Supplemental Files

Title: Catalase Activity is Critical for *Proteus mirabilis* Biofilm Development, EPS Composition, and Dissemination During Catheter-Associated Urinary Tract Infection

Running title: Contribution of catalase to biofilm development

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Supplemental Materials and Methods

RNA Isolation. Biofilms of wild-type *P. mirabilis* and the Δ*katA* mutant were established as described in the main text (materials and methods) and incubated at 37°C for up to 20 hours. At 2, 4, 6, 12, and 20 hours, the supernatants were removed and the biofilms were resuspended in 500 µl of STAT60 (TEL-TEST, INC.) and transferred to 1.5 ml microfuge tubes. 0.2 ml of chloroform: iso-amyl alcohol 24:1 (Calbiochem) was added to each aliquot, microfuge tubes were inverted several times to mix, and centrifuged at 18,000 × g for 30 min at 4°C. The aqueous layer was removed and transferred to a new microfuge tube, to which 0.5 ml of isopropanol was added (Fischer Scientific), mixed by inversion and incubated overnight at −20°C to precipitate RNA. The next day, samples were centrifuged at 18,000 × g for 30 min at 4°C, supernatants were discarded and the nucleic acid pellet was washed three times with 1 ml of 75% ethanol and centrifuged at 18,000 × g each wash for 10 min. After the final wash, the supernatant was carefully removed and the tubes were placed on a 55°C heat block for ~20 sec to remove excess ethanol. The remaining pellets were dissolved in 30 µL of molecular biology grade water (Corning). Nucleic acid concentration was measured by Nanodrop and adjusted to 200 ng/µl in 30 µl of total volume with water. DNA was removed via the Invitrogen DNA-Free Kit, according to manufacturer's protocol. DNAse-treated RNA was frozen at −20°C until use.

cDNA Synthesis and qRT-PCR. DNAse-treated RNA was used to generate cDNA via the iScript cDNA Synthesis Kit (BioRad) per manufacturer's instructions. Two technical

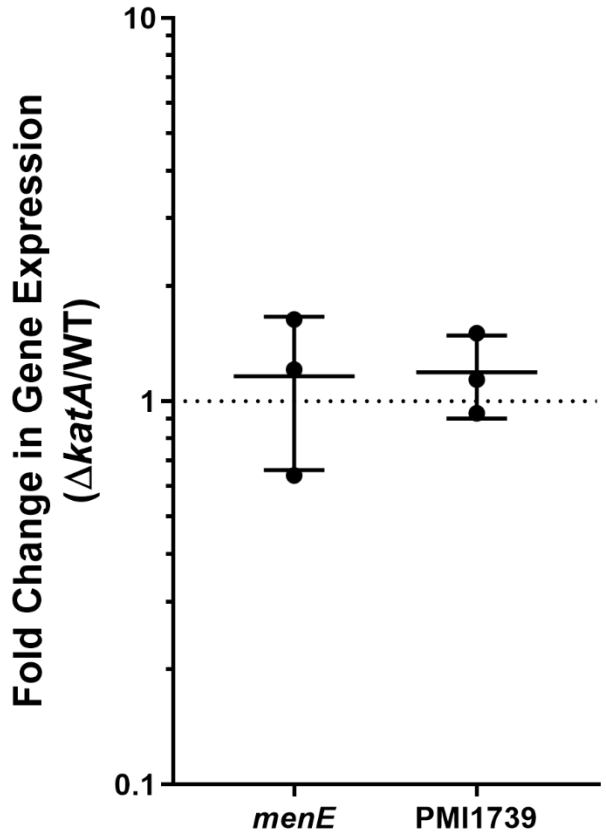
replicate reactions for each strain (wild-type and Δ*katA* isolated at various timepoints in biofilm formation: 2, 4, 6, 12, and 20 h) were prepared by combining 2 µl of cDNA (approximately 10 ng of RNA template), 0.8 µl of each 10 µM primer stock, 6.4 µl of water and 10 µl of qPCR-Bio SyGreen Blue Mix Lo-Rox (PCR Biosystems). Primer sequences are provided in Supplementary Table 1. Reactions were initiated on a BioRad CFX-Connect Real Time system programmed to acquire data on the SYBR Green Channel with the following cycles: 1 cycle at 95°C for 3 min, 40 cycles at 95°C for 10 sec, and 40 cycles at 60°C for 30 sec. For analysis, *rpoA* was chosen as the reference gene for normalization between samples as it exhibited low variation (Fig. S4G). Specific target gene expression was determined for the following genes involved in ROS detoxification: *ahpC1213*, *ahpC0073*, *sodA*, *sodB*, and *sodC*. For expression in wild-type biofilms overtime, the data were analyzed according to the Relative Quantification (RQ) method by Pfaffl et al 2001, in which the 2 h time point of biofilm formation was considered the 'Control' and each subsequent time point was the 'Sample'. The same method was used to analyze expression in the Δ*katA* mutant biofilms overtime in comparison to the expression in wild-type biofilms. For this comparison the wild-type strain was considered the 'Control' and the Δ*katA* mutant was considered the 'Sample'. The full equation shown below:

