In Staphylococcus aureus , the acyl-CoA synthetase MbcS supports branched-chain fatty acid synthesis from carboxylic acid and aldehyde precursors

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Abstract

In the human pathogen *Staphylococcus aureus*, branched-chain fatty acids (BCFAs) are the most abundant fatty acids in membrane phospholipids, and strains deficient for their synthesis experience BCFAs auxotrophy in laboratory culture and attenuated virulence during infection. Thus, membrane integrity is essential for *S. aureus* pathogenesis. Furthermore, the membrane of *S. aureus* is among the main targets for antibiotic therapy. Therefore, determining the mechanisms involved in BCFAs synthesis is critical to manage *S. aureus* infections. Here, we report that overexpression of the bona fide acyl-CoA synthetase gene *mbcS* (formerly SAUSA300_2542) restores BCFAs synthesis in strains lacking the canonical biosynthetic pathway catalyzed by the branched-chain a-keto acid dehydrogenase (BKDH) complex. We demonstrate that the acyl-CoA synthetase activity of MbcS activates branched-chain carboxylic acids, and is required by *S. aureus* to utilize the isoleucine derivative 2-methylbutyraldehyde to restore BCFAs synthesis in *S. aureus*. Based on the ability of some staphylococci to convert branched-chain aldehydes into their respective branched-chain carboxylic acids and our findings demonstrating that branched-chain aldehydes are in fact BCFAs precursors, we propose that MbcS promotes the scavenging of exogenous branched-chain carboxylic acids (BCCAs) and mediates branched-chain fatty acids synthesis via a *de novo* alternative pathway.

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Summary

In the human pathogen *Staphylococcus aureus* , branched-chain fatty acids (BCFAs) are the most abundant fatty acids in membrane phospholipids, and strains deficient for their synthesis experience BCFAs auxotrophy

in laboratory culture and attenuated virulence during infection. Thus, membrane integrity is essential for *S. aureus*pathogenesis. Furthermore, the membrane of *S. aureus* is among the main targets for antibiotic therapy. Therefore, determining the mechanisms involved in BCFAs synthesis is critical to manage *S. aureus* infections. Here, we report that overexpression of the bona fide acyl-CoA synthetase gene *mbcS* (formerly SAUSA300_2542) restores BCFAs synthesis in strains lacking the canonical biosynthetic pathway catalyzed by the branched-chain α-keto acid dehydrogenase (BKDH) complex. We demonstrate that the acyl-CoA synthetase activity of MbcS activates branched-chain carboxylic acids, and is required by *S. aureus* to utilize the isoleucine derivative 2-methylbutyraldehyde to restore BCFAs synthesis in *S. aureus* . Based on the ability of some staphylococci to convert branched-chain aldehydes into their respective branched-chain carboxylic acids and our findings demonstrating that branched-chain aldehydes are in fact BCFAs precursors, we propose that MbcS promotes the scavenging of exogenous branched-chain carboxylic acids (BCCAs) and mediates branched-chain fatty acids synthesis via a *de novo* alternative pathway.

Introduction

Bacterial cytoplasmic membranes serve as the interface between the cell and the extracellular environment. They are essential for cell integrity in the face of environmental stress (Denichet al., 2003; Sun et al., 2012; Wang et al. , 2014). They also serve as the signaling platform for coordinating cellular responses to achieve cell homeostasis (Gohrbandt et al., 2022; Jung et al., 2018; Schneider et al., 2015). In many Gram-positive bacteria, branched-chain fatty acids (BCFAs) are the predominant fatty acids for membrane phospholipids under normal growth conditions (de Mendoza et al., 1993; Kaneda, 1991; Singh et al., 2008; Sun et al., 2012). In the human opportunistic pathogen*Staphylococcus aureus* , BCFA content is as high as 70% (Whittaker et al., 2005), with the balance mostly straight chain fatty acids and lower levels of cardiolipin and carotenoids (Schleifer & Kropenstedt, 1990; Sen et al., 2016). *S. aureus* cannot synthesize unsaturated fatty acids or desaturate saturated fatty acids. Thus BCFAs fluidize staphylococcal membranes to avoid membrane phase separation, protein segregation, and cell death (Gohrbandt et al., 2022). While the organism can incorporate host unsaturated fatty acids to support growth, it is unclear if this salvaging is sufficient to maintain full virulence during infection, since specific BCFAs promote the production of secreted and surface-associated factors via major regulators of virulence (Pendleton & Yeo et al., 2022).

The synthesis of BCFAs starts with the branched-chain amino acids isoleucine, leucine, and valine. Following transamination by IlvE, the respective α-keto acids undergo oxidative decarboxylation to branched-chain carboxylic acids and subsequent activation to their acyl-CoA derivatives. This series of reactions is catalyzed by the branched-chain α-keto acid dehydrogenase complex (BKDH). These acyl-CoAs are primed by FabH, catalyzing condensation of acyl-CoA with malonyl-ACP. The resulting β-ketoacyl-ACP is elongated by the type II fatty acid synthase (FASII) prior to incorporation into phospholipids**(Fig 1)**(Parsons & Rock 2013). BCFAs can be classified as iso (*i*) or anteiso (*a*) depending on the location of the methyl group in their structure and the fatty acids *a* 15:0 and *a* 17:0 derived from isoleucine are the most abundant in the *S. aureus* membrane (Kaneda, 1991).

The BKDH-dependent route of BCFA synthesis has been studied in multiple Gram-positive bacterial species including *S. aureus,* and is essential for growth in laboratory culture (Brinsmade & Sonenshein, 2011; Gohrbandt et al., 2022; Pendleton & Yeo et al., 2022; Singh et al., 2008; Ward et al., 1999). In *S. aureus* the BKDH complex is composed of four enzymes: a dehydrogenase (E1α), a decarboxylase (E1β), a dihydrolipoamide acyltransferase (E2), and a dihydrolipoamide dehydrogenase (E3), encoded by *bkdA1* , *bkdA2* , *bkdB* , and *lpdA* , respectively (Singh et al., 2008). We and others demonstrated that disruption of *lpdA* results in a significant decrease in the percentage of membrane BCFAs and attenuates virulence due to a loss of virulence regulator activity, indicating that a functional BKDH complex is indispensable for BCFA synthesis and full virulence gene expression (Pendleton & Yeo et al., 2022; Singh et al., 2008). Given their importance in bacterial physiology and virulence in*S. aureus* , we wondered whether the canonical BKDH pathway was the sole source of BCFA synthesis. In the present study, we take advantage of the *lpdA* mutant and looked for mutants that bypassed BCFA auxotrophy. We report that suppressor mutations that increase promoter activity of a putative acyl-CoA synthetase restore BCFA synthesis during laboratory growth in rich,

complex medium. We present genetic and biochemical evidence that the gene product we now call MbcS is a member of the AMP-forming acyl-CoA synthetases and catalyzes the activation of branched-chain carboxylic acids to their acyl-CoAs with a preference for*i* C_4 and *a* C_5 substrates. We show that the branched-chain aldehyde 2-methylbutyraldehyde can also restore BCFA synthesis in the *lpdA* mutant, revealing the existence of an alternative route to BCFA synthesis. Our data have implications for colonization of human skin as well as interspecies interactions during skin and soft tissue infection.

Results

Mutations in the *mbcS* promoter restore growth of a*lpdA* mutant during laboratory growth. A *lpdA* mutant lacks the dihydrolipoamide dehydrogenase subunit of the BKDH complex and is deficient for BCFA synthesis (Teoh et al., 2021). As expected, the strain is not viable in rich, complex medium (tryptic soy broth [TSB]) and does not achieve an optical density beyond ˜0.1 after ˜7-8 h unless supplemented with a mixture of the branched-chain carboxylic acids (BCCAs; 2-methylbutyric acid [*a* C_5], 3-methylbutyric acid [*i* C_5], and isobutyric acid [*i* C_4]) or with *a* 15:0 BCFA **(Fig 2A-C** , compare squares to circles**)** . However, we observed that the *lpdA* mutant formed small colonies upon prolonged incubation on tryptic soy agar (TSA) in the absence of BCCAs. We reasoned that these colonies harbor additional mutations that bypass the requirement for the BKDH complex. To test this, independently isolated mutants were submitted for whole genome sequencing and the sequences of the apparent suppressor mutants and the parent strain were compared with BRESEQ (Barrick et al., 2009). Interestingly, nearly all mutants harbored either single nucleotide polymorphisms or insertion-deletions in the promoter region of SAUSA300_2542,which is annotated to encode an acyl-CoA synthetase **(Table 1, Fig 3A).** We refer to these alleles hereafter as *mbcS* alleles to reflect the *m* ethyl*b* utyri*c* acid supplementation bypassed in these strains. We first analyzed growth behavior of a representative *lpdA* mutant with a mutant *mcbS* allele (*i.e., mbcS1*). This strain achieved a doubling time and cell yield essentially identical to that of the WT strain, even in the absence of BCFAs or their carboxylic acid precursors **(Fig 2A-C,**compare triangles and squares to circles**)** .

