Host protease activity on bacterial pathogens promotes complement- and antibiotic-directed killing

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Running title: Protease promotes complement-mediated bacteria-killing

ABSTRACT

Our understanding of how the host immune system thwarts bacterial evasive mechanisms remains incomplete. Here, we show that host protease neutrophil elastase acts on *Acinetobacter baumannii* and *Pseudomonas aeruginosa* to destroy factors that prevent serum-associated, complement-directed killing. The protease activity also enhances bacterial susceptibility to antibiotics in sera. These findings implicate a new paradigm where host protease activity on bacteria acts synergistically with the host complement system and antibiotics to defeat bacterial pathogens.

Keywords: Host immune system, Protease neutrophil elastase, Bacterial pathogens, Multi-drug resistant bacteria, Host complement system, Antibiotics

The human immune system deploys distinct innate immune mechanisms to thwart bacterial pathogens. These mechanisms include the host complement system and host proteases that are present at sites of bacterial infection. The complement system, a network of proteins in sera that are activated by microbial patterns, provides a first line of immune defense (1). Activation results in the deposition of complement proteins on bacterial surfaces, thereby labeling bacteria for phagocytic uptake and subsequent killing. In addition, complement deposition on bacteria drives the formation of the membrane attack complex on bacterial surfaces, which kills Gram-negative bacteria via pore formation. Host proteases, released by immune cells, also contribute to bacterial killing by compromising the integrity of bacterial cell walls (2). In addition, these proteases can destroy virulence factors and thereby thwart bacterial pathogenesis (3). To survive within the human host, bacteria have evolved systems to circumvent, subvert or evade these innate immune defense mechanisms (4). However, the ways in which the host immune system can overcome these immune-

evasive bacterial factors constitute a gap in our understanding. Here, we demonstrate that host protease activity on bacterial cell surfaces can destroy bacterial-associated complement inhibitory activities, thereby rendering resistant bacteria susceptible to complement-directed killing and sensitive to frontline antibiotics. Our studies featured the use of three clinically or agriculturally significant bacterial species: *Pseudomonas aeruginosa* (Pa), *Acinetobacter baumannii* (Ab) and *Brucella melitensis* (Bm). Pa and Ab are opportunistic bacterial pathogens that constitute significant threats to civilian and warfighter personnel, as well as patients with underlying disease, including cystic fibrosis (5, 6). Importantly, a significant proportion of clinical isolates of these pathogens display resistance to killing by normal human serum (HS) (7). Moreover, we analyzed a vaccine strain of Bm, the world's most prevalent bacterial zoonotic agent that displays complement resistance (8).

To test the hypothesis that protease activity on bacteria confers enhanced sensitivity to complement killing in HS, we used a checkerboard strategy (9) to assess synergistic interactions. Briefly, we co-incubated the above-mentioned bacterial strains at 1×10^7 CFU/mL in the presence of various concentrations (0 ~ 20%) of pooled human complement serum (HS, Innovative Research, ICSER100) and neutrophil elastase (NE, EMD Millipore, 324681) (0 ~ 0.3 U/mL) at 37°C. We then determined bacterial growth by measuring the OD_{600} of the culture using a plate reader (BioTek, Inc., VT, USA) at 16 (Pa and Ab) or 72 (Bm) hr post-inoculation (h.p.i.) to assess the inhibitory and/or synergistic activity of HS and NE on the survival or growth of the tested bacteria. Pa strain PAO1 displayed reduced turbidity in 7.5% HS and did not grow when treated with 0.05 U/mL of NE (**Fig. S1A, B**). In 5% HS and 0.1 U/mL of NE, the strain displayed poor growth; however, the turbidity of the cultures was significantly reduced by 0.3 U/mL of NE (**Fig. 1A; Fig. S1A, B**). Pa strain PA14 was weakly resistant to 2.5% HS and growth of this strain was strongly inhibited when treated with greater than 0.1 U/mL NE at this concentration of HS (**Fig. S1A, C**). To explore the hypothesis that NE targeting

