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Increased flexibility in the use of exogenous lipoic acid by *Staphylococcus aureus*

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Summary

Lipoic acid is a cofactor required for intermediary metabolism that is either synthesized *de novo* or acquired from environmental sources. The bacterial pathogen *Staphylococcus aureus* encodes enzymes required for *de novo* biosynthesis, but also encodes two ligases, LplA1 and LplA2, that are sufficient for lipoic acid salvage during infection. *S. aureus* also encodes two H proteins, GcvH of the glycine cleavage system and the homologous GcvH-L encoded in an operon with LplA2. GcvH is a recognized conduit for lipoyl transfer to α-ketoacid dehydrogenase E2 subunits, while the function of GcvH-L remains unclear. The potential to produce two ligases and two H proteins is an unusual characteristic of *S. aureus* that is unlike most other Gram positive *Firmicutes* and might allude to an expanded pathway of lipoic acid acquisition in this microorganism. Here, we demonstrate that LplA1 and LplA2 facilitate lipoic acid salvage by differentially targeting lipoyl domain-containing proteins; LplA1 targets H proteins and LplA2 targets α-ketoacid dehydrogenase E2 subunits. Furthermore, GcvH and GcvH-L both facilitate lipoyl relay to E2 subunits. Altogether, these studies identify an expanded mode of lipoic acid salvage used by *S. aureus* and more broadly underscore the importance of bacterial adaptations when faced with nutritional limitation.

Graphical Abstract

Lipoic acid is a metabolic cofactor required for intermediary metabolism. *Staphylococcus aureus* acquires lipoic acid through either *de novo* biosynthesis or salvage. We show that *S. aureus* adapts to lipoic acid limitation through an expanded salvage pathway including two lipoic acid ligases with divergent targets; intrinsic lipoamidase activity that extracts inaccessible lipoic acid from

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Author contributions

IL, WPT, and FA conceived and designed components of the study; IL, WPT, SF, JPG, AZ, and FA assisted in the acquisition, analysis, and interpretation of the data; IL, WPT, and FA wrote the manuscript; all authors commented on the final version manuscript.

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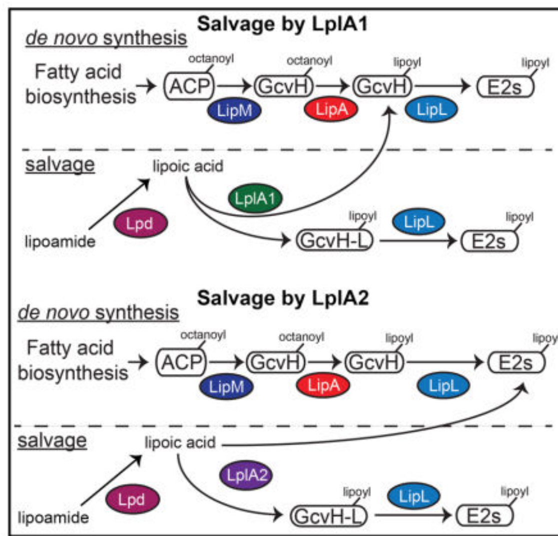
peptide-bound sources; and an accessory lipoyl-protein that serves as an additional source of lipoic acid for transfer to metabolic enzyme subunits.



Keywords

lipoic acid; ligase; *S. aureus* ; metabolic adaptation; virulence; nutrient acquisition

Introduction

Staphylococcus aureus is an opportunistic pathogen that causes disease in a wide range of tissues including skin, kidney, bone, lung, and heart (Tong *et al.*, 2015, Liu *et al.*, 2011). Each infectious niche poses unique challenges to *S. aureus* survival due to significant differences in nutrient bioavailability at each site. In order to thrive in such a wide range of environments, *S. aureus* harnesses key adaptive traits that permit trace metal/ nutrient acquisition, maintenance of metabolic homeostasis, and survival in nutritionally deficient or otherwise inhospitable conditions (Cassat & Skaar, 2012, Hood & Skaar, 2012, Richardson *et al.*, 2015, Grim *et al.*, 2017, Kelliher *et al.*, 2018, Beasley & Heinrichs, 2010, Mazmanian *et al.*, 2003). Aside from a relatively well-developed understanding of trace metal acquisition, the mechanisms used by *S. aureus* to acquire other scarce nutrients remains comparatively understudied.

In addition to the necessity for trace metal scavenging for optimal metabolic activity and bacterial survival, *S. aureus* also requires the essential cofactor lipoic acid (Zorzoli *et al.*, 2016, Grayczyk *et al.*, 2017). Lipoic acid, a derivative of the medium-chain fatty acid octanoic acid, is a sulfur-containing cofactor that is covalently attached to conserved lysine residues in multi-subunit enzyme complexes needed for aerobic metabolism, fatty acid biosynthesis, glycine detoxification, and maintenance of redox homeostasis (Spalding & Prigge, 2010, Cronan, 2016). Five lipoylated enzyme complexes are used in bacteria: pyruvate dehydrogenase (PDH) catalyzes the oxidative decarboxylation of pyruvate to acetyl CoA; 2-oxoglutarate dehydrogenase (OGDH) converts α-ketoglutarate to succinyl-CoA;

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branched-chain 2-oxoacid dehydrogenase (BCODH) degrades branched chain amino acids to make a branched chain CoA intermediate needed for fatty acid biosynthesis; acetoin dehydrogenase (AoDH) is similar to the PDH complex and also catalyzes the conversion of pyruvate to acetyl CoA; and the glycine cleavage system (Gcs) catalyzes the reversible decarboxylation of glycine (Cronan, 2016, Spalding & Prigge, 2010, Perham, 2000). *S. aureus* encodes PDH, OGDH, and BCODH α-ketoacid dehydrogenase complexes, as well as the Gcs, but not AoDH (Spalding & Prigge, 2010, Zorzoli *et al.*, 2016). The α-ketoacid dehydrogenases are comprised of multiple copies of three different subunits referred to as E1, E2, and E3, whereas the glycine cleavage system uses subunits referred to as P (pyridoxal phosphate-containing protein), H (hydrogen carrier protein), T (tetrahydrofolate-containing protein), and L (lipoamide dehydrogenase) proteins (Douce *et al.*, 2001, Kikuchi *et al.*, 2008). The lipoic acid cofactor is attached by an amide bond to a conserved lysine residue on E2 and H subunits and acts to channel substrates through the active sites of the enzyme complexes (Douce *et al.*, 2001, Perham, 2000). The necessity of lipoic acid for the activity of the α-ketoacid dehydrogenase complexes and glycine cleavage system demands a strategy to synthesize and/or salvage lipoic acid from the environment, as well as a method to attach lipoic acid to the conserved lysine within the lipoyl domains of E2 and H subunits.

A growing body of literature suggests that lipoic acid biosynthesis and salvage can have a major role in facilitating the pathogenesis of microorganisms (Spalding & Prigge, 2010). Lipoic acid salvage in parasites such as *Plasmodium falciparum*, the causative agent of malaria, is crucial for survival and growth of the parasite during the blood and liver stage (Afanador *et al.*, 2014, Gunther *et al.*, 2009, Storm & Muller, 2012, Falkard *et al.*, 2013). In addition, disruption of the lipoic acid biosynthesis and salvage pathway attenuates *Burkholderia pseudomallei* virulence in an intranasal mouse infection model (Pilatz *et al.*, 2006). In *Pseudomonas aeruginosa*, a functional lipoylated PDH enzyme complex is important for the expression of the type three secretion system (Dacheux *et al.*, 2002). Furthermore, disruption of *dlaT*, a gene encoding the E2 PDH subunit in *Mycobacterium tuberculosis*, results in increased susceptibility to macrophage killing and oxidative stress (Bryk *et al.*, 2013, Bryk *et al.*, 2002). *Listeria monocytogenes,* a lipoic acid auxotroph, uses one of two lipoic acid salvage enzymes to facilitate acquisition of lipoic acid within the intracellular environment (Christensen *et al.*, 2011a, Keeney *et al.*, 2007, O'Riordan *et al.*, 2003). Overall, these examples demonstrate the range of effects that lipoic acid acquisition and lipoic acid-dependent enzyme activities have on promoting pathogen survival within an infected host. Our studies with *S. aureus* suggest niche specific requirements for *de novo* biosynthesis and salvage during infection, where *de novo* biosynthesis is required to infect the heart and salvage is necessary for optimal infection of the kidney, highlighting the relevance of both pathways during infection (Zorzoli *et al.*, 2016). In addition, we have identified a novel activity for lipoyl-E2-PDH in the extracellular environment where it acts to blunt macrophage activation by toll-like receptor ligands, further expanding the relevance of protein lipoylation in the context of infection (Grayczyk *et al.*, 2017).