A recent study reported that TSB and other standard laboratory media contain trace amounts of branched-chain carboxylic acids (Whaley et al., 2023) **.** We wondered whether *mbcS* was required to scavenge these BCFA precursors and promote BCFA synthesis in the absence of *lpdA* . To test this, we constructed an *lpdA mbcS* double mutant using available mutants from the Nebraska Transposon Mutant Library (Fey et al., 2013) and compared the strain's growth behavior to the *lpdA* single mutant. Unlike the *lpdA* mutant, whose growth was restored with branched-chain carboxylic acids, the *lpdA mbcS* double mutant failed to grow beyond an optical density of ˜0.1 unless *a* 15:0 was included in the medium (**Fig 2** , compare inverted triangles to squares). These data indicate that the mutations in the *mbcS* promoter suppress the BCFA auxotrophy, and that *mbcS* is required to restore growth when BCCAs are provided exogenously in the growth medium.

Promoter-up mutations in*mbcS* alter the membrane fatty acid profile in *S. aureus*.Many of the changes we observed in the *mbcS* promoter of our mutants either decrease the spacing between the -35 and -10 boxes from 18 nucleotides to the typical 17 nucleotides found in *S. aureus* , or refine the -35 and -10 sequences toward a consensus σ^A-dependent promoter **(Table 1, Fig 3A)**(Helmann, 1995). We hypothesized that these changes would increase*mbcS* expression. To test this, we introduced a *gfp*reporter plasmid containing either the wild-type (WT) promoter of*mbcS* or the promoters isolated from two independently isolated mutants into wild-type cells and measured promoter activity **(Fig 3A, B)** . During growth in TSB, *mbcS* expression is relatively low in WT cells. Mutation of the *mbcS* promoter resulted in a 3- to 4-fold increase in promoter activity (**Figure 3B)** . We then wondered whether overexpressing *mbcS* altered the membrane fatty acid profile, supporting growth in TSB. To address this question, we grew the WT and our two selected *lpdA* suppressor mutants to exponential phase and subjected the cells to gas chromatography fatty acid methyl ester analysis (GC-FAME). As expected, WT cells have a relatively high proportion of *a* 15:0 BCFAs in their membranes with lower levels of *i* 14:0 BCFAs. Interestingly, we measured a large increase in *i* 14:0 BCFAs and relatively low levels of *a* 15:0 BCFAs in the suppressor mutants (**Fig 3C**). These data strongly suggest that overexpressing *mbcS* rescues BCFA auxotrophy by promoting the synthesis of iso but

not anteiso BCFAs.

MbcS is a branched-chain acyl-CoA synthetase. SAUSA300_2542 is annotated to encode an acyl-CoA synthetase we call MbcS. Three facts support this annotation. First, MbcS is 56% similar to (end-to-end)*S. aureus* acetyl-CoA synthetase (Burckhardt et al., 2019). Second, MbcS is 43-47% similar to other bona fide acyl-CoA synthetases from *Salmonella enterica* and *Rhodopseudomonas palustris*(Crosby & Escalante-Semerena, 2014; Crosby et al., 2012; Starai & Escalante-Semerena, 2004). Third, close inspection of the predicted amino acid sequence of MbcS reveals a conserved motif and catalytic lysine residue among representative members of the AMP-forming family of acyl-CoA synthetases (**Fig 4A**) (Burckhardt et al., 2019; Crosby & Escalante-Semerena, 2014; Crosby et al., 2012; Starai et al., 2002). To begin to test the hypothesis that MbcS has acyl-CoA synthetase activity, we complemented a *lpdA mbcS* double mutant with integrative plasmids coding for either the wild-type *S. aureus mbcS* (*SambcS* $^{+}$) or a variant substituting the catalytic lysine residue with alanine (K510A) under the control of an anhydrotetracycline inducible promoter and the Tet repressor (TetR) and assessed growth behavior in TSB. When we introduced the empty vector into the *mbcS lpdA* double mutant, the cells were unable to achieve an optical density beyond ˜0.1 after ˜6-8 h **(Fig 4B** , compare triangles to circles**)** . Growth was restored in this mutant when we introduced a wild-type copy of *mbcS* but not the mutant allele coding for the catalytically inert MbcS^{K510A} variant, strongly suggesting that MbcS-dependent acyl-CoA synthetase activity was necessary and sufficient for growth in TSB **(Fig 4B** , compare inverted triangles and diamonds to circles**)** . Interestingly, we measured no obvious growth defect in the single *mbcS* mutant. To test genetically if MbcS indeed supports growth of the *lpdA*mutant by catalyzing acyl-CoA synthetase activity, we complemented the*lpdA mbcS* double mutant with an integrative plasmid coding for the bona fide acyl-CoA synthetase IbuA from *R. palustris*(*RpibuA*$^{+}$), and assessed growth behavior**(Fig 4C, D)** . Unlike *SambcS*$^{+}$,*RpibuA*$^{+}$ does not complement the *lpdA mbcS* mutant for growth in TSB in the absence of inducer **(Fig 4C)** . However, adding 25 ng ml^{-1} of anhydrotetracycline (aTc) to the growth medium was sufficient to restore growth of the *lpdA mbcS* strain to a level similar to the WT strain **(Fig 4D)** . Taken together, our genetic data indicate that, in the absence of the BKDH complex, the acyl-CoA synthetase activity of MbcS bypasses the BCFA auxotrophy of the *lpdA*mutant.

To support the *in vivo* data above, we cloned, overexpressed, and purified *N*- terminal hexahistidine (His_6)-tagged MbcS protein (*Sa* MbcS). Following tag removal, we reisolated the protein to apparent homogeneity and conducted a coupled spectrophotometric assay with Mg*ATP, coenzyme A (CoA) and a variety of carboxylic acids as substrates as described in *Experimental Procedures* . *Sa* MbcS activated short, straight and branched-chain monocarboxylic acids and typically preferred branched substrates over straight substrates with the same carbon length. *Sa* MbcS had the highest activity with isobutyrate (*i* C_4)**(Fig 5A)** . We saw no activity when *a* 15:0 was used as substrate in the reaction (data not shown).

The coupled assay does not provide a direct identification of the product formed. Therefore, we used LC-MS analysis to authenticate isobutyryl-CoA (*i* C_4-CoA) formation from isobutryate (*i* C_4). Briefly, we incubated*Sa* MbcS with Mg*ATP, isobutyrate (IB), and CoA in buffer. As a negative control, *Sa* MbcS was denatured at 85°C, and its activity was tested. Using LC-MS, we successfully separated, detected, and confirmed the identities of isobutyryl-CoA and CoA in reaction mixtures containing recombinant *Sa* MbcS. We failed to detect IB-CoA formation when substrates were incubated with denatured MbcS, demonstrating product formation was enzyme-dependent **(Fig 5B)** .

