Staphylococcus aureus adapts to the host nutritional landscape to overcome tissue-specific branched-chain fatty acid requirement

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During infection, pathogenic microbes adapt to the nutritional milieu of the host through metabolic reprogramming and nutrient scavenging. For the bacterial pathogen *Staphylococcus aureus*, virulence in diverse infection sites is driven by the ability to scavenge myriad host nutrients, including lipoic acid, a cofactor required for the function of several critical metabolic enzyme complexes. *S. aureus* shuttles lipoic acid between these enzyme complexes via the amidotransferase, LipL. Here, we find that acquisition of lipoic acid, or its attachment via LipL to enzyme complexes required for the generation of acetyl-CoA and branched-chain fatty acids, is essential for bacteremia, yet dispensable for skin infection in mice. A *lipL* mutant is auxotrophic for carboxylic acid precursors required for synthesis of branched-chain fatty acids, an essential component of staphylococcal membrane lipids and the agent of membrane fluidity. However, the skin is devoid of branched-chain fatty acids. We showed that *S. aureus* instead scavenges host-derived unsaturated fatty acids from the skin using the secreted lipase, Geh, and the unsaturated fatty acid–binding protein, FakB2. Moreover, murine infections demonstrated the relevance of host lipid assimilation to staphylococcal survival. Altogether, these studies provide insight into an adaptive trait that bypasses de novo lipid synthesis to facilitate *S. aureus* persistence during superficial infection. The findings also reinforce the inherent challenges associated with targeting bacterial lipogenesis as an antibacterial strategy and support simultaneous inhibition of host fatty acid salvage during treatment.

Staphylococcus aureus | branched-chain fatty acid | lipoic acid | membrane | virulence

The gram-positive bacterium *Staphylococcus aureus* is notorious for its capacity to cause widespread pathology in nearly every organ and tissue during infection (1). Much of this success can be attributed to the ability of *S. aureus* to extract essential nutrients from the host milieu (2). Hence, understanding the adaptive traits that allow *S. aureus* to exploit in situ resources for survival is critical to devising effectual treatments for staphylococcal diseases.

Lipoic acid is an organosulfur compound derived from an early-stage intermediate of fatty acid biosynthesis that is required for carbon shuttling in central metabolism via a redox-sensitive dithiolane ring at its distal end (3). In a previous study, we found that shuttling of lipoic acid to metabolic enzyme complexes by the amidotransferase, LipL, was paramount for *S. aureus* survival in a murine model of bacteremia but not skin infection (4). At minimum, LipL appears to be required for the transfer of lipoic acid to E2 subunits of the pyruvate dehydrogenase (PDH) and branched-chain α-ketoacid dehydrogenase (BCODH) complexes (4, 5). The absence of lipoic acid renders each complex nonfunctional (4, 5). The discrepancy in demand for lipoic acid attachment in these two infection sites suggests that the nutritional environment of the skin eases the requirement for lipoic acid–dependent metabolic processes, especially those that require PDH or BCODH complex activity. PDH is responsible for the generation of acetyl-CoA upon exit from glycolysis, while BCODH is essential for the synthesis of saturated branched-chain fatty acids (BCFAs), a major component of staphylococcal membrane phospholipids (6). Like unsaturated fatty acids (UFAs), BCFAs provide membrane fluidity to staphylococci, which neither synthesize UFAs nor encode a fatty acid (FA) desaturase that converts saturated fatty acids (SFAs) to unsaturated products (7). Since a lack of membrane fluidity triggers cell death as a result of lipid phase separation and protein segregation, bacterial survival hinges on the fine-tuning of membrane lipid composition to adapt to fluctuating environments (8).

Given the importance of de novo FA synthesis to ensure bacterial membrane integrity, enzymes that are involved in this metabolic process serve as attractive targets for antibacterial drug discovery. One such drug, triclosan, binds to the enoyl-acyl carrier protein reductase, FabI, a key enzyme in the type II FA synthesis (FASII) system found in archaea and bacteria (9). FASII restriction induces FA starvation and initiates the stringent response, where high concentrations of the alarmone (p)ppGpp inhibit synthesis of the FASII substrate malonyl-CoA (10). Nevertheless, mounting evidence suggests that *S. aureus* can resolve triclosan interference by incorporating host-derived UFAs into its membrane (11–13). During anti-FASII–adaptive outgrowth, dissipation of (p)ppGpp levels replenishes the malonyl-CoA pool and skews distribution of the FASII substrate to favor binding with FapR, a global repressor of phospholipid synthesis genes in *S. aureus* (10). Ultimately, this interaction sequesters FapR to allow expression of *plsX* and *plsC*, which encode enzymes that use host UFAs to

Significance

Lipoylation is a posttranslational modification critical for the function of several metabolic enzymes. In the bacterial pathogen *Staphylococcus aureus*, lipoylation deficiency compromises growth and causes tissue-specific virulence defects. Perturbation of lipoylation causes attenuation in part due to disruption of the enzyme complex required for the synthesis of branched-chain fatty acids, an essential constituent of *S. aureus* membrane. *S. aureus* overcomes branched-chain fatty acid auxotrophy in the skin by acquiring host unsaturated fatty acids. This work underscores the adaptability of *S. aureus* when faced with nutrient scarcity and the relevance of lipoic acid sufficiency during infection. Our findings support the view that versatility in *S. aureus* membrane biogenesis must be considered when devising therapeutics.

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The authors declare no competing interest.