The mechanisms of lipoic acid acquisition and synthesis can vary considerably among bacteria, fungi, and protozoans (Cronan, 2016). Many of these organisms either encode a *de novo* biosynthesis pathway, salvage pathway, or both. Each confers a range of acquisition strategies that presumably have evolved to satisfy the unique lipoic acid requirements

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of that particular microorganism within its replicative niche. Most studies of lipoic acid biosynthesis and salvage in bacteria were conducted in *Escherichia coli, Bacillus subtilis*, and *L. monocytogenes* (Cronan, 2016, Christensen & Cronan, 2010, Christensen *et al*., 2011a, Christensen *et al*., 2011b, Hermes & Cronan, 2009, Jordan & Cronan, 2003, Martin *et al*., 2011, Miller *et al*., 2000, Morris *et al*., 1995, Vanden Boom *et al*., 1991, Zhao *et al*., 2003). These studies highlight the diversity of lipoic acid biosynthesis and salvage strategies between bacterial species. Recently, our lab used a genetic approach to define the lipoic acid acquisition pathways of *S. aureus* (Zorzoli et al., 2016). This work determined that *S. aureus* has a complex lipoic acid biosynthesis and salvage pathway that bears similarities to that of *B. subtilis* and *L. monocytogenes* combined. *S. aureus* encodes the three characteristic enzymes needed for *de novo* biosynthesis in *B. subtilis*, LipM, LipA, and LipL, as well as iron sulfur cluster biogenesis proteins, SufCDSUB and SufT, needed for incorporation of sulfur into the lipoic acid molecule (Christensen & Cronan, 2010, Martin *et al*., 2011, Christensen *et al*., 2011b, Mashruwala *et al*., 2016, Roberts *et al*., 2017). However, it also encodes two lipoic acid ligases, LplA1 and LplA2, needed for salvage during infection and two H proteins, GcvH and GcvH-Like (GcvH-L), which we suspect may be used as a source of lipoyl protein for subsequent transfer to E2 subunits by an amidotransferase (Christensen *et al*., 2011a, Zorzoli *et al*., 2016, Cao *et al*., 2018).

As with *B. subtilis*, lipoic acid synthesis in *S. aureus* begins with transfer of octanoic acid, a medium chain fatty acid, from its acyl carrier protein (ACP) to GcvH by the octanoyl transferase, LipM (Christensen & Cronan, 2010, Zorzoli *et al*., 2016). The lipoyl synthetase, LipA, then converts the octanoyl moiety to lipoic acid, followed by transfer of the lipoyl group from GcvH to the E2 subunits of PDH, BCODH, and OGDH by LipL (Zorzoli *et al*., 2016, Christensen *et al*., 2011b, Martin *et al*., 2011). *S. aureus* contains two additional enzymes that have activities associated with lipoic acid salvage from the host environment, LplA1 and LplA2 (Zorzoli *et al*., 2016). This is reminiscent of *L. monocytogenes*, which also encodes two lipoic acid ligases, but unlike *S. aureus,* has no *de novo* biosynthesis enzymes (Christensen *et al*., 2011a, Keeney *et al*., 2007). In *L. monocytogenes,* LplA1 is integral for lipoic acid acquisition *in vitro* and *in vivo*, whereas LplA2 only facilitates acquisition *in vitro* (Keeney *et al*., 2007). In a similar vein, *S. aureus* LplA1, but not LplA2, was found to be crucial for the salvage of lipoic acid *in vitro* (Zorzoli *et al*., 2016). However, *in vivo* studies indicate that either LplA1 or LplA2 is sufficient to promote kidney infection during murine systemic infection. Because the LplA2 ligase does not have a discernable function *in vitro* - unless bacteria are cultured in the presence of fetal bovine serum (FBS) - its exact mode of action in lipoic acid salvage is unknown (Zorzoli *et al*., 2016). Furthermore, LplA2 is encoded in an operon containing an ADP-ribosyltransferase, macrodomain protein, luciferase-like monooxygenase, and the H subunit-like protein GcvH-L (Zorzoli *et al*., 2016, Rack *et al*., 2015). This operon is postulated to participate in lipoic acid-dependent maintenance of redox homeostasis, which suggests that LplA2 may have roles that extend beyond lipoic acid salvage (Rack *et al*., 2015). Nonetheless, the presence of a potential alternative H protein encoded within this operon may indicate an unusual layer of complexity should lipoyl GcvH-L indeed act as a substrate in lipoic acid transfer (Cao *et al*., 2018). Based on this information, we hypothesized that LplA1 and LplA2 in *S. aureus* use alternative lipoylated substrates and/or the enzymes have different lipoylation targets,

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thereby providing *S. aureus* with an increased degree of flexibility in its mechanisms of lipoic acid acquisition in lipoic acid-limiting environments.

In this study, we used a biochemical approach to interrogate the activity of both LplA1 and LplA2 of *S. aureus*. We found that LplA1 attaches lipoic acid primarily to the H subunit, GcvH, and the H subunit-like protein, GcvH-L, whereas LplA2 attaches lipoic acid to E2 subunits, as well as operon-linked GcvH-L. Each ligase functions independently, with limited overlap in protein targets. Furthermore, both ligases have the capacity to use octanoic acid as a substrate for attachment to GcvH and GcvH-L. Neither enzyme uses lipoyl tripeptides as a substrate unless co-incubated with crude lysates of *S. aureus*, which harbors lipoamidase activity. Furthermore, GcvH and GcvH-L both provide a source of lipoyl protein for subsequent transfer to E2 subunits, despite a postulated role for GcvH-L in orchestrating resistance to host-derived redox stress (Rack *et al.*, 2015). Taken together, these studies highlight the importance of the divergent functions of LplA1 and LplA2, as well as the contributions of both GcvH and GcvH-L to protein lipoylation and likely explains why *S. aureus* thrives so well when faced with low levels of free lipoic acid during host infection.

Results

S. aureus LplA1 promotes lipoic acid salvage and stimulates bacterial replication in broth culture

Our genetic analyses of lipoic acid biosynthesis and salvage in *S. aureus* have led to a model whereby the enzymes LipM, LipA, and LipL comprise a functional *de novo* lipoic acid biosynthesis pathway that acts *in vitro* and within certain tissues *in vivo* (Figure 1A) (Zorzoli *et al.*, 2016). In addition, *S. aureus* produces two lipoic acid salvage enzymes, LplA1 and LplA2, implying potential for flexibility in lipoic acid salvage. However, biochemical studies to date suggest the activity of LplA2 is limited to lipoylation of GcvH-L (Figure 1A) (Rack *et al.*, 2015). In contrast, our genetic analyses indicate that LplA1 and LplA2 are both sufficient to promote survival of a lipoic acid auxotroph during infection, implying some degree of functional overlap between LplA1 and LplA2, at least in the host environment (Zorzoli *et al.*, 2016). To begin to interrogate the ligase activities of LplA1 and LplA2, we conducted a series of growth curves using *S. aureus* strains harboring deletions of *lipA* and one or both lipoic acid ligases, followed by complementation with *lplA1* or *lplA2* driven by a constitutive promoter. When inoculated into medium lacking free lipoic (RPMI) or supplemented with the *de novo* biosynthesis precursor octanoic acid (OA), only the wild type strain was able to replicate (Figure 1B–C). In contrast, supplementation of the medium with lipoic acid restored wild type growth characteristics to *lipA* *lplA2* and *lipA* *lplA1* *lplA2* + *lplA1* strains, while *lipA* *lplA1* and *lipA* *lplA1* *lplA2* + *lplA2* strains were unable to replicate (Figure 1D). These data support our prior studies and suggest that LplA1, but not LplA2 scavenges free lipoic acid to support bacterial replication (Zorzoli *et al.*, 2016).

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Both LplA1 and LplA2 are sufficient to promote lipoylation of E2 and H subunits

The fact that LplA2 is unable to support replication of lipoic acid auxotrophs in the presence of lipoic acid was unusual given our observation that LplA2 is sufficient to promote kidney infection by *lplA1 lplA2* and *lipA lplA1 lplA2* + *lplA2* strains (Zorzoli *et al.*, 2016). Furthermore, a *lipA lplA1 lplA2* + *lplA2* strain constitutively expresses high levels of LplA2 in broth culture as determined by immunoblot with polyclonal rabbit α-LplA2 antibody (Figure 2A). Thus, although restoration of growth was not observed, we reasoned that we might still detect ligase activity for LplA2 by monitoring protein lipoylation. To this end, we tested whether or not the *lipA lplA1 lplA2* + *lplA2* strain is capable of lipoylating E2 and H proteins despite the inability of LplA2 to restore growth in broth culture. WT, *lipA lplA1 lplA2*, *lipA lplA1 lplA2* + *lplA1*, *lipA lplA1 lplA2* + *lplA2* strains were grown in medium containing the branched chain fatty acid precursors isobutyric acid, isovaleric acid, and 2-methylbutyric acid (BCFA), to bypass the requirement of lipoic acid for growth (Martin *et al.*, 2011, Martin *et al.*, 2009). The medium was supplemented with either lipoic acid or octanoic acid, followed by immunoblot of whole cell lysates to ascertain LplA1 and LplA2-dependent protein lipoylation. Contrary to its inability to grow in the presence of free lipoic acid, the *lipA lplA1 lplA2* + *lplA2* strain resembled a *lipA lplA1 lplA2* + *lplA1* strain in its lipoylation of E2-PDH, E2-OGDH, and GcvH with modest reductions in the lipoylation on E2-BCODH (Figure 2B and Figure S1). Lipoylation of these same subunits when grown in BCFA medium or BCFA supplemented with octanoic acid was only observed for the wild type strain (Figure 2B and Figure S1). In summary, LplA1 and LplA2-dependent salvage of lipoic acid occurs in broth culture.