We then determined a limited set of kinetic parameters for *Sa* MbcS and compared them to bona fide isobutyryl-CoA synthetase from *R. palustris* (*Rp* IbuA) using the same continuous spectrophotometric assay. Because our interest lay in the specificity of the enzyme for substrates relevant to BCFA synthesis, we focused on carboxylic acid substrates and used a fixed amount of coenzyme A and ATP co-substrates. Under our *in vitro* conditions, the apparent affinity of*Sa* MbcS for *i* C_4 and*a* C_5 were essentially identical (K_m values were calculated to be 8.9 ± 2.1 μM and 5.5 ± 1.0 μM, respectively). *Sa* MbcS displayed a significantly lower affinity for *i* C5 (> 500 μM) **(Table 2)** . MbcS displayed a measurably higher affinity for *i* C4 compared to*Rp* IbuA. This seemed to be offset by the faster rate of reaction catalyzed by *Rp* IbuA compared to *Sa* MbcS using*i*

C_4 as substrate **(Table 2** , compare K_{cat} values for *Rp* IbuA and*Sa* MbcS**)** . This resulted in comparable catalytic efficiencies for the two enzymes (compare K_{cat}/K_m values). As expected, the catalytically inert *Sa* $MbcS^{K510A}$ variant exhibited no detectable acyl-CoA synthetase activity **(Table 2)** . Taken together, these data indicate that *Sa* MbcS is a bona fide methylbutyryl-CoA synthetase with a preference for*i* C_4 as substrate and participates in branched-chain fatty acid synthesis.

mbcS **supports the utilization of 2-methylbutyraldehyde as a precursor for BCFA synthesis.** The BKDH complex catalyzes an oxidative decarboxylation reaction, converting the branched-chain α-keto acids to their cognate BCCAs. These BCCAs are activated to acyl-CoAs that feed fatty acid synthesis (Frank et al., 2021; Sen et al., 2015; Singh et al., 2008; Ward et al., 1999). Blocking BKDH function revealed a second enzyme that performs this activation step. *S. aureus* produces aldehydes during isoleucine, leucine, and valine catabolism in peptide-rich environments (Bos et al., 2013; Filipiak et al., 2012). These aldehydes are conceivably oxidized to carboxylic acids and activated by MbcS. Indeed, staphylococci used in the food industry, such as *S. carnosus* , oxidize branched-chain aldehydes to their respective BCCAs to synthesize BCFAs (Beck, 2005; Beck et al., 2002). To test if this is also true for *S. aureus* , we grew cells in chemically defined medium (CDM) (Sheldon et al., 2014) supplemented with the isoleucine-derived aldehyde 2-methylbutyraldehyde (2MA), the BCCA 2-methylbutyric acid (*a* C_5), and the BCFA*a* 17:0. As expected, the *lpdA* single mutant failed to achieve a final optical density beyond ˜0.1 in the absence of supplementation. Both the *lpdA* mutant and the*lpdA mbcS1* suppressor mutant grew to similar levels in medium containing the aldehyde and the carboxylic acid (albeit not to WT levels for the *lpdA mbcS1* strain). BCCA- and aldehyde-dependent growth was dependent on *mbcS* **(Fig 6** , compare pink and orange bars, *lpdA::kan*$^+$ *mbcS::* φNΣ vs.*lpdA::kan*$^+$ **)** . Indeed, the double mutant only grew in the presence of *a* 17:0 BCFA, indicating that lack of viability was due to insufficient intracellular branched-chain acyl-CoA precursors.

S. aureus heavily suppresses branched-chain amino acid (BCAA) synthesis during rapid growth (Kaiser et al., 2018; Waters et al., 2016). Rather, BCAAs are preferentially imported during growth as either free amino acids or as peptides and play critical roles for cell physiology. As abundant amino acids found in proteins, BCAAs can be used directly for protein synthesis and drive folding via the hydrophobic effect (Brosnan & Brosnan, 2006; Dill 1990). BCAAs are also readily interconverted to their α-keto acids that serve as precursors for pantothenate and coenzyme A, and for the BCFAs. Thus, the metabolism of BCAAs controls membrane phospholipid composition (Frank et al., 2021; Richardson et al., 2015). *Lactococcus lactis* strains are capable of decarboxylating BCAAs, particularly leucine, to their corresponding aldehyde 3-methylbutanal. These aldehydes are important for flavor formation in certain cheeses by *Lactococcus* spp. (Rijnen et al., 2003; Smit et al., 2005; Smit et al., 2004). We wondered whether*S. aureus* might also be capable of generating BCFAs *de novo* through a pathway parallel to that formed by the BKDH complex. To test this, we compared the growth behavior of the *lpdA* mutant to the WT parent strain, with and without *mbcS* overexpressed. Unlike the *lpdA* mutant, the *lpdA mbcS1* mutant grew, albeit poorly, in CDM medium without BCFA or precursor supplementation. Again, this growth was abrogated in the *lpdA mbcS* double mutant**(Fig 6** , grey and black bars, mutant strains compared to WT**)** .

Metabolites from *S. epidermidis* restore growth of*lpdA* mutant in an MbcS-dependent manner. The human skin is an important habitat for microbial colonization. Metagenomic studies show that different species of bacteria, fungi and viruses compose the skin microbiota (Byrd et al., 2018). Coagulase negative staphylococci (CoNS) such as *S. epidermidis* , *S. hominis* , *S. haemolyticus* , and *S. lugdunensis* are the most abundant skin colonizers and serve as a barrier to protect against local and systemic infections caused by pathogenic species like *S. aureus* (Brown & Horswill, 2020; Byrd et al., 2018; Zipperer et al., 2016). Indeed, skin commensals successfully compete with *S. aureus* when resources become limited in the skin environment. For instance, *S. lugdunensis* synthesizes the antimicrobial peptide lugdunin that effectively inhibits the growth of *S. aureus in vitro* and interferes with nasal colonization in a mouse model (Zipperer et al., 2016).

Microbial cells within natural communities also can exchange metabolites with one another, working together to distribute labor before interactions potentially become antagonistic (Pande et al., 2014). Considering the

interactions between *S. aureus* and staphylococci in the skin, we wondered if *S. epidermidis* could feed our*S. aureus lpdA* mutant given its propensity to make volatile compounds derived from the branched-chain amino acids. To address this, we streaked the *lpdA* single mutant on medium lacking branched-chain fatty acids next to *S. epidermidis.* If*S. epidermidis* can cross-feed *S. aureus,* we expect to observe growth adjacent to S. *epidermidis,* with the zone supporting growth proportional to the amount of the nutrient being secreted and diffusing through the medium. Interestingly, the*lpdA* mutant grew only in close proximity to *S. epidermidis,* and this growth was abrogated when we streaked the*lpdA mbcS* double mutant next to *S. epidermidis*(**Fig 7A**). We then quantified the effect using *S. epidermidis* conditioned medium. As expected, the *lpdA* single mutant was chemically complemented with a mix of branched-chain carboxylic acids or *a* 17:0 BCFAs **(Fig 7B** , compare grey, orange bars to black bars, respectively**)** . We then determined that 10% conditioned medium from *S. epidermidis* restored near WT growth. The effect was lost when the cells were supplemented with decreasing concentrations of conditioned medium (**Fig 7B** , compare green, pink, and blue bars). Only *a* 17:0 supplementation restored growth of the *lpdA mbcS* double mutant**(Fig 7B)** . Taken together, these data indicate *S. aureus*can salvage isoleucine-derived, exogenous branched-chain aldehydes in addition to branched-chain carboxylic acids in a *mbcS*- dependent manner for BCFA synthesis. Our data also strongly suggest that *S. epidermidis* can support *S. aureus* growth by providing these same essential precursors for BCFA synthesis.

Discussion

Branched-chain fatty acids, specifically *a* 15:0 and *a* 17:0, are the predominant fatty acids in Gram-positive bacterial membranes. They are essential for growth and virulence in pathogenic species including *S. aureus* (Annous et al., 1997; Beck 2005; Pendleton & Yeo et al., 2022; Singh et al., 2008; Whaley et al., 2023). Indeed,*a* 15:0 fatty acid is essential for full activity of the major*S. aureus* virulence two-component regulatory system SaeRS, and*lpdA* mutant cells are attenuated for virulence (Pendleton & Yeo et al., 2022). Given their critical roles, it is difficult to imagine that there is no redundancy in the pathways that lead to their synthesis. On the other hand, disrupting the BKDH complex results in BCFA auxotrophy. In the present study we took advantage of this phenotype and looked for mutants that might reveal an alternative route to BCFA synthesis. Extragenic suppressor mutants isolated in TSB medium map to the regulatory region of a putative acyl-CoA synthetase gene (now referred to as *mbcS*). These mutations result in overexpression of*mbcS* and resolve the BCFA auxotrophy **(Table 1, Figs 2-4)** . Using genetic and biochemical approaches, we demonstrate herein that MbcS is an acyl-CoA synthetase with specificity for short, branched carboxylic acid substrates, and MbcS activity is required for salvaging exogenous carboxylic acids and aldehydes for BCFA synthesis when the BKDH complex is inactivated **(Table 2, Figs 4-7)** .