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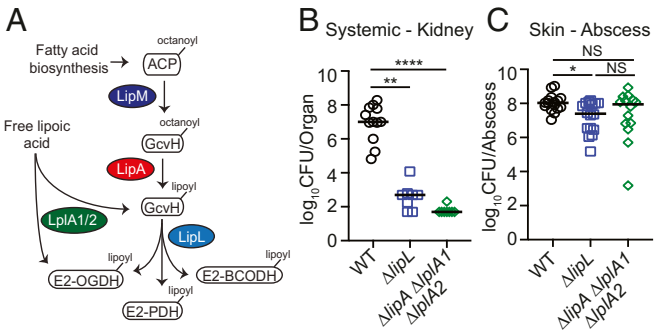

Fig. 1. Differential requirement for lipoic acid by *S. aureus* during infection. (*A*) Lipoic acid synthesis and salvage pathways in *S. aureus*. De novo synthesis of lipoic acid by *S. aureus* requires the octanoyltransferase, LipM, and the lipoyl synthase, LipA. The amidotransferase, LipL, shuttles the lipoyl moiety to the E2 subunits of α-ketoacid dehydrogenase complexes that require the cofactor for function. *S. aureus* also encodes two salvage enzymes, LplA1 and LplA2, to scavenge free lipoic acid from the environment. ACP, acyl carrier protein; GcvH, H subunit glycine cleavage complex; E2-PDH, E2 subunit pyruvate dehydrogenase complex; E2-OGDH, E2 subunit 2-oxoglutarate dehydrogenase complex; E2-BCODH, branched-chain α-ketoacid dehydrogenase complex. (*B*) Bacterial burden ($\log_{10}$ CFU) in kidneys of mice at 96 h postinfection with 1×10^7 CFU WT ($n = 12$), Δ*lipL* ($n = 8$), and Δ*lipA* Δ*lplA1* Δ*lplA2* ($n = 8$) strains. (*C*) Bacterial burden ($\log_{10}$ CFU) in skin abscesses of mice at 120 h postinfection with 1×10^7 CFU WT ($n = 16$), Δ*lipL* ($n = 16$), and Δ*lipA* Δ*lplA1* Δ*lplA2* ($n = 14$) strains. *P* values were determined by a nonparametric one-way ANOVA (Kruskal–Wallis test) with Dunn's posttest. $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$, and NS, not significant.

synthesize phospholipids (10, 14). UFAs are commonly found esterified as triglycerides and cholesterol esters in vertebrates (15). In humans, both compounds form the lipid core of the five major groups of lipoproteins (chylomicron, VLDL, LDL, IDL, and HDL) that transport lipids throughout the body (15). Triglycerides are also the major constituents of adipocytes and exist to a lesser extent in nonadipose cells (16, 17). It has been suggested that *S. aureus* secretes the glycerol ester hydrolase Geh to liberate esterified FAs from triglycerides and LDL for use as FA substrates as a nutrient acquisition strategy, although this phenomenon has not yet been tested in vivo (18, 19).

Despite increasing understanding of FASII bypass by *S. aureus* in recent years, the overall mechanism of rescue continues to be investigated. Here, we draw upon insights derived from the study of *S. aureus* lipoic acid synthesis and acquisition to make connections between BCFA synthesis and bacterial survival during infection. We report that de novo BCFA synthesis by *S. aureus* is the primary enzymatic process that necessitates lipoic acid in vitro and during skin infection. However, the requirement for BCFA synthesis in the skin is bypassed through assimilation of UFAs from host tissue. We also provide genetic, in vivo, and chemical evidence, via treatment with the over-the-counter lipase inhibitor, orlistat, that suggests UFA acquisition depends on the secreted lipase, Geh, and the UFA binding protein, FakB2. Finally, we demonstrate that ablating both BCFA synthesis and exogenous FA uptake significantly improves staphylococcal infection outcome in mice.

Results

Tissue-Specific Requirement for Lipoic Acid during *S. aureus* Infection in Mice. We previously characterized a putative *S. aureus* amidotransferase, LipL, and demonstrated that it is capable of shuttling lipoic acid between lipoyl-domain–containing proteins that require the cofactor for function (Fig. 1*A*) (4). Additionally, we showed that a Δ*lipL* mutant is highly attenuated for renal colonization in a murine sepsis model but exhibited a modest decrease in infectivity in a murine skin and soft tissue infection model (4). Therefore, we wondered if this difference in bacterial load reflects a distinct demand for lipoic acid between tissue sites. To this end, we infected mice intravenously or intradermally with either the wild-type (WT) strain, Δ*lipL,* or Δ*lipA* Δ*lplA1* Δ*lplA2* mutant strain that lacks the lipoyl synthase, LipA, and both lipoic acid salvage enzymes, LplA1 and LplA2 (5, 20). At 96 h postintravenous infection, kidneys of Δ*lipL* and Δ*lipA* Δ*lplA1* Δ*lplA2* mutant–infected animals had ∼10,000 to 100,000-fold fewer colony-forming units (CFU) than those infected with WT (Fig. 1*B*). At 120 h postintradermal infection, ∼fivefold fewer CFU were recovered from abscesses of Δ*lipL*, and no CFU difference was observed for Δ*lipA* Δ*lplA1* Δ*lplA2* mutant-infected animals compared to those infected with WT (Fig. 1*C*). Thus, a Δ*lipA* Δ*lplA1* Δ*lplA2* mutant is not attenuated, and a Δ*lipL* mutant is modestly attenuated in the skin, whereas both strains are markedly attenuated in the kidney, suggesting that *S. aureus* is largely refractory to lipoic acid deficiency in murine skin but not kidneys.

BCFA Auxotrophy Is Responsible for the Growth Defect of a Lipoic Acid–Deficient Strain. We surmised that lack of lipoylation on the E2 subunit of one or more metabolic enzyme complexes causes the reduced survival of the Δ*lipL* and Δ*lipA* Δ*lplA1* Δ*lplA2* mutant strains. To evaluate the lipoylation capabilities of these strains, we performed an anti-lipoic acid immunoblot on whole-cell lysates from each strain grown in lipoic acid bypass medium, which consists of Roswell Park Memorial Institute (RPMI) medium supplemented with sodium acetate (NaAc) and branched-chain carboxylic acids that are BCFA precursors (BCFAP). The supplemented precursors include 2-methylbutyric acid (2MB), isovaleric acid (IV), and isobutyric acid (IB) (21, 22). The Δ*lipL* mutant was unable to lipoylate E2-PDH and E2-BCODH, whereas the Δ*lipA* Δ*lplA1* Δ*lplA2* mutant could not lipoylate the E2 subunit of 2-oxoglutarate dehydrogenase and the glycine cleavage system H protein, GcvH, in addition to E2-PDH and E2-BCODH (Fig. 2*A*). Supplementation with exogenous lipoic acid or NaAc failed to rescue growth of Δ*lipA* Δ*lplA1* Δ*lplA2* and Δ*lipL* mutant strains in RPMI (Fig. 2*B* and 4, 5, 20). However, addition of any one BCFAP (2MB, IV, or IB) was sufficient to restore viability of both strains (Fig. 2*B*). Taken together, these observations suggest that the dearth of lipoyl-E2-BCODH, which is involved in BCFA synthesis, renders a lipoic acid–deficient strain nonviable in medium lacking this cofactor.