Interrogating the functional activities of LplA1 and LplA2

Thus far, our data indicate that LplA1 and LplA2 behave as lipoic acid ligases. However, their exact biochemical function and protein targets remain unclear. In *E. coli,* the lipoic acid ligase is a promiscuous enzyme and attaches lipoic acid or octanoic acid to most H proteins as well as E2 subunits from a variety of species (Green *et al.*, 1995, Morris *et al.*, 1994). In contrast, lipoic acid attachment in *B. subtilis* and *L. monocytogenes* occurs on the H subunit of the glycine cleavage system followed by transfer to E2 subunits by the amidotransferase LipL (Christensen *et al.*, 2011a, Christensen *et al.*, 2011b). These enzymes do not appreciably target E2 subunits *in vitro* (Christensen *et al.*, 2011a, Christensen *et al.*, 2011b). The diversity of functions in the ligase family of enzymes argues that the biochemical functions, protein targets, or substrates could vary between LplA1 and LplA2 (Cronan, 2016). Therefore, to interrogate the activities of the ligases in greater detail, we purified recombinant *S. aureus* LplA1 and LplA2 from *E. coli* for use in *in vitro* lipoylation assays with purified *S. aureus* E2-PDH, E2-OGDH, E2-BCODH, GcvH, and GcvH-L (Figure 3A).

LplA1 and LplA2 targeting of GcvH and GcvH-L

S. aureus contains a canonical GcvH protein important for glycine cleavage, but also encodes a GcvH-Like protein (GcvH-L), with 29% amino acid identity (54% similarity) to GcvH. GcvH-L is encoded in a putative operon with the *lplA2* gene - the last gene in

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the operon (Zorzoli *et al.*, 2016). Our genetic evidence supports a model where, in addition to its role in glycine cleavage, GcvH acts as a major conduit for transfer of lipoic acid to E2 subunits by the amidotransferase LipL (Zorzoli *et al.*, 2016). GcvH-L, like LplA2, does not appear to be expressed in broth culture, precluding direct assessment of its activity (Zorzoli *et al.*, 2016, Rack *et al.*, 2015, Nobre & Saraiva, 2013). Based on their proposed assignment as H proteins, we reasoned that both GcvH of the glycine cleavage system and GcvH-L are targets for lipoylation by one or both *S. aureus* ligases. Support from this notion comes from Rack *et al*, who demonstrated that a 1:1 to 1:2 molar ratio of *S. aureus* LplA1 or LplA2 and GcvH-L is sufficient to promote lipoylation (Rack *et al.*, 2015). To begin to decipher the biochemical mechanisms of lipoic acid salvage in *S. aureus*, we first tested the ability of LplA1 and LplA2 to attach free lipoic acid to one of these two H proteins. Lipoylation assays conducted with LplA1 or LplA2 and GcvH (molar ratio of enzyme to substrate 1:20) with free lipoic acid showed complete lipoylation of GcvH by LplA1, as evidenced by immunoblot with α-lipoic acid antibody and a shift in apparent molecular weight presumably due to a loss of positive charge at the conserved lysine of the lipoyl domain after attachment (Figures 3B and Figure S2A, left) (Martin *et al.*, 2011, Posner *et al.*, 2009). In contrast, no lipoylation was detected for reactions containing LplA2 and GcvH. When LplA1 and LplA2 were used in lipoylation reactions with GcvH-L, lipoylation by immunoblot and shifts in apparent molecular weight were observed regardless of the ligase used (Figure 3B and Figure S2A, right) (Rack *et al.*, 2015). When octanoic acid was used in place of lipoic acid, we found that LplA1 could target both GcvH and GcvH-L for octanoic acid attachment, whereas LplA2 only targeted GcvH-L (Figure 3C and Figure S2B). Taken together, these data indicate that LplA1 is indiscriminate in its ability to attach lipoic acid and octanoic acid to *S. aureus* H proteins, whereas LplA2 attaches lipoic acid and octanoic acid to GcvH-L.

LplA1 and LplA2 targeting of E2 proteins

Our prior genetic data and H protein lipoylation studies presented here suggest that *S. aureus* LplA1 and LplA2 functions diverge in such a way that each has different targets for lipoyl attachment (Zorzoli *et al.*, 2016). A scenario such as this would increase the likelihood of sufficient lipoylation in the absence of *de novo* biosynthesis, allow for fine-tuning of protein lipoylation in cases where requirements for exogenous lipoic acid are high, or facilitate lipoylation of a particular enzyme complex over another. To test the range of E2 protein targets of LplA1 and LplA2, we conducted lipoic acid attachment assays with purified lipoyl domain-containing E2 proteins of *S. aureus* - E2-PDH, E2-OGDH, and E2-BCODH. In a reaction containing E2-PDH and LplA2, a prominent lipoyl protein band was detected after immunoblot with α-lipoic acid antibody (Figure 4A and Figure S3A). Modest, but detectable lipoylation over that of the control reaction containing only E2-PDH and lipoic acid was observed in reactions with LplA1. In reactions containing E2-OGDH, lipoylation was seen in the presence of LplA1 and LplA2 although LplA2-dependent attachment was more robust than that caused by LplA1 (Figure 4B and Figure S3B). In contrast, lipoic acid attachment to E2-BCODH was only observed in the presence of LplA2, but not LplA1 (Figure 4C and Figure S3C). Although a faint band was observed in lanes from reactions with LplA1, this band was also present in control wells containing only E2-BCODH and lipoic acid (Figure 4C). These data suggest that LplA1-mediated lipoic acid attachment to

E2 subunits is limited, whereas LplA2 is promiscuous and targets all three E2 subunits for lipoic acid attachment.

Use of lipoyl peptides as a source of lipoic acid requires lipoamidase activity and LplA1 or LplA2

Thus far, our data indicate that the lipoic acid ligases of *S. aureus* are capable of attaching free lipoic acid to E2 and H protein subunits with different efficiencies. However, free lipoic acid is limiting within the host environment and is likely to be present attached to lysine residues within conserved lipoyl domains via an amide bond. Previous studies with *L. monocytogenes* suggest that lipoamide can be used as a source of lipoic acid to stimulate growth, by virtue of the activity of an *L. monocytogenes*-encoded lipoamidase (Christensen *et al.*, 2011a). We wondered whether or not peptide-bound lipoic acid might facilitate growth of *S. aureus* lipoic acid auxotrophs. We first tested if supplementation of RPMI medium with porcine PDH was sufficient to stimulate growth of gene deletion mutants lacking one or both ligases in a *lipA* mutant background. Addition of PDH was unable to promote growth of any mutant strain tested (Figure 5A). In contrast, when PDH was first co-incubated with proteinase K agarose beads prior to supplementation, the *lipA* *lplA2* and *lipA* *lplA1* *lplA2* + *lplA1* strains grew identically to the WT strain (Figure 5B). Because commercial porcine PDH is unlikely to be uniformly pure, we synthesized a series of tripeptides [Asp-Lys-Ala (DKA), Asp-Lys^{Lipoyl}-Ala (DK^{L}A), Asp-Lys-Thr (DKT), and Asp-Lys^{Lipoyl}-Thr (DK^{L}T)] that resemble the minimum peptides generated by digestion of porcine PDH with proteinase K (Keeney *et al.*, 2007). These tripeptides are sufficient to stimulate the growth of *L. monocytogenes* in the absence of free lipoic acid (Christensen *et al.*, 2011a, Keeney *et al.*, 2007). We monitored the growth of the indicated *S. aureus* strains after supplementation with DKA, DK^{L}A, DKT, and DK^{L}T into RPMI medium. Growth was restored for the *lipA* *lplA2* and *lipA* *lplA1* *lplA2* + *lplA1* strains, but only when the peptide containing the lipoyl moiety was present (Figure 5C–F). Consistent with prior observations with lipoic acid, lipoyl peptides were unable to promote growth of *lipA* *lplA1* and *lipA* *lplA1* *lplA2* + *lplA2* strains (Figure 5C–F).