of protease-labile components on bacterial cell surfaces promoted complement directed killing, we performed similar experiments using PAO1 strains that harbored mutations in the *Ecotin*, *Wzz* or *AprI* genes. *Ecotin* and *Wzz* contribute to complement resistance (10, 11). AprI is an inhibitor of AprA that protects PAO1 from complement killing. We found that the strains harboring deletions in *Ecotin* and *Wzz* were sensitive to 5% HS (**Fig. 1B-C**), indicating that both *Ecotin* and *Wzz* genes were required for Pa resistance to complement-directed killing. In addition, the PAO1Δ*AprI* mutant displayed enhanced resistance to complement killing and grew well in 5% HS, but was weakly inhibited in 10% HS (**Fig. 1D**). Ab strain Ab5075 displayed resistance to 5% HS but increased sensitivity when treated with NE from 0.1 to 0.3 U/mL (**Fig. 1E**). When treated with 0.3 U/mL NE, the strain displayed reduced growth in 1.25% HS, strong growth inhibition in 2.5% HS, and no growth in 5% HS (**Fig. 1E**). The Bm vaccine strain Bm16MΔ*vjbR* displayed more resistance to complement killing than Ab5075 and PAO1. Bm16MΔ*vjbR* displayed growth in 15% HS in the presence of NE concentrations of ≤ 0.05 U/mL; however, NE treatment increased bacterial sensitivity to HS. When treated with more than 0.1 U/mL of NE, the growth of the Bm strain was inhibited (**Fig. 1F**). Interestingly, Bm16MΔ*vjbR* grew better in HS (< 10%) than non-HS containing medium (**Fig. 1F**). To verify that a heat-labile proteinaceous component of HS was mediating the observed killing activity, we measured bacterial survival in reaction mixtures that contained heat-treated HS. Briefly, HS was heat-inactivated at 55°C for 0.5 h or 65°C for 1 h and then incubated with PAO1 or Ab5075. Under these conditions, no inhibition of bacterial growth was observed, and the synergistic effect observed with non-heat-killed HS was eliminated (**Fig. 1G, H; Fig. S1E**). Interestingly, NE simultaneous coincubation with HS results in better bacterial growth inhibition than subsequent addition of HS (**Fig. 1I, J**), suggesting that a longer period of NE and HS coincubation yields a better synergistic effect in HS (2.5~5%).

Taken together, the data support the hypothesis that synergistic interactions between protease activities and complement-directed killing promote the destruction of Gram negative bacterial pathogens. We were intrigued whether these findings could be extended by determining whether protease activity on multi-drug resistant bacteria could enhance sensitivity to front-line antibiotics. Toward this end, we measured the growth of bacteria in the presence of tobramycin (TCI American, Portland, OR, USA). Ab5075 was weakly resistant to 25 µg/mL tobramycin; however, both PAO1 and PA14 displayed sensitivity to tobramycin at this concentration (**Fig. 2A-C**). NE (0.1 to 0.3 U/mL) treated Ab5075 was susceptible to 25 µg/mL tobramycin in 2.5% HS and this treatment displayed a synergistic effect on bacterial killing; NE-treated PAO1 and PA14 were susceptible to 12.5 µg/mL tobramycin (**Fig. 2D-F**). Collectively, these data demonstrated a synergistic interaction between protease activity on bacteria and antibiotic treatment in driving the killing of bacterial pathogens; and providing a new avenue for bacterial disease management.

Data availability

This study did not generate/analyze datasets or code.