Host-Derived UFAs Relieve Lipoic Acid Requirement of *S. aureus*. Our findings so far led us to hypothesize that the murine skin environment is enriched in certain nutrients that can restore viability

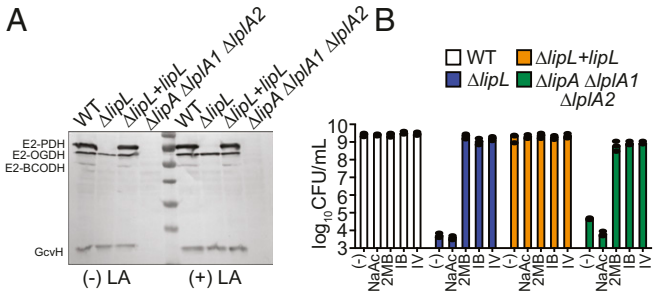

Fig. 2. Lipoic acid deficiency causes BCFA auxotrophy in *S. aureus*. (*A*) In vivo lipoylation profiles of WT, Δ*lipL*, Δ*lipL*+*lipL*, and Δ*lipA* Δ*lplA1* Δ*lplA2* strains after 9 h subculture growth in RPMI + BCFAP [10 mM IB, 9 mM 2MB, 9 mM IV + 10 mM NaAc], with or without 5 μM lipoic acid (LA), were assessed by immunoblotting SDS-PAGE–resolved whole-cell lysates with rabbit α-lipoic acid antibody. Presented blot is representative of at least three independent experiments. (*B*) Growth ($\log_{10}$ CFU/mL) of WT, Δ*lipL*, Δ*lipL*+*lipL*, and Δ*lipA* Δ*lplA1* Δ*lplA2* strains after 24 h growth in RPMI only or RPMI + 10 mM NaAc or any one BCFAP (9 mM 2MB, 9 mM IV, or 10 mM IB).

to the Δ*lipL* and Δ*lipA* Δ*lplA1* Δ*lplA2* strains. Since these strains are auxotrophic for BCFA, which provides fluidity to staphylococcal membrane, we predict that the likely substrate is either BCFA or UFA. BCFAs are present in negligible quantities on murine skin, whereas UFAs, which contribute to membrane fluidity in many other bacteria including *Escherichia coli*, are of high abundance (23). Hence, to address whether UFAs can restore growth of a Δ*lipL* mutant in RPMI, we supplemented the medium with one of three UFAs [palmitoleic acid (16:1), oleic acid (18:1), or linoleic acid (18:2)] and evaluated growth of the mutant compared to the WT strain by quantifying CFUs. All three UFAs successfully restored growth of the Δ*lipL* mutant as well as the Δ*lipA* Δ*lplA1* Δ*lplA2* mutant to WT levels (Fig. 3 *A* and *B*). Conversely, supplementation with SFAs [palmitic acid (16:0) or stearic acid (18:0)] failed to reestablish bacterial growth (Fig. 3*C*). Considering most UFAs in the host originate from triglyceride stores and cholesterol esters (16, 17), we also assessed the ability of saturated and unsaturated triglycerides [glyceryl tripalmitate (GTP, 16:0) and glyceryl trioleate (GTO, 18:1)] or human low-density lipoprotein (LDL) to serve as FA substrates in RPMI. As expected, GTO and LDL but not the saturated triglyceride, GTP, successfully reestablished viability of these strains (Fig. 3*D*). The FA moieties from GTO and LDL are esterified to glycerol and glycerol/cholesterol, respectively, although some free FA may exist in purified human LDL. Therefore, we reasoned the acquisition of UFAs from GTO and LDL would require the major secreted lipase, Geh, to liberate free FAs before use. We generated a Δ*geh* Δ*lipL* double mutant and a Δ*geh* Δ*lipL* + *geh* complementation strain and monitored growth in RPMI or RPMI supplemented with GTP, GTO, and LDL. As predicted, the growth of the Δ*geh* Δ*lipL* double mutant was no longer rescued by supplementation with GTO or LDL, while complementation with *geh* partially restored growth in the presence of these substrates (Fig. 3*E*). To ascertain a role for Geh in liberating UFA to promote survival in vivo, we intradermally infected mice with WT, Δ*geh*, Δ*lipL*, and Δ*lipL* Δ*geh* strains and quantified CFU in abscesses after 120 h. A Δ*geh* mutant was not attenuated to any measurable degree in the skin after 120 h, whereas a Δ*lipL* mutant was modestly attenuated (Figs. 1*C* and 3*F*). Compared to the Δ*lipL* mutant, infection with Δ*lipL* Δ*geh* double mutant led to recovery of 10-fold fewer CFU. These results suggest that lipase activity is partially responsible for the bypass of lipoic acid requirement in the skin.