$$\textbf{Relative Quantification Ratio} = \frac{(\mathbf{E_{target}})^{\Delta \mathbf{CPtarget\ (control-sample)}}}{(\mathbf{E_{reference}})^{\Delta \mathbf{CPreference\ (control-sample)}}}$$

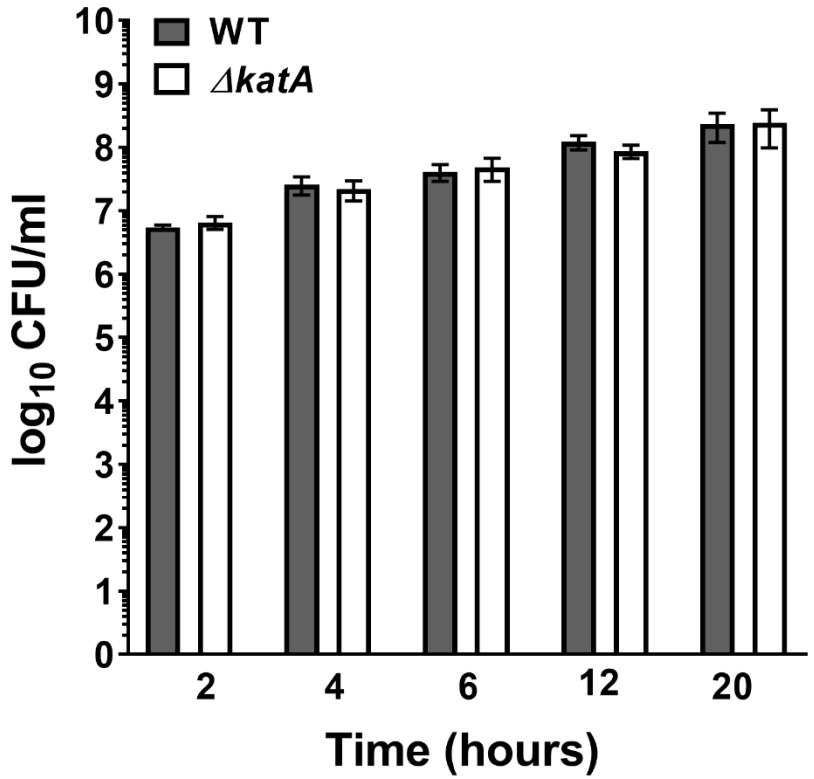
In this equation, E refers to the primer efficiency of the target gene (E_{target}) or the reference gene ($E_{reference}$), and CP refers to the Cycle Point, or the cycle number at which the signal exceeds the threshold.

Name	Sequence
katA-699s-EBS2(A)	TGAACGCAAGTTTCTAATTTCGATTGCTTCTCGATAGAGGAAAGTGTCT
katA-699s-IBS(A)	AAAAAAGCTTATAATTATCCTTAGAAGCCCTAGTAGTGCGCCCAGATAGGGTG
katA-699s-EBS1 d(A)	CAGATTGTACAAATGTGGTGATAACAGATAAGTCCTAGTAGGTAACTTACCTTTCTTTGT
EBS Universal Primer(A)	CGAAATTAGAAACTTGCGTTCAGTAAAC
katA_ver2_F(A)	TAAGGCTGTTCGCTTCGTTT
kat_ver2_R(A)	GGGTGAAAGGGCAACAAGTA
katA_CF_Fwd(B)	TTCTGAAACTCTTCATGCTGAAACACGCCAATTAAGTC
katA_CF_Rev(B)	GGCATGCCCTGCAGGAATTCAACACGGGATAGAGGTTG
pGEN_Fwd(B)	GAATTCCTGCAGGGCATG
pGEN_REV(B)	CAGCATGAAGAGTTTCAG
katA qPCR F(C)	GCGGAAGCTCTAGTAGGTAAAG
katA qPCR R(C)	TGTAACTTCCAACGCGGATAA
ahpC PMI1213 qPCR F(C)	CCAGCTGACTTTACCTTTGTTTG
ahpC PMI1213 qPCR R(C)	GCTGCTATGCCATGCTTTATG
ahpC PMI0073 qPCR F(C)	GCTATCGATGATGGCGGTATT
ahpC PMI0073 qPCR R(C)	CACCCGCTTCTGGATGTT
sodA qPCR F(C)	CCTGCACTCCCTTATGCTTAT
sodA qPCR R(C)	AACGCAGTATTGGTATTGTTCAC
sodB qPCR F(C)	GGCAAACACCACCAAACTTAC
sodB qPCR R(C)	GTTCCAAACTTGAGCAGCATTAT
sodC2 qPCR F(C)	GCTCTTGTGAGCCAGATATGAA
sodC2 qPCR R(C)	GGCCCTAAGTGAACACCTTTAT
menE qPCR F(C)	TGCGAGGTTACTCGATCTTTC
menE qPCR R(C)	GACCTGATGATCCAGAGGTTAAG
PMI1739 qPCR F(C)	GAATGTGCAAGCGGGTAAAC
PMI1739 qPCR R(C)	TCACTACGTGCCCAATTACC