Stimulation of growth after supplementation with lipoyl peptides suggests that *S. aureus* is capable of acquiring lipoic acid from its lysine-bound state. The ability to do so would either require intrinsic amidotransferase activity within LplA1 or LplA2, or the presence of an enzyme with lipoamidase activity. To test whether or not LplA1 or LplA2 harbors amidotransferase activity, we conducted biochemical assays with recombinant LplA1 or LplA2 with GcvH, GcvH-L, E2-PDH, E2-OGDH, E2-BCODH and DK^{L}A. Neither LplA1 nor LplA2 was able to use DK^{L}A to lipoylate H or E2 subunits (Figure 6A and Figure S4A–B). This finding suggests that like *L. monocytogenes, S. aureus* must also produce an enzyme with lipoamidase activity in order to liberate free lipoic acid for subsequent ligation by LplA1 and LplA2. To test this possibility, we set up a series of lipoylation reactions with DK^{L}A, LplA1, LplA2, GcvH, or GcvH-L in the presence or absence of crude lysates derived from a *lipA* *lipM* *lipL* *lplA1* *lplA2* mutant lacking all lipoic acid *de novo* biosynthesis and salvage enzymes. Indeed, GcvH and GcvH-L were now partially lipoylated by LplA1 (Figure 6B and Figure S5A), while GcvH-L was partially lipoylated by LplA2 in the presence of crude lysates (Figure 6C and Figure S5B). Altogether, these data indicate

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that LplA1 and LplA2-dependent utilization of lipoyl peptides requires the presence of an endogenous lipoamidase produced by *S. aureus*.

GcvH-L is sufficient to restore growth to a lipoic acid auxotroph in the absence of free lipoic acid

LplA1 and LplA2 both attach lipoic acid to GcvH-L, while LplA1 only lipoylates GcvH. Although GcvH-L has been implicated in promoting resistance to redox stress during infection, its classification as a lipoyl domain H protein and recent evidence for evolutionary maintenance of H proteins as lipoyl reservoirs suggests that it may also promote lipoic acid biosynthesis and salvage (Rack *et al.*, 2015, Cao *et al.*, 2018). In Gram-positive *Firmicutes*, the H subunit of the glycine cleavage system is a critical conduit for the transfer of lipoic acid to E2 subunits, an activity that is mediated by the LipL amidotransferase (Christensen *et al.*, 2011a, Christensen *et al.*, 2011b, Martin *et al.*, 2011). A *gcvH* mutant is unable to replicate in the absence of free lipoic acid due to an inability to shuttle lipoic acid from GcvH to E2 proteins (Zorzoli *et al.*, 2016, Christensen *et al.*, 2011b). To test whether or not GcvH-L is sufficient to restore growth to a *gcvH* mutant in the absence of free lipoic acid – and hence restore lipoyl transfer to E2 proteins – we generated *gcvH* + *gcvH*, *gcvH* + *gcvH-L*, and *gcvH* + *gcvH-L** (containing a single mutation K56A to remove the conserved lysine of the lipoyl domain (Rack *et al.*, 2015)) strains, wherein the expression of *gcvH*, *gcvH-L*, and *gcvH-L** is driven by the constitutive P_{HELP} promoter. We subsequently monitored growth on solidified RPMI medium, growth in RPMI liquid culture, and lipoylation of E2 and H proteins in cell lysates. Expression of either GcvH or GcvH-L permitted growth on solid agar plates lacking lipoic acid, while expression of GcvH-L* displayed poor growth (Figure 7A). Furthermore, *gcvH* + *gcvH* and *gcvH* + *gcvH-L* strains grew to similar final optical densities in broth culture, although the *gcvH* + *gcvH-L* strain had a slightly reduced growth rate compared to WT and *gcvH* + *gcvH* strains (Figure 7B). In contrast, the *gcvH* + *gcvH-L** strain grew poorly and resembled the *gcvH* mutant. Supplementation of lipoic acid into the culture medium restored WT growth characteristics to all strains due to limited lipoic acid attachment to E2-OGDH and subsequent transfer to other E2 subunits as seen in our prior studies (Figure 7C and (Zorzoli *et al.*, 2016)). Immunoblots of whole cell lysates derived from the *gcvH* + *gcvH* strain in BCFA medium lacking lipoic acid yielded prominent bands corresponding to lipoylation of all E2 proteins (Figure 7D, left and Figure S6). Similarly, lysates derived from the *gcvH* + *gcvH-L* strain contained a dominant lipoyl E2-PDH band with detectable, albeit reduced, levels of lipoylation on E2-OGDH and E2-BCODH (Figure 7D, left). Lysates derived from the *gcvH* + *gcvH-L** strain had low levels of lipoylation on E2 subunits that were similar to the background levels seen in a *gcvH* mutant (Figure 7D, left). Lipoylation of all E2 subunits was observed when the BCFA medium was supplemented with exogenous lipoic acid (Figure 7D, right and Figure S6). A band corresponding to lipoyl GcvH-L was not detected in any condition after immunoblot (Figure 7D). Identical results were obtained when experiments were repeated with a *gcvH* *gcvH-L* double mutant and *gcvH* *gcvH-L* + *gcvH* or *gcvH* *gcvH-L* + *gcvH-L* strains (Figure S7) Together, these data indicate that both GcvH and GcvH-L are sufficient to promote lipoyl transfer in *S. aureus* with important implications for both *de novo* biosynthesis and salvage of lipoic acid since H proteins are required for *de novo* biosynthesis, but are also direct targets of LplA1 and LplA2 (Figure 3).

Discussion

Microbial pathogens depend on a wide range of adaptive traits to promote their survival in nutrient-limited conditions. Trace nutrient acquisition pathways constitute a key bacterial survival strategy during infection. In this study, we used a biochemical approach to dissect the functional overlap between two lipoic acid ligases and two H proteins of *S. aureus* as it relates to flexibility in lipoic acid acquisition. Our findings indicate that LplA1 and LplA2 indeed harbor canonical lipoic acid ligase activity, but differ in their protein targets as outlined in Figure 8. LplA1 primarily lipoylates H proteins, whereas LplA2 has the capacity to target a wider range of proteins including the three E2 subunits of PDH, OGDH, and BCODH (Figure 8A–B). Neither of these ligases harbors secondary amidotransferase activity that permits the use of substrates other than free lipoic acid. However, *S. aureus* appears to make a soluble lipoamidase that allows use of peptide-bound lipoic acid by these ligases (Figure 8A–B). Furthermore, the ability to produce two H proteins – one that is expressed in culture and one that is posited to be upregulated *in vivo* or during periods of redox stress – provides an additional source of protein bound lipoic acid for transfer to E2 subunits. Together, these findings expand our understanding of lipoic acid salvage in *S. aureus,* and suggest an adaptive strategy that makes this pathogen extremely adept at lipoic acid acquisition.

The lipoic acid ligase of *B. subtilis,* LplJ, targets GcvH but not other E2 subunits, while LplA of *E. coli* is indiscriminate in its recognition of lipoyl domain lysines (Christensen *et al.*, 2011a, Christensen *et al.*, 2011b, Green *et al.*, 1995, Morris *et al.*, 1994). This function for LplA in *E. coli* is logical because prior lipoyl attachment to GcvH and subsequent transfer to E2 subunits is not required for synthesis of lipoic acid (Cronan, 2016). In *B. subtilis* and *L. monocytogenes* on the other hand, GcvH serves as an important conduit for lipoic acid transfer to E2 subunits due to the amidotransferase activity of LipL (Christensen *et al.*, 2011a, Christensen *et al.*, 2011b, Martin *et al.*, 2011). Direct lipoylation of H subunits in this scenario allows GcvH to serve as a source of lipoic acid for the bacterial cell. However, herein lies a potential bottleneck for these Gram positive species during periods of lipoic acid starvation; salvage and transfer to E2 subunits depends on passing through a GcvH intermediate, rather than direct lipoylation of the target E2 protein. In *S. aureus,* this limitation is eliminated by LplA2, which lipoylates all E2 subunits without the requirement of first passing through GcvH. However, *S. aureus* still maintains the LplA1 ligase, which behaves similarly to LplJ of *B. subtilis* and incidentally has greater amino acid sequence identity (57% compared to 39% for LplA2). The presence of two ligases with different lipoyl targets likely confers a level of added flexibility that may allow *S. aureus* to overcome severe limitations in bioavailable lipoic acid by directing trace amounts of the cofactor to the most critical complexes. A similar scenario may be true in *L. monocytogenes*, which also encodes two lipoic acid ligases, though technical difficulties in the purification of LplA2 have prevented an in-depth assessment of its biochemical activity (Christensen *et al.*, 2011a).