Our data are in strong agreement with a recent report by Whaley *et al.* , demonstrating MbcS is an AMP-forming acyl-CoA synthetase that selectivity catalyzes the activation of isobutyrate and 2-methylbutyrate. In that manuscript, they clearly show extracellular carboxylic acids are converted to their CoA derivatives and flow into the FASII elongation cycle, and argue that MbcS serves in a salvaging capacity **(Figs 4-5)** (Whaley et al., 2023). Our suppressor screen also points to MbcS, and kinetic analysis of the enzyme indicates a high (*i.e.,* low micromolar) affinity for*i* C_4 and *a* C_5 substrates. This is consistent with the finding that TSB medium contains a trace amount of these compounds. Interestingly, unlike in TSB, the *lpdA*single mutant does not exhibit auxotrophy for BCFAs when grown in lysogeny broth, prompting Whaley *et al.* to hunt for *mbcS.*This is likely due to the relative levels of carboxylic acids in these two media. Indeed, this accounts for the distinct membrane fatty acid profiles seen for WT cells (Sen et al., 2016; Whaley et al., 2023). Moreover, BCFA auxotrophy is resolved in our suppressor mutants by incorporating *i* 14:0 BCFA into membranes derived from*i* C_4. This makes physiological sense - while FabH prefers *a* C_5-CoA to initiate fatty acid synthesis, incorporating iso even fatty acids into the bilayer reflects not only the affinity of MbcS for substrate, but also the availability of the acyl-CoA precursor pool (Whaley et al., 2023). We note that in contrast to the Whaley *et al.* study where they report a ~5-fold increase in affinity for*a* C_5 over *i* C_4, we did not measure a significant difference in MbcS affinity for*i* C_4 or *a* C_5. The discrepancy may be explained by the assay used to determine kinetic constants. Our coupled assay may simply be not sensitive enough to resolve the K_m values below 5 μM.

Our data validate and expand the idea that *S. aureus* salvages BCFA precursors by showing that branched-chain aldehydes are catabolized**(Figs 6-7)** . At the same time, it is intriguing that strains lacking a functional BKDH complex with heightened MbcS enzyme activity are able to grow, albeit poorly, in unsupplemented, defined medium. We posit this growth is the result of a cryptic alternative *de novo*synthesis pathway. Previous studies investigating BCFA synthesis in related Gram-positive bacteria inform our working model for an alternative route to BCFAs synthesis **(Fig 8)** . For instance, the Gram-positive bacterium *Lactococcus lactis* , which is largely used for the fermentation of dairy products, harbors an α-keto acid decarboxylase KdcA that converts the branched-chain α-keto acids into their respective branched-chain aldehydes, and these are recognized to be important for flavor formation (Smit et al., 2005). In USA300 strains, SAUSA300_0190 is annotated to encode an indole-pyruvate decarboxylase (IpdC). IpdC is 64% similar to *L. lactis* KdcA that has highest activity for the α-keto acid of valine (Smit et al., 2005), consistent with our GC-FAME data showing incorporation of valine derived, *i1* 4:0 BCFA in the *lpdA mbcS1* and *lpdA mbcS2* strains **(Fig 3)** . We propose that IpdC is a branched-chain α-keto acid decarboxylase. Three additional facts support this hypothesis: i) IpdCs are known to have broad specificity (Parsons et al., 2015); ii) *ipdC* expression is repressed by *S. aureus* CodY, which controls BCFAs synthesis by regulating the genes that direct the synthesis of the short BCFAs precursors (Waters et al., 2016); and iii) *S. aureus* synthesizes tryptophan but, unlike other organisms, does not catabolize the amino acid (Proctor & Kloos,1973; Spaepen et al., 2007;). AlsSD (acetolactate synthase/decarboxylase) and CidC (pyruvate oxidase) also have significant similarity to KdcA. However, how these enzymes would fit into the model is unclear.

As described above, other staphylococcal species are commonly used in the food industry due to their ability to form aldehydes, which are important for flavor formation (Beck, 2005; Beck et al., 2002). *S. aureus* itself was described to synthesize nine different aldehydes among them the branched chain 2-methylbutyraldehyde, 3-methylbutanal, and 3-methylpropanal (Bos et al., 2013; Filipiak et al., 2012). Mass spectrometry data from *S. xylosus* showed these cells can convert 3-methylbutanal to its respective carboxylic acid, the 3-methylbutanoic acid (Beck et al., 2002). In addition, human commensals like*Cutibacterium acnes* and *S. epidermidis* also produce these compounds on skin (Bos et al., 2013; Duffy & Morrin, 2019; Tait et al., 2014; Verhulst 2011) and salvage them in laboratory culture in an MbcS-dependent manner **(Fig 7)** , suggesting these compounds are salvaged by *S. aureus* using this pathway during infection. Taken together, data from our lab and other labs indicate that *S. aureus* oxidizes the branched-chain aldehyde to the carboxylic acid. The*S. aureus* genome encodes several potential aldehyde dehydrogenases (Alds); some of which have been characterized and are known to have broad specificity (Imber et al., 2018). Thus, we posit that one or more of these Alds converts the aldehyde derivatives of the branched-chain α-keto acids to their cognate carboxylic acids. MbcS then would serve to activate the carboxylic acid to its acyl-CoA to feed fatty acid synthesis. The α-keto acid decarboxylase and the aldehyde dehydrogenase(s) involved in this proposed alternative pathway are still to be found but this is currently an active focus of our laboratory.

Why has this pathway remained hidden until now? Overexpression of*mbcS* (either by mutation of the native promoter or by artificial overexpression using an inducible promoter) restores growth in the BKDH-deficient strain **(Figs 2-4)** . This suggests that MbcS activity in laboratory cultures is normally too low to support growth. Interestingly, MbcS is a member of the AMP-forming family of acyl-CoA synthetases. These enzymes (including *Rp* IbuA used in this study) are known to be regulated by reversible lysine modification of a conserved lysine residue by acetylation (Crosby & Escalante, 2014; Crosby et al., 2010; Gardner et al., 2006). Acetylation down-regulates enzyme activity (Gardner et al., 2006; Starai et al., 2002). It is conceivable that MbcS is acetylated under our laboratory conditions, and overproducing MbcS increases enzyme activity because the fraction of MbcS that escapes the acetylation machinery is increased. Indeed, the GCN5-related *N*- acetyltransferase AcuA was recently shown to acetylate acetyl-CoA synthetase in *S. aureus* (Burckhardt et al., 2019). The role of AcuA in bacterial physiology is unknown. Whether MbcS is acetylated and is a substrate for AcuA is not known. We are actively pursuing these questions in our laboratory.

Experimental Procedures

Bacterial strains, growth media, and culture conditions

Strains used in the present study are listed in **Table S1** . To routinely cultivate *S. aureus* strains, tryptic soy broth (TSB) containing 0.25% (wt/vol) dextrose (BD Biosciences) or complete chemically defined medium (CDM, pH 6.5) were used (Sheldon et al., 2014). Briefly, CDM medium was formulated with alanine (672 μM), arginine (287 μM), aspartic acid (684 μM), cysteine (166 μM), glutamic acid (680 μM), glycine (670 μM), histidine (129 μM), isoleucine (228 μM), leucine (684 μM), lysine (342 μM), methionine (20 μM), phenylalanine (240 μM), proline (690 μM), serine (285 μM), threonine (260 μM), tryptophan (50 μM), tyrosine (275 μM), valine (684 μM), thiamine (56 μM), nicotinic acid (10 μM), biotin (0.04 μM), pantothenic acid (2.3 μM), $MgCl_2$ (1,000 μM), $CaCl_2$(100 μM), monopotassium phosphate (40,000 μM), dipotassium phosphate (14,700 μM), sodium citrate dehydrate (1,400 μM), magnesium sulfate (400 μM), ammonium sulfate (7,600 μM), and glucose (27,753 μM). Blood agar plates were used to propagate the *lpdA mbcS* double mutant and its derivatives to mitigate the severe growth defect on TSB.*Escherichia coli* strains were grown in lysogeny broth (LB) medium without glucose (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, and 10 g L^{-1}sodium chloride) (Bertani, 1951). When necessary, media were solidified with agar at 1.5% [w/v] and supplemented with antibiotics at the following concentrations to maintain selection: ampicillin (Ap) 100 μg ml^{-1}, chloramphenicol (Cm) 10 μg ml^{-1}, erythromycin (Em) 10 μg ml^{-1}, kanamycin (Km) 100 μg ml^{-1} or tetracycline (Tc) 1.5 μg ml^{-1}. When required, media were supplemented with the short branched-chain carboxylic acids*i* C_4, *i* C_5, and*a* C_5 (Sigma-Aldrich), with the branched-chain fatty acids *a* 15:0 (Avanti Polar Lipids) and *a* 17:0 (Sigma-Aldrich), or with the branched-chain aldehyde 2-methylbutyraldehyde (Sigma-Aldrich) to a final concentration of 0.5 mM. For genetic complementation, TSB was supplemented with 50 ng ml^{-1} anhydrotetracycline (aTc). Unless otherwise noted, all strains were grown at 37°C. A double-back dilution scheme was used to grow cells for growth curves and GC-FAME analysis to ensure steady-state growth. Briefly, for growth curve experiments, overnight cultures were used to inoculate precultures in disposable 16x125 mm borosilicate glass tubes (Fisher Scientific) to an optical density at 600 nm (OD_{600}) of 0.05 and incubated with rotation. Cell growth was monitored by measuring OD_{600} using an Amersham Ultraspec 2100 Pro UV-visible spectrophotometer. After streaking from frozen stocks, overnight cultures were inoculated from single colonies and were used to initiate pre-cultures that were grown to exponential phase to an OD_{600} of 1.0 and diluted into fresh TSB to an OD_{600} of 0.05 and continued to grow. Exponential-phase samples were collected at an OD_{600} of 0.5±0.2. Samples were inoculated into a fresh medium in a 96-well plate to an OD_{600} of 0.05 and cell density was monitored over time in a computer-controlled Synergy H1 plate reader (BioTek/Agilent) running Gen5 software ver3.14. Cells for GC-FAME analysis were grown following the same scheme as above but in 125-mL DeLong shake flasks with 12.5 mL TSB (10:1 flask/medium ratio) and incubated in a gyratory water bath shaking. Exponential-phase samples were collected at an OD_{600} of 0.5 ± 0.1. Cells were grown to stationary phase in disposable borosilicate glass tubes for reporter and chemical complementation assays.