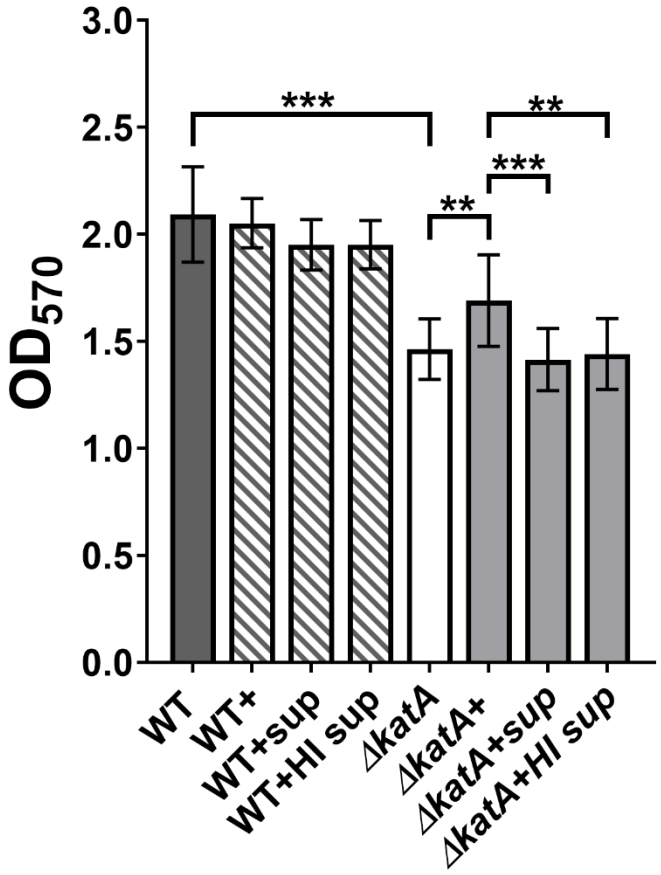
Supplementary Table 1: ***Primer sequences.*** Name and sequence listed for primers used in the generation and verification of (A) the Δ*katA* mutant, (B) Δ*katA*+ complemented strain, and (C) the assessment of gene expression via RT-qPCR.



