concentration of 1 μg/mL to further assess the role of PKF in serum resistance.

**Alternative Complement Pathway Assay**

As previously shown [9], *A. baumannii* activates the alternative complement pathway. Activity of the alternative pathway was assayed by diluting NHS, incubating with rabbit erythrocytes, and determining the volume required to lyse 50% of those cells present (AP50). Ethylene glycol tetracetic acid was used in a concentration of 5 mM to chelate Ca²⁺ needed for the classical and lectin pathways, making the assay specific for the alternative pathway. A maximum lysis buffer (5% saponin) was used to calculate 50% lysis, and the volume of serum
correlating to that number was used to determine the AP50. To determine the effect of PKF on serum, 1 mL of NHS was incubated with 1 μg of recombinant PKF for 3 hours at 37°C and then used in the alternative pathway assay.

Biofilms
As previously described [9], overnight cultures were used to inoculate 3 mL of LB broth, and the culture was grown to approximately 10^7 cells/mL. In triplicate, 50 μL of this culture was used to inoculate 50 μL of fresh LB broth in the wells of a sterile 96-well polystyrene microtiter plate. In some experiments, recombinant protease was added to this reaction, and the volumes were adjusted to 150 μL with PBS. In other experiments, protease inhibitor AEBSF was added in varying concentrations. Plates were incubated at 37°C overnight. Plates were then washed 4 times by immersion in ddH2O, and 100 μL of 0.1% crystal violet was added to each well at room temperature for 30 minutes. Plates were washed 4 times with ddH2O, 200 μL of 95% EtOH was added to each well, and 125 μL of the solution was transferred to a fresh microtiter plate. Absorbance was measured at 540 nm. For a more qualitative analysis, bacteria were grown in the well of a culture slide in 100 μL of LB broth for 15 hours. This was then washed, fixed, stained, and observed under a light microscope.

To determine whether PKF could dissociate biofilms that were already formed, recombinant PKF was added 15 hours after inoculation and the plates were incubated at 37°C for 6 hours before washing and staining.

Statistical Analysis
All experiments were performed a minimum of 3 times independently. Statistical analyses were performed using INSTAT (Graphpad Software, San Diego, CA). Comparisons between groups were performed using the Mann–Whitney 2-sample rank test. P values of <.05 were considered statistically significant.

RESULTS
Secreted Serine Proteases Affect Serum Resistance
The focus of this study was a putative secreted serine protease of A. baumannii. This putative protease was detected in the culture supernatant of A. baumannii LK41 using mass spectrometry (data not shown; GenBank accession no. JF729315). As seen in Figure 1, this protease has several conserved domains and has homology to proteins in the HtrA-like family, specifically a mucD precursor found in Pseudomonas aeruginosa. However, PKF of A. baumannii is secreted into the culture supernatant, while the mucD precursor in P. aeruginosa is a cytoplasmic factor. Additionally, the nucleotide sequence that encodes PKF is present in many of the searchable A. baumannii genomes.

To investigate secreted proteases of A. baumannii, protease inhibitors were used as a treatment on LK41 in combination and individually. LK41 is a serum-resistant strain of A. baumannii and normally has almost complete survival following a 3-hour incubation in NHS. When treated with a protease inhibitor cocktail containing several different classes of protease inhibitors, though, survival in 40% NHS was reduced to <20% (Figure 2A). When individual classes of protease inhibitors were used as a treatment before incubation with serum, only the serine protease inhibitor AEBSF had a significant effect on survival, inhibiting survival almost to the level achieved with the cocktail (Figure 2B). This suggests that a secreted serine protease performs some function in serum resistance, possibly by cleaving complement components or regulators.

Serine Protease PKF Contributes to Serum Resistance
The gene encoding PKF was cloned from LK41 and the recombinant protein was expressed in E. coli. A recombinant protein of approximately 50 kDa was produced. An inactivation mutant that did not express PKF was also created in LK41 using insertional mutagenesis to disrupt the gene. The PKF-negative LK41.3 was used in a serum survival assay and...
survival was reduced significantly, indicating that this protein is involved in serum resistance (Figure 3A). We attempted to genetically complement LK41.3 by electroporation of an E. coli–A. baumannii shuttle plasmid containing the entire coding region of pkf. While this plasmid could be electroporated into LK41 and E. coli, we were not successful in establishing the plasmid in LK41.3. Since genetic complementation was not successful, we investigated the role of PKF in serum resistance using exogenously added recombinant PKF (rPKF). As seen in Figure 3B, when rPKF (1 μg/mL) was added to this assay, serum resistance was restored.

To assess the affect of rPKF on complement, we used a previously described hemolytic assay [19, 20] to examine complement activity following exposure to rPKF. The addition of 1 μg/mL of rPKF reduced the complement activity of NHS by almost 50% after a 3-hour incubation (Figure 4). This is the maximum level of inhibition, because adding more recombinant PKF in a subsequent incubation failed to decrease the activity further (data not shown).