Genetic techniques

Oligonucleotides used in this study were synthesized by Integrated DNA Technologies (IDT; Coralville, IA) and are listed in **Table S2.**Genomic DNAs (gDNAs) were sequencing using Illumina whole genome sequencing (SeqCenter; Pittsburgh, PA) and results were analyzed using BRESEQ software (Barrick et al., 2009). Plasmids used in this study are listed in **Table S3** and were constructed using Gibson assembly as described previously (Gibson et al., 2009) and verified by Sanger sequencing (Azenta Life Sciences) or whole plasmid sequencing (Plasmidsaurus; Eugene, OR). *E. coli* NEB 5α (NEB) was used as a host for plasmid constructions, which were then transferred into*S. aureus* strain RN4220 by electroporation as previously described (Schenk & Laddaga, 1992). Plasmids and marked mutations were moved between *S. aureus* strains via Φ85-mediated transduction (Novick, 1991).

Construction of the lpdA::kan$^+$ strain*:*To construct the *lpdA::kan$^+$* strain (SRB3000), we moved the transposon insertion containing the Km^Rmarker from the *S. aureus* SH1000 strain VKS102 (Singh et al., 2008) into the *S. aureus* USA300 LAC SRB2421 (*lpdA* ::φNΣ) via Φ85-mediated transduction (Novick, 1991). Recombinants were selected on TSA Km medium and scored for Em susceptibility. We then confirmed the

allele by PCR.

Plasmids construction

(i) Construction of gfp reporter plasmids

To create the P_{mbcS}-*gfp* reporter fusion, a 236-bp fragment upstream of the translation initiation codon of SAUSA300_2542 (*i.e.* , *mbcS*) was amplified from *S. aureus* LAC with Q5 DNA polymerase (NEB) using oAP41 and oAP42 as primers. In parallel, the promoterless *gfp* reporter plasmid pMRSI (Waters et al., 2016) was amplified and linearized using oAP39 and oAP40 as primers. PCR products were treated with DpnI (NEB), subjected to Gibson Assembly, and inserted into *E. coli* NEB 5α (NEB). The resulting plasmid was named pAP4 (*mbcSp*$^{+}$*-gfp*). Plasmids pAP10 (*mbcS1p-gfp*), and pAP11 (*mbcS2p-gfp*), containing the same*mbcS* regulatory region but with point mutations identified during suppressor analysis, were constructed in a similar way by cloning the fragments into pMRS1.

(ii) Construction of new vectors

pWY51 (pCT1). A 821-bp fragment containing the *tetR* gene encoding the TetR repressor and the *tetR* promoter region (*tetR*$^{+}$ -P_{tet}) was PCR-amplified from pYJ335 (Ji et al.,1999) using primers oWY466 and oWY467. pCL55 (Lee et al., 1991) was used as the backbone plasmid and was amplified by PCR using primers oWY468 and oWY469. The DNA fragments were assembled by the Gibson method (Gibson et al., 2009), inserting the*tetR*$^{+}$ -P_{tet} cassette at the multicloning site of pCL55, and the DNA mixture was transformed into *E. coli* DH5α.

pWY52 (pCT2), and pWY53 (pCT3). A 2225-bp fragment containing the*tetM*· gene from pTET (Bose et al., 2013) was amplified by PCR using primers oWY486 and oWY487. pCL55 or pWY51 as a backbone plasmid was amplified by PCR using primers oWY488 and oWY489 .The DNA fragments were assembled by the Gibson method and the DNA mixture was transformed into *E. coli* DH5α, resulting in pWY52 (pCT2) or pWY53 (pCT3) that confer tetracycline resistance instead of chloramphenicol resistance.

(iii) Construction of complementation plasmids

1. pWY54 (P_{tet}-*mbcS*$^{+}$). A 1648-bp fragment containing the *mbcS* Shine Delgarno sequence and coding sequence was PCR-amplified from the USA300 LAC* chromosome using primer pair oWY470/oWY471. This also appends the sequence (DYKDDDDK) for a C-terminal FLAG epitope tag (Hopp et al., 1988). pWY53 as a backbone plasmid was amplified with primer pair oWY472/oWY473. The DNA fragments were assembled by the Gibson method and the DNA mixture was transformed into *E. coli* DH5α, resulting in pWY54. This places the production of *Sa* MbcS-FLAG under the control of the inducible P_{tet} promoter by anhydrotetracycline addition.
2. pWY55 (P_{tet}-*mbcS* [K510A]). The 1523-bp fragment containing the *mbcS* Shine Delgarno sequence (25bp) and coding sequence (1498bp) was PCR-amplified from the USA300 LAC* chromosome using the primer pair oWY470 and oWY500. A point mutation in the active site (K510A) of *mbcS* was generated by a gblock (125bp, oWY499). As before, pWY53 as a backbone plasmid was amplified with primer pair oWY469 and oWY473. The DNA fragments were assembled by the Gibson method and the DNA mixture was transformed into *E. coli* DH5α, resulting in pWY55. This results in the production of*Sa* $MbcS^{K510A}$-FLAG under the control of the inducible P_{tet} promoter by anhydrotetracycline addition.
3. pWY75 (P_{tet}-*RpibuA*$^{+}$). A 1716-bp fragment containing the coding sequence of Rp*ibuA* $^{+}$ and a FLAG tag at the C-terminus from*R. palustris* was optimized and synthesized by GenScript and the RBS and ORF from *SambcS* were utilized. This fragment was inserted into plasmid pWY53 and verified by GenScript. The resulting plasmid was transformed into *E. coli* DH5α, resulting in pWY75. The plasmid directs the synthesis of *Rp* IbuA-FLAG under the control of the inducible P_{tet} promoter by anhydrotetracycline addition.

(iv) Construction of plasmids used for protein overproductio n

pFM01. A 1636-bp fragment containing the*SambcS*$^{+}$ coding sequence was amplified from*S. aureus* LAC using oWY543 and oWY544. In parallel, pTEV5 (Rocco et al., 2008) was amplified from plasmid pRpIBUA1 using oWY545 and oWY546. Both DNA fragments were assembled via the Gibson method (Gibson et al., 2009) and the product was transformed into *E. coli* DH5α, resulting in pFM01.

pFM03. The *SambcS* allele coding for the*Sa* MbcS^{K510A} variant protein was amplified from pWY55 using oWY543 and oWY544. In parallel, pTEV5 was amplified using oWY545 and oWY546. The DNA fragments were assembled via the Gibson method (Gibson et al., 2009) and the mixture was transformed into *E. coli* DH5α, resulting in pFM03.

