Table S1

Strain or plasmid	Description	Source or reference
Strains:		
***S. aureus*:**		
USA300 LAC	Community associated MRSA; wild type strain cured of resistance plasmids	(1)
RN4220	r_K^- m_K^+; capable of accepting foreign DNA	(2)
USA300Δ*graS*	USA300 with markerless *graS* deletion	(3)
USA300Δ*farER*	USA300 with markerless *farER* deletion	(4)
USA300Δ*fakA*	USA300 with markerless *fakA* deletion	(4)
USA300Δ*pro* (Δ*sspABC-aur::lacZ)*	USA300 deficient in Aureolysin metalloprotease and *sspABC* serine protease operon; *Erm*^{r} *Tc*^{r}	(1)
USA300*mprF*::*Tn*	φNΣ1360 allele from Nebraska transposon library transduced into USA300 LAC*; Erm*^{r}	(3, 5)
E. coli:		
DH5α	F$^-$ Φ80*lacZ*ΔM15 *recA1 endA1 gyrA96 thi-1 hsdR17* ($r_K^-m_K^+$) *supE44 relA1 deoR* Δ(*lacZYA-argF*)*U169 phoA*	Invitrogen
Plasmids:		
pALC2073	Shuttle vector used for expression of genes under control of tetracycline-inducible $P_{xyl/tetO}$ promoter in *S. aureus*; genes are expressed at a basal level in absence of induction	(6)
p*graS*	Promoterless *graS* gene under transcriptional control of $P_{xyl/tetO}$ promoter of pALC2073	(3)

p*graS*$^{3D>G}$	p*graS* after mutagenesis with *graS*-SDM-F and *graS*-SDM-R primers; Asp to Gly substitution at D35, D37, and D41	This study
p*mprF*	Promoterless *mprF* gene amplified by PCR with primers *mprF*-pALC-F and *mprF*-pALC-R, and cloned into *BamHI* site of pALC2073	This study
p*mprF*^{K547A}	p*mprF* after mutagenesis with *mprF*-SDM-F and *mprF*-SDM-R primers; Lys to Ala substitution at K547 to inactivate lysyl-transferase activity	This study

Table S2

Oligonucleotide	Description
graS-SDM-F	CATTAGTCTAATCGGTTATGGTTTTCCAATAGGCAGTTTATTTTATATTGTTTC
graS-SDM-R	GAAACAATATAAAATAAACTGCCTATTGGAAAACCATAACCGATTAGACTAATG
mprF-pALC-F	GATTTATAACAGAAAGGATCCGAGGAGGTGTGAAAAAATGAATCAGGAAG
mprF-pALC-R	TTTGGATCCCGCATCAGGCATAACTGT
mprF-SDM-F	GATATATAGTGGTGACGCGCAGTTTTTCACTAATGA
mprF-SDM-R	GCTGTTTTATTTTCATTAGTGAAAAACTGCGCGTCA

References:

1. **Arsic B**, **Zhu Y**, **Heinrichs DE**, **McGavin MJ**. 2012. Induction of the staphylococcal proteolytic cascade by antimicrobial fatty acids in community acquired methicillin resistant *Staphylococcus aureus*. PLoS One **7**.
2. **Novick RP**. 1991. Genetic systems in staphylococci. Methods Enzym **204**:587–636.

3. **Flannagan RS**, **Kuiack RC**, **McGavin MJ**, **Heinrichs DE**. 2018. *Staphylococcus aureus* uses the GraXRS regulatory system to sense and adapt to the acidified phagolysosome in macrophages. MBio **9**:e01143-18.

4. **Alnaseri H**, **Kuiack RC**, **Ferguson KA**, **Schneider JET**, **Heinrichs DE**, **McGavin MJ**. 2019. DNA binding and sensor specificity of FarR, a novel Tetr family regulator required for induction of the fatty acid efflux pump FarE in *Staphylococcus aureus*. J Bacteriol **201**:1–16.