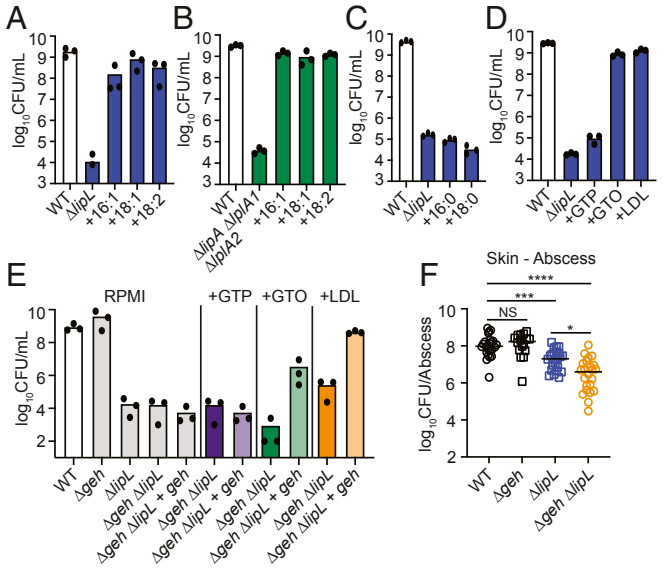

Fig. 3. UFA substrates bypass requirement for lipoic acid by *S. aureus*. (*A* and *B*) 24 h subculture growth (log_{10} CFU/mL) of (*A*) Δ*lipL* and (*B*) Δ*lipA* Δ*lplA1* Δ*lplA2* strains in RPMI only or RPMI supplemented with 50 μM of each palmitoleic acid (16:1), oleic acid (18:1), or linoleic acid (18:2). (*C* and *D*) 24 h subculture growth (log_{10} CFU/mL) of Δ*lipL* strain in RPMI supplemented with (*C*) saturated FAs [50 μM palmitic acid (16:0) or 50 μM stearic acid (18:0)] or (*D*) covalently bound FA substrates (16.67 μM GTP, 16.67 μM GTO, or 0.34 mg/mL human LDL). (*E*) 24 h subculture growth (log_{10} CFU/mL) of Δ*geh* Δ*lipL* and Δ*geh* Δ*lipL* + *lipL* strains in RPMI supplemented with GTP, GTO, or LDL as indicated in (*D*). WT Δ*geh*, Δ*lipL*, Δ*geh* Δ*lipL*, and Δ*geh* Δ*lipL* + *lipL* strains were grown in RPMI without lipid supplementation as controls. (*F*) Bacterial burden (log_{10} CFU) in skin abscesses of mice at 120 h postinfection with 1×10^7 CFU WT ($n = 24$), Δ*geh* ($n = 16$), Δ*lipL* ($n = 24$), and Δ*geh* Δ*lipL* ($n = 24$) strains. *P* values were determined by a nonparametric one-way ANOVA (Kruskal–Wallis test) with Dunn's posttest. $*P < 0.05$, $***P < 0.001$, and $****P < 0.0001$.

Liberation of UFAs Relieves BCFA Auxotrophy. To confirm that uptake of UFAs by *S. aureus* sidesteps the requirement for BCFA synthesis, we generated a Δ*bmfBB*::*kan* mutant, which lacks the E2 subunit of BCODH, and tested the ability of the mutant strain to use free FAs (18:0, 18:1, and 18:2) as substrates for growth in RPMI. Similar to the Δ*lipL* mutant, the Δ*bmfBB*::*kan* mutant grew in RPMI supplemented with UFA but not SFA substrates (Fig. 4*A*). Deleting *pdhC*, which encodes E2-PDH, did not change the requirement of USFA for the growth of a Δ*bmfBB*::*kan* mutant (Fig. 4*B*). We also assessed the ability of the unsaturated triglyceride, GTO (18:1), or human LDL to serve as substrates for the Δ*bmfBB*::*kan* mutant in RPMI. Similar to the Δ*lipL* mutant, the Δ*bmfBB*::*kan* mutant grew in RPMI supplemented with GTO and LDL (Fig. 4*A*). To test the role of Geh in liberating 18:1 from GTO, we attempted to generate a Δ*bmfBB* Δ*geh* double mutant but were not successful after numerous attempts. Instead, we acquired an over-the-counter human pancreatic lipase inhibitor, orlistat, which was recently cocrystalized with *S. aureus* Geh (24) and tested its ability to inhibit Geh activity and prevent restoration of growth to the Δ*bmfBB*::*kan* mutant in the presence of GTO. When purified recombinant Geh was coincubated with orlistat and GTO, it was unable to hydrolyze the esterified 18:1 FA, whereas Geh alone was able to completely hydrolyze GTO (Fig. 4*C*). Consistent with its successful inhibition of Geh activity, orlistat prevented growth of a Δ*bmfBB*::*kan* mutant in the presence of GTO (Fig. 4*D*). To ascertain the relevance of strain-to-strain variability that has been proposed to affect UFA assimilation upon FASII inhibition (10–13, 25–28), we generated Δ*bmfBB*::*kan* mutations in the strains used in these studies as well as methicillin-resistant *S. aureus* strains that either harbor a WT *geh* allele or a naturally occurring phage disruption within the *geh* allele (*SI Appendix*, Table S1) and monitored growth in the presence of 18:1 and GTO. Our data indicate that Δ*bmfBB*::*kan* mutants of all strains were able to grow on 18:1 by 24 h, while Δ*bmfBB*::*kan* mutants in strains Newman and COL (both contain phage disruptions in *geh*) were unable to grow on GTO (Fig. 4*D*). RN6390 Δ*bmfBB*::*kan* was slower to adapt to 18:1 and GTO as evidenced by its limited growth at 8 h but reached a similar final CFU at 24 h, consistent with prior FASII inhibition studies (Fig. 4*D*). In sum, these observations demonstrate that host ester-linked UFAs are released by Geh-mediated ester hydrolysis and can functionally compensate for BCFA auxotrophy in strains with an intact *geh* allele.

FakB2 Is Required for UFA-Dependent Growth of a BCFA Auxotroph. *S. aureus* encodes two FA-binding proteins, FakB1 and FakB2 (29). FAs bound to either protein serve as substrates for the FA kinase FakA, which phosphorylates the FA acyl chains (29). These activated acyl-phosphates eventually get incorporated into membrane phospholipids (14). While both FA-binding proteins can bind SFAs, only FakB2 can bind UFAs (29, 30). Therefore, we predict that the Δ*bmfBB*::*kan* mutant requires FakB2 for UFA-dependent growth in RPMI. To determine the requirement of

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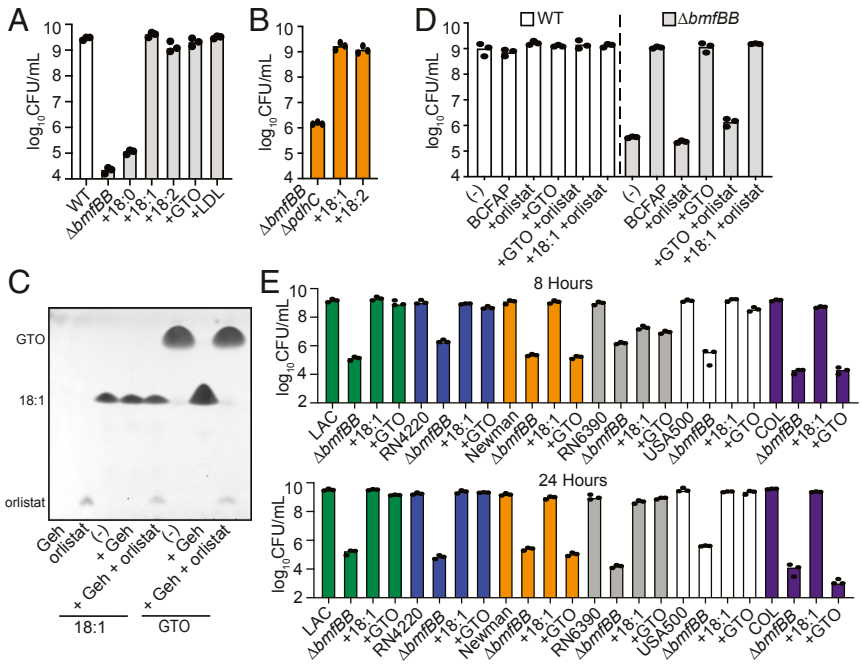