Identification of suppressor mutants

To screen for spontaneous mutations, the *lpdA* mutant was streaked from frozen stocks on TSA plate supplemented with a mixture of the branched-chain carboxylic acids *a* C5, *i* C5, and *i* C4. Independent colonies were then grown overnight in borosilicate glass tubes containing TSB supplemented with the mixture of the branched-chain carboxylic acids. A double-back dilution was performed in unsupplemented medium, and once cells reached an OD_{600} of 0.1 (growth arrest) they were serial diluted, plated on TSA without supplementation, and incubated at 37°C. Colonies (one per liquid culture) were picked, restreaked, and growth in the absence of carboxylic acids was confirmed. Following confirmation, gDNAs were purified and submitted for whole genome sequencing as a fee for service at SeqCenter. Analysis of spontaneous mutations was performed using BRESEQ software (Barrick et al., 2009).

Analysis of fatty acid composition

S. aureus strains were grown to exponential phase (OD_{600} ˜ 0.5), at which time 10-50 ml of each culture (˜100 mg of wet cells) were pelleted, washed twice with phosphate-buffered saline (PBS), and stored at -80°C. Fatty acids were saponified and methylated, and then analyzed by gas chromatographic analysis of fatty acid methyl esters (GC-FAME) as a fee for service at the Center for Microbial Identification and Taxonomy (Norman, OK).

GFP reporter assays

Single colonies of cells carrying the indicated reporter fusions were inoculated into sterile borosilicate glass tubes 16x125 mm (Fisher Scientific) containing 2 ml of TSB with appropriate antibiotic to maintain selection. Cells were grown to a stationary phase overnight. Pellets were collected and washed twice with PBS and resuspended in the same buffer. 100 µL of the cell suspension was transferred into a flat bottom black 96-well plate (Corning). Fluorescence was measured using a Synergy H1 plate reader (BioTek/Agilent) tuning the monochromator to 485 nm and 535 nm (excitation/emission, respectively). Relative fluorescence units (RFUs) were calculated by subtracting the fluorescence of plain PBS and dividing by OD_{600} to correct for cell density.

Protein Purification

rTEV. The plasmid encoding rTEV protease was transformed into*E. coli* BL21 Codon Plus and purified as described (Blommel & Fox, 2007).

Acyl-CoA synthetases . Plasmids containing the coding sequences for *Sa* MbcS^{WT},*Sa* MbcS^{K510A}, and *Rp* IbuA, all fused to cleavable *N* -terminal hexahistidine (His_6) tags, were overproduced and purified using a two-step procedure as described (Crosby et al., 2012). Briefly, the plasmids were introduced into*E. coli* BL21 C41λ (DE3) cells. The resulting strains were grown in LB medium supplemented with 100 mg L^{-1} Ap at 37°C first overnight in borosilicate tubes. The overnights were then subcultured at a 1:20 ratio with shaking until early stationary phase (OD_{600} ˜2). A subculture was prepared by diluting the initial culture at a 1:100 ratio into 500 mL of LB medium supplemented with 100 mg L^{-1} Ap, and cells were grown with shaking until reaching an OD_{600} of 0.6. Expression was induced using 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), followed by overnight incubation at 30°C. Cells were harvested by centrifugation at 8000 x g for 12 minutes, and the resulting pellets were stored at -80°C. Pellets were resuspended in 30 mL of buffer 1 (50

mM Tris-HCl pH 8.0, 300 mM NaCl, 10 mM imidazole). Cells were lysed using a Digital Sonifier (Branson) for 2- pulses separated by 5-s pauses on wet ice (25% amplitude) for 1.5-2 minutes. Cellular debris was removed by centrifugation at 38000 x g for 30 minutes at 4°C, and the soluble fraction was filtered using a 0.45 μm filter. The protein was purified by two-step Ni^{2+} affinity chromatography via a 4 mL bed volume of nickel-nitrilotriacetic acid resin (Thermo Scientific) pre-equilibrated with buffer 1 for 10 column volumes. The lysate was loaded on the column, and the column was washed with wash buffer 2 (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 20 mM imidazole) containing 1 mg ml^{-1} lysozyme (Thermo Scientific) and 0.2mM phenylmethanesulfonyl fluoride (PMSF) (Thermo Scientific). for a total of seven column volumes. Proteins were eluted with buffer 3 (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 250 mM imidazole) over six column volumes. Eluted protein fractions were analyzed using SDS-PAGE, and those fractions containing the target protein were pooled. The His_6 tag was cleaved with rTEV protease using a 1:100 [v/v] rTEV to protein ratio overnight on at 4°C ice. Following the cleavage, proteins were dialyzed sequentially against buffer 4 (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 0.5 mM EDTA), dialysis buffer 5 (50mM Tris-HCl pH 8.0, 300 mM NaCl), and finally against buffer 1. The proteins were reapplied to a pre-equilibrated Ni^{2+}-NTA column to capture the His_6tag and rTEV. Untagged target proteins were isolated to apparent homogeneity from the flow-through and dialyzed against buffer 6 (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.5 mM EDTA, 20% [v/v] glycerol) and then buffer 7 (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 20% glycerol). Proteins were concentrated, drop-frozen in liquid nitrogen, and stored at -80°C until used. Protein purity was evaluated using SDS-PAGE.

In vitro acyl-CoA synthetase activity assay

The activities of MbcS and IbuA were evaluated using a coupled and continuous spectrophotometric assay based on NADH consumption, as described (Crosby et al., 2012). Briefly, 100 μL reactions were composed of 50 mM HEPES pH 7.5, 1 mM tris (2-carboxyethyl) phosphine (TCEP), 2.5 mM ATP, 5 mM $MgCl_2$, 0.5 mM coenzyme A, 3 mM phosphoenolpyruvate, 0.1 mM NADH, 1 unit of pyruvate kinase, 5 units of myokinase, 1.5 units of lactate dehydrogenase, and 60 nM MbcS (calculated for monomeric enzyme) (or 30 nM IbuA). The reactions were initiated by adding 2 μL of the indicated carboxylic acid substrate to the 98 μL reaction mixture. The changes in the absorbance, measured at 340 nM, were recorded over time at 37°C degrees in a computer-controlled Synergy H1 plate reader running Gen5 software ver3.14 (Agilent/BioTek). Each experiment was performed in triplicate. All reagents and enzymes were purchased from Sigma, except for lactate dehydrogenase, which was purchased from Worthington Biochemical Corporation. The specific activity of MbcS for different carboxylic acids as substrates was calculated using the molar extinction coefficient of NADH (6220 M^{-1} cm^{-1}) and taking into account that two moles of NADH are oxidized for every mole of AMP being produced (Garrity et al., 2007). Specific activity was reported as μmol of AMP per minute per milligram of MbcS.

Liquid Chromatography-Mass Spectrometry (LC-MS)

Reagents and chemicals

The following reagents were purchased: water, acetonitrile, isopropanol, and methanol from Optima grade (Fisher Scientific); high purity formic acid (Thermo-Scientific); 3-HPMA-d6 and Isobutyril-Coenzyme A were purchased from Cayman chemicals.

Preparation of standards and internal standard stock solutions

CoA was resuspended in HEPES buffer pH 7.5 to generate a 3.83 mg ml^{-1} stock solution (stock A). A 1 mg mL^{-1} solution (stock B) of Isobutyryl-Coenzyme A (IB-CoA) was prepared by dissolving IB-CoA in HEPES buffer pH 7.5. The stock A and B were diluted in extraction buffer (3-HPMA-d6 was dissolved in methanol to achieve a 1 mg mL^{-1} stock solution, and subsequently diluted to a final concentration 500 ng mL^{-1} of IS) to generate stock C (20 μg ml^{-1} of CoA and 10 μg ml^{-1} IB-CoA). Stock C was then serially diluted to generate calibration curve standards ranging from 20 ng ml^{-1} to 10 μg ml^{-1} (IB-CoA) and 40 ng ml^{-1} to 20 μg ml^{-1} (CoA).

Sample preparation

MbcS acyl-CoA activity was assessed via LC-MS. The reaction mixture consisted of 50 mM HEPES pH 7.5, 2.5 mM Mg-ATP, 493 μM CoA, 73 μM isobutyric acid, and 60 nM MbcS. MbcS was denatured through incubation at 85°C for 10 minutes and used as a negative control. The reactions were initiated by adding isobutyric acid and incubated at 37°C for 2 hours, followed by placing the samples on ice. For IB-CoA measurements, to the 40 μl of samples was added 360 μl of extraction buffer and vortexed. For CoA measurements, the samples prepared for IB-CoA measurements were diluted 10-fold using extraction buffer. The samples were transferred to the MS vials for data acquisition.