5. **Fey PD**, **Endres JL**, **Yajjala VK**, **Widhelm TJ**, **Boissy RJ**, **Bose JL**, **Bayles KW**. 2013. A genetic resource for rapid and comprehensive phenotype screening of nonessential *Staphylococcus aureus* genes. MBio **4**.

6. **Bateman BT**, **Donegan NP**, **Jarry TM**, **Palma M**, **Cheung AL**. 2001. Evaluation of a tetracycline-inducible promoter in *Staphylococcus aureus in vitro* and *in vivo* and its application in demonstrating the role of *sigB* in microcolony formation. Infect Immun **69**:7851–7857.

7. **Alnaseri H**, **Arsic B**, **Schneider JET**, **Kaiser JC**, **Scinocca ZC**, **Heinrichs DE**, **McGavin MJ**. 2015. Inducible expression of a Resistance-Nodulation-Division-type efflux pump in *Staphylococcus aureus* provides resistance to linoleic and arachidonic acids. J Bacteriol **197**:1893–905.

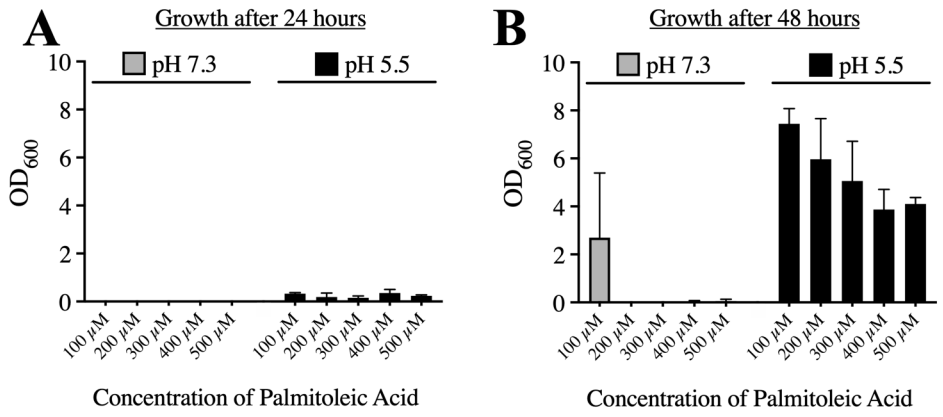

Figure S1. Following an extended lag phase, acidic pH makes *S. aureus* more resistant to C16:1 antimicrobial uFFA. Minimum inhibitory concentration assay with palmitoleic acid in TSB and TSB pH 5.5 after 24 hours **(A)** and 48 hours **(B)** of growth. Each data point represents the mean ± SE of triplicate 3mL cultures.

