Heme cross-feeding can augment Staphylococcus aureus and Enterococcus faecalis dual species biofilms

Jun-Hong Ch'ng, Mugil Muthu, Kelvin K. L. Chong, Jun Jie Wong, Casandra A. Z. Tan, Zachary J. S. Koh, Daniel Lopez, Artur Matysik, Zeus J. Nair, Timmy Barkham, Yulan Wang and Kimberly A. Kline

© The Author(s) 2022

INTRODUCTION

Biofilms consist of a sessile community of microbes embedded within a matrix of extracellular polymeric substances. Biofilms represent the dominant mode of bacterial life, and biofilm cells exhibit different patterns of behaviour compared to planktonic cells [1]. Characteristically, biofilms confer protection to chemical and physiological stresses [2], shear forces [3, 4], predation [5], and to antibiotic-mediated clearance [6]—rendering biofilm-associated infections difficult to treat. Behaviourally, biofilm-embedded cells are extremely heterogeneous, exhibiting varying levels of metabolic activity (from very active to quiescent) [7, 8], transcriptional profiles (depending on the microenvironment) [9, 10] and higher frequencies of genetic exchange (via transformation, conjugation and transduction) [11, 12].

Biofilms are frequently polymicrobial, comprising of different species, phyla or kingdoms interacting within the complex community [13]. Typically categorized as commensal, antagonistic or synergistic for simplicity, these multi-species interactions are multifaceted and evolve temporally with fluctuations in the microenvironment such as pH, temperature, oxygen, nutrient and waste levels, and quorum-sensing signals [14]. Although in-depth characterization of molecular interactions in polymicrobial biofilms is limited, controlled studies have enabled the identification of critical mediating compounds important for interspecies interactions. Notable mediators include c-di-GMP [15, 16], Al-2 [17, 18], alarmone ppGpp [19, 20], bacteriocins [21–23], siderophores [24], L-ornithine [25], lactic acid [26], lipoteichoic acid [27, 28], glycans [29] and indole [30, 31], and these have been reviewed elsewhere [14, 32–42].

E. faecalis and S. aureus are both opportunistic pathogens and are among the leading causes of nosocomial infections [43, 44]. The biofilm-forming potential of each species is well documented [33, 45] and both have been implicated in biofilm-associated infections such as endocarditis [46, 47], urinary tract infections [48, 49] and chronic wounds [50, 51]. Although, E. faecalis and S. aureus are commonly co-isolated in chronic wounds such as diabetic foot ulcers [52], venous leg ulcers [53] and pressure wounds [54], studies of their interactions are largely limited to the transfer of vancomycin resistance genes from E. faecalis to S. aureus in clinical settings [55–57]. Therefore, in this study, we explored the molecular interactions between E. faecalis and S. aureus in biofilms.

Aerobic respiration in E. faecalis requires exogenous heme as a cofactor for cytochrome bd [58, 59]. Enterococci are unable to...
synthesize heme due to an absent TCA cycle that prevents the formation of porphyrin precursors. Through a yet unknown importer, heme is taken into the cells and incorporated into cytochrome bd (CydB), which then converts terminal demethylquinol (DMKH₂) to demethylmenaquinone (DMK), consuming O₂ and releasing H₂O in the process [60]. DMK is reduced by NADH: quinone oxidoreductase back into DMKH₂ consuming NADH. Importantly, Enteroococi do not express other membrane-embedded electron carriers like ubiquinone or menaquinone [60], but only make DMK which is a modified menaquinone lacking a 2-methyl group [61]. Cytochrome bd is the key respiratory enzyme for E. faecalis and contains two subunits (CydA and CydB) with three cytochromes, b₃, b₅₅₈, and b₅₆₄. The translocation of a proton by cytochrome bd establishes a proton motive force that, when coupled with F0F1-ATP synthase, generates ATP [62, 63]. Additionally, cydC and cydD are necessary for cytochrome bd production and have been suggested to be involved in heme transport and/or cytochrome bd assembly [60]. Interestingly, reduction of O₂ by DMK increases extracellular superoxide production, the latter of which was inhibited by exogenous heme [64], while a functional electron transport chain sensitizes E. faecalis to oxidative burst and decreased its survival in human blood [65].

In this study, we show that S. aureus-derived heme is required to activate E. faecalis aerobic respiration, leading to augmented E. faecalis growth and augmented dual-species biofilm production. We speculate that this interspecies cross-feeding of heme, where one species consumes metabolic end-products from another, may affect mixed species infection outcomes in heme-restricted host and environmental niches.

**Materials and Methods**

**Bacterial strains and growth conditions**

Strain, isolate and transposon library details are available in Supplementary Information. Overnight cultures of E. faecalis were grown in Brain Heart Infusion broth (Becton-Dickinson, United States) whereas S. aureus grown in Tryptone Soy Broth (Oxoid, England). Agar Technical Powder No. 3 (Oxoid, England) was used for agar plates. Strains were cultured under static conditions at 37 °C. Overnight cultures were spun down and washed once in phosphate-buffered saline prior to normalization. An OD₆₀₀ of 1.3 and 3.0 for E. faecalis and S. aureus respectively gave about 1 × 10⁹ CFU/ml. MRSA Select II agar (Bio-Rad Laboratories, United States) was used to select S. aureus USA300LAC from mixed-species cultures whereas rifampicin (25 μg/ml) was added to BHI agar to select for OG1RF. Rifampicin (Sigma-Aldrich, United States) was dissolved in DMSO to make a stock of 25 mg/ml whereas human hemoglobin (Sigma-Aldrich) was dissolved in dH₂O and stored at 4 °C. Hemin (Sigma-Aldrich) was dissolved in DMSO to make a stock of 25 mg/ml and 10 μg/ml respectively was used unless otherwise stated.

**Biofilm assays**

Normalized cultures of E. faecalis and S. aureus were mixed (with equal CFU) in the ratio of 1:1 for dual-species biofilms and 8 μl of single- or dual-species cultures were inoculated in 200 μl of Tryptone Soy Broth in a 96-well flat transparent plates (Thermo Fisher Scientific, United States) and incubated under static conditions at 37 °C for 5 days unless otherwise stated. For anoxic experiments, plates were instead incubated in an anaerobic jar (Merck, Singapore) with a gas pack (Becton Dickinson, United States) and incubated at 37 °C for five days. Details on Crystal Violet staining and CFU determination is available in Supplementary Information. For biofilm oxygen consumption rate (OCR) assays, 5-day biofilms were grown directly in Seahorse XFe96 FluxPak 96-well plates with 80 μl of inoculated media added per well. Planktonic cells were removed with three washes of 80 μl PBS using a 96-channel pipettor to prevent cross-contamination and the remaining biofilms were resuspended in 80 μl of PBS. After three baseline measurements were taken, 30 μl of fresh TSB was injected into the wells and OCR measured for 1 h using standard parameters.