LC-MS methods

This method was designed to measure CoA and Isobutyryl-CoA by UPLC-MS. The samples were resolved on a HSS T3, 1.8 μm, 2.1 x 100 mm column online with a triple quadrupole mass spectrometer (Xevo-TQ-S, Waters Corporation) operating in the multiple reaction monitoring (MRM) mode. The LC gradient method started with 98% of mobile phase A (10 mM ammonium formate in water) that involved a gradient change from 2% B (ACN:water [95:5] with 10 mM ammonium formate) to 98% phase B in 2.5 minutes after an initial lag phase of 0.5 minutes with flow rate 0.6 ml/min. The column was maintained at 25 °C and injection volume was kept at 5 μL. The autosampler was maintained at 5 °C. The column eluent was introduced directly into the TQS mass spectrometer by electrospray operating in positive mode at a capillary voltage of 3.0 kV and a sampling cone voltage of 83 V. The desolvation gas flow was set to 1000 l/h and the desolvation temperature was set to 500 °C. The cone gas flow was 150 l/h and the source temperature was set to 150 °C.

The sample cone voltage and collision energies were optimized for the analyte to obtain maximum ion intensity for parent and daughter ions using "IntelliStart" feature of MassLynx software (Waters Corporation). The instrument parameters were optimized to gain maximum specificity and sensitivity of ionization for the parent and daughter ions. Signal intensities from the MRM Q1>Q3 ion pairs for the CoA (768.1>261.1), IB-CoA (838.1>331.1) and 3-HPMA-d6 (228>169) were ranked to ensure selection of the most intense precursor and fragment ion pair for MRM-based quantitation. This approach resulted in selection of cone voltages and collision energies that maximized the generation of each fragment ion species. The details for the calibration curves for standards are included in Supplementary Materials **(Table S4)** . CoA was detectable as low as 5.0 ng ml^{-1} (limit of detection [LOD]) and showed 250 ng ml^{-1} as the lowest limit of quantification (LLOQ). While IB-CoA showed the LOD and LLOQ as 25 ng ml^{-1} and 50 ng ml^{-1}, respectively. The quantification range (QR) for CoA and IB-CoA were 250 ng ml^{-1} to 20 μg ml^{-1} and 50 ng ml^{-1} to 10 μg ml^{-1}, respectively. As ascertained by blanks run on either side of sample sets, no sample-to-sample carryover was observed. Each experiment was conducted in triplicate. Blanks (solvent alone) were injected on either side of test samples to assess sample carryover. Data was processed using Target Lynx 4.1. The relative quantification values of analytes were determined by calculating the ratio of peak areas of transitions of samples normalized to the peak area of the internal standard.

Kinetic analysis

Reactions were performed using the acyl-CoA synthetase activity assay described above. Pseudo-first order kinetic parameters were calculated using Prism version 9.0 (GraphPad Software), fitting the data to Equation 1,

$V_o = V$ x [S] / (K_m + [S]) (Equation 1)

where V_o is initial velocity, [S] is substrate concentration, V is maximum velocity, and $K_M$is the Michaelis-Menten constant (Segel, 1975).

Cross-feeding assay

Single colonies of *S. epidermidis* 1457 were inoculated into sterile, 16x125 mm borosilicate glass tubes containing 2 ml of CDM. Cells were grown to stationary phase overnight. The liquid culture was centrifuged

at 5450 x g for 15 minutes and the supernatant was filter-sterilized using 0.45 μm membranes (Avantor Sciences) to prepare conditioned medium. A single colony each of *S. aureus* WT,*lpdA* single mutant, and *lpdA mbcS* double mutant cells was inoculated to OD_{600} 0.05 in CDM, CDM supplemented with either 0.5 mM short branched-chain carboxylic acids or*a* 17:0 branched-chain fatty acid (Sigma-Aldrich), or decreasing concentrations of conditioned medium as indicated in Fig 7. Cells were grown to stationary phase overnight and OD_{600} was measured using Amersham Ultraspec 2100 Pro UV-visible spectrophotometer.

Statistical analyses

Prism version 9.0 (GraphPad Software) was used to perform all statistical analyses. Statistical significance was calculated using measurements from at least three biological replicates and one-way or two-way analysis of variance (ANOVA); for time course experiments, two-way ANOVA with repeated measures was used to assess differences and, if significant, analyses were followed by Tukey's honestly significant difference (Tukey's HSD) tests for pairwise comparisons. Statistical significance was assumed at a P value of <0.05.

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Author Contributions

SRB conceived the study; MCDSF and SRB conceptualized the research goals and aims; MCDSF, AP, WY, FCMG, DC, and MM performed the investigations and analyzed the data, SRB, MCDSF, WY, FCMG, DC, and MM prepared the original manuscript draft; SRB and MCDSF prepared the final manuscript.

Figure legends and tables

Table 1. Extragenic suppressor mutations map to the promoter region of *mbcS* .

mbcS **Allele**	**Location of Mutation**	**Mutation Effect**
mbcS1	Deletion at 105/C->T at 109	Shortens spacing between -10 and -35 to 17 bp/Unknown Effect
mbcS2	C-> T at 126 in -10 Box	Unknown Effect
mbcS4	Deletion at 102 in -35 Box	Shortens spacing between -10 and -35 to 17 bp
mbcS5	A->G at 109	Unknown Effect
mbcS8 (x2)[a]	Deletion at 108	Shortens spacing between -10 and -35 to 17 bp
mbcS11	Deletion at 120	Shortens spacing between -10 and -35 to 17 bp
mbcS19	Deletion at 121	Shortens spacing between -10 and -35 to 17 bp
mbcS21	A-> C at 104 in -35 Box	Unknown Effect
mbcS22	Deletion at 105	Shortens spacing between -10 and -35 to 17 bp
mbcS25	G-> A at 126 in the -10 Box	Creates perfect -10 Box (TATAA)
mbcS30	A-> C at 103 in -35 Box	Unknown Effect
mbcS31	AG->TC at 117 and 118	Unknown Effect

[a]Independently isolated twice.

Table 2. Comparison of kinetic parameters for *Sa* MbcS and*Rp* IbuA.

Protein	**Substrate**	$\mathbf{K_m}$ (μM)	$\mathbf{V_{max}}^a$	$\mathbf{K_{cat}}$ (s^{-1})	$\mathbf{K_{cat}}$/ $\mathbf{K_m}$ (s^{-1} μM^{-1})
*Sa*MbcS	isobutyrate (*i*C4)	8.9 ± 2.1	1.6 ± 0.3	0.43 ± 0.09	0.049
	2-methylbutyrate (*a*C5)	5.5 ± 1.0	0.8 ± 0.2	0.23 ± 0.05	0.042
	isovalerate (*i*C5)	> 500	ND[b]	ND[b]	ND[b]
*Sa*MbcS^{K510A}	2-methylbutyrate (*a*C5)	ND[b]	ND[b]	ND[b]	ND[b]
*Rp*IbuA^{WT}	isobutyrate (*i*C4)	72.3 ± 26.9	5.4 ± 1.6	3.01 ± 0.87	0.042
All values are mean ± standard deviation [a]μM of AMP produced min^{-1} [b]Not determined; enzyme is catalytically inactive.	*All values are mean ± standard deviation* [a]μM of AMP produced min^{-1} [b]Not determined; enzyme is catalytically inactive.				

Figure 1. Canonical pathway for the synthesis of branched-chain fatty acids (BCFA) via the BKDH complex. The branched-chain amino acids isoleucine (Ile), leucine (Leu), and valine (Val) are converted into their respective α-ketoacids by the transaminase IlvE; α-KMV (α-keto-β-methylvalerate), α-KIC (α-ketoisocaproate), and α-KIV (α-ketoisovalerate) undergo oxidative decarboxylation catalyzed by the α-keto acid dehydrogenase (BKDH) complex and the respective acyl-CoA primers, once condensed with malonyl CoA via 3-ketoacyl-ACP synthase III (FabH), are elongated into their respective BCFAs through the type II fatty acid synthase (FASII).