Acknowledgments

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Figure and Figure legends

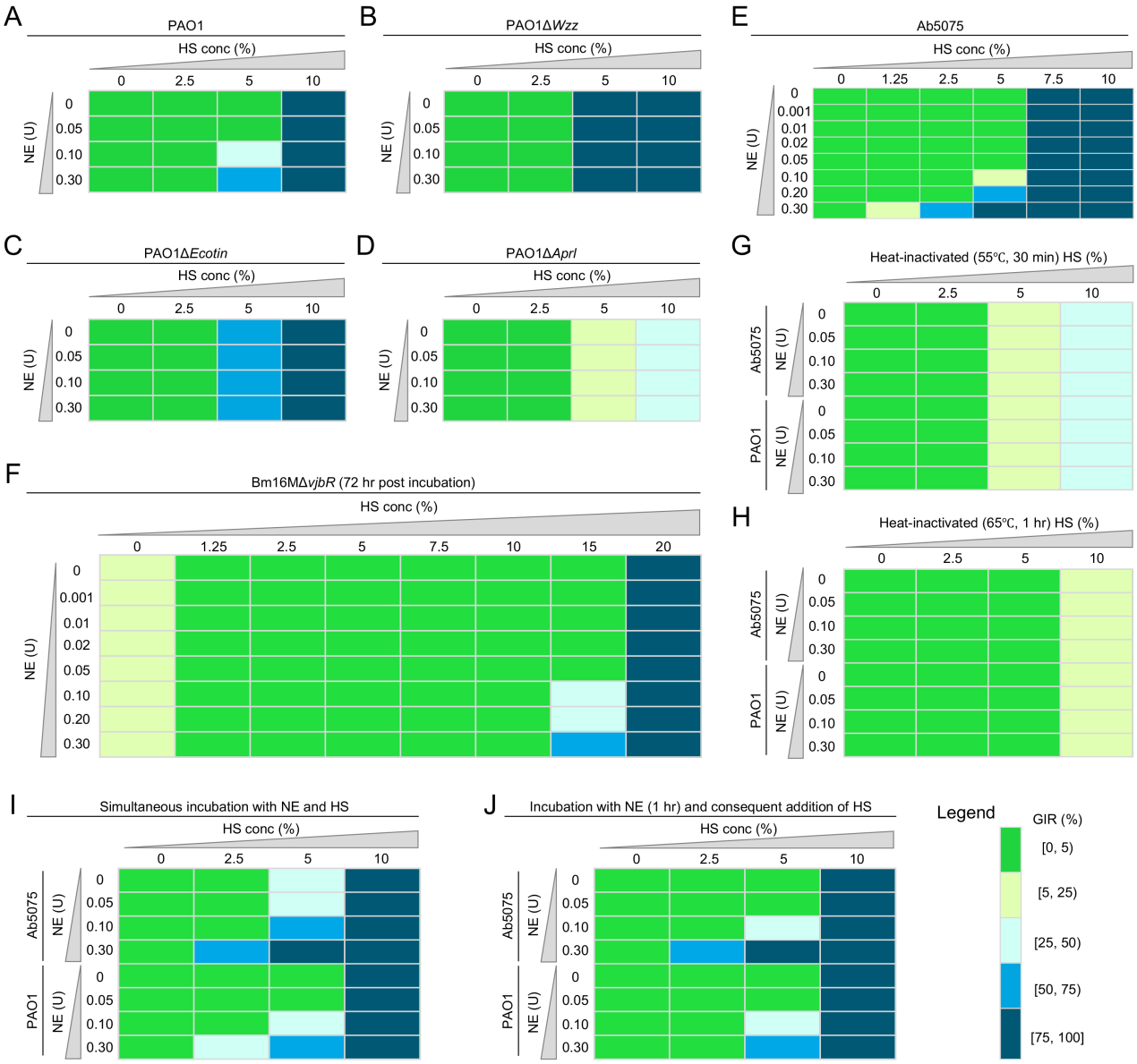


Figure 1. Synergistic effect of neutrophil elastase (NE) and human serum (HS) on bacterial growth. (A-D) Synergy of protease and serum on bacterial pathogens *P. aeruginosa* PAO1 (A) and its derived mutants PAO1Δ*Ecotin* (B), PAO1Δ*Wzz* (C), PAO1Δ*AprI* (D). (E, F) NE promotes *A. baumannii* Ab5075 (E) or *B. melitensis* Bm16MΔ*vjbR* (F) killing in the presence of HS. (G, H) Bacterial killing effect of NE decreases in the presence of heat-treated serum at 55°C, 30 min (G) or at 65°C for 1 h (H). (I, J) Addition of NE in HS at the same time (I) or at 1 h post incubation of HS and bacteria (J) varies the synergistic effect. Growth inhibition rate (GIR, %) = [(Contrl OD_{600} – treatment OD_{600})/Contrl OD_{600}]×100%. "[" or "]" and "(" or ")" indicate inclusion and exclusion, respectively.