**Results**

**Growth kinetics**

Normalized cells were diluted 100× in PBS and 8 μl of diluted cells were added to 200 μl TSB in a 96-well plate. The plate was incubated in Tecan Infinite M200 PRO Spectrophotometer at 37 °C for 20 h and absorbance (600 nm) recorded for every 15 min after shaking the plate for 3 s.

**Transposon library screen and transposon mutants**

The E. faecalis Transposon Library was provided by Gary M. Dunny [66] and S. aureus transposon mutants were from Nebraska Transposon Library [67] provided by the Network on Antimicrobial Resistance in Staphylococcus aureus (NARSA) and distributed by BEI resources, NIAID. Details on the screening of the E. faecalis transposon library [66, 68] are available in Supplementary Information. The primary screen of dual-species biofilms was performed by adding 5 μl of transposon mutants grown overnight and 3 μl of USA300LAC overnight cultures to 200 μl TSB in the 96-well plate. Each plate had controls of parental strains of OG1RF – USA300LAC. Plates were incubated at 37 °C for five days in a humidified incubator and biofilms quantified by crystal violet. E. faecalis transposon mutants that showed reduced staining were selected as hits. Confirmation of primary screen hits was done via a secondary screen (three biological replicates) and secondary screen hits that consistently showed reduced dual-species biofilms were subject to further validation. Transposon mutants without defects in growth kinetics and single-species biofilms, but with growth defects in dual-species biofilms, were shortlisted as validated hits.

**LC-MS for heme quantification from cell pellet**

E. faecalis strains (OG1RF, cydA::Tn, cydD::Tn and cydC::Tn) were grown overnight in 20 ml of TSB with or without supplementation of 5 μg/ml hemin. Cell pellets were washed twice with PBS before being normalized by OD₆₀₀. Cell pellets were mixed with 100 μl of lysozyme (10 mg/ml), incubated in a water bath at 37 °C for 1 h and then stored at −80 °C until LC-MS quantification.

S. aureus strains (USA300LAC, hemA::Tn, hemL::Tn, hemB::Tn and hemE::Tn) were grown overnight in 20 ml of TSB and were resuspended in 950 μl of P1 Buffer. 50 μl of lysostaphin (5 mg/ml) was added and incubated at 37 °C for 1 hr and then stored at −80 °C until LC-MS quantification.

**Statistics**

Statistical analyses used GraphPad Prism 7 software (version 7.03) (GraphPad, United States). The data was analyzed through unpaired students T-test or one-way analysis of variance (ANOVA) with Tukey’s post-hoc testing for multiple inter-group comparison, or with Dunnett’s post-hoc testing if comparing to a specific control group. Significance for p < 0.05 are reported.

**Ethics**

All procedures were performed in accordance with Nanyang Technological University Research Integrity Policy. Anonymized and pure bacterial strains used in this study did not require IRB approval.
Data shows mean and SD (ratio of 1:1, dual-species bio following inoculation of bio and S. aureus) biomass accumulation occurred at day 4 biomass from day 1 and peaked at day 4. Since the greatest significance in dual-species biomass accumulation occurred at day 4 and 5, we chose day 5 for subsequent assays.

Tukey combination (1:1) over the greatest virulence studies and used throughout this study bio isolate OG1, commonly used in molecular manipulation and virulence studies and used throughout this study) biofilm biomass peaked at day 1 and plateaued for the remaining four days, whereas S. aureus (strain USA300LAC) formed very poor biofilms under these experimental conditions (Fig. 1A). By contrast, following inoculation of E. faecalis together with S. aureus at a ratio of 1:1, dual-species biofilms showed significantly greater biomass from day 1 and peaked at day 4. Since the greatest difference in dual-species biomass accumulation occurred at day 4 and S. aureus strain variation also affects augmentation observed during co-culture with S. aureus strains/isolates showed a significant biofilm biomass increase when co-cultured with E. faecalis OG1RF (Fig. 2A and Supplementary Table 1). Newman strain and isolate C37 did not augment augmentation of S. aureus USA300LAC was much more heterogenous with only six out of 32 strains/isolates showing augmented biofilms (Fig. 2B and Supplementary Table 2). Of these six strains, the degree of augmentation observed varied between 2.06–3.10 fold (Supplementary Table 2). Of the 26 that did not produce augmented biofilms, 17 showed single-species biofilm levels that were lower.

(a rifampicin and fusidic acid resistant derivative of a human oral isolate OG1, commonly used in molecular manipulation and virulence studies and used throughout this study) biofilm biomass contributed to the augmented biomass, biofilms were manually disrupted and CFU determined for both single and dual species biofilms. In day 5 biofilms, we observed a statistically significant 5-fold increase in E. faecalis CFU within dual-species biofilms compared to mono-species biofilms (Fig. 1B) which correlated with the increased biomass observed by crystal violet staining. E. faecalis out-numbered S. aureus by approximately 60-fold in dual-species biofilms. Surprisingly, although S. aureus produced very little biofilm biomass compared to E. faecalis (Fig. 1A), CFU equivalent to that of E. faecalis were recovered, suggesting that E. faecalis may produce more abundant biofilm matrix than S. aureus. Compared to the increased E. faecalis biomass and CFU in the presence of S. aureus, there were no significant differences in CFU sampled from non-adherent (planktonic) volume of the same biofilm wells, suggesting that E. faecalis growth augmentation by S. aureus is specific to biofilms (Fig. 1C).

**Fig. 1** Single- and dual-species in vitro biofilms. A E. faecalis (Ef) and S. aureus (Sa) biofilms were grown in 96-well plates alone or in combination (1:1) over five days before biofilm was quantified by crystal violet (CV). Day 0 refers to 1 h post-inoculation. Data shows mean and SD (N ≥ 3) with ***p < 0.001 and ****p < 0.0001 when compared to E. faecalis-only biofilms of the same time point using 1-way ANOVA with Tukey’s post-hoc test. B Day 5 biofilm and (C) planktonic cells of Ef or Sa or both were collected and CFU/well determined using selective agar. Data shows mean and SD (N ≥ 6) with ***p < 0.001 and **** p < 0.0001 using 1-way ANOVA and Tukey’s post-hoc test.

**Dual-species biofilm augmentation is strain dependent**

To investigate whether S. aureus augmentation of E. faecalis biofilm is a phenomenon unique to the strains used in the initial studies (E. faecalis strain OG1RF and S. aureus strain USA300LAC), we assayed for biofilm augmentation using five additional commonly used S. aureus laboratory strains, as well as ten clinical wound isolates. We observed that 14 out of the 16 tested S. aureus strains/isolates showed a significant biofilm biomass increase when co-cultured with E. faecalis OG1RF (Fig. 2A and Supplementary Table 1). Newman strain and isolate C37 did not augment mixed species biofilm biomass, potentially because of anti-enterococcal activity or biofilm-restricting properties of these strains that are discussed later. Together, the data indicates that S. aureus strain variation influences the nature of the dual-species relationship with E. faecalis vis-à-vis biofilm development.