Figure 2. The putative acyl-CoA synthetase gene *mbcS* contributes to BCFAs synthesis in the absence of an active BKDH complex . Wild-type (WT), *lpdA* mutant, *lpdA* suppressor mutant with a modified *mbcS* promoter (*lpdA mbcS1)* and *lpdA mbcS* double mutant cells were grown in **(A)** TSB,**(B)** TSB supplemented with a mixture of 0.5 mM BCCAs *i* C4,*i* C5, and *a* C5 or **(C)** TSB supplemented with 0.5 mM*a* 15:0 fatty acid, and growth behavior was monitored over time as an increase in optical density at 600 nm (OD_{600}). Data are plotted as mean ± SD of three biological replicates. ****p<0.0001, using two-way ANOVA with Tukey's multiple comparison test at 5-8 h. In panel A, asterisks indicate that *lpdA* single mutant and *lpdA mbcS* double mutant are significantly different compared to WT and *lpdA mbcS1* . In panels B and C, asterisks indicate that *lpdA mbcS* double mutant is significantly different compared to WT, *lpdA* single mutant, and *lpdA mbcS1* .

Figure 3. Mutations in the *mbcS* promoter increase its activity and result in synthesis of *i*14:0 BCFAs. (A)Selected alleles identified during suppressor analysis are compared to the WT allele of the *mbcS* promoter region. Changes are highlighted in magenta, and -10 and -35 boxs are indicated by the gray boxes. **(B)** WT cells harboring plasmids with either WT or mutant *mbcS* promoter regions fused translationally to *gfp*(P_{mbcS}-*gfp*) were grown to stationary phase in TSB, at which time cells were pelleted and washed with PBS. Promoter activity was then measured (relative fluorescence units [RFUs]; GFP/OD_{600}). **** p<0.000,

one-way ANOVA with Tukey's multiple comparison test. **(C)** Suppressor mutants with the indicated *mbcS* alleles were grown to exponential phase in TSB. Cells were washed with PBS, and membrane fatty acid content was analyzed by GC-FAME. Data are presented as mean ± SD from three biological replicates. **** p<0.0001, ***p<0.001, two-way ANOVA with Tukey's multiple comparison test; ns, not significant.

Figure 4. Acyl-CoA synthetase activity of MbcS restores growth of a *S. aureus lpdA mbcS* strain in rich, complex medium lacking BCFAs.

(A) Clustal Omega (Sievers et al., 2011) was used to align MbcS with amino acid sequences of previously characterized acyl-CoA synthetases. Amino acids that constitute a conserved motif in the C-terminal catalytic domain are shown. Amino acids strictly conserved in the selected proteins are shaded; the conserved lysine residue required for the catalytic activity of acyl-CoA synthetases is highlighted in yellow. Acs, acetyl-CoA synthetase from *Salmonella enterica*(Starai & Escalante-Semerena, 2004); Acs2, acetyl-CoA synthetase from*Saccharomyces cerevisiae* (Starai & Escalante-Semerena, 2004); AcsA, acetyl-CoA synthetase from *B. subtilis* (Gardner et al., 2006); IbuA, isobutyryl-CoA synthetase from *R. palustrus* (Crosby & Escalante-Semerena, 2014; Crosby et al., 2012); FcsA, fatty acyl-CoA synthetase from *R. palustris* (Crosby & Escalante-Semerena, 2014). **(B)** WT, *mbcS,* or *lpdA mbcS* strains containing either the empty integration vector pCT3 (vector-only control [VOC]), pCT3 containing the WT allele of *S. aureus mbcS* under the control of the anhydrotetracycline inducible *tet* promoter (p*SambcS* $^{+}$), or the allele of *mbcS* that codes for a lysine-to-alanine substitution at the residue 510 [p*SambcS* (K510A)] were grown in TSB and growth (OD_{600}) was monitored over time. **(C, D)** WT or*lpdA mbcS* strains with the empty vector control or the wild-type allele of *ibuA* from *R. palustris*(p*RpibuA* $^{+}$) **(C)** with or **(D)**without 25 ng ml^{-1} of anhydrotetracycline (aTc) as gratuitous inducer were grown in TSB and OD_{600} was monitored over time. Data are plotted as mean ± SD from three biological replicates. **** p<0.0001, two-way ANOVA with Tukey's multiple comparison test. ns, not significant. In panel B, asterisks indicate that *lpdA mbcS* $^{\mathrm{VOC}}$ and *lpdA mbcS* $^{\mathrm{p}SambcS\ (\mathrm{K510A})}$ are statistically different from $\mathrm{WT}^{\mathrm{VOC}}$,*mbcS* $^{\mathrm{VOC}}$ and*lpdA mbcS* $^{\mathrm{pS}ambcS\ +}$. In panel C, asterisks indicate that *lpdA mbcS* $^{\mathrm{VOC}}$ and *lpdA mbcS* $^{\mathrm{pRp}ibuA\ +}$ are statistically different from $\mathrm{WT}^{\mathrm{VOC}}$ and *mbcS* $^{\mathrm{VOC}}$. In panel D, asterisks indicate that *lpdA mbcS* $^{\mathrm{VOC}}$ is statistically different from $\mathrm{WT}^{\mathrm{VOC}}$,*mbcS* $^{\mathrm{VOC}}$ and *lpdA mbcS* $^{\mathrm{pRp}ibuA\ +}$.

Figure 5. MbcS is a methylbutyryl-CoA synthetase. (A) The activity of MbcS was tested *in vitro* with several short, straight and branched carboxylic acids. Acids are indicated as C_X , where X denotes the carbon length. Data are plotted as the mean specific activity of the enzyme ± SD of at least three independent trials. **(B)** *Sa* MbcS was incubated with 2.5 mM MgATP, 493 μM coenzyme A (CoA) and 73 μM isobutyric acid (IB) to produce isobutyryl-CoA as described in *Experimental Procedures* . Detection and quantification of CoA and IB-CoA was measured via LC-MS. HK: heat killed enzyme. Traces are representative of three independent trials.

Figure 6. *S. aureus* utilizes branched-chain aldehydes to produce BCFAs in a MbcS-dependent manner . WT, *lpdA* mutant,*lpdA mbcS1* suppressor mutant, and *lpdA mbcS* double mutant cells were inoculated into chemically defined medium (CDM) (black) or CDM supplemented with vehicle (DMSO; gray), *i* C_4(2MB; pink), 2-methylbutyraldehyde (2MA; orange), and *a* 17:0 fatty acid (green). Cell density (OD_{600}) was measured after overnight incubation (16-18 h of growth). Data are plotted as mean ± SD from three biological replicates. **** p<0.0001, ***p<0.001, **p<0.01 two-way ANOVA with Tukey's multiple comparison test for each genotype; ns, not significant.

Figure 7. Metabolites from *S. epidermidis* support growth of *S. aureus* in an MbcS-dependent manner. (A) The *lpdA*mutant (Left) and the *lpdA mbcS* double mutant (Right) were inoculated ˜5 mm apart from *S. epidermidis* on TSA plates and incubated for 24 h. Arrow head indicates the point of inoculation. (B) WT, *lpdA* mutant, and *lpdA mbcS* double mutant cells were inoculated into chemically defined medium (CDM) (black) or CDM supplemented with a mixture of *a* C5, *i* C4, and *i* C5 (gray), *a* 17:0 fatty acid (orange), and 10% (green), 1% (pink) or 0.1% (blue), of conditioned CDM from *S. epidermidis* (i.e., cell-free supernatant). Cell density (OD_{600}) was measured after overnight incubation (16-18 h of growth). Data are plotted as mean ± SD from three biological replicates. **** p<0.0001, *p<0.05, two-way ANOVA with

Tukey's multiple comparison test; ns, not significant.

Figure 8. Working model for the synthesis of BCFAs in *S. aureus* in a BKDH-independent manner. In a *lpdA* mutant the BKDH complex is inactive and during laboratory cultivation the synthesis of BCFAs is blocked. *S. aureus* strains with high MbcS enzyme activity (*i.e.,* overexpression of *mbcS*) can synthesize BCFAs independent of the BKDH complex using exogenous or endogenous precursors. We propose α-keto acids are converted into their respective branched-chain aldehydes by an α-keto acid decarboxylase, followed by a reaction catalyzed by an aldehyde dehydrogenase to generate branched-chain carboxylic acids. MbcS-dependent acyl-CoA synthesis feeds FASII to generate BCFAs for incorporation into membrane phospholipids. Whether the two pathways operate in parallel or function under specific conditions is a focus of ongoing research. BrnQ, branched-chain amino acid permease; IlvE, branched-chain amino acid aminotransferase.

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