

**Supplementary Information for**  
**Restriction of Arginine Induces Antibiotic Tolerance in**  
***Staphylococcus aureus***

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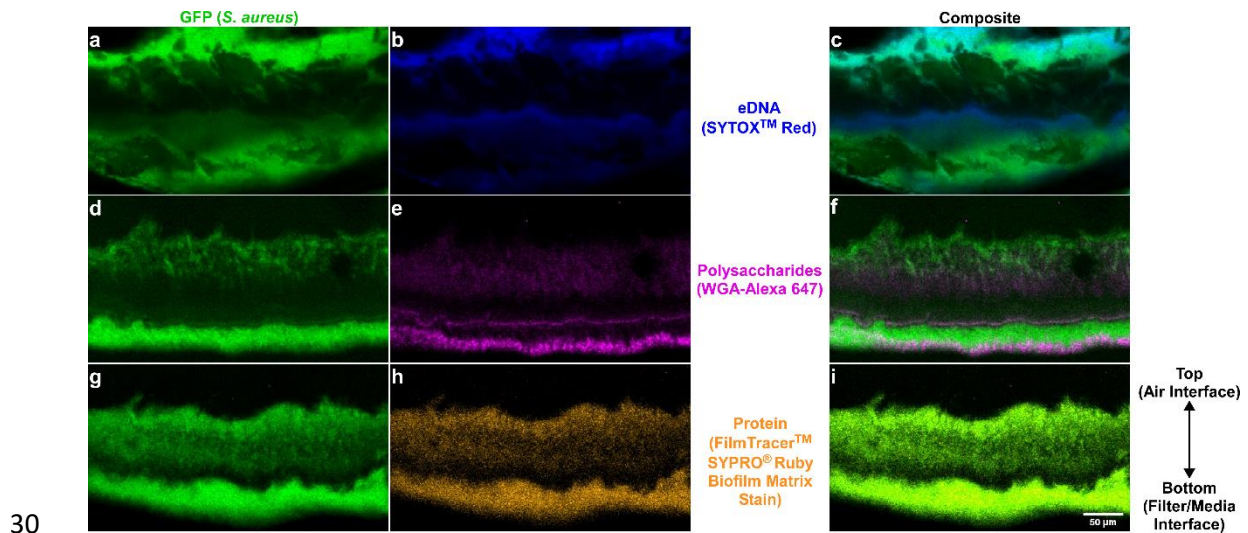
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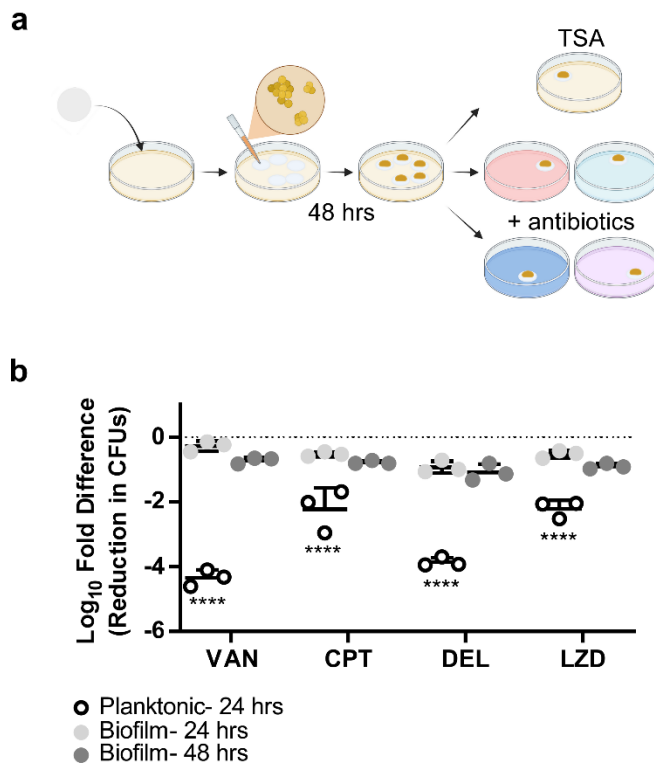
Figures S1 to S12