We next investigated if the E. faecalis strain variation also affects dual-species biofilm augmentation by testing three additional E. faecalis laboratory strains and 28 clinical isolates derived from bloodstream (VRE isolates), wound (TSHW-EF05 to EF43), urinary tract infections (UTIEF isolates), or the healthy gastrointestinal tract of children (HCG isolates). In contrast to the relative consistent ability of S. aureus strains to augment dual-species biofilms, augmentation observed during co-culture with S. aureus USA300LAC was much more heterogenous with only six out of 32 strains/isolates showing augmented biofilms (Fig. 2B and Supplementary Table 2). Of these six strains, the degree of augmentation observed varied between 2.06–3.10 fold (Supplementary Table 2). Of the 26 that did not produce augmented biofilms, 17 showed single-species biofilm levels that were lower.
than OG1RF under the conditions used (defined arbitrarily as <0.5× the biofilm levels of OG1RF), five were defined as having high biofilm levels (>2× the biofilm levels of OG1RF), and four had similar levels of biofilm to OG1RF (>0.5× but <2× of OG1RF) (Supplementary Table 2). Taken together, we conclude that many E. faecalis isolates are not susceptible to biofilm augmentation by USA300LAC. This is in keeping with other studies showing strain differences (not just species composition) profoundly affects the nature of microbial interactions [71–73].

**E. faecalis** transposon screen identifies menA and cydA to be crucial for dual-species biofilm augmentation

To determine the mechanism by which E. faecalis CFU are increased in the presence of S. aureus, leading to dual-species biofilm augmentation, we screened a near-saturated E. faecalis transposon mutant library [66] for mutants that displayed altered dual-species biofilm accumulation, using the same microtiter CV biofilm assay. After secondary screening to eliminate any mutants that were attenuated in biofilm formation, we validated nine mutants in seven unique genes that reduced dual-species biofilms (Fig. 3A).

Amongst these was epaOX that encodes a glycosyltransferase involved in the production of cell wall rhamnopolysaccharide enterococcal polysaccharide antigen (Epa), is responsible for E. faecalis biofilm structure and stability in response to antibiotic stress [74], and is a determinant of biofilm-associated antibiotic resistance [75]. Both the transposon mutant and a clean deletion mutant of epaOX showed a reduction in dual-species biofilms relative to the single species control which was restored to parental strain levels in the plasmid-complemented strain (Supplementary Fig. 1a) after correcting for variations in single species biofilms (Supplementary Fig. 1b). As such, it seems likely that epaOX is involved in augmented dual-species biofilms through EPS production.

The roles of four additional validated gene products identified in the transposon screen (an ABC transporter, SufB, RpiR, and a Ser/Thr Phosphatase, Fig. 3A) were not pursued. The essentiality of sufB had been suggested in some studies [58] but deemed otherwise by others [58], and its involvement in respiration, via FeS cluster assembly, merits future attention. Most importantly, we identified three unique menA (OG1RF_11661) and one cydA (OG1RF_11666) transposon mutants that failed to undergo dual species augmentation. Both genes encode proteins participating in oxidative respiration (Fig. 3B) [77] prompting closer investigation into this process.

---

Fig. 2 Augmentation of biofilms with multiple E. faecalis and S. aureus laboratory strains and clinical isolates. A Six S. aureus laboratory strains (USA300LAC–ISP479) and ten patient isolates (C01–C50) were grown alone or with E. faecalis (OG1RF) for five days before biofilms were quantified by crystal violet (CV). Results show biofilm levels normalized to OG1RF-only control, with mean and SD displayed (N ≥ 3). Points colored green are significantly different to respective single species biofilms by p < 0.05 using 1-way ANOVA with Bonferroni’s post-hoc test (details in Supplementary Table 1). B Four laboratory strains of E. faecalis (OG1RF-V583), together with 28 E. faecalis patient isolates (VRE122-TTSHW-EF43) were grown for five days alone or with S. aureus (USA300LAC) before biofilm was quantified by CV. Results show biofilm levels normalized to OG1RF-only controls, with mean and SD (N ≥ 3). Points colored green are significantly different to respective single species biofilms by p < 0.05 using 1-way ANOVA with Bonferroni’s post-hoc test (details in Supplementary Table 2).
E. faecalis OG1RF biofilm augmentation by S. aureus, heme and hemoglobin requires O2 and cydABCD

We hypothesized that E. faecalis respiration is necessary for dual-species biofilm augmentation, and to test this, biofilms were cultured in oxic or anoxic conditions. As predicted, dual-species biofilm augmentation was not observed in anoxic conditions (Fig. 4A) but was increased by over 2-fold in oxic conditions (Fig. 4B), demonstrating that oxygen is required for dual-species biofilm augmentation.

Since the utilization of oxygen by E. faecalis is dependent cytochrome bd which requires the incorporation of exogenous heme as a cofactor (E. faecalis does not synthesize heme [63, 78, 79]), we directly investigated the effect of free and conjugated heme (hemin and haemoglobin respectively) on E. faecalis biofilms. Supplementation with either source significantly increased biofilm biomass by over two-fold in oxic conditions (Fig. 4B) and was accompanied by an increase in oxygen consumption rate (Supplementary Fig. 2). Additionally, E. faecalis biofilm growth kinetics with hemin supplementation were measured, with a rapid increase in biofilm staining observed to plateau after day 1 (Supplementary Fig. 3). Under anoxic conditions, hemin supplementation minimally impacted biofilm levels and hemoglobin had no effect (Fig. 4A).

Cytochrome bd complex consists of two subunits that are encoded by cydA and cydB. The same operon (Fig. 5A insert) includes cydC and cydD, which encode an ATP-binding cassette (ABC)-type transporter required for the expression of a functional cytochrome bd complex [63, 80]. As a functional cydABCD operon was presumed to be required for aerobic respiration to occur, we tested if disruptions of cydB, cydC and cydD would also attenuate the E. faecalis biofilm response to both S. aureus and hemin. Biofilm augmentation by both hemin and S. aureus was significantly impaired in all the tested transposon mutants of the four genes (Fig. 5A), indicating that that a functional operon was required for augmentation. Deletion mutants of cydB and cydD likewise failed to augment in the presence of hemin and S. aureus, with augmentation completely restored in the complementary strains (Fig. 5A). Additionally, the absence of heme-
E. faecalis biofilms augmented under oxic and anoxic conditions. E. faecalis (Ef) biofilms were grown for five days under A anoxic and (B) oxic conditions, alone or in the presence of S. aureus (Sa), hemin or hemoglobin (Hb) before biofilm was quantified by crystal violet (CV). Data shows mean and SD of CV absorbance. ***p < 0.0001 when compared to Ef-alone control using Dunnett’s post-hoc test (N ≥ 4).