An added layer of complexity in our understanding of how LplA1 and LplA2 promote lipoic acid salvage exists in the varied gene expression of the two enzymes. *lplA1* is expressed both *in vitro* and *in vivo* as evidenced by: (1) the ability of a *lipA lplA2* mutant to grow in the presence of exogenous lipoic acid in broth culture; and (2) the

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fact that *lplA2* and *lipA lplA1 lplA2* + *lplA1* strains infect the kidneys of mice at similar levels to WT *S. aureus*, while *lplA1 lplA2* and *lipA lplA1 lplA2* strains are markedly attenuated (Zorzoli *et al.*, 2016). This is not true for a *lipA lplA1* strain, which is defective for growth and lipoylation in broth culture even in the presence of free lipoic acid – unless cultured in the presence of FBS (Zorzoli *et al.*, 2016). When *lplA2* expression is driven by a constitutive promoter, lipoylation in the presence of exogenous lipoic acid is observed, although the levels of lipoylation on the E2 subunit of BCODH appear to be slightly lower when compared to complementation with *lplA1* (Figure 2). Together, these observations strongly suggest that *lplA2* is not normally expressed under standard growth conditions. In contrast, a *lplA1* mutant is fully virulent *in vivo*, while a *lplA1 lplA2* mutant is significantly attenuated, indicating that *lplA2* is expressed and sufficient to promote salvage *in vivo* (Zorzoli *et al.*, 2016). These prior observations, coupled with our new understanding of LplA1 and LplA2 targeting, suggest that variations in both gene regulation and lipoyl protein targeting are likely to greatly expand the breadth and efficiency of lipoic acid attachment in appropriate environmental conditions. Identification of an *in vitro* growth condition that leads to high expression of the operon containing *lplA2* has yet to be uncovered despite prior suggestion that redox stress is an inducing signal (Nobre & Saraiva, 2013). Although limited LplA2 activity is observed in the presence of FBS, this growth condition may eventually prove useful in the characterization of the gene regulatory programs of the *lplA2* operon. Current work in the laboratory aims to decipher how gene regulation and the newly identified divergent activities of LplA1 and LplA2 interface with one another to promote diversification in lipoic acid acquisition.

In this study, we determined that *S. aureus* harbors lipoamidase activity and permits the salvage of lysine-bound lipoic acid. An acquisition strategy such as this is likely imperative to an infectious microbe such as *S. aureus*, which can reside in host environments that have negligible free lipoic acid levels (Zorzoli *et al.*, 2016, Akiba *et al.*, 1998), but instead contain significant amounts of the cofactor covalently attached to enzyme complex subunits. *Enterococcus faecalis* is the only bacterium with a fully characterized lipoamidase that is required for removal of lipoic acid from covalently attached lysines (Spalding & Prigge, 2009, Jiang & Cronan, 2005). BLAST of this sequence for similar proteins in *S. aureus* yielded no hits. Interestingly, Cristea and colleagues have determined that sirtuins of mammalian cells and bacteria can harbor lipoamidase activities that allow them to fine-tune the activity of major metabolic enzyme complexes (Rowland *et al.*, 2017, Mathias *et al.*, 2016, Mathias *et al.*, 2014). It stands to reason that such a sirtuin might also exist in *S. aureus*. In a recent study, the *E. coli* sirtuin, CobB, a previously characterized deacetylase, was shown to have lipoamidase activity that can regulate the function of PDH and OGDH (Rowland *et al.*, 2017). SrtN, the sirtuin homolog in *B. subtilis* also acts as a lipoamidase (Rowland *et al.*, 2017). A homolog of this sirtuin exists in *S. aureus* (SAUSA300_2157), but deletion of this gene has no effect on the ability of a *S. aureus lipA* strain to grow in the presence of DK^{L}A, suggesting that another, or more than one, lipoamidase exists to carry out this activity (Teoh, unpublished).

One unusual outcome of this study was the observation that expression of *lplA2* via a constitutive promoter restored lipoylation to lipoic acid auxotrophs in the presence of free lipoic acid, but was insufficient to restore bacterial growth in the presence of free lipoic

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acid. A similar phenotype is observed in yeast where supplementation of the growth medium with free lipoic acid does not restore aerobic growth to a *LIP5* (LipA homolog) mutant, but is sufficient to promote lipoylation of target proteins (Sulo & Martin, 1993, Hermes & Cronan, 2013). At present, we suggest two possible explanations for this outcome. First, the observation that lipoylation of E2-BCODH is reduced in broth culture after complementation with *lplA2* compared to WT suggests deficiencies in E2-BCODH activity may hamper the generation of branched chain fatty acids important for *S. aureus* membrane fluidity and replication. Indeed, supplementation of RPMI medium with BCFA restores growth to the *lipA lplA1* and *lipA lplA1 lpla2* + *lplA2* strains *in vitro*, supporting the notion that perturbations in branched chain fatty acid metabolism are at least partially responsible for the growth deficit observed in our study (Figure S8). Our own efforts to generate an *e2-BCODH* mutant in *S. aureus* have been unsuccessful suggesting that the activity of this enzyme is critical for bacterial replication (Zorzoli *et al.*, 2016). This is further supported by the poor growth of a transposon insertion mutant that disrupts BCODH activity (Mashruwala *et al.*, 2016). The growth of this BCODH null strain is improved by supplementing BCFA to the culture medium. Other experimental perturbations that reduce the demand for lipoic acid-dependent enzyme function - oxygen depletion or mutation of *clpC* – are likely to yield the same outcome (Mashruwala *et al.*, 2016). Alternatively, an inability of LplA2 to complement replication defects *in vitro* may suggest an unappreciated role for the other enzymes in the *lplA2*-encoding operon for restoration of growth. It is interesting to note that GcvH-L is also encoded in this operon where it is posited to have functions related to redox homeostasis that are regulated by lipoylation-dependent ADP-ribosylation by an operon-encoded sirtuin (Rack *et al.*, 2015). Certainly, a deeper understanding of this locus and its biological activities is warranted and we are currently exploring the relationship between its primary activities and how they relate to lipoic acid salvage. Nonetheless, our data clearly indicate that despite the putative functions of GcvH-L and LplA2 that may be related to redox homeostasis and ADP-ribosylation, GcvH-L can also participate in lipoyl relay to E2 subunits, thereby expanding the functional lipoic acid biosynthesis and salvage pathways of *S. aureus*. Recent studies from Cao *et al.* corroborate this notion and provide evidence that many H and H-Like proteins, even those whose activities may not be directly involved in glycine cleavage, have evolved in an unusual way to participate in lipoyl relay (Cao *et al.*, 2018).

Altogether, this study has expanded our understanding of the lipoic acid salvage strategies of *S. aureus* and highlights a unique adaptive strategy that is likely to contribute significantly to survival in nutrient deficient environments. Our prior demonstration that LplA1 and LplA2 can compensate for one another during murine infection underscores the biological significance of this point (Zorzoli *et al.*, 2016). Further, the work herein places this observation within a mechanistic framework by highlighting the divergent targets of these two enzymes and the expanded acquisition pathways imparted by lipoamidase and GcvH-L. Thus, expansion of lipoic acid salvage mechanisms by *S. aureus* represents a critical adaptive trait likely used for survival in a wide range of nutrient limited environments, including host tissues.

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Experimental Procedures

Bacterial strains and growth conditions

All bacterial strains used in this manuscript are listed in Table 1. *E. coli* strains were routinely grown in Lysogeny Broth (LB) (Amresco) with antibiotics added as necessary. *S. aureus* strains were grown in either rich medium, Tryptic Soy Broth (TSB) (Criterion), or in defined medium, Roswell Park Memorial Institute medium (RPMI) (Corning) supplemented with 1% casamino acids (Amresco). All strains were grown overnight at 37°C at a 45° angle with shaking unless stated otherwise. For growth curves, *S. aureus* overnight cultures were grown in RPMI containing branched chain carboxylic acids (10 mM isobutyric acid, 9 mM 2-methylbutyric acid, 9 mM isovaleric acid) and 10 mM sodium acetate (Sigma) in order to bypass the requirement of lipoic acid or octanoic acid for replication (Sigma) (Martin *et al.*, 2011, Martin *et al.*, 2009). When needed, cultures were supplemented with the following agents for selection: 100 μg ml^{-1} ampicillin (Amp), 3 μg ml^{-1} erythromycin (Erm), 10 μg ml^{-1} chloramphenicol (Cam), 50 μg ml^{-1} kanamycin (Kan), 50 μg ml^{-1} neomycin (Neo), and 0.2 mM cadmium chloride.