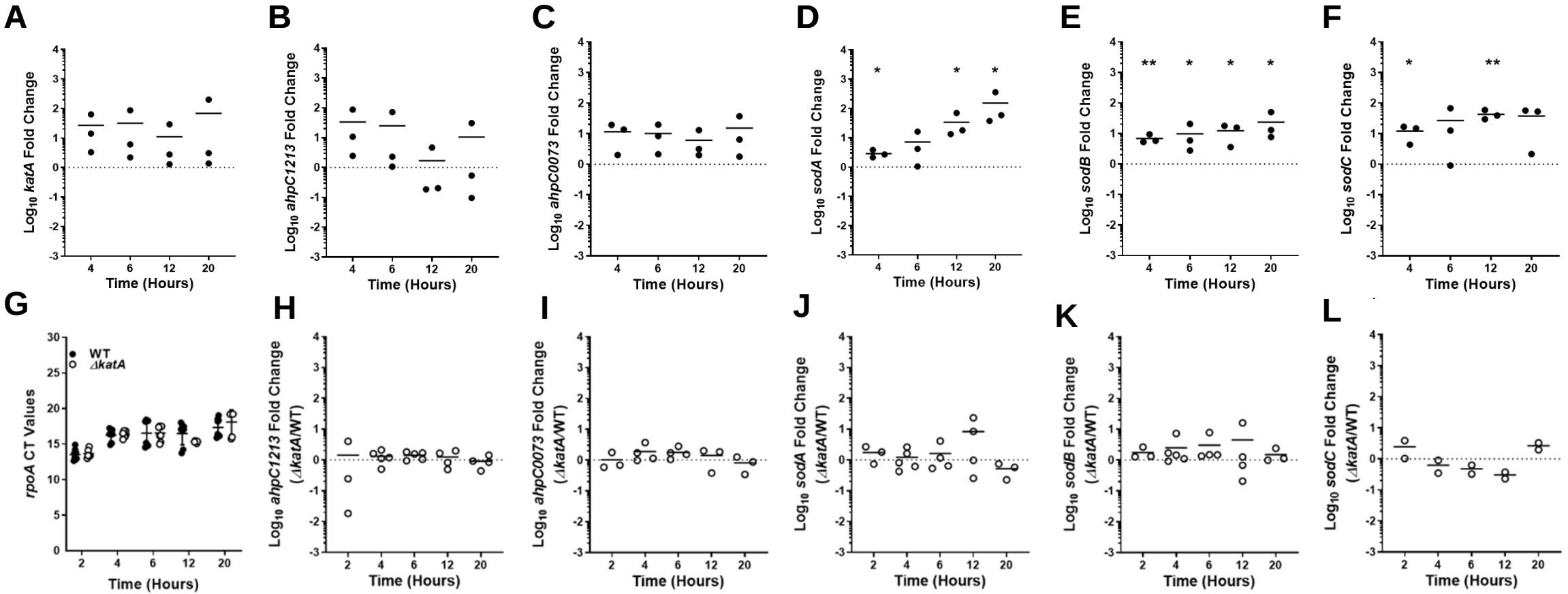
Supplementary Figure 1: ***Disruption of katA does not impact expression of adjacent genes.*** Expression level of genes surrounding Δ*katA* (*menE* and *PMI1739*) were assessed by qRT-PCR using RNA isolated from 6 h biofilms of both the wild-type (WT) and Δ*katA* mutant grown in LB at 37°C. Error bars represent the mean and standard deviation (SD) of 3 independent experiments with 2 technical replicates each. No significant differences detected by one-sample t test.



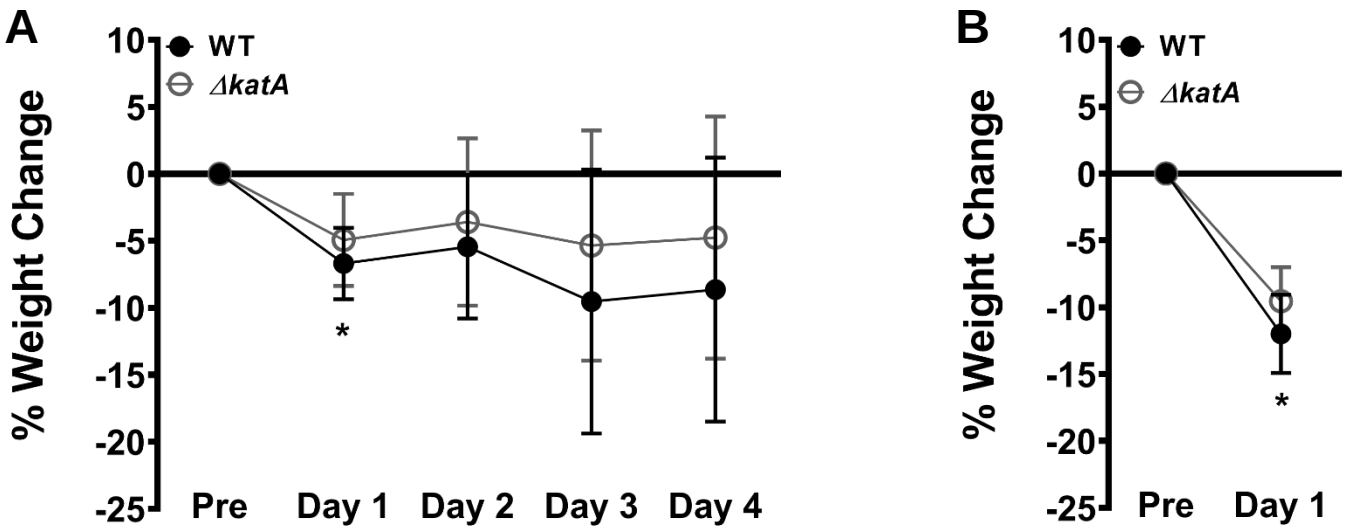
Supplementary Figure 2: ***The ΔkatA mutant does not exhibit a decrease in biofilm viability.*** Colony forming units (CFUs) of WT and *ΔkatA* biofilms grown in LB at 37°C were assessed at 2, 4, 6, 12, and 20 h. Error bars represent mean and standard deviation (SD) of 3 independent experiments with 3 technical replicates. No significant differences were detected by Student's t-test.