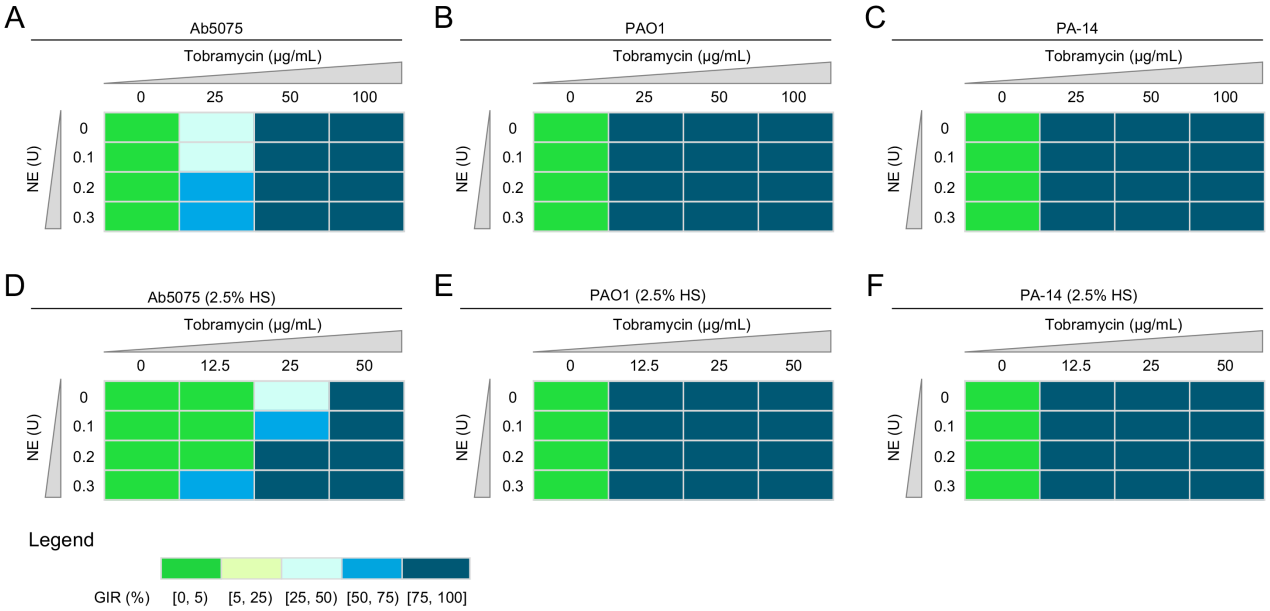
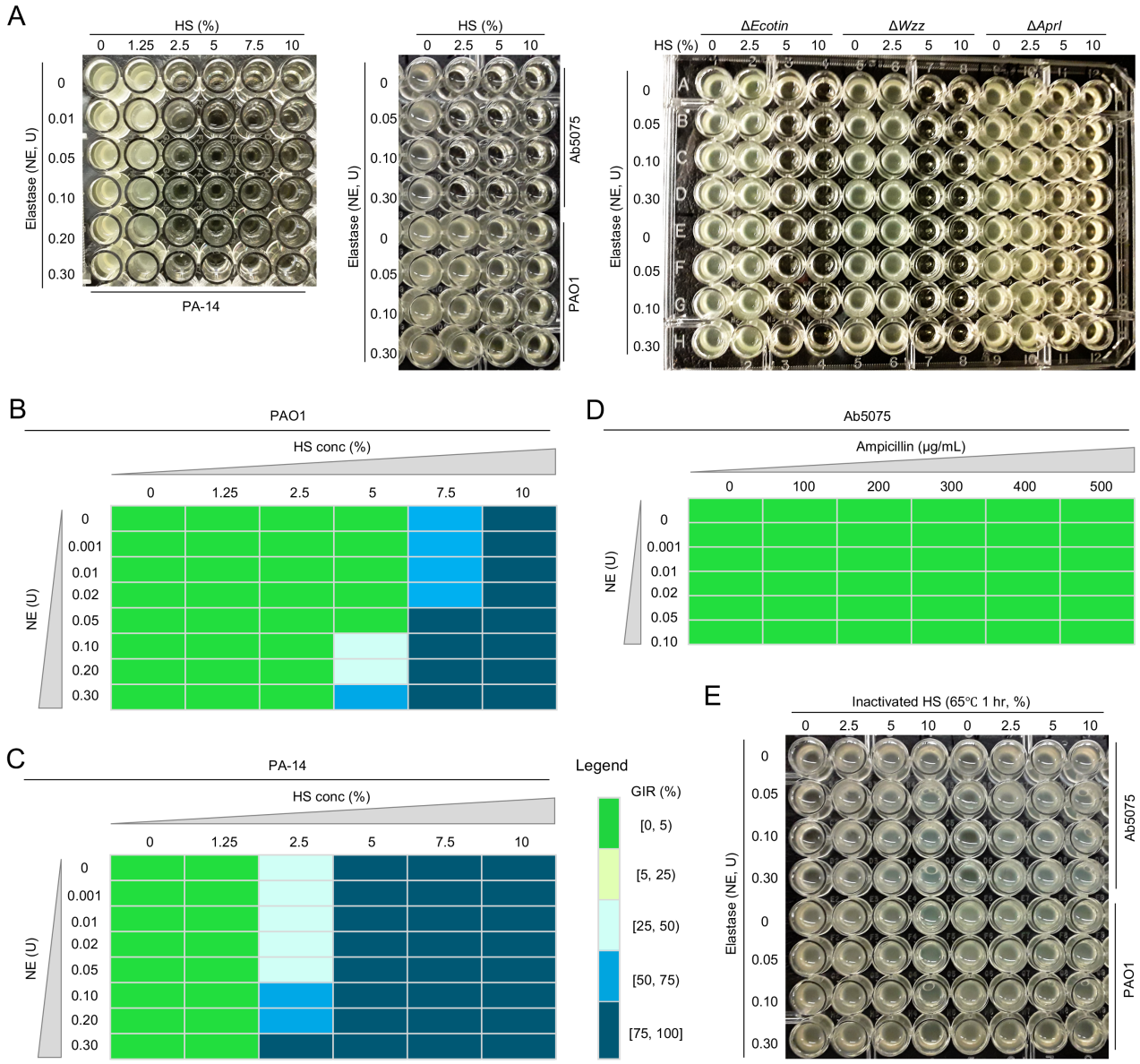