Fig. 4. UFA substrates rescue BCFA auxotrophy. (*A*) An 8 h subculture growth (log_{10} CFU/mL) of Δ*bmfBB*::*kan* strain in RPMI supplemented with different 18-carbon-long FA substrates [50 μM stearic acid (18:0), 50 μM oleic acid (18:1), 50 μM linoleic acid (18:2), or 16.67 μM GTO or 0.34 mg/mL human LDL]. (*B*) An 8 h subculture growth (log_{10} CFU/mL) of Δ*bmfBB* Δ*pdhC*::*kan* strain in RPMI only or RPMI supplemented with 50 μM 18:1 or 50 μM 18:2. (*C*) TLC of in vitro lipase activity assays containing 400 μM FA substrates [oleic acid (18:1) or GTO] in the presence or absence of 1 μM Geh ± 400 μM orlistat. (*D*) The effect of 50 μM orlistat on 8 h subculture growth (log_{10} CFU/mL) of WT and Δ*bmfBB*::*kan* strains in RPMI supplemented with 50 μM GTO or 50 μM 18:1. (*E*) An 8 and 24 h subculture growth (log_{10} CFU/mL) of the indicated Δ*bmfBB*::*kan* (Δ*bmfBB*) strains in RPMI supplemented with 50 μM oleic acid (18:1) or 16.67 μM GTO.

either FA-binding proteins for growth in RPMI supplemented with 18:1 or 18:2 UFA, we deleted *fakB1* or *fakB2* from the Δ*bmfBB* mutant strain. The Δ*bmfBB*::*kan* Δ*fakB2* strain, but not the Δ*bmfBB*::*kan* Δ*fakB1* strain, failed to replicate in RPMI supplemented with either of the aforementioned UFA substrates (Fig. 5*A*). This growth defect was reversed in the Δ*bmfBB*::*kan* Δ*fakB2* + *fakB2* strain (Fig. 5*A*). The Δ*bmfBB*::*kan* Δ*fakB2* double mutant also failed to replicate in RPMI medium supplemented with GTO or LDL (Fig. 5*B*). Together, these findings underpin the role of FakB2 in scavenging exogenous UFAs to support growth of a BCFA auxotroph.

***S. aureus* Displays Tissue-Specific BCFA Dependency during Infection in Mice.** We showed that a Δ*bmfBB*::*kan* mutant is defective for growth in RPMI but that cell growth can be restored in a Geh- and FakB2-dependent manner by addition of UFAs and triglycerides containing UFAs. Given that a Δ*bmfBB*::*kan* mutant phenocopies a Δ*lipL* mutant for viability defects in broth culture, we surmised that a Δ*bmfBB*::*kan* mutant would also be compromised for systemic disease but not superficial skin infection in mice, as FakB2 would be critical for this survival advantage in the skin on account of UFA uptake. To this end, we initially infected mice intravenously or intradermally with WT, Δ*fakB1*, and Δ*fakB2* mutant strains and monitored infection in the kidney (systemic) or superficial abscess (skin). No differences in recovered CFU were observed (Fig. 6*A*). We then infected mice intravenously or intradermally with WT, Δ*bmfBB*::*kan*, Δ*bmfBB*::*kan* Δ*fakB2*, and Δ*bmfBB*::*kan* Δ*fakB2* + *fakB2* strains. At 96 h postintravenous infection, kidneys of Δ*bmfBB*::*kan* mutant-infected animals had ∼10,000-fold fewer CFUs than those infected with WT, while renal CFUs of Δ*bmfBB*::*kan* Δ*fakB2* mutant-infected animals were reduced an additional ∼10-fold (Fig. 6*B*). Complementation of the double mutant with *fakB2* restored renal CFUs to nearly WT levels (Fig. 6*B*). At 120 h postintradermal infection, WT and Δ*bmfBB*::*kan* strains infected to similar levels (Fig. 6*C*). However, ∼100-fold fewer CFUs were recovered from the abscesses of Δ*bmfBB*::*kan* Δ*fakB2* mutant-infected animals (Fig. 6*C*). Complementation of the double mutant with *fakB2* restored CFUs to levels of WT and Δ*bmfBB*::*kan* strains (Fig. 6*C*). The differences in infection efficiency of these four strains were reflected in abscess severity at 96 h post infection (Fig. 6*C*). While we observed well-demarcated dermonecrotic abscesses in animals infected with WT, Δ*bmfBB*::*kan*, and Δ*bmfBB*::*kan* Δ*fakB2* + *fakB2* strains, infection with the Δ*bmfBB*::*kan* Δ*fakB2* strain failed to induce abscess formation and only displayed minor redness at the site of injection (Fig. 6*C*). Thus, a Δ*bmfBB*::*kan* mutant mimics a Δ*lipL* mutant in terms of its differential infectivity in murine sepsis and skin disease models. These results also further cement the importance of FakB2 in supporting *S. aureus* infection when BCFA synthesis is hindered.

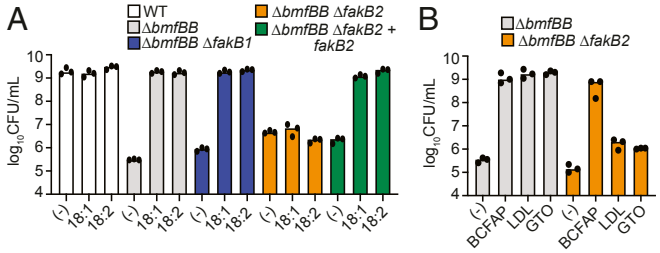

Fig. 5. Use of UFA substrates requires FakB2. (*A*) An 8 h subculture growth (log_{10} CFU/mL) of WT, Δ*bmfBB*::*kan*, Δ*bmfBB*::*kan* Δ*fakB1*, Δ*bmfBB*::*kan* Δ*fakB2*, and Δ*bmfBB*::*kan* Δ*fakB2* + *fakB2* strains in RPMI only or RPMI supplemented with 50 μM oleic acid (18:1) or 50 μM linoleic acid (18:2). (*B*) An 8 h subculture growth (log_{10} CFU/mL) of Δ*bmfBB*::*kan* and Δ*bmfBB*::*kan* Δ*fakB2* strains in RPMI supplemented with BCFAP (10 mM IB, 9 mM 2MB, 9 mM IV), 0.34 mg/mL human LDL, or 16.67 μM GTO.