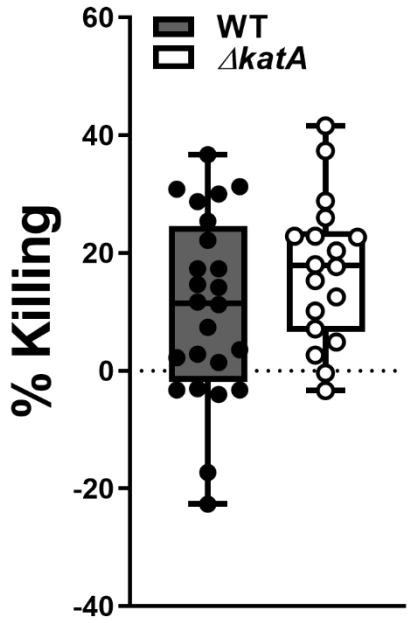
Supplementary Figure 3: ***Active catalase enzyme is required for complementation of ΔkatA mutant biofilms.*** 20 h Biofilms of the WT and Δ*katA* strain were grown at 37°C in LB under both untreated (gray, white respectively) and catalase treated conditions (gray stripes, light gray respectively). Catalase treatments consist of the following: active bovine catalase (strain+), 10 kDa filtrate from active bovine catalase (strain+sup), and heat-inactivated 10 kDa filtrate (strain+HI sup). Heat inactivation of the commercial catalase solution was performed at 95°C for a 30 min duration and resulting enzyme inactivation was verified by a catalase foam height assay. Biofilm biomass was assessed via crystal violet staining and detected by OD_{570}. Error bars represent mean and SD of 3 independent experiments with 3 replicates each. ** P<0.01, ***P<.001 by two-way ANOVA with Tukey's test for multiple comparisons.



Supplementary Figure 4: *Gene expression by qRT-PCR.* (A-F) $\log_{10}$ fold change in expression of genes involved in ROS detoxification (*katA*, *ahpC1213*, *ahpC0073*, *sodA*, *sodB*, and *sodC*) in wild-type biofilms over time. (G) Raw CT values for reference gene *rpoA* over time during all qRT-PCR experiments. (H-L) $\log_{10}$ fold change in expression of *ahpC1213*, *ahpC0073*, *sodA*, *sodB*, and *sodC* in the Δ*katA* mutant biofilms compared to the wild-type biofilms over time. Fold change was quantified using the Pfaffl Method of relative quantification to account for differences in primer efficiencies. Data is representative of at least 2 independent experiments with 2 technical replicates each. *P<0.05, ** P<0.01 by One-sample test.



Supplementary Figure 5: ***Weight Loss during CAUTI.*** All mice were weighed prior to infection ("Pre") and daily thereafter. Percent weight change relative to pre-infection weight is displayed for both (A) standard infection and (B) pre-colonized catheter infection with either the wild-type (WT, black) or the Δ*katA* mutant (gray). *P<0.05 by Two-way ANOVA with Sidak's test for multiple comparisons.



Supplementary Figure 6: Opsonophagocytic (OPH) killing assay The ability of neutrophils to kill wild-type *P. mirabilis* (gray) and the Δ*katA* mutant (white) ex vivo was measured using a well-established opsonophagocytic (OPH) killing assay as previously described (Siwapornchai et al., 2020; Standish & Weiser, 2009). Briefly, 1 × 10^5 neutrophils (isolated from female C57BL/6 mice) were incubated with 5 × 10^4 CFU of bacteria grown to mid-log phase and opsonized with 3% mouse sera in 100 µl reactions of HBSS/0.1% gelatin. Reactions were rotated for 40 min at 37°C. Percent killing was determined by plating on low salt LB agar and calculated in comparison to a no-neutrophil control under the exact same conditions. Box and whisker plot displays combined results from at least 3 independent experiments with 6 technical replicates each. No significant differences detected by Mann-Whitney test.