Staphylococcus aureus exhibits altered growth and response to antibiotics in a polymicrobial hypoxic biofilm model

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INTRODUCTION: Periprosthetic joint infection (PJI) is a growing clinical complication and a primary indicator for revision arthroplasty, affecting 1-2% of the more than 2 million total joint replacement patients each year [1]. The joint environment is profoundly hypoxic [2], and many PJI cases involve polymicrobial infections that complicate diagnosis and treatment [3]. However, *in vitro* PJI studies are largely conducted under normoxia with a single strain of bacteria, limiting their relevance to the clinical environment. We investigated the behavior of *Staphylococcus aureus*, the primary PJI pathogen, in hypoxia and under stress from co-culture with *Escherichia coli*, a common Gram-negative pathogen in polymicrobial infections [4].

METHODS: Stainless steel coupons were inoculated with 10^5 colony forming units (CFU) of methicillin-sensitive *S. aureus* (MSSA, strain 12600), methicillin-resistant *S. aureus* (MRSA, strain L1101), or *E. coli* (strain 25922). Bacteria were applied individually or as a 1:1 co-culture of *E. coli* and one *S. aureus* strain and grown under hypoxic conditions (2% oxygen) in tryptic soy broth for 6 or 24 hours. For biofilm quantification, coupons were washed after incubation and sonicated for 40 min at 40 kHz to disrupt the biofilms. The resulting suspensions were serially diluted and plated on selective agar for CFU quantification. Biofilm growth experiments were performed on n=9 total samples in three independent experiments and analyzed using Welch's ANOVA and a Games-Howell post hoc test for multiple comparisons. For minimum biofilm eradication concentration (MBEC) calculation, biofilms were grown on stainless steel coupons for 6 hours and then exposed to a range of concentrations of gentamicin for 24 hours, after which remaining bacteria were quantified as described above. MBEC experiments were performed on n=4 total samples for each antibiotic concentration in two independent experiments and analyzed using a *t*-test. *P*-values less than 0.05 were considered significant.

RESULTS: At 24 hours, co-culture with *E. coli* significantly reduced MSSA and MRSA adhesion (5.1-log and 4.8-log reductions, respectively; p = 0.003 and p = 0.01; Figures 1A and 1B) compared to *S. aureus* monoculture. The MBEC of gentamicin for MSSA was significantly decreased when cultured under polymicrobial conditions (13.5-fold reduction, p=0.003, Table 1).

DISCUSSION: These results mirror previous findings under normoxia [5], suggesting *E. coli* competitively inhibits *S. aureus* in both oxygen conditions. Interestingly, both *S. aureus* and *E. coli* formed more robust biofilms in hypoxia, likely due to adaptation to low oxygen stress [6]. The inhibitory effect of *E. coli* might suggest a secreted factor with anti-staphylococcal activity. Additionally, we found that the MBEC of gentamicin was slightly reduced in hypoxia for both MSSA and *E. coli* compared to normoxia [5], and that the MBEC for MSSA, but not *E. coli*, was significantly reduced when cultured under polymicrobial conditions. Ongoing work will further investigate this effect and assess the mechanisms by which hypoxic polymicrobial biofilm conditions influence antibiotic susceptibility. These experiments illustrate the importance of developing *in vitro* models that more faithfully represent clinical conditions to accurately understand bacterial infection in patients.

SIGNIFICANCE/CLINICAL RELEVANCE: These findings concerning biofilm growth under hypoxia contribute to a more clinically relevant model of the joint space in PJI and may inform future therapeutic strategies to treat polymicrobial infections.

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	MBEC (ug/mL)
MSSA	75-200
E. coli	5-10
MSSA from polymicrobial biofilm	<5-10
***E. coli* from polymicrobial biofilm**	10-50

Table 1 (Left): Minimum Biofilm Eradication Concentration range of gentamicin for bacteria grown for 6 hours in mono- or polymicrobial conditions in TSB.

Figure 1 (Below): (A) Adherent bacteria (CFU/mL) +/- standard error for MSSA, *E. coli*, or both in co-culture at 0, 6, and 24 hours. (B) Adherent bacteria (CFU/mL) +/- standard error for MRSA, *E. coli*, or both in co-culture at 0, 6, and 24 hours. *p<0.05, **p<0.005.