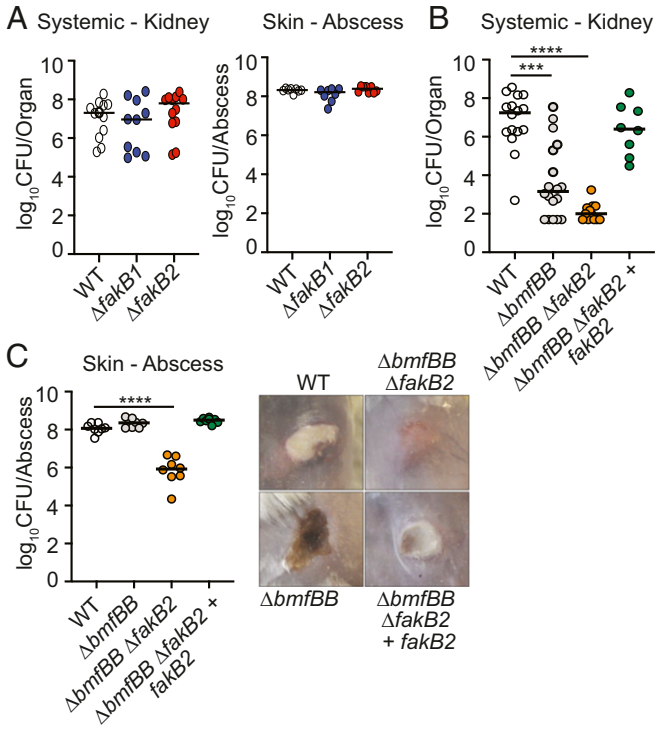

Fig. 6. FakB2 is important for bypass of BCFA requirement during *S. aureus* infection. (*A*) Bacterial burden ($\log_{10}$ CFU) in kidneys of mice at 96 h postinfection with 1×10^7 CFU WT ($n = 12$), Δ*fakB1* ($n = 10$), and Δ*fakB2* ($n = 12$) strains; and bacterial burden ($\log_{10}$ CFU) in skin abscesses of mice at 120 h postinfection with 1×10^7 CFU WT ($n = 8$), Δ*fakB1* ($n = 8$), and Δ*fakB2* ($n = 8$) strains. (*B*) Bacterial burden ($\log_{10}$ CFU) in kidneys of mice at 96 h postinfection with 1×10^7 CFU WT ($n = 16$), Δ*bmfBB*::*kan* ($n = 18$), Δ*bmfBB*::*kan* Δ*fakB2* ($n = 12$), and Δ*bmfBB*::*kan* Δ*fakB2* + *fakB2* ($n = 8$) strains. (*C*) Bacterial burden ($\log_{10}$ CFU) in skin abscesses of mice at 120 h postinfection with 1×10^7 CFU WT ($n = 8$), Δ*bmfBB*::*kan* ($n = 8$), Δ*bmfBB*::*kan* Δ*fakB2* ($n = 8$), and Δ*bmfBB*::*kan* Δ*fakB2* + *fakB2* ($n = 8$) strains, and representative corresponding images of skin lesions. *P* values were determined by a nonparametric one-way ANOVA (Kruskal–Wallis test) with Dunn's posttest. ***$P < 0.001$ and ****$P < 0.0001$.

Discussion

Evidence to support the link between lipoic acid metabolism and virulence of bacterial pathogens is growing (31). Indeed, we previously determined that lipoic acid synthesis and salvage supports *S. aureus* colonization during murine bloodstream infection (5) and restricts macrophage generation of reactive nitrogen and reactive oxygen species in a peritonitis infection model (32). We also found that autolysis-dependent release of *S. aureus* lipoyl E2-PDH suppresses macrophage activation by antagonizing Toll-like receptor (TLR) 1/2 signaling (33). Yet, the relative contributions of lipoic acid–dependent metabolic processes to *S. aureus* infection have not been explored. Here, we present evidence that BCODH, which converts transaminated branched-chain amino acids to precursors for BCFA synthesis, is arguably the most vital enzyme complex for optimal *S. aureus* colonization in mice during systemic disease but not skin and soft tissue infection. A similar tissue-specific requirement for LipL led us to determine that host-derived UFAs can rescue BCFA auxotrophy in the skin, and by extension, circumvent demand for lipoic acid for effective *S. aureus* infection.

We previously found that a *S. aureus* strain defective for lipoic acid salvage (Δ*lplA1* Δ*lplA2*) is markedly attenuated in the kidneys of systemically infected mice (5). Therefore, lipoic acid salvage is instrumental for survival in this organ despite an intact de novo synthesis pathway. This is perhaps due to lower expression of lipoic acid synthesis enzymes or a higher demand for lipoyl proteins in the kidneys. Nevertheless, considering the findings in this study, we postulate that lipoic acid salvage is required in the kidney in order to augment BCODH activity since *S. aureus* appears unable to acquire sufficient UFAs from the renal milieu. However, our data do not rule out the alternative possibility that other lipoic acid–containing enzymes might also be required for survival in the kidney, namely E2-PDH. We are currently testing this hypothesis.

To make BCFA, BCODH-synthesized precursors in the form of short branched-chain acyl CoAs are elongated by FASII. Since this pathway is not found in mammals, which uses FASI (34), FASII enzymes were thought to be excellent targets for antibacterials. These include inhibitors of FabI (triclosan, AFN-1252, and MUT056399) (9, 35, 36), FabF (platensimycin and fasamycins A and B) (37, 38), and FabH (amycomicin) (39). While anti-FASII drugs remain potent antimycobacterial agents due to a dearth of exogenous very long chain FAs specific to members of this genus (40), Firmicutes, such as staphylococci and streptococci, were later found to be capable of exploiting host UFAs to evade FASII inhibition (11–13). Therefore, our findings further stress the sophisticated adaptive trait of acquiring a fail-safe system to maintain membrane homeostasis in the absence of BCFA.