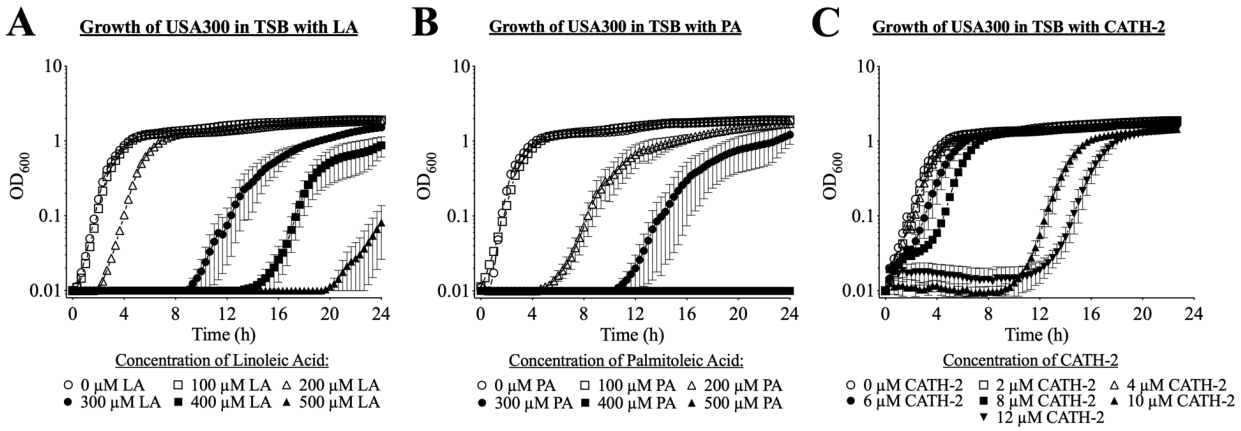

Figure S2. Determining the inhibitory concentrations of LA, PA, and CATH-2 for *S. aureus* USA300 in a 96-well plate assay. Growth of USA300 in TSB with varying concentrations of LA **(A)**, PA **(B)**, and CATH-2 **(C)**. Inhibitory concentrations were used for assays in Figure 3. Each data point represents mean ± SE of triplicate cultures.

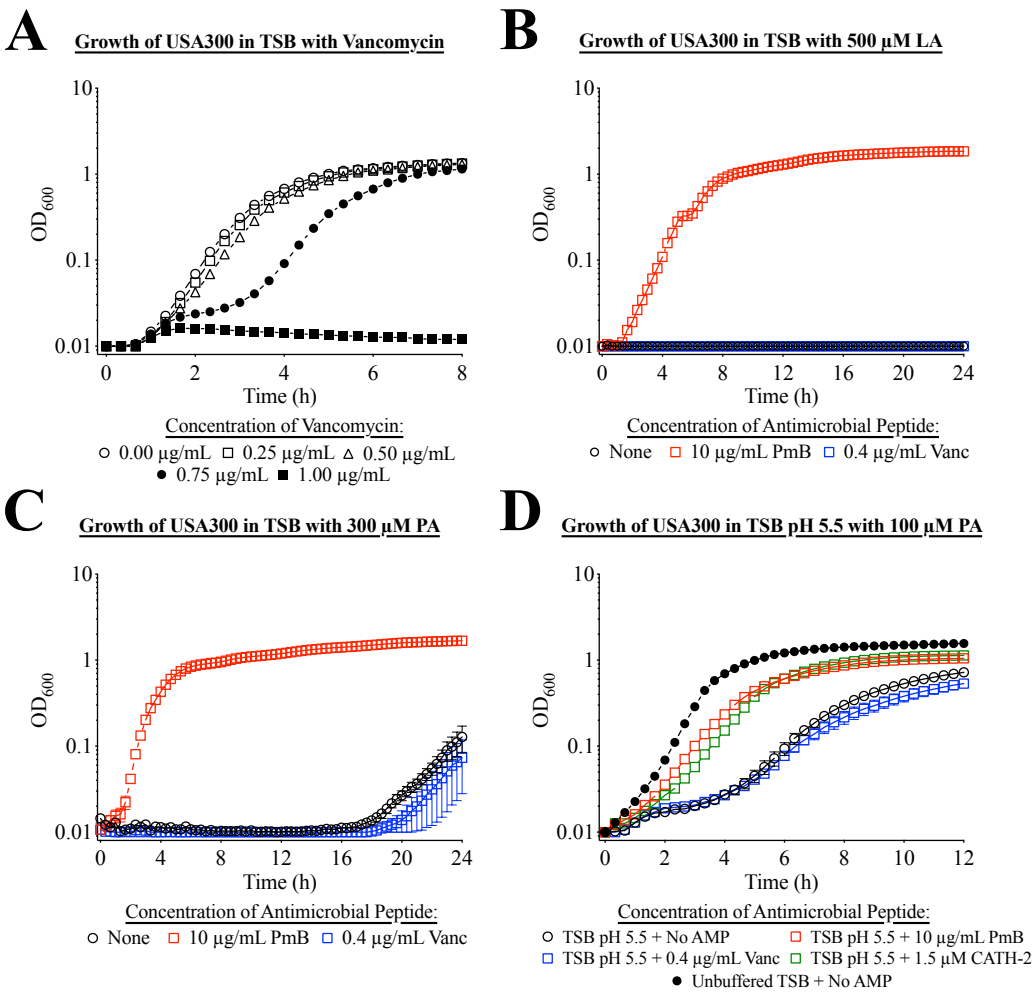

Figure S3. Subinhibitory vancomycin does not stimulate resistance to antimicrobial uFFA at neutral or acidic pH conditions. (A) Growth of USA300 in TSB with varying concentrations of vancomycin. **(B)** Growth of USA300 in 500 µM linoleic acid, supplemented with no antimicrobial peptide, or with subinhibitory concentrations of Polymyxin B or vancomycin. **(C)** Growth of USA300 in 300 µM palmitoleic acid, supplemented with no antimicrobial peptide, or with subinhibitory concentrations of Polymyxin B or vancomycin. **(D)** Growth of USA300 in TSB pH 5.5 with 100 µM PA, supplemented with no antimicrobial peptide, or with subinhibitory concentrations of Polymyxin B, vancomycin, or CATH-2. Growth in unbuffered TSB + 100 µM PA with no antimicrobial peptides is also included for comparison. Growth as assessed in 96 well plate format, and each data point represents mean ± SE of quadruplicate cultures.

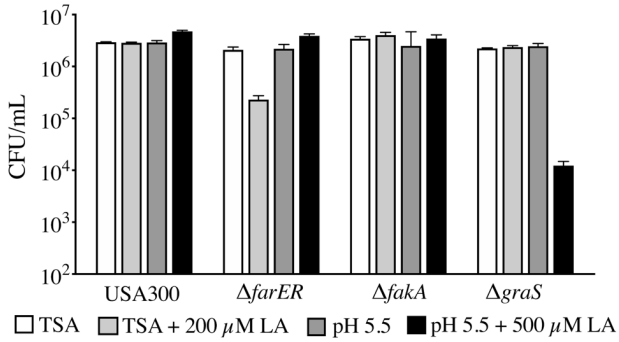

Figure S4. Genetic requirement for resistance to antimicrobial uFFA differs depending on the pH of the media. Viability (CFU/ml) of USA300 and isogenic Δ*farER*, Δ*fakA* or Δ*graS* mutants after plating on TSA, TSA + 200 μM LA, TSA pH 5.5, and TSA pH 5.5 + 500 μM LA. Mid-exponential phase triplicate cultures grown in TSB were diluted in fresh TSB to OD_{600} = 0.01. Serial dilutions were then prepared and plated on different TSA formulations as indicated, to determine viability after 24h of incubation. Each set of serial dilutions were done in triplicate, and the average CFU/ml for each culture was calculated. Data graphed as mean ± SE of triplicate cultures.

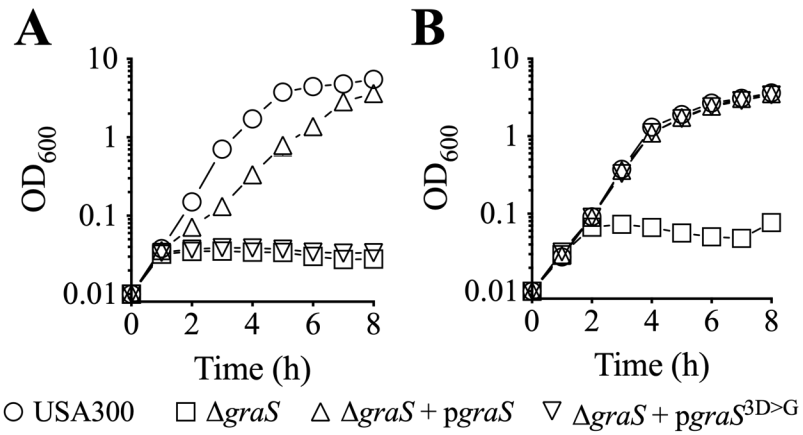

Figure S5. Role of GraS and GraS$^{3D>G}$ in resistance to PmB at neutral or acidic pH growth conditions. Growth of USA300 and isogenic Δ*graS* mutant complemented with pALC2073, p*graS,* or p*graS*$^{3D>G}$ in TSB + 10 μg/mL PmB **(A)** or TSB pH 5.5 + 20 μg/mL PmB **(B)**. OD_{600} was measured at hourly intervals for the first 8 hours of the end point growth assay represented in Figure 5A. Each data point represents the mean ± SE of triplicate cultures.