Figure 2. Synergistic effect of NE and tobramycin and/or HS on bacterial killing. (A-C) NE promotes *A. baumannii* Ab5075 (A), *P. aeruginosa* PAO1 (B), or PA-14 (C) killing in the presence of tobramycin. (C-D) Synergy of NE and tobramycin on *A. baumannii* Ab5075 (D), PAO1 (E), or PA-14 (F) killing in the presence of 2.5% HS.

1 **Supplemental Information**



2 **Figure S1. Effect of NE and HS or Ampicillin on bacterial killing.** (A) Growth inhibition assays
3 of the bacterial pathogens *P. aeruginosa* PA-14, PAO1, and *A. baumannii* Ab5075 in the indicated
4 concentrations of neutrophil elastase (NE) and normal human serum (HS). (B-C) Synergy of NE and
5 HS on the tested bacterial strains PAO1 (B) and PA-14 (C). (D) NE fails to promote Ab5075 killing
6 in the presence of Ampicillin at the indicated concentrations. (E) Heat-inactived HS fails to promote
7 NE bacterial killing. Growth inhibition rate (GIR, %) = [(Contrl OD_{600} – treatment OD_{600})/Contrl
8 OD_{600}]×100%. "[" or "]" and "(" or ")" indicate inclusion and exclusion, respectively. Pictures froms
9 a representative experiments of at least three independent experiments.