**Heme biosynthesis in S. aureus is responsible for dual-species biofilm augmentation**

Since E. faecalis cannot synthesize heme [63, 79], we hypothesized that S. aureus-derived heme might enable the activation of cytochrome bd, giving rise to augmented dual-species biofilms. If so, S. aureus mutants deficient in heme biosynthesis should be unable to augment E. faecalis biofilms. We identified S. aureus transposon mutants in four key heme biosynthesis enzymes (hemA, hemB, hemE and hemL) in the S. aureus Nebraska transposon mutant library [67]. To verify the loss of heme biosynthetic activity, we performed growth assays to query the expected growth defect when exogenous heme was limited [81] and quantified heme in cell pellets by LC-MS. Only hemB::Tn displayed a growth defect (Supplementary Fig. 5a) that was restored upon heme supplementation (Supplementary Fig. 5b). The hemB mutant was also the only one that showed a 40-fold reduction in heme within cell pellets (Supplementary Fig. 5c). These data indicated that the hemB transposon mutant was the only bona fide heme-defective mutant of the four. The hemA, hemE and hemL mutants may have acquired secondary mutations, a documented caveat to this library [82], enabling them to bypass these mutations.

Consistent with our hypothesis, the S. aureus hemB mutant defective in heme biosynthesis did not give rise to augmented dual-species biofilms (Fig. 6). Notably, the single-species parental S. aureus biofilm levels were comparable to the hemB mutant, suggesting that growth restriction of this mutant in the absence of heme supplementation is unlikely to account for the drastic reduction in dual-species biofilms. However, this does not rule out the possibility that the hemB mutant is outcompeted by E. faecalis during biofilm formation. Overall, the data indicate that S. aureus and E. faecalis dual-species biofilm augmentation is dependent on heme biosynthesis in S. aureus.
As AtLA is necessary for cell lysis during \textit{S. aureus} biofilm formation \cite{83}, we asked whether autolysis contributed to heme release by \textit{S. aureus} to augment dual-species biofilms. When \textit{E. faecalis} and the \textit{S. aureus} atLA transposon mutant were co-cultured, they produced less biofilm than when \textit{E. faecalis} was co-cultured with the \textit{S. aureus} parental strain, but the biomass were still significantly augmented compared to the \textit{E. faecalis}-only control (Fig. 6). This suggests that AtLA contributes to, but is not exclusively required, for heme release from \textit{S. aureus}.

Recognizing the importance of \textit{S. aureus}-derived heme in driving dual-species biofilm biomass augmentation, we sought to determine if heme and hemoglobin similarly augmented \textit{E. faecalis} biofilm CFU as observed in Fig. 1B. As expected, the un-augmented \textit{cydA::Tn} biofilms had similar CFU to the \textit{E. faecalis} parental strain single species biofilm (Supplementary Fig. 6). Interestingly, while the \textit{E. faecalis} parental strain underwent augmented biomass upon supplementation with hemin or hemoglobin (Fig. 4B) this was not reflected in increased CFU (Supplementary Fig. 6). These data suggest that the mechanism of biofilm augmentation by heme and hemoglobin may be distinct from that by \textit{S. aureus}, where heme and hemoglobin elicit increased extracellular matrix production rather than elevating cell numbers. Further work to understand these differences is worthwhile and could inform the induction of \textit{E. faecalis} stress responses to heme/hemoglobin.

\textit{E. faecalis} Gelatinase E (GelE) is involved in using heme synthesized by \textit{S. aureus}

Having elucidated the role of heme in dual-species biofilm augmentation, we re-examined the \textit{S. aureus} and \textit{E. faecalis} strains and isolates to understand combinations that did not give rise to augmented biofilms (Fig. 2A, B). We assayed intracellular \textit{S. aureus} heme levels in Newman strain and isolate C37 (both failed to augment dual-species biofilms, Fig. 2A) and found them to have comparable levels as USA300LAC (Supplementary Fig. 7). This suggests that heme biosynthesis in \textit{S. aureus}, though important, may not be the dominant factor governing dual-species biofilm augmentation. For instance, strain differences in heme or hemoprotein release from \textit{S. aureus} would affect the efficiency of cross-feeding, whereas production of biofilm or growth inhibitors might curtail \textit{E. faecalis} biofilm formation to begin with.

Of the \textit{E. faecalis} strains and isolates, it was notable that OG1X, a sister clone of OG1RF lacking in gelatinase activity \cite{84,85}, was not augmented by USA300LAC (Fig. 2B). We therefore investigated whether gelE was responsible for use of USA300LAC-derived heme. Significant biofilm augmentation of \textit{E. faecalis} OG1RF::gelE only occurred during supplementation with hemin, but not \textit{S. aureus} or hemoglobin (Fig. 7). This result demonstrates that free heme (in the form of hemin), but not conjugated heme (in the form of hemoglobin) could be used to augment biofilms in the gelE mutant. Moreover, the inability of the gelE mutant to augment biofilms in the presence of USA300LAC (Fig. 7) suggests...
that heme produced by USA300LAC is also in a conjugated hemoprotein form. Additional post-hoc testing also revealed that hemin-augmented biofilms of *E. faecalis* ΔgelE were lower than those of WT, likely due to the established role of GelE in *E. faecalis* biofilm [33] that extend beyond its hemoproteolytic activity.

We then selected a subset of *E. faecalis* strains and isolates (20 out of the original 32, and including OGI1RFΔgelE as control) to assay for biofilm augmentation by hemin and hemoglobin. Hemin supplementation augmented biofilms in 17 of the 21 tested strains/isolates (Supplementary Fig. 8 and Supplementary Table 3). The four that showed little to no augmentation by hemin (HCG5A4, HCG9A2, TTSHW-EF12 and TTSHW-EF30) also did not show augmentation with hemoglobin and *S. aureus*, consistent with the requirement for a functional respiratory chain in *E. faecalis* biofilm augmentation by hemoglobin and *S. aureus*. For TTSHW-EF30, intrinsically high biofilm levels (≥ four-fold that of OGI1RF) may have been less amenable to further augmentation. Of the 17 that were augmented by hemin, 10 were also augmented by hemoglobin (Supplementary Fig. 8 and Supplementary Table 3). Of these ten, eight (80%) were gelatinase-positive whereas two (HCG8E9 and TTSHW-EF16) were gelatinase-negative (Supplementary Table 3). In contrast, out of the seven that were augmented by hemin but not by hemoglobin, only VS83 was gelatinase-positive. Together these data suggest that gelatinase activity is important for heme digestion (heme acquisition) but may not be the exclusive enzyme for this activity and may not be sufficient for heme acquisition.