**PKF Suppresses Biofilm Formation**

Biofilm formation is a significant factor in A. baumannii infections. LK41 has been previously shown to produce very low levels of biofilm [9], but when LK41.3 was used in a biofilm assay, LK41.3 produced significantly higher levels of biofilm as compared to wild-type A. baumannii (Figure 5A). In fact, LK41.3 produced more biofilm than LK88, which we had previously shown to be one of our higher biofilm-producing strains.
strains. Light microscopy (Figure 5D) confirmed a lack of biofilm formation by LK41, compared with LK41.3, after 15 hours growth on a plastic slide. This biofilm production could be inhibited in a dose-dependent manner by addition of the rPKF to the reaction (Figure 5B). When rPKF was added after the biofilm had completely formed at 15 hours, however, it did not disperse the biofilm (Figure 5C). The addition of serine protease inhibitor AEBSF was shown to induce biofilm production in wild-type A. baumannii, confirming that the presence of PKF acts to either inhibit or dissociate biofilm in the initial steps (Figure 6).

**DISCUSSION**

There are many mechanisms of serum resistance in addition to the binding of fluid-phase complement regulators, such as factor H. Some of these include destroying complement by degrading key factors in the cascade, such as C3 and C5, or regulatory factors, like properdin. Many organisms, including...
Streptococcus pyogenes, Salmonella enterica, and Enterococcus faecalis, use this mechanism and degrade C3, properdin, and C5a, C3, C4, and C5, and C3, respectively [13]. This can lead to inflammation due to cleavage products acting as chemotactants, but often the complement system is inactivated completely [13]. We demonstrated that a serine protease inhibitor could abolish A. baumannii serum resistance. An A. baumannii mutant that did not express PKF was unable to survive incubation in serum, unlike A. baumannii wild-type LK41, which had nearly 100% survival after 3 hours in serum. The serum resistance of the mutant could be restored by the addition of exogenous rPKF. This suggests that PKF is important in A. baumannii serum resistance, perhaps by the cleavage of complement components. Because the activity of NHS was only reduced by 50%, this suggests that a secondary protein such as properdin may be inhibited. By affecting properdin, which is responsible for stabilization of the C3 convertase, PKF could be indirectly inhibiting C3, an essential component of all 3 complement cascades, leading to the dissociation of the cascade and subsequent survival of the bacterium. It is also important to note that A. baumannii shows pathology most often in patients that are severely immunocompromised, so a 50% inactivation of the complement immune defense could allow infection to proceed.

Another important factor for A. baumannii is the production of biofilm. Many colonizations and subsequent infections are preceded by intubation or catheter use where bacteria are able to form substantial biofilms. These biofilms shield bacteria from both host defenses and antibiotics, a possible contributing factor in the difficulty involved in treatment of these organisms. Biofilms are also important in wound infections caused by A. baumannii, resulting in delayed healing and recurrent infection [21]. As in serum resistance, proteases often play an important role in biofilm formation. Proteases can be responsible for the establishment of biofilms, as in Enterococcus faecalis, for which a gelatinase serves to cleave substrates involved in cell-surface adhesion, facilitating biofilm formation [22]. Secreted proteases have also been shown to have the opposite effect, as in some Staphylococcus species, for which a protease from Staphylococcus epidermidis is known to inhibit formation of and destroy S. aureus biofilms [23]. We demonstrated that PKF inhibited biofilm formation by A. baumannii.

We hypothesize that PKF may be inhibiting biofilm formation in the primary stages of attachment or dispersing it before a complex matrix is formed. In these first stages of attachment, the biofilm structure begins to form largely by cell-to-cell adhesion as bacteria pile atop one another while one layer is attached to a surface [24]. Proteases are often involved in dispersal of bacteria from a biofilm [25], and PKF may be cleaving proteins involved in cell-to-cell adhesion, thus preventing the complex structure from forming. By producing a protein that controls the formation of biofilm, A. baumannii may be able to regulate its behavior in a host by forming biofilm when conditions call for it or by producing PKF to inhibit biofilm formation when it would be advantageous to disperse to different sites. Since PKF prevents biofilm formation but seems to lack the ability to disperse biofilms that are already fully formed, this suggests that PKF plays a role in the initial stages of biofilm formation.

Proteases are often involved in regulation of bacterial processes. They are able to transmit signals, activate or inactivate cascades, or control how proteins or cells react with one another [26–29]. PKF appears to protect A. baumannii from the innate immune system via serum resistance and to control biofilm formation. By further examining this protein, its specific effects, and its regulation, we may be able to further elucidate its complex role in pathogenesis of this organism.

Notes

Acknowledgment. We thank Dr Mary Marquart for experimentation and editorial advisement.

Financial support. This work was supported by the University of Mississippi Medical Center and the University of Texas Health Science Center at Tyler.

Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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