Molecular genetic techniques

When necessary for validation of mutant strains, chromosomal DNA was isolated from *S. aureus* using the Wizard Genomic DNA purification kit (Promega) following the manufacturer's protocol with minor modifications. Overnight cultures were started in 5 ml TSB and 1.5 ml was pelleted by centrifugation at 21,000 x g for 3 min the following day. Bacterial pellets were resuspended in 200 μl of TSM (50 mM Tris, 0.5 M Sucrose, 10 mM $MgCl_2$, pH 7.5). In order to disrupt the cell wall, 2.5 μl of lysostaphin (2 mg ml^{-1} in 0.5 Tris, pH 8.0) was added to the resuspended cell pellet and incubated for 15 min at 37°C. Following incubation, the bacteria were pelleted at 21,000 x g for 3 min and the supernatant was discarded. The remaining steps to purify genomic DNA from *S. aureus* were completed using the manufacturer's protocol. Recombinant plasmids were extracted using QIAGEN mini and midi prep kits with the following modifications for plasmid isolation from *S. aureus* when necessary. An overnight culture of *S. aureus* was grown in 5 ml TSB and pelleted at 3,000 x g for 10 min the following day. The bacterial pellet was resuspended in 400 μl TSM (50 mM Tris, pH 7.5, 0.5 M Sucrose, 10 mM $MgCl_2$) followed by the addition of 20 μl of lysostaphin. This mixture was incubated for 10 min at 37°C to digest the cell wall and then pelleted at 16,000 x g for 2 min after which the supernatant was discarded. The remaining procedures were completed as suggested by the manufacturer's protocol. Polymerase chain reaction (PCR) products were either gel-extracted or purified using QIAGEN QIAquick gel extraction and PCR purification kits. All PCRs were conducted using Phusion high-fidelity DNA polymerase (NEB).

E. coli competent cell preparation

An overnight culture of *E. coli* was grown in 3 ml LB at 37°C with shaking. The next day, the bacteria were subcultured 1:55 into a 250 ml flask and grown for an additional ~2.5 hours at 37°C with shaking until the culture reached an optical density at 600 nm (OD600) of 0.3–0.4. The following steps were all completed on ice. Cultures were aliquoted into 50 ml conical tubes and chilled on ice for 10 min. Afterwards, bacteria were pelleted by

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centrifugation at 3000 x g for 10 min and the bacterial pellet was washed twice with 10 ml TFB-1 (20 mM KOAc, 100 mM $RbCl_2$, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% glycerol, adjusted to pH 5.8 using 0.2 M Acetic acid) and incubated on ice for 10 min between washes. Bacteria were then pelleted at 3,000 x g for 10 min and resuspended in 1/25 of the original culture volume in TFB-2 (10 mM MOPS, 75 mM $CaCl_2$, 10 mM $RbCl_2$, 15% glycerol, adjusted to pH 6.5 using KOH). 100 µl of the competent cells were aliquoted into 1.5 mL tubes and stored at −80°C.

E. coli heat transformation

5 µl of ligation mix or 1 µl of purified plasmid were added to 50 µl of competent *E. coli.* The mixture was incubated on ice for 30 min, heat shocked at 42°C for 45 seconds, and then incubated for an additional 2 minutes on ice. 250 µl of super optimal broth with catabolite repression (SOC) medium (0.5% tryptone, 0.5% yeast extract, 0.05% NaCl, and 250 mM KCl adjusted to pH 7.0 with 5 M NaOH followed by addition of 20 mL 1M glucose) was added and the bacteria were incubated for 2 hours at 37°C with shaking. 100 µl of the bacterial suspension was plated onto the appropriate selection medium and incubated overnight at 37°C.

Preparation of S. aureus electrocompetent cells

An overnight culture of *S. aureus* was grown in 5 ml TSB at 37°C with shaking. The following day the bacteria were subcultured 1:100 in 30 ml TSB and incubated for an additional 3 hours at 37°C until the culture reached an OD600 of 0.5. The bacterial culture was pelleted by centrifugation at 3,000 x g for 10 min. All subsequent steps were carried out on ice. After pelleting, the bacteria were washed three times by resuspending in 30 ml ice cold 10% glycerol and pelleted by centrifugation at 3,000 x g for 10 min. After the last wash, the bacteria were suspended in 3 ml 10% glycerol, aliquoted into 1.5 ml tubes, and stored at −80°C.

Transformation of S. aureus

Frozen competent cells were thawed at room temperature for 5 min. 2 µl of plasmid DNA was then added to 50 µl of *S. aureus* LAC competent cells and incubated at room temperature for 30 min. The competent cell mixture was transferred to sterile 2 mm electroporation cuvettes and pulsed at 1800 V, 10 µF, and 600 Ω. After electroporation, the bacteria were resuspended in 750 µl of TSB or TSB + BCFA and incubated at 37°C or 30°C for 1.5 hours. After incubation, the bacteria were pelleted at 10,000 x g for 2 min and resuspended in 100 µl of TSB or TSB + BCFA, plated on TSA or TSA + BCFA plates containing antibiotic and incubated at 37°C or 30°C for 1–2 days.

Generation of a lipA lipM lipL lplA1 lplA2 mutant

Strain FA-S1178 (*lipA lplA1 lplA2*) was used as the recipient strain for transductions with generalized transducing phage φ11 harboring marked mutations in *lipL* (FA-S953 - *lipL::kan*) and *lipM* (NE1334 – *lipM::erm*). In order to package φ11 with donor DNA, a 3 ml overnight culture of the marked donor strain (FA-S953 or NE1334) was started in TSB +/ – BCFA:LB (1:1) supplemented with 5 mM $CaCl_2$ and 5 mM $MgSO_4$ and grown overnight

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with shaking at 37°C. The following day, the overnight strain was subcultured 1:100 into 10 ml TSB +/– BCFA:LB (1:1) supplemented with 5 mM $CaCl_2$ and 5 mM $MgSO_4$ and grown for 2.5 – 4 hours with shaking at 37°C until the culture reached an OD600 of 0.3 to 0.9. 500 μl of the bacterial culture was incubated with 10-fold serial dilutions of φ11 phage stock in TMG (10 mM Tris pH 7.5, 5 mM $MgCl_2$, 0.01% gelatin (v/v)), vortexed gently, and incubated at room temperature for 30 minutes. After 30 minutes, tubes containing the bacteria and phage dilutions were mixed with 3 ml CY Top agar (casamino acids 3 g L^{-1}, yeast extract 3 g L^{-1}, NaCl 6 g L^{-1}, 7.5 g L^{-1} agar, +/– BCFA as needed) supplemented with 5 mM $CaCl_2$ and 5 mM $MgSO_4$, cooled to 55°C, and poured onto TSA +/– BCFA plates. After the top agar solidified, plates were incubated at 30°C overnight face up. The next day the top agar from 2–3 plates with confluent plaques was scraped off the plate using a sterile scoopula and resuspended in 2 ml TMG buffer per plate followed by extensive vortexing. The top agar was pelleted by centrifugation at 16,000 x g for 15 minutes. The supernatant containing φ11 phage was filtered twice using a 0.2 μm filter and then an additional two times with a 0.45 μm filter. All phage stocks were stored at 4°C.

To transduce marked mutations, the recipient strain FA-S1178 (*lipA lplA1 lplA2*) was cultured overnight with shaking at 37°C in 20 ml TSB+BCFA:LB (1:1) supplemented with 5 mM $CaCl_2$. The following day, FA-S1178 was pelleted by centrifugation at 16,000 x g for 15 min and resuspended in 3 ml of TSB+BCFA:LB (1:1) supplemented with 5 mM $CaCl_2$. 500 μl of the recipient bacteria were serial diluted and incubated with 100 μl of the φ11 phage (10^8–10^9 PFU) collected as described above or 100 μl of TMG buffer as an uninfected control for 30 min at room temperature, inverting the tubes every 10 min. After 30 min the bacteria/phage suspension was supplemented with 40 mM sodium citrate and incubated for an additional 30 min, inverting the tubes every 10 min. The bacteria were pelleted by centrifugation at 16,000 x g for 3 min and washed twice with 500 μl TSB+BCFA:LB (1:1) supplemented with 10 mM sodium citrate. Washed bacterial pellets were resuspended in 250 μl of TSB+BCFA:LB (1:1) supplemented with 10 mM sodium citrate and 200 μl was plated on TSA + BCFA containing 10 mM sodium citrate supplemented with erythromycin or kanamycin/neomycin as needed. Plates were incubated at 37°C for 24–48 hours until bacterial colonies were detected. All mutants were verified by PCR.

Generation of gcvH-L and gcvH::kan gcvH-L mutants

Regions of homology corresponding to 498 bp upstream and 492 bp downstream of *gcvH-L* were amplified using primer pairs gcvH-L-SOE1 and gcvH-L-SOE2 or gcvH-L-SOE3 and gcvH-L-SOE4 (Table 2). The amplicons corresponding to the upstream and downstream regions of homology flanking *gcvH-L* were used as template in a splicing by overlap extension PCR reaction with primers gcvH-L-SOE1 and gcvH-L-SOE4 followed by sub-cloning the resulting amplicon into the allelic replacement plasmid pIMAY to generate pIMAY-gcvH-L (Monk *et al.*, 2012). pIMAY-gcvH-L was introduced into WT AH-LAC and mutagenesis was carried out as previously described to obtain an in-frame deletion of *gcvH-L* that removed the entire coding region (Zorzoli *et al.*, 2016). A marked *gcvH::kan* mutation was subsequently transduced into the *gcvH-L* strain, as described above, to generate a *gcvH::kan gcvH-L* double mutant.