29 **Figure S1**



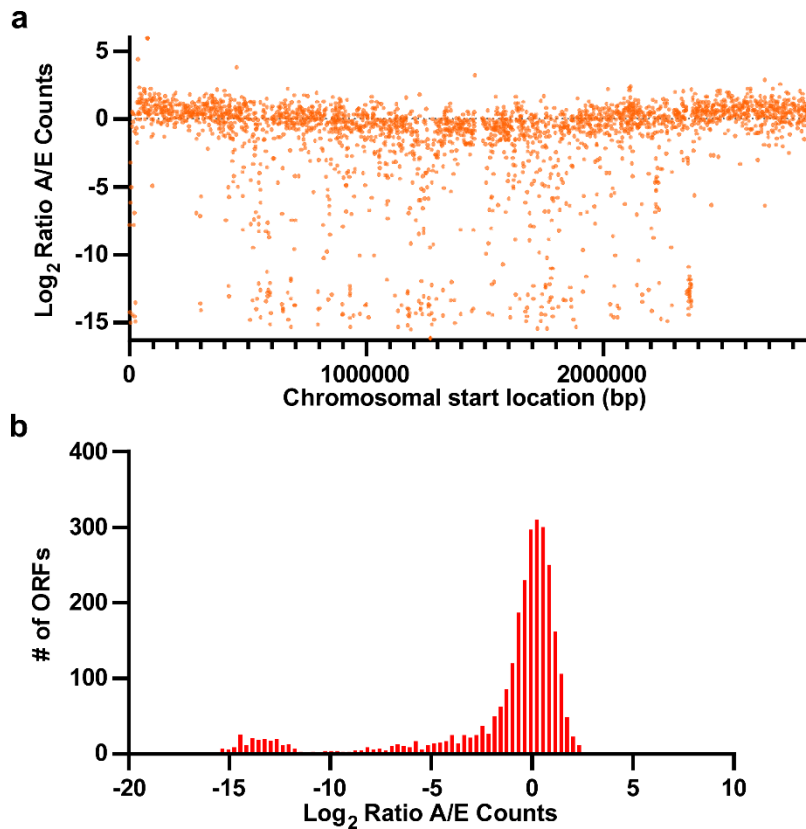
31 **Figure S1. Colony filter biofilms have a complex extracellular matrix consisting of eDNA,**  
 32 **polysaccharides, and protein.** A *S. aureus* USA300 with constitutive GFP expression was grown  
 33 on polycarbonate filters on TSA plates to form colony filter biofilms. After 48 hours of growth the  
 34 filter biofilms were embedded in a CMC-gelatin matrix and stained with either SYTOX™ Red (**a-c**),  
 35 WGA-Alexa 647 (**d-f**), or FilmTracer™ SYPRO® Ruby biofilm matrix stain (**g-i**). The embedded  
 36 and stained biofilms were frozen prior to sectioning. Cross sections of the biofilms were imaged  
 37 using confocal microscopy.

38 **Figure S2**



39 **Figure S2. Colony filter biofilms exhibit increased levels of antibiotic tolerance.** **a** *S. aureus*  
 40 strain JE2 was grown for 48 hours on polycarbonate filters on TSA plates before being transferred  
 41 to TSA plates with or without antibiotics added at the indicated concentrations. **b** Biofilms were  
 42 homogenized after 24 and 48 hours of antibiotic exposure and plated to determine CFUs remaining  
 43 versus immediately prior to antibiotic exposure. Data represent technical replicates of biological  
 44 triplicates. The corresponding reduction in CFUs for a planktonic culture (mid-exponential phase)  
 45 that was exposed to the same concentration of antibiotics for 24 hours is also superimposed. The  
 46 difference in the reduction in CFUs between planktonic and biofilm cultures after 24 hours of  
 47 antibiotic exposure was tested for significance using a 2-way ANOVA with Šídák multiple  
 48 comparisons test; \*\*\*\*= $p < 0.0001$ . The reduction in CFUs after 48 hours in biofilm culture is shown  
 49 for reference, but no statistical analysis was performed on 48 hour timepoints. Figure S2/panel a  
 50 Created with BioRender.com released under a Creative Commons Attribution-NonCommercial-  
 51 NoDerivs 4.0 International license (<https://creativecommons.org/licenses/by-nc-nd/4.0/deed.en>).

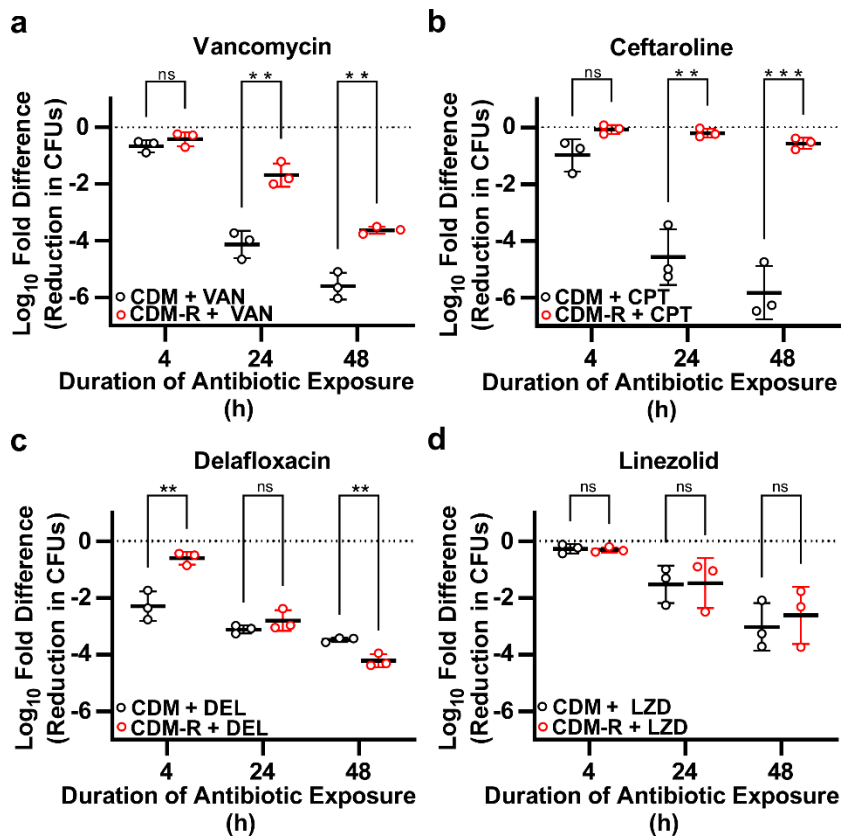
53 **Figure S3**



54

55 **Figure S3. Analysis of high density TnSeq library.** Analysis of the TnSeq library generated for  
 56 showed 150,000 independent transposon insertions covering nearly 55% of all TA sites including  
 57 at least 1 TA site in 2619 out of 2807 annotated open reading frames in the *S. aureus* USA300  
 58 FPR\_3757 genome. **a** Log<sub>2</sub> transformed ratios of the actual number of sequencing reads per  
 59 gene/expected number of sequencing reads (A/E) is shown for each annotated open reading frame  
 60 across the *S. aureus* USA300 FPR\_3757 genome (A/E ratio). **b** Histogram showing the distribution  
 61 of the Log<sub>2</sub> transformed A/E counts for all 2807 annotated open reading frames.

62 **Figure S4**

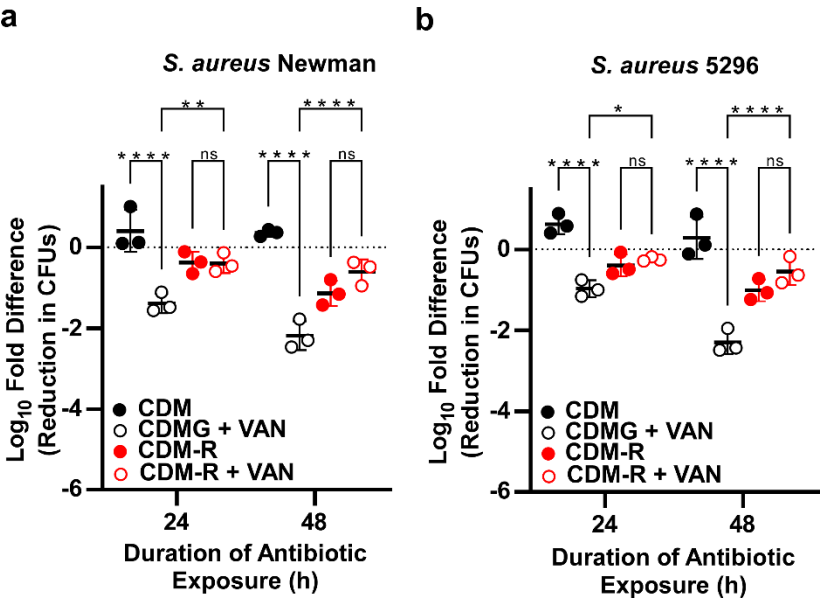


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64 **Figure S4. Induction of antibiotic tolerance is more variable during planktonic growth.**

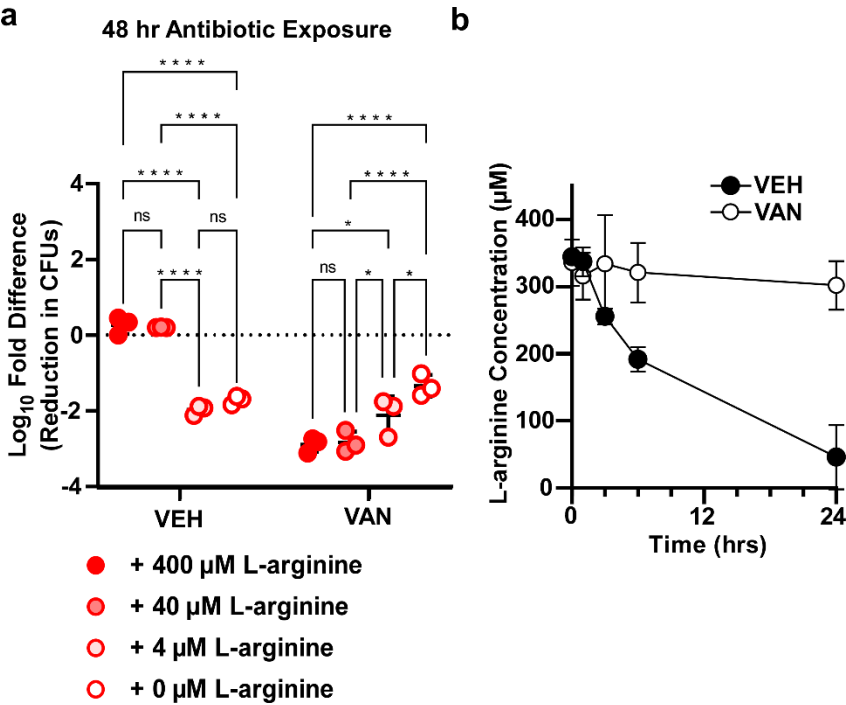
65 Overnight cultures of JE2 were diluted 1:100 in CDM broth and grown to mid-log phase before  
 66 being washed, split into fresh CDM or CDM-R broth with or without the addition of **a** vancomycin,  
 67 **b** ceftaroline, **c** delafloxacin, or **d** linezolid, and grown for an additional 48 hours under planktonic  
 68 growth conditions. Data represent technical replicates of biological triplicates, mean  $\pm$  SD shown.  
 69 Two-sided Student T-test; \*= $p < 0.05$ , \*\*= $p < 0.005$ , \*\*\*= $p < 0.0005$ , \*\*\*\*= $p < 0.0001$ , ns=not significant,  
 70 VAN=vancomycin (400  $\mu$ g/ml), CPT=ceftaroline (20  $\mu$ g/ml), DEL=delafloxacin (9  $\mu$ g/ml),  
 71 LZD=linezolid (20  $\mu$ g/ml).

**Figure S5**



**Figure S5. Arginine deprivation induces tolerance in multiple strains of *S. aureus*.** The MSSA strain Newman **a** and the MRSA clinical isolate 5296 **b** were grown for 48 hours on polycarbonate filters on CDM agar plates before being homogenized and transferred to liquid CDM or CDM-R media with or without vancomycin added at a concentration of 400  $\mu\text{g/ml}$ . Data represent technical replicates of biological triplicates, mean  $\pm$  SD shown. 2-way ANOVA with Tukey multiple comparisons test; \* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.0005$ , \*\*\*\* $p < 0.0001$ , ns=not significant, VAN=vancomycin.

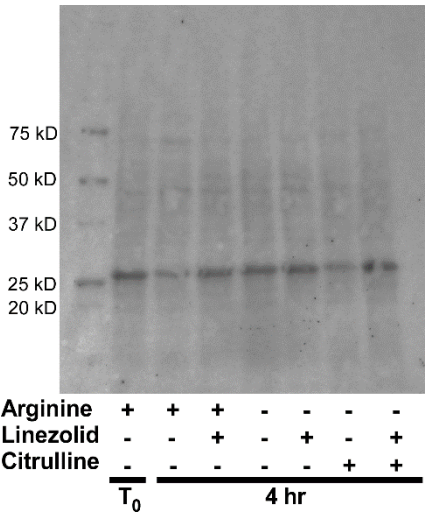
Figure S6



**Figure S6. Arginine influences antibiotic tolerance in a concentration dependent manner. a**

The *S. aureus* strain JE2 was grown for 48 hours on polycarbonate filters on CDM agar plates before being homogenized and transferred to liquid CDM-R media with or without vancomycin added at a concentration of 400 μg/ml. Arginine was added at concentrations ranging from 0 μM to 400 μM (the concentration in full CDM). Data represent technical replicates of biological triplicates. 2-way ANOVA with Tukey multiple comparisons test, mean ± SD shown; \*= $p < 0.05$ , \*\*= $p < 0.005$ , \*\*\*= $p < 0.0005$ , \*\*\*\*= $p < 0.0001$ , ns=not significant, VAN=vancomycin. **b** The depletion of extracellular arginine was measured by sampling extracellular media at various time points from homogenized biofilm samples in CDM with a starting arginine concentration of 400 μM. Data represent biological triplicates, mean ± SD shown.

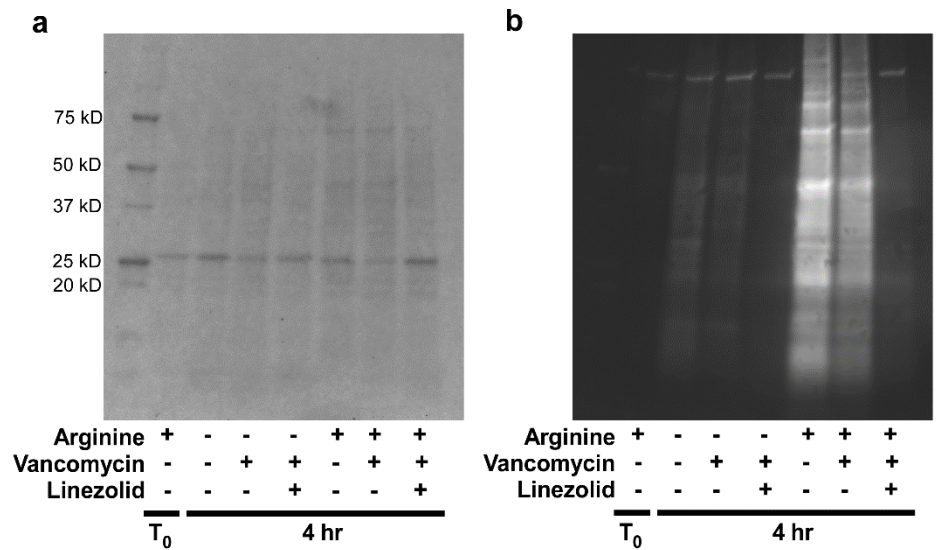
**Figure S7**



**Figure S7. Ponceau S Stain of Total Protein.** Total protein was isolated from homogenized biofilm cultures at T<sub>0</sub> and 4 hrs after incubation with the methionine analog, L-HPG. L-HPG incorporated into nascent protein was labelled with biotin via click chemistry and the resulting protein samples were separated via SDS-PAGE gel. Protein was transferred to a nitrocellulose membrane and prior to western blotting total protein was visualized using a Ponceau S stain. A representative Ponceau S stained membrane is shown here. The first lane contains Bio-Rad Precision Plus Protein Ladder with the individual bands labeled with their respective molecular weights.

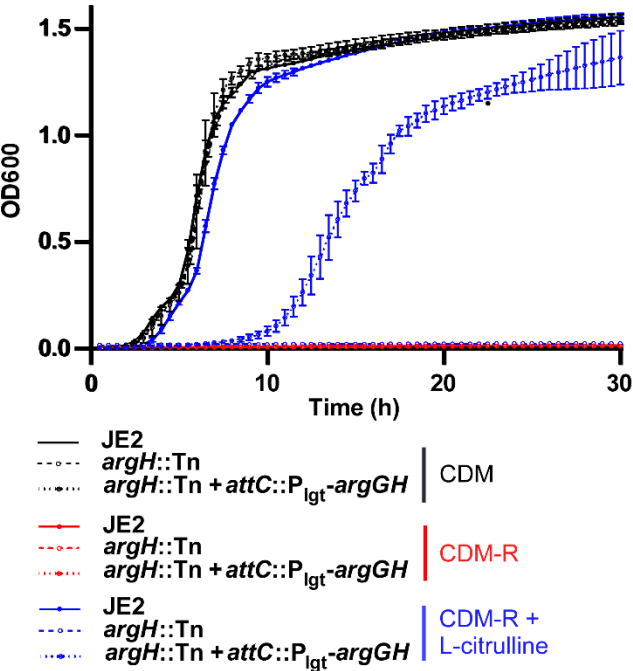


**Figure S8**



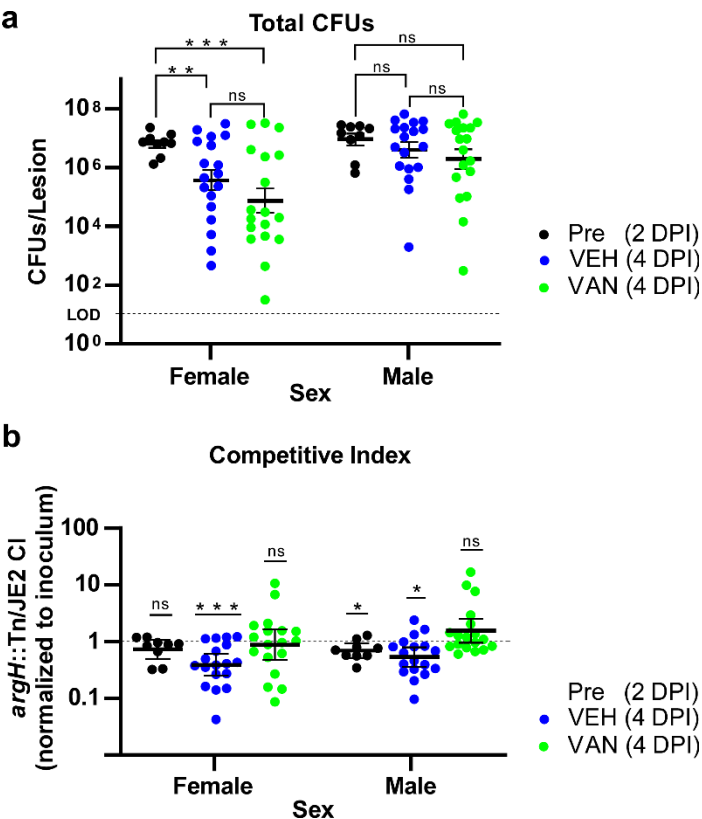
**Figure S8. Nascent protein labeling in the presence of antibiotics.** Total protein was isolated from homogenized biofilm cultures at  $T_0$  and 4 hrs after incubation with the methionine analog, L-HPG in the presence or absence of vancomycin. L-HPG incorporated into nascent protein was labelled with biotin via click chemistry and the resulting protein samples were separated via SDS-PAGE. Protein was transferred to a nitrocellulose membrane and prior to western blotting with an IRDye 680RD Streptavidin probe, total protein was visualized using a Ponceau S stain. A representative Ponceau S stained membrane is shown in **a**, and the corresponding western blot is shown in **b**. The first lane contains Bio-Rad Precision Plus Protein Ladder with the individual bands labeled with their respective molecular weights. Data from this experiment along with two other independent experiments were quantified and are represented in Figure 4E.

Figure S9



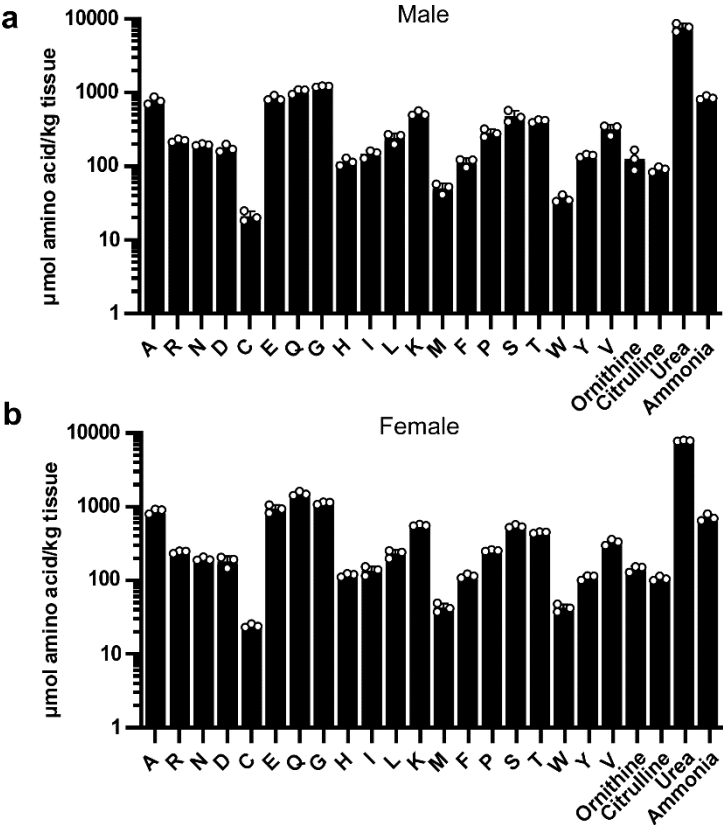
**Figure S9. ArgH is required to utilize citrulline for growth in the absence of arginine.** Growth of JE2 is inhibited in CDM in the absence of arginine, but this auxotrophy can be chemically complemented with the addition of citrulline. An *argH::Tn* mutant, however, is unable to use citrulline to complement an arginine auxotrophy. Data represent biological triplicates, mean  $\pm$  SD shown.

Figure S10



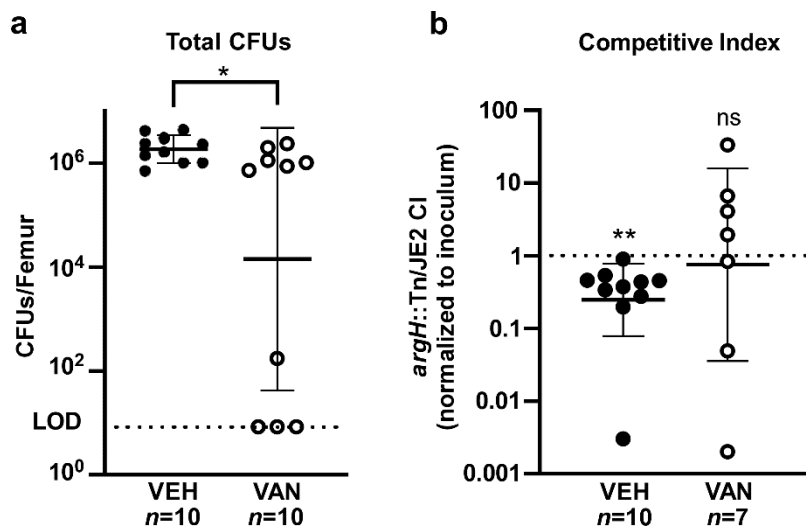
**Figure S10. Differences in the response to vancomycin treatment in a murine SSTI model by sex.** Both male and female C57BL/6 mice were infected with a mixture of JE2 and *argH*::Tn using a superficial skin infection model of a SSTI. **a** Infected female mice exhibited significant decreases in CFUs between the 2 and 4 DPI, regardless of treatment group, while males only showed a significant decrease in CFUs between 2 and 4 DPI for the vehicle control group. **b** Changes in the competitive index (CI) were similar between the two sexes with both males and females showing a numerical increase in the CI to favor the mutant during vancomycin treatment, and this difference was significant for males. Data are combined from three independent experiments with a total of 9 mice per sex at 2 DPI and 18 mice per sex for each of the 4 DPI treatment groups. **a** Two-sided Multiple T-test with Holm-Šídák multiple comparisons test, mean  $\pm$  SD shown. **b** Two-sided Wilcoxon Signed Rank Test, median with 95% CI shown; \*= $p$ <0.05, \*\*= $p$ <0.005, \*\*\*= $p$ <0.0005 ns=not significant, VEH= no antibiotic vehicle control, VAN=vancomycin, DPI=days post infection.

Figure S11



**Figure S11. Concentrations of amino acids in exposed mouse skin.** **a** Male and **b** female C57BL/6 mice were euthanized directly after tape-stripping in preparation for a superficial skin infection, prior to inoculation with bacteria. The newly exposed skin lesions were sent for full amino acid profiling. Data represent the averages from 3 mice for each sex. Mean  $\pm$  SD shown.

Figure S12



**Figure S12. ArgH contributes to antibiotic tolerance in a murine osteomyelitis model.** Female C57BL/6 mice were infected with a 2:1 mixture of JE2 and *argH*::Tn using a murine osteomyelitis and treatment model. Mouse femurs were harvested after 7 days of treatment with vancomycin (VAN) or a vehicle control (VEH). **a** Total CFUs recovered per femur from each group. Three mice in the vancomycin treated group did not have any detectable bacteria in the femurs, suggesting clearance of the infection. **b** Competitive indices were calculated for the mice with recoverable bacteria (10 in the vehicle treated group and 7 in the vancomycin treated group). **a** Two-sided Student T-test, mean  $\pm$  SD shown,  $p=0.017$ . **b** Two-sided Wilcoxon Signed Rank Test, geometric mean  $\pm$  95% CI shown; vehicle  $p=0.002$ , vancomycin  $p=0.375$ ;  $*$ = $p<0.05$ ,  $**$ = $p<0.005$ , ns=not significant, VEH= no antibiotic vehicle control, VAN=vancomycin.