Of the eight gelatinase-positive strains and isolates that were augmented by both hemin and hemoglobin, five were also augmented by *S. aureus* whereas three were not (Supplementary Fig. 8 and Supplementary Table 3). The three that were not augmented by *S. aureus* (TTSHW-EF9, TTSHW-EF18 and TTSHW-EF28) all produced higher levels of biofilms in the unsupplemented *E. faecalis*-only controls (2.6–2.9× more biofilms than OGI1RF). This implies that the ability to extract heme from hemoglobin does not necessarily result in dual-species biofilm augmentation. Though there appear to be additional factors that contribute to the response of *E. faecalis* clinical isolates to *S. aureus*, it remains clear that gelE facilitates heme acquisition by *E. faecalis* from hemoglobin and *S. aureus* hemoproteins, highlighting a previously unappreciated role for this established virulence factor in *E. faecalis* polymicrobial biofilm formation.

**DISCUSSION**

Despite their co-isolation in biofilm associated infections, the only studies characterizing the molecular interaction between *S. aureus* and *E. faecalis* pertain to vancomycin resistance transfer [55–57]. In this study, we show that *S. aureus* and *E. faecalis* synergize to produce more biofilm and demonstrate that this is dependent on the synthesis of heme by *S. aureus* and cross-feeding by *E. faecalis* to exploit this resource for growth in an oxygen-dependent manner.

To appreciate these findings and provide context to the current literature, it is helpful to understand how *E. faecalis* and *S. aureus* interact with other species. *E. faecalis* produces bacteriocin which reduces growth of *C. perfringens* [21] and also hinders the secretion of botulinum neurotoxin of *C. botulinum* [86]. In oropharyngeal candidiasis, *E. faecalis* bacteriocin, EntV, prevents hyphal morphogenesis of *C. albicans* to suppress hyphal-dependent cytotoxicity [87]. Under iron-limiting conditions, *E. faecalis* secretes L-ornithine which promote siderophore synthesis in *E. coli* [25, 88]. Furthermore, *E. faecalis* suppresses host NF-κB-dependent immune activity to promote the virulence of uropathogenic *E. coli* [89], and in early biofilm stages, AI-2 of *E. faecalis* acts as a chemo-attractant and augments *E. coli* biofilms [90]. *E. faecalis* also promotes biofilm matrix production in *P. aeruginosa* [91] through increasing production of exopolysaccharide Pel. In these studies, the effect of *E. faecalis* on other species is highlighted, yet details on how other species influence *E. faecalis* is conspicuously lacking.

The literature on *S. aureus* interactions with other co-colonizing species is more abundant and has been reviewed [92]. In biofilm-associated infections with *Candida albicans*, *S. aureus* exhibits increased resistance to vancomycin [93], increased mucosal adhesion and pathogenicity [94–96], and increased binding to hyphae via hyphal protein Alp3p [97]. By contrast, *S. aureus* interactions with other bacteria appear competitive: *Staphylococcus epidermidis* and *Bacillus subtilis* interferes with *S. aureus* quorum sensing by inhibiting the accessory gene regulatory (*agr*) system, resulting in suppression of virulence genes [98–100]. Additionally, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae* and lactic acid bacteria (LAB) produces toxic products such as pyocyanin, LasA and hydrogen peroxide (H₂O₂) to inhibit the growth of *S. aureus* [101–104]. LAB also inhibit the colonization of *S. aureus* by competing for host adhesion sites [105]. As countermeasures, *S. aureus* forms small-colony variants to counter *P. aeruginosa* toxins and produces bacteriocins to inhibit LAB [106, 107]. By contrast, *S. aureus* promotes colonization of *Haemophilus influenzae* within the nasal cavity through hemolysis of erythrocytes and the subsequent release of heme and NAD that drives *H. influenza* growth [108, 109]. Notably, *S. aureus* responds to hemoglobin in nasal secretions to increase colonization by dampening *agr* expression [110].

One challenge in microbial ecology is in understanding how strain differences contribute to mixed species interactions. Here we show that most *S. aureus* strains augment *E. faecalis* biofilm formation but that the converse is not true. Of the 16 tested *S. aureus* strains grown with *E. faecalis*, only Newman and C37 did not show any biofilm augmentation. In the case of Newman, supernatants inhibit biofilm formation by other *S. aureus* strains via a proteinase-sensitive, heat-tolerant and soluble protein [111]. It is possible that this same biofilm-inhibitory protein could also interfere with *E. faecalis* biofilm formation giving rise to antagonism such that dual-species biofilm levels were lower than *E. faecalis*-only biofilms. Of 31 *E. faecalis* strains tested, only five were amenable to biofilm augmentation in dual-species biofilms.
For several strains, this may be due to their intrinsically lower biofilm producing capacity in the first place. However, the majority appeared to be able to form biofilms yet behaved differently from OG1RF under the conditions tested and restricting the generalizability of dual-species augmentation. This emphasizes the importance of strain differences and raises questions about intrinsic differences in heme uptake and/or aerobic respiration capacity in different E. faecalis strains.

The majority of transposon insertion mutants identified as deficient in undergoing biofilm augmentation in the presence of S. aureus were in menA (1,4-dihydroxy-2-naphthoate isoprenyltransferase, which converts 1,4-dihydroxy-2-naphthoate to demethylmenaquinone) and cydA (cytochrome d ubiquinol oxidase subunit I), both of which encode proteins involved in oxidative respiration [60]. For respiration to occur in E. faecalis, oxygen and heme are required [60, 112], the latter serving as an essential cofactor for cytochrome bd (made up of CydA, CydB and three moieties of cofactor heme) and which acts as the terminal demethylquinolin (DMKH2) oxidase, generating H2O from O2 and establishing a proton motive force to generate ATP [113]. Since E. faecalis is unable to produce porphyrins required to synthesize heme [78], heme must be supplied exogenously. In our experiments, heme was supplied in the form of hemin (free heme) or hemoglobin (protein-conjugated heme). Both hemin and hemoglobin augmented E. faecalis biofilms only underoxic conditions, leading to the conclusion that heme augments E. faecalis biofilms by enabling aerobic respiration. Clinically, this could translate to dual-species biofilm being more prominent in sites where oxygen is available, such as superficial wounds, and less of a concern in anoxic regions like the bladder [114, 115].

Through a very different mechanism, the shift from anaerobic to aerobic respiration because of interspecies interactions has been described for Aggregatibacter actinomyctetomitis. Though primarily fermentative, the opportunistic oral pathogen A. actinomycetomitis switched to respiratory metabolism when grown in the presence of Streptococcus gordonii in response to enhanced O2 bioavailability during coinfection [116]. Although it was not determined how O2 levels were augmented, the assumed provision of electron-acceptors (O2) from S. gordonii (which requires O2 to produce H2O2) resulted in increased A. actinomycetomitis growth and persistence, giving rise to the term “cross-respiration”. Opposite to this, S. aureus has been shown to increase biofilms when grown in anoxic conditions via a SrrAB-dependent programmed cell lysis, whereas the inactivation of the heme production (hemB) permitted greater biofilm growth underoxic conditions [83]. Differences between the previous S. aureus study and ours could be due to differences in biofilm assay conditions, in particular the duration of incubation (22 hr used previously compared to 5 d used in our study).

Although exogenous heme utilization has been established for both oxidative respiration and catalase (KatA) stability and activity in E. faecalis [117, 118], no heme import machinery has been defined. Incidental to our main findings is the discovery that cydDC is essential for heme import. Previously shown to be essential for cytochrome bd assembly in E. coli, the CydDC proteins are ABC transporters that export redox-active thiol compounds such as cysteine and glutathione [119–122]. In E. coli, it has also been suggested that CydDC may bind heme to enhance ATP hydrolysis and thiol export [78, 123–125]. Given that no intracellular heme could be detected in E. faecalis cydD or cydC mutants cultured in heme-supplemented media, we propose that the CydDC heterodimer may also function as a key, if not the sole, heme importer in this species. However, others have suggested that CydDC is not involved in heme import or cytochrome assembly based on everted E. coli membrane vesicles involving a cydD1 point mutant that showed similar patterns of hemin uptake as those in the parental strain and may instead be important for maintenance of a suitable redox environment in the periplasm for conversion to heme d [126, 127]. Further work is needed to rule out the possibility that cytochrome bd assembly requires CydDC, which could also impact heme levels in the membrane, or that other import mechanisms may exist since heme-dependent KatA activity has been described in cydABDC deletion mutants [78]. Moreover, we only performed heme quantification on whole-cell lysates, and fractionation experiments could ascertain if heme is internalized into the cytosol or directly incorporated into the cell membrane.

Loss-of-function mutations to the cydABDC operon are enriched in E. faecalis isolates resistant to killing in the hemoglobin-rich environment of whole blood [128] and this has been attributed to the induction of respiration in parental strain of E. faecalis in the presence of heme which increased vulnerability to neutrophil killing [65]. It is therefore possible, in the context of host infection, that only within the immune-shielded environment of biofilms can heme utilization by E. faecalis safely take place without concomitant increasing their susceptibility to immune clearance - hence the importance of E. faecalis augmenting biofilms in response to heme.

In vitro, it is unclear why E. faecalis augmentation by S. aureus occurs in biofilms but not planktonic cells. It has been reported by others (and our own studies agree, data not shown) that E. faecalis planktonic growth is unaffected by heme supplementation [118]. We speculate that the spatially confined microenvironment of biofilms, or the unique transcriptional profile of biofilm cells, results in sensitivity to heme-based growth augmentation.

Biofilm augmentation of many E. faecalis strains and isolates by heme but not by hemoglobin or heme-producing USA300LAC suggests that an important secondary E. faecalis determinant is involved in this mixed species interaction. Comparative genomics led us to identify GelE as required for E. faecalis OG1RF biofilm augmentation in the presence of hemoglobin and USA300LAC. Free heme could augment biofilm formation in the gelE deletion mutant, but this strain was unable to extract heme from hemoglobin or S. aureus hemoproteins. Together with its established role in biofilms, GelE has previously been shown to hydrolyse hemoglobin [129] which would result in heme release and subsequent import, potentially by CydDC, and incorporation into cytochrome bd of E. faecalis to allow aerobic respiration to augment biofilms. We speculate that S. aureus hemoproteins, whether secreted or released during cell death, may likewise need to be hydrolysed for heme release and subsequent uptake by E. faecalis.

In summary, we show that augmentation of S. aureus and E. faecalis dual-species biofilms requires the biosynthesis of heme by S. aureus which facilitates oxidative respiration in E. faecalis. In most cases, successful cross-feeding of heme (likely in the form of secreted hemoproteins) will require gelatinase-mediated heme acquisition by E. faecalis. It is conceivable that the increased energy derived from aerobic respiration enables production of complex and “expensive” extracellular biofilm-associated proteins like EpaOX or surface-anchored pili (EbpABC) [130]. Since epaOX (along with fslr, gelE and epal) correlate with biofilm-associated antibiotic resistance [75], the potential impact of this inter-species interaction on antibiotic efficacy is worth considering, especially since this has been demonstrated for other mixed species interactions [131, 132]. The findings of this study highlight the importance of understanding inter-species interactions in biofilms and underscore the usefulness of identifying potentiating determinants, like heme, to develop interventions relevant in a complex host setting.

REFERENCES
1. Watnick P, Kolter R. Biofilm, city of microbes. J Bacteriol. 2000;182:2675–9.
2. Yin W, Wang Y, Liu L, He J. Biofilms: the microbial “protective clothing” in extreme environments. Int J Mol Sci. 2019;20:3423.
3. Islam N, Kim Y, Ross JM, Marten MR. Proteomic analysis of Staphylococcus aureus biofilm cells grown under physiologically relevant fluid shear stress conditions. Proteome Sci. 2014;12:21.
4. Thomen P, Robert J, Monmeyran A, Bitbol AF, Douarche C, Henry N. Bacterial biofilm under flow: first a physical struggle to stay, then a matter of breathing. PLoS One. 2017;12:1–24.
5. Debaring S, Pender-Vandervelde M, Scheidegger A, Pronk W, Morgenroth E. Predation influences the structure of biofilm developed on ultratransparent membranes. Water Res. 2012;46:3323–33.
6. Sharma D, Misra L, Khan AU. Antibiotics versus biofilm: an emerging battleground in microbial communities. Antimicrob Resist Infect Control. 2019;8:76.
7. Solokhina A, Bruckner D, Bonkat G, Braissant O. Metabolic activity of mature biofilms of Mycobacterium tuberculosis and other non-tuberculous mycobacteria. Sci Rep. 2017;7:29225.
8. Wang N, Wang H, Ng CK, Mukherjee M, Ren D, Cao B, et al. Bacterial metabolism during biofilm growth investigated by 13C tracing. Front Microbiol. 2018;9:2657.
9. Qi Z, Chen L, Zhang W. Comparison of transcriptional heterogeneity of eight genes between batch Desulfovibrio vulgaris biofilm and planktonic culture at a single-cell level. Front Microbiol. 2016;7:597.
10. Nett JE, Lepak AJ, Marchillo K, Andes DR. Time course global gene expression analysis of in vivo Candida biofilm. J Infect Dis. 2009;200:307–13.
11. Ghigo JM. Natural conjugative plasmids induce bacterial biofilm development. Nature. 2001;412:442–5.
12. Molin S, Tolker-Nielsen T. Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stalk formation of the biofilm structure. Curr Opin Biotechnol. 2003;14:245–61.
13. Gabriška RA, Rumbaugh KP. Biofilm models of polymicrobial infection. Future Microbiol. 2015;10:1997–2015.
14. Toyofuku M, Inaba T, Kiyokawa T, Obana N, Yawata Y, Nomura N. Environmental factors that shape biofilm formation. Biosci Biotechnol Biochem. 2016;80:7–12.
15. Lori C, Ozaki S, Steiner S, Bohn R, Abel S, Dubey BN, et al. Cyclic di-GMP acts as a cell cycle oscillator to drive chromosome replication. Nature. 2015;523:236–9.
16. Mills E, Petersen E, Kulaška RE, Miller SI. A direct screen for c-di-GMP modulators reveals a Salmo nea Typhimurium periplasmic c-arginine-sensing pathway. Sci Signal. 2015;8:ra57.
17. Ahmed NA, Petersen FC, Scheie AA. Al2+/LuxS is involved in increased biofilm formation by Streptococcus intermedius in the presence of antibiotics. Antimicrob Agents Chemother. 2009;53:4285–63.
18. Antonova ES, Hammer BK. Quorum-sensing autoinducer molecules produced by members of a multispecies biofilm promote horizontal gene transfer to Vibrio cholerae. FEMS Microbiol Lett. 2011;322:68–76.
19. Chavez de Paz LE, Lemos JA, Wickstrom C, Sedgley CM. Role of (p)ppGpp in biofilm formation by Enterococcus faecalis. Appl Environ Microbiol. 2012;78:1627–30.
20. Abranches J, Martinez AR, Kajfasz JK, Chavez V, Garsin DA, Lemos JA. The molecular alarmone (p)ppGpp mediates stress responses, vancomycin tolerance, and virulence in Enterococcus faecalis. J Bacteriol. 2009;191:2248–56.
21. Han SK, Shin MS, Park HE, Kim SY, Lee WK. Screening of bacteriocin-producing Enterococcus faecalis strains for antagonistic activities against Clostridium perfringens. Korean J Food Sci Anim Resour. 2014;43:614–21.
22. Lemme A, Grobe D, Reck M, Tomasch J, Wagner-Dobler I, et al. Cyclic di-GMP as a determinant of competence-stimulating-peptide-induced Streptococcus mutans. J Bacteriol. 2011;193:1863–77.
23. van der Ploeg JR. Regulation of bacteriocin production in Streptococcus mutans by the quorum-sensing system required for development of genetic competence. J Bacteriol. 2005;187:3980–9.
24. Kang D, Kirienko NV. Interdependence between iron acquisition and biofilm formation in Pseudomonas aeruginosa. J Bacteriol. 2018;196:449–57.
25. Keogh D, Tay WH, Ho YY, Dale JL, Chen S, Umashanker S, et al. Enterococcal metabolite cues facilitate interspecies niche modulation and polymicrobial infection. Cell Host Microbe. 2016;20:493–503.
26. Kubota H, Senda S, Nomura N, Tokuda H, Uchiyama H. Biofilm formation by lactic acid bacteria and resistance to environmental stress. J Biosci Bioeng. 2008;106:381–6.
27. Ahn KB, Baik JE, Yun CH, Han SH. Lipoteichoic acid inhibits Staphylococcus aureus biofilm formation. Front Microbiol. 2018;9:327.
28. Fabretti I, Theilacker C, Baldassarri L, Kaczynski Z, Kropec A, Holst O, et al. Alanine esters of enterococcal lipoteichoic acid play a role in biofilm formation and resistance to antimicrobial peptides. Infect Immun. 2006;74:4164–71.
29. Wu H, Zeng M, Fives-Taylor P. The glycan moieties and the N-terminal poly-peptide backbone of a fimbria-associated adhesin, Fap1, play distinct roles in the biofilm development of Streptococcus parasanguinis. Infect Immun. 2007;75:2181–8.
30. Lee J, Jayaraman A, Wood TK. Indole is an inter-species biofilm signal mediated by SdaA. BMC Microbiol. 2007;7:42.
70. O
80. Winstedt L, Yoshida K, Fujita Y, von Wachenfeldt C. Cytochrome...
67. Fey PD, Endres JL, Yajjala VK, Widhelm TJ, Boissy RJ, Bose JL, et al. A genetic...
61. Baum RH, Dolin MI. Isolation of 2-solanesyl-1,4-naphthoquinone from...
62. Ritchey TW, Seely HW Jr. Distribution of cytochrome-like respiration in strep...
66. Kristich CJ, Nguyen VT, Le T, Barnes AM, Grindle S, Dunny GM. Development and...
76. Garsin DA, Urbach J, Huguet-Tapia JC, Peters JE, Ausubel FM. Construction of an...
77. Huycke MM, Moore D, Joyce W, Wise P, Shepard L, Kotake Y, et al. Extracellular...
58. Gilmore MS, Salamzade R, Selleck E, Bryan N, Mello SS, Manson AL, et al. Genes...
64. Huycke MM, Moore D, Joyce W, Wise P, Shepard L, Kotake Y, et al. Extracellular...
73. Vanhommerig E, Moons P, Pirici D, Lammens C, Hernalsteens JP, De Greve H, ...
40. – Toole GA, Pratt LA, Watnick PI, Newman DK, Weaver VB, Kolter R. Genetic...
17. – Hammer ND, Skaar EP. Powerful genetic resource for the study of methicillin-
20. – Haemophilus influenzae. J Bacteriol. 1983;80:5369–73.
86. Kruger M, Shehata AA, Schrodl W, Rodloff A. Glyphosate suppresses the...
13. – Keys to the understanding of phagocytosis. J Leukoc Biol. 2006;80:581–5.
22. – Haemophilus influenzae in the presence of haematin. J Gen Microbiol. 1978;104:15–20.
88. – Vermont. J. Gen Microbiol. 1976;93:195–203.
75. Dale JL, Cagnazzo J, Phan CQ, Barnes AM, Dunny GM. Multiple roles for...
33. – lms. Methods Enzymol. 1999;310:91–105.
34. – S. aureus and E. coli. Proc Natl Acad Sci USA. 1983;80:5369–73.
84. – Craig RA, White BA, Yagi Y, Clewell DB. Modification of Streptococcus...
2. – S. aureus by a M-variant superoxide dismutase. J Bacteriol. 1997;179:4706–12.
82. – ND, Skaar EP. Powerful genetic resource for the study of methicillin-
40. – Toole GA, Pratt LA, Watnick PI, Newman DK, Weaver VB, Kolter R. Genetic...
17. – Keys to the understanding of phagocytosis. J Leukoc Biol. 2006;80:581–5.
competition and interactions with host's immune response. BMC Microbiol. 2010;10:59.

109. Artman M, Domenech E, Weiner M. Growth of Haemophilus influenzae in simulated blood cultures supplemented with hemin and NAD. J Clin Microbiol. 1983;18:376–9.

110. Pymonen M, Stephenson RE, Schwartz K, Hernandez M, Boles BR. Hemoglobin promotes Staphylococcus aureus nasal colonization. PLoS Pathog. 2011;7:e1002104.

111. Cue D, Junecoo JM, Lei MG, Blevins JS, Smeltzer MS, Lee CY. SaeRS-dependent inhibition of biofilm formation in Staphylococcus aureus Newman. PLoS One. 2015;10:e0123027.

112. Ritchey TW, Seeley HW. Cytochromes in Streptococcus faecalis var. zymogenes grown in a haematin-containing medium. J Gen Microbiol. 1974;85:220–8.

113. Hederstedt L, Gorton L, Pankratova G. Two routes for extracellular electron transfer in Enterococcus faecalis. J Bacteriol. 2020;202:e00782–19.

114. Martin-Gutierrez G, Rodriguez-Beltran J, Rodriguez-Martinez JM, Costas C, Aznar J, Pascual A, et al. Urinary tract physiological conditions promote ciprofloxacin resistance in low-level-quinolone-resistant Escherichia coli. Antimicrob Agents Chemother. 2016;60:4252–8.

115. Giannakopoulou X, Evangelou A, Kalfakakou V, Gramenitis E, Papandropoulos I, Charalambopoulos K. Human bladder urine oxygen content: implications for urinary tract diseases. Int Urol Nephrol. 1997;29:393–401.

116. Stacy A, Fleming D, Lamont RJ, Rumbaugh KP, Whiteley M. A commensal bacterium promotes virulence of an opportunistic pathogen via cross-respiration. mBio. 2016;7:e00782–16.

117. Frankenberg L, Brugna M, Hederstedt L. Enterococcus faecalis heme-dependent catalase. J Bacteriol. 2002;184:6351–6.

118. Bauredry M, Barane E, Hederstedt L. In vitro assembly of catalase. J Biol Chem. 2014;289:28411–20.

119. Yamashita M, Shepherd M, Booth WI, Xie H, Postis V, Nyathé Y, et al. Structure and function of the bacterial heterodimeric ABC transporter CydABC: stimulation of ATPase activity by thiol and heme compounds. J Biol Chem. 2014;289:23177–88.

120. Hederstedt L, Barane E, Bauredry M. In vitro assembly of catalase. J Biol Chem. 2014;289:28411–20.

121. Pittman MS, Corker H, Wu G, Binet MB, Moir AJ, Poole RK. Cysteine is exported from the Escherichia coli cytoplasm by CydABC, an ATP-binding cassette-type transporter required for cytochrome assembly. J Biol Chem. 2002;277:49841–9.

122. Shepherd M. The CydABC ABC transporter of Escherichia coli: new roles for a reductant efflux pump. Biochem Soc Trans. 2015;43:908–12.

123. Borisov VB, Gennis RB, Hemp J, Verkhovsky ML. The cytochrome b respiratory oxygen reductases. Biochim Biophys Acta. 2011;1807:1398–413.

124. Bauredry M, Hederstedt L. Heme proteins in lactic acid bacteria. Adv Micro Physiol. 2013;62:1–43.

125. Poole RK, Cozens AG, Shepherd M. The CydDC family of transporters and their roles in oxidase assembly and homeostasis. Adv Micro Physiol. 2015;66:1–53.

126. Goldman BS, Kranz RG. ABC transporters associated with cytochrome c biogenesis. Res Microbiol. 2019;170:407–16.

127. Cook GM, Poole RK. Oxidase and periplasmic cytochrome assembly in Escherichia coli K-12: CydDC and CymAB are not required for haem-membrane association. Microbiol (Read). 2000;146:527–36.

128. Van Tyne D, Manson AL, Huycke MM, Karanicolas J, Earl AM, Gilmore MS. Impact of antibiotic treatment and host innate immune pressure on enterococcal adaptation in the human bloodstream. Sci Transl Med. 2019;11:eaat8418.

129. Makinen PL, Clewell DB, An F, Makinen KK. Purification and substrate specificity of a strongly hydrophobic extracellular metalloendopeptidase ("gelatinase") from Streptococcus faecalis (strain OG1-R). J Biol Chem. 1989;264:3325–34.

130. Smith DR, Chapman MB. Economical evolution: microbes reduce the synthetic cost of extracellular proteins. mBio. 2010;1:e00031–10.

131. Radlinski L, Rowe SE, Karchtnner LB, Maire R, Caims BA, Vitko NP, et al. Pseudomonas aeruginosa exoproduits determine antibiotic efficacy against Staphylococcus aureus. PLoS Biol. 2015;13:e1002081.

132. Little W, Black C, Smith AC. Clinical implications of polymicrobial synergism effects on antimicrobial susceptibility. Pathogens. 2021;10:144

ACKNOWLEDGEMENTS

We are grateful to the following people for input and resources provided: Eric Skaar for input on the study, Yap Shao Jun Nicole for contributing unpublished data to the initial characterization of the interaction, and Singapore Phenome Center (Tham Wai Kin, Cheah Yeong Cheng, Liang Xu) for measurement of heme. Bacterial strains, isolates, mutants, and transposon mutant libraries were generously shared with us by Gary Dunny, Jennifer Dale, Haiyin V. Nielsen, Scott J. Hultgren, Philip I. Tarr, Stéphane Mesnager, Alexander R. Horswill, Yang Liang, Eric P. Skaar, Lynn Hancock, Barnes-Jewish Hospital microbiology laboratory in St. Louis, Tan Tock Seng Hospital in Singapore, American Type Culture Collection, Network on Antimicrobial Resistance in Staphylococcus aureus (NARSA), BEI resources and NIAID.

AUTHOR CONTRIBUTIONS

KKLC and KAK conceived the study. JHC, KKLC, and KAK designed the experiments. JHC and MM conducted and analysed all experiments, with contributions from JIW, CAZT, ZJSK, AM, ZIN and DL. KAK supervised the research. TMSB provided clinical isolates. YW guided the mass spectrometry analyses. JHC wrote the manuscript, with contributions from KKLC, MM, JIW, CAZT, AM, and KK. All co-authors critically reviewed and approved the submitted manuscript.

COMPETING INTERESTS

This work was carried out at the Singapore Centre for Environmental and Life Science Engineering (SCELSE), whose research is supported by the National Research Foundation Singapore, Ministry of Education, to Nanyang Technological University and the National University of Singapore under its Research Centre of Excellence Programme. This work was also supported by supported by The Lee Foundation Green, Department of Surgery, Yong Loo Lin School of Medicine, National University of Singapore, Singapore.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41396-022-01248-1.

Correspondence and requests for materials should be addressed to Jun-Hong Ch’ng or Kimberly A. Kline.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party materials in this article are included in the article’s Creative Commons license and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2022