In this study, we demonstrated that the free or triglyceride form of UFAs and human LDL were able to revitalize BCFA-deficient cells in broth medium. However, prior studies indicate that most UFAs exert potent antimicrobial activity on *S. aureus* by distorting membrane architecture (18, 41, 42). For example, while 18:1 can be directly incorporated into *S. aureus* phospholipids, allowing the bacterium to bypass FASII, 16:1 and 18:2 trigger membrane depolarization and release of low-molecular–weight proteins from cells (41). To counter these deleterious effects, *S. aureus* hydrates 16:1 and 18:2 to nontoxic hydroxy-16:0 and hydroxy-18:1, respectively, with the oleate hydratase, OhyA (43), or elongates 16:1 by two carbon units for assimilation into membrane lipids (41). Production of the surface protein IsdA by *S. aureus* also decreases its cellular hydrophobicity, in turn conferring resistance to the bactericidal actions of skin FAs (44). Moreover, *S. aureus* secretes FA-modifying enzymes that esterify free FAs with cholesterol or short-chain alcohols to inactivate these cytotoxic compounds (45). In addition, concomitant attachment of UFAs to lipoproteins renders *S. aureus* susceptible to the TLR2 immune response (46). Such mechanisms of detoxification are likely at play in our studies, allowing incorporation of less deleterious UFAs such as 18:1 and hydroxy-18:1 to promote survival in the skin.

Prior studies on adaptation to anti-FASII antibiotics highlighted strain-dependent variabilities in the assimilation of host FAs (10–13, 25–28). Using BCFA auxotroph mutants, we did not observe such variation after supplementation with free UFAs but did uncover growth defects upon GTO supplementation when using strains that harbor a phage disruption in the *geh* gene. Approximately 15% of sequenced isolates harbor this disruption, thus our current study implies the presence or absence of *geh* may have biologically meaningful consequences on nutritional adaptation in addition to previously described effects on immune function (47). RN6390, a derivative of strain 8325 (48, 49), was the only strain to exhibit a notable delay in UFA utilization upon deletion of *bmfBB*. This may be due to known genetic variations in this lineage that affect *fakB1*, among other regulatory and metabolic loci (10, 11, 49). Nevertheless, our findings argue that assimilation of free UFAs by *S. aureus* BCFA auxotrophs is likely conserved, whereas the ability to use triglycerides is predicted by the status of the *geh* gene.

Our combined approach of genetic and animal studies substantiates the model whereby FakB2 is responsible for binding exogenous UFAs, thus enabling *S. aureus* to tolerate anti-FASII treatment (11, 29, 30). We recapitulated the effects of this class of drugs by homing in on BCFA synthesis and genetically disrupting BCODH activity and determined that deletion of *fakB2*

abrogates the compensatory role of host UFAs in sustaining growth and infectivity of BCFA-deficient cells. It is notable that while chromosomal complementation of *fakB2* in a Δ*bmfBB*::*kan* Δ*fakB2* mutant restored colonization to WT levels during murine skin infection (Fig. 6*C*), the complemented mutant colonized the kidneys better than a Δ*bmfBB* mutant during systemic infection (Fig. 6*B*). We ascribe this outcome to the constitutive expression of complemented *fakB2*, which likely elevates incorporation of host UFAs and enhances renal infection. Since synthesis of 12-methyltetradecanoic acid (anteiso-15:0), the major BCFA species found in *S. aureus* membrane, requires BCODH, the ability of a Δ*bmfBB* mutant to grow in the presence of UFAs supports the view that the preferential occupancy of anteiso-15:0 at the *sn*-2 position of phosphatidylglycerol can be overcome to undermine anti-FASII efficacy (11–13, 27, 28, 50). In sum, the results of our studies emphasize the importance of the host environment in establishing an adaptive niche for survival and the synergistic potential of blocking host FA incorporation and de novo FA synthesis as a therapeutic approach for staphylococcal infections.

Materials and Methods

Bacterial Strains and Growth Conditions. All bacterial strains used in this manuscript are listed in *SI Appendix*, Table S1. *E. coli* strains were routinely grown in Miller's lysogeny broth (BD), while *S. aureus* strains were grown in either tryptic soy broth (BD) or in RPMI medium (Corning) supplemented with 1% casamino acids (VWR). When needed, cultures were supplemented with the following agents for selection: 100 μg · mL^{-1} ampicillin (GoldBio), 10 μg · mL^{-1} chloramphenicol (Amresco), 150 μg · mL^{-1} kanamycin (Amresco), and 0.1 mM cadmium chloride (Alfa Aesar). The following FA substrates were used for various assays throughout this study: 10 mM IB (Sigma-Aldrich), 9 mM 2-methylbutyric acid (Alfa Aesar), 9 mM IV (Sigma-Aldrich), 50 μM palmitic acid (MP Biomedicals), 50 μM palmitoleic acid (Sigma-Aldrich), 50 μM stearic acid (MP Biomedicals), 50 μM oleic acid (Sigma-Aldrich), 50 μM linoleic acid (VWR), 16.67 μM glyceryl tripalmitate (Sigma-Aldrich), 16.67 μM glyceryl trioleate (Sigma Aldrich), 0.34 μg/μL purified human LDL (Kalen Biomedical), and 50 μM orlistat (Alli, GlaxoSmithKline).

Murine Infection Models. Murine systemic and skin and soft tissue infections were performed as previously described (5, 51) with WT, Δ*lipL*, Δ*geh*, Δ*lipL* Δ*geh*, Δ*lipA* Δ*lplA1* Δ*lplA2*, Δ*fakB1*, Δ*fakB2*, Δ*bmfBB*::*kan*, Δ*bmfBB*::*kan* Δ*fakB2*, and Δ*bmfBB*::*kan* Δ*fakB2* + *fakB2* strains.

Determination of Protein Lipoylation. Proteins from optical density (OD)-normalized whole-cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 12% polyacrylamide gels at 120 V. Separated proteins were transferred from gels to 0.2 μm Immobilon polyvinylidene difluoride membranes (Millipore Sigma) at 100V for 30 min in a Criterion Blotter (Bio-Rad). After transfer, membranes were incubated for 1 h in Tris-buffered saline + 0.1% TWEEN 20 (Amresco) (TBST) supplemented with 5% bovine serum albumin (BSA) (GoldBio). Rabbit polyclonal α-lipoic acid antibody (Calbiochem) was added to the membranes at a 1:7,500 dilution followed by incubation for 1 h and three subsequent 15 min washes in ∼20 mL of TBST. Alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (H+L) (Invitrogen) was then added at a 1:5,000 dilution in 5% BSA in TBST for 45 min followed by three 15 min washes in ∼20 mL of TBST and another 5 min wash in AP Buffer (100 mM Tris pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). Membranes were developed with 5-bromo-4-chloro-3-indoyl-phosphate/nitro blue tetrazolium color development substrate (GoldBio).

Generation of Δ*bmfBB::kan* and Δ*bmfBB* Strains. The kanamycin resistance cassette, *aphA3*, was amplified from the plasmid pBTK (52) with primer pairs kanF-KasI and kanR-KasI (*SI Appendix*, Table S2), followed by subcloning into pIMAY harboring the ∼500-base-pair upstream and downstream regions of homology that flank the *bmfBB* gene using a KasI restriction site introduced between the two flanking regions (53). Mutagenesis was performed as previously described (5). An unmarked in-frame deletion of *bmfBB* was also generated with the same method but without using *aphA3*. Absence of any secondary mutations was verified by whole-genome sequencing.

Generation of Δ*lipL* Δ*geh* and Δ*bmfBB* Δ*pdhC* Strains. Marked Δ*geh*::*kan* and Δ*pdhC*::*kan* mutations were transduced into Δ*lipL* and Δ*bmfBB* strains, respectively, as described previously (5), to generate Δ*lipL* Δ*geh* and Δ*bmfBB* Δ*pdhC* strains.

Generation of Δ*lipL* Δ*geh* + *Geh* Strains. The pJC1112-*geh* construct (47) was transduced into the Δ*lipL* Δ*geh* strain as described previously (5).

Generation of Δ*fakB1* and Δ*fakB2* Strains. A total of ∼500-base-pair regions of homology upstream and downstream of *fakB1* and *fakB2* genes were amplified from WT *S. aureus* genomic DNA (a list of primers is provided in *SI Appendix*, Table S2). The amplicons were used in a splicing by overlap extension (SOE) PCR to obtain the final amplicon, which was subcloned into the pIMAY plasmid (53). Mutagenesis was performed as previously described (5).

Generation of RN4220 Δ*bmfBB::kan*, RN6390 Δ*bmfBB::kan*, Newman Δ*bmfBB::kan*, COL Δ*bmfBB::kan*, BK2395 Δ*bmfBB::kan*, Δ*bmfBB::kan* Δ*fakB1*, and Δ*bmfBB::kan* Δ*fakB2* Strains. The Δ*bmfBB*::*kan* mutation was transduced into the indicated *S. aureus* strains, as described previously (5).

Generation of Δ*bmfBB::kan* Δ*fakB2* + *fakB2* Strain. The Δ*bmfBB*::*kan* Δ*fakB2* + *fakB2* strain was generated using the pJC1111 plasmid (54). Using primers listed in *SI Appendix*, Table S2, the *fakB2* gene was amplified from WT *S. aureus* genomic DNA, while the constitutive P_{HELP} promoter was amplified from pIMAY plasmid (53). The resulting amplicons were subjected to SOE PCR to obtain P_{HELP}-*fakB2*, which was cloned into pJC1111 at the PstI and SalI restriction sites. The recombinant construct was propagated in *E. coli* DH5-α and transformed into SaPI-1-integrase–expressing *S. aureus* (RN9011) to allow single-copy integration at the SaPI-1 site in the chromosome (54, 55). Bacteriophage Φ-11 was used to package the integrated complementation plasmid from RN9011 and was subsequently transduced into the Δ*bmfBB*::*kan* Δ*fakB2* strain. Δ*bmfBB*::*kan* Δ*fakB2* + *fakB2* transductants were selected for based on cadmium chloride resistance and confirmed by PCR.

Bacterial Viability Assay. *S. aureus* strains were grown in RPMI containing BCFAP and 10 mM NaAc (Sigma-Aldrich) overnight at 37 °C with shaking. Bacteria were washed three times and resuspended in the same volume of fresh RPMI before being subcultured 1:100 into 990 μL of the same medium supplemented with any one of aforementioned FA substrates in a 15 mL conical tube at 37 °C with shaking for 24 h. Bacterial viability was assessed by enumerating CFU on tryptic soy agar.

Purification of Geh-6xHis. Geh-6xHis was purified as described previously (47).

Orlistat Activity Assay. To assay the inhibitory activity of orlistat against Geh, 500 μL reactions were carried out at 37 °C in phosphate-buffered saline, with 400 μM FA substrate (oleic acid or glyceryl trioleate), 1 μM Geh-6xHis, and 400 μM orlistat for 6 h. A total of 5 μL of each reaction were applied onto a thin-layer chromatography (TLC) Silica gel 60 with concentrating zone 20 × 2.5 cm (Merck). The TLC plate was developed with hexane (Fisher):ethyl ether (VWR):acetic acid (Fisher) (80:20:2) as previously described (56).

Ethics Statement. All experiments were performed following the ethical standards of the Institutional Biosafety Committee and the Institutional Animal Care and Use Committee (IACUC) at Loyola University Chicago Health Sciences Division. The institution is approved by Public Health Service (PHS; A3117-01 through 02/28/2022), is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (000180, certification dated 11/17/2016), and is registered/licensed by the United States Department of Agriculture (USDA) (33-R-0024 through 08/24/2023). All animal experiments were performed in animal biosafety level 2 facilities with IACUC-approved protocols (IACUC #2017028) under the guidance of the Office of Laboratory Animal Welfare following USDA and PHS policy and guidelines on the humane care and use of laboratory animals.

Quantification and statistical analyses. All experiments were repeated at least three independent times. For in vitro growth assays, data shown are representative experiments conducted in triplicate that were repeated on a minimum of three independent occasions. All statistical significance was analyzed using GraphPad Prism version 9.0 with statistical tests specified in

the figure legends. All animal studies include combined data from at least two independent experiments.

Data Availability. All study data are included in the article and/or *SI Appendix*.

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