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Generation of gcvH::kan + gcvH, gcvH::kan + gcvH-L, gcvH-L + gcvh-L, gcvH::kan gcvH-L + gcvH, and gcvH::kan gcvH-L + gcvH-L strains

gcvH::kan + *gcvH*, *gcvH::kan* + *gcvH-L*, *gcvH-L* + *gcvh-L*, *gcvH::kan gcvH-L* + *gcvH*, and *gcvH::kan gcvH-L* + *gcvH-L* complementation strains were generated using plasmid pJC1111, which stably integrates into the SaPI-1 site of the *S. aureus* chromosome after passage through *S. aureus* strain RN9011 containing pRN7203 expressing the SaPI-1 integrase, leading to single-copy stable integration of the plasmid (Chen *et al.*, 2014). We first generated pJC1111 plasmids that drive expression of the *gcvH* or *gcvH-L* genes under the control of the constitutive P_{HELP} promoter obtained from allelic replacement plasmid pIMAY (Monk *et al.*, 2008, Monk *et al.*, 2012, Riedel *et al.*, 2007). The oligonucleotides listed in Table 2 were used to amplify the P_{HELP} promoter and the open reading frames corresponding to *gcvH* and *gcvH-L* including ~150 nucleotides downstream of the stop codon. The P_{HELP} amplicon was combined with either the *gcvH* or *gcvH-L* amplicon and the two fragments were joined by splicing by overlap extension PCR. The resulting P_{HELP}-*gcvH* and P_{HELP}-*gcvH-L* amplicons were subsequently purified and cloned into pJC1111 after digestion with restriction endonucleases PstI and SacI and subsequent ligation into similarly digested pJC1111, generating pJC1111-P_{HELP}-*gcvH* and pJC1111- P_{HELP}-*gcvH-L*.

Both pJC1111-P_{HELP}-*gcvH* and pJC1111- P_{HELP}-*gcvH-L* were propagated in *E. coli* DC10B, followed by isolation and subsequent electroporation into *S. aureus* RN9011 and plating on TSA plates supplemented with chloramphenicol and $CdCl_2$. Plasmid integrants were used as donors to package and transduce the complementation allele into the AH-LAC *gcvH::kan*, *gcvH-L*, and *gcvH::kan gcvH-L* strain backgrounds. Final complementation strains were selected based on their $CdCl_2$ resistance and validated via PCR. This resulted in the generation of the strains designated *gcvH::kan* + *gcvH*, *gcvH::kan* + *gcvH-L*, *gcvH-L* + *gcvh-L*, *gcvH::kan gcvH-L* + *gcvH*, and *gcvH::kan gcvH-L* + *gcvH-L*.

Generation of gcvH::kan + gcvH-L*

Using the complementation plasmid pJC1111-P_{HELP}-*gcvH-L* as template, a K56A substitution was introduced into GcvH-L. K56 corresponds to the conserved lysine residue of the GcvH-L lipoyl domain that is targeted for lipoylation. The mutation was generated by introducing a two-nucleotide change in the center of the 5′-phosphorylated forward primer gcvH-L-K56A–F (Table 2). The 5′ phosphorylated reverse primer gcvH-L-K56A–R was designed so that the 5′ ends of the two primers anneal back-to-back. After exponential amplification with Phusion® High-Fidelity DNA Polymerase (NEB), the PCR reaction was treated with the methylation-dependent restriction enzyme DpnI (NEB) for one hour at 37°C to eliminate the original, non-mutated plasmid template. The blunt-end PCR products were then ligated with T4 DNA ligase (NEB) at room temperature for two hours and the reaction was transformed into DC10B competent cells and plated on LB agar with 100 μg ml^{-1} ampicillin (Monk *et al.*, 2012). Plasmids were purified from transformants and sequenced with the external primers used to generate P_{HELP}-*gcvH-L* (Table 2) to validate the substitution (K56A). The resultant pJC1111-P_{HELP}-*gcvH-L** was introduced into the *gcvH::kan* strain as described above, yielding *gcvH::kan+gcvH-L**

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Whole cell lysate preparation

Wild type and mutant *S. aureus* strains were grown overnight at 37°C with shaking in 15 mL conical tubes containing 5 mL of RPMI + BCFA medium. 50 μL of these overnight cultures was inoculated into 15 mL conical tubes containing 5 mL of RPMI + BCFA; RPMI + BCFA + 5 μM α-lipoic acid, or RPMI + BCFA + 150 μM octanoic acid. Samples were incubated with shaking at 200 rpm for 9 hours and bacterial growth was determined by measuring OD600 using a Genesys 10S UV-Vis spectrophotometer (Thermo Scientific). The remaining culture volume was centrifuged at 3,000 x g for 15 minutes, the supernatant was discarded, and the bacterial pellets were suspended in 250 μL of PBS and transferred to screw cap microcentrifuge lysing tubes (Fisher Scientific) containing 250 μL of 0.1 mm glass cell disruption beads (Scientific Industries, Inc.). Cells were lysed using a Fast Prep-24 5G (MP Biomedicals) bead disruption system in two sequential steps, at 5.0 speed for 20 seconds and at 4.5 speed for 20 seconds, each separated by a 5 minute incubation period on ice. After cell disruption, samples were centrifuged at 19,000 x g for 15 minutes. 45 μL of the supernatant were collected in microcentrifuge tubes containing 15 μL of 6X SDS sample buffer and subsequently boiled for 10 minutes prior to storage at −20°C.

Determination of protein lipoylation and LplA2 levels in whole cell lysates

Protein samples from OD-normalized whole cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 12% polyacrylamide gels at 120 volts for approximately 4 hours. Gel staining was performed to evaluate protein patterns and equivalent loading of samples using GelCode Blue stain reagent (Thermo Scientific) with PageRuler Prestained Protein Ladder (Thermo Scientific) used as a molecular weight marker. Protein lipoylation and LplA2 levels were assessed via immunoblot. Briefly, resolved proteins were transferred from polyacrylamide gels to 0.2 μm PVDF membranes (Immobilon, Roche) at 70 V for 1 hour and 15 minutes in a Trans-Blot® Cell (Bio-Rad). After transfer, membranes were incubated for 1 hour with TBST (0.1% Tween-20 in Tris Buffered Saline) supplemented with 5% bovine serum albumin (BSA) at room temperature. A 1:3,000 dilution of rabbit polyclonal α-lipoic acid antibody (Calbiochem) or 1:50,000 dilution of rabbit polyclonal α-LplA2 antibody (Pacific Immunology) was added to the membrane followed by incubation for 1 hour and three subsequent washes in ~20 mL of TBST. AP-conjugated Goat anti-Rabbit IgG (H+L) (Invitrogen) was then added at a 1:5000 dilution in TBST for 45 minutes followed by three 15 minute washes in ~20 mL of TBST. Membranes were developed with BCIP/NBT (5-bromo-4-chloro-3-indoyl-phosphate/nitro blue tetrazolium) color development substrate (VWR).

Preparation of Proteinase K agarose beads and digestion of porcine PDH

Proteinase K agarose beads and porcine pyruvate dehydrogenase (PDH) were purchased from Sigma. The Proteinase K beads were activated by suspending 40 mg beads in 1 ml activation buffer (20 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.4) and incubated for 2 hours at room temperature. Before protein digestion, the beads were washed three times by centrifugation for 3 min at 400 x g followed by resuspension in 800 μl activation buffer. PDH (16.0 mg ml^{-1}) was exchanged from its storage buffer into 20 mM Tris-HCl, 1 mM $CaCl_2$ pH 7.4. Briefly, 2 ml PDH solution was diluted into 18 ml of 20 mM Tris-HCl, 1 mM $CaCl_2$

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pH 7.4 followed by concentration to 250 µl using a 10 kDa cut-off Amicon Ultra-15 spin column (Milipore). This step was repeated 5 times. After buffer exchange, 2.5 mg ml^{-1} PDH was digested overnight with 400 µl Proteinase K agarose bead suspension. The following morning, the beads were removed from the digestion reaction by centrifugation at 16,000 x g for 10 min and the supernatant was used in subsequent growth curves.

Growth curves

Overnight cultures were grown in 200 µl of RPMI + BCFA in a 96-well plate at 37°C with shaking. The next day, the strains were pelleted for 10 min at 2,000 x g at 4°C. The strains were washed three to five times with 200 µl of RPMI in order to remove any remaining BCFA that might stimulate growth. Each strain was grown in RPMI supplemented with 5 µM lipoic acid (Sigma), 25 µM octanoic acid (Sigma), 0.25 mg ml^{-1} PDH (Sigma) or the volume equivalent of proteinase K digested PDH, and 100 µM DK^{L}A or 100 µM DKA tripeptides (Anaspec). All growth curves were conducted in a 96-well plate at 37°C for 8–10 hours until reaching stationary phase. Changes in turbidity were recorded every hour by measuring OD550 on a BioTek plate reader.

Generation of 6x-Histidine tagged protein expression plasmids

The coding sequences of *lplA1*, *lplA2 e2-PDH*, *e2-OGDH*, *e2-BCODH*, *gcvH*, and *gcvH-L*, were amplified using the following primer pairs: His-LplA1-F/His-LplA1-R (*lplA1*), His-LplA2-F/His-LplA2-R (*lplA2*), His-E2PDH-F/His-E2PDH-R (*e2-PDH*), His-E2OGDH-F/His-E2OGDH-R (*e2-OGDH*), His-E2BCODH-F/His-E2BCODH-R (*e2-BCODH*), His-GcvH-F/His-GcvH-R (*gcvH*), and His-GcvH-L-F/His-GcvH-L-R (*gcvH-L*) (See Table 2). The resulting amplicons were sub-cloned into protein expression vector pET-15b using NdeI and BamHI restriction endonucleases to generate expression plasmids containing each gene with an N-terminal 6x-Histidine tag. Plasmids containing 6x-His-*lplA1* and 6x-His-*lplA2* were transformed into *lysY/I*^{q} *E. coli* and plasmids containing 6x-His-*e2-PDH*, 6x-His-*e2-OGDH*, 6x-His-*e2-BCODH*, 6x-His-*gcvH*, and 6x-His-*gcvH-L* were transformed into *lipA::kan lysY/I*^{q} *E. coli* to ensure each subunit would be purified in its apo form (Grayczyk *et al.*, 2017).

Purification of LplA1 and LplA2

LplA1 and LplA2 were purified using Ni^{2+} affinity chromatography. *lysY/I*^{q} *E. coli* strains containing pET15b-*6x-His-lplA1*, and pET15b-*6x-His-lplA1* were grown in 5 ml LB with 100 µg ml^{-1} ampicillin at 37°C with shaking overnight. The following day, bacteria were subcultured 1:100 and allowed to grow for 3 hours at 37°C until reaching an OD600 of 0.25–0.3. Protein expression was induced upon addition of 0.1 mM IPTG followed by incubation for 16 hours at 16°C with shaking. The next day, bacteria were pelleted by centrifugation at 13,000 x g for 10 min at 4°C followed by storage at −80°C. To purify the recombinant proteins, bacterial pellets were thawed at 37°C and resuspended in lysis buffer (25 mM imidazole, 50 mM Tris-HCl, 300 mM NaCl, pH 8) supplemented with 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF). The bacteria were lysed at a constant rate of 0.8 seconds per pulse and an output of 340 W in 20 second intervals for 15 min on ice using a Branson S-450A large tip sonicator. Debris from lysed bacteria was pelleted by centrifugation for 30 min at 16,000 x g followed by filtration of

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the clarified lysate using a 0.45 μm syringe filter. The lysate was then incubated with 1 ml nickel-NTA resin (Qiagen) while rocking for 1 hour at 4°C, washed with wash buffer (50 mM imidazole, 1 mM DTT, 50 mM Tris-HCl, 300 mM NaCl, pH 8.0), followed by elution of the bound protein using the same buffer containing 500 mM imidazole. Samples were dialyzed with 10 kDa molecular weight cut-off (MWCO) snakeskin dialysis tubing (Thermo Scientific) using the following scheme: the sample was first placed into 1 L buffer containing 100 mM imidazole, 50 mM Tris-HCl, 300 mM NaCl, pH 8 for 3 hours, then into buffer containing 25 mM imidazole, 50 mM Tris-HCl, 300 mM NaCl, pH 8 overnight, followed by an additional 4 hours in buffer containing 50 mM Tris-HCl, 300 mM NaCl, pH 8 the following day. The concentration of the purified protein was measured using a bicinchoninic acid (BCA) kit (Thermo Scientific) and stored at −80°C. Protein purity was confirmed by resolving ~2 μg of purified protein on SDS-PAGE gels followed by staining with GelCode Blue. Where necessary, proteins were further purified to homogeneity using fast protein liquid chromatography (FPLC) on a Superdex 100 Increase 3.2/300 size exclusion column.

Purification of E2 and H subunits of lipoylated enzyme complexes

The E2-subunits of PDH, BCODH, OGDH, GcvH, and GcvH-L were purified from a *lipA::kan lysY/I^q^ E. coli* strain using Ni^{2+} affinity chromatography. *lipA::kan lysY/I^q^ E. coli* strains containing pET15b-*6x-His-e2-PDH*, and pET15b-*6x-His-E2-OGDH*, pET15b-*6x-His-e2-BCODH*, pET15b-*6x-His-gcvH*, and pET15b-*6x-His-gcvH-L* were grown overnight in 30 ml LB with 100 μg ml^{-1} ampicillin at 37°C with shaking. The following day, strains were subcultured 1:100 into LB medium containing 100 μg ml^{-1} ampicillin and grown for 20 hours at 37°C with shaking. The next day, protein expression was induced with 0.5 mM IPTG for 4 hours at 37°C with shaking. After induction, the bacteria were pelleted by centrifugation at 13,000 x g for 10 min at 4°C and stored at −80°C overnight. The remaining purification of E2 and H subunits followed the same protocol used for LplA1 and LplA2 described above.

Lipoylation and octanoylation assays

Lipoylation assays were set up as described by Martin *et al* with some modifications (Martin *et al.*, 2011). All assays were conducted in 50 μl reaction volumes in reaction buffer (50 mM Tris-HCl, 300 mM NaCl, pH 8.0) containing 6 mM ATP, 1 mM DTT, 1 mM $MgCl_2$, 1 μM purified LplA1 or LplA2, and 20 μM substrate (E2-PDH, E2-OGDH, E2-BCODH, GcvH, or GcvH-L). The reactions were incubated with or without lipoic acid (2.4 mM), octanoic acid (2.4 mM), or $DK^{L}A$ (2.4 mM) for 2 hours at 37°C with shaking. After incubation, the reaction mixtures were resolved on parallel 12% SDS-PAGE gels at 120 V for approximately 3 hours. One gel was stained with GelCode Blue (Thermo Scientific) and the other was used for immunoblotting. The resolved reactions were transferred to 0.2 μM PVDF membranes at 1000 mA for 1 hour. After transfer, the membrane was blocked overnight at 4°C in Tris-buffered saline + 0.1% Tween (TBST) containing 5% bovine serum albumin (BSA). The following day, rabbit α-lipoic acid antibody was added to the membrane at a dilution of 1:7,500 in TBST + 5% BSA for 1 hour at room temperature with rocking. The membrane was washed three times in TBST followed by addition of goat α-rabbit IgG AP conjugate to the membrane at a 1:5,000 dilution in TBST + 5%

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BSA for 1 hour at room temperature. The membrane was washed three times in TBST at 15 min intervals followed by visualization of lipoylated proteins using colorimetric detection upon addition of 66 μl of nitro-blue tetrazolium (NBT) (50 mg NBT in 1 ml 70% dimethylformamide (DMF)/30% H_2O) and 35 μl of 5-bromo-4-chloro-3′-indolyphosphate (BCIP) (50 mg BCIP in 1 ml DMF) to 10 ml AP Buffer (100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5) and incubating the membrane with this solution for ~2 min while rocking at room temperature. Development of the blot was stopped by washing the membrane with water and allowing it to dry at 37°C for 15 min.

Lipoamidase activity assay

Overnight cultures of the *lipA lipM lipL lplA1 lplA2 S. aureus* strain lacking all enzymes involved in *de novo* biosynthesis and salvage of lipoic acid was grown in 5 ml RPMI + BCFA at 37°C with shaking. The following day, the strain was subcultured 1:100 in 6 ml of RPMI + BCFA and grown for 9 hours at 37°C with shaking. The bacteria were pelleted by centrifugation at 3,000 x g for 15 min, the supernatant discarded, and remaining pellet stored at −80°C. The following day, the bacterial pellet was thawed on ice for 10 min, resuspended in 250 μl 1X PBS, and transferred to microcentrifuge screw-cap tubes containing ~250 μl 0.1 mm glass cell disruption beads (Scientific Industries, Inc.). The bacteria were lysed using a Fast Prep-24 5G (MP Biomedicals) at speed 5.0 for 20 seconds followed by speed 4.5 for an additional 20 seconds with 5 min incubation on ice between lysis steps. After lysis, the samples were centrifuged at 18,000 x g for 15 min and clarified lysates were collected and immediately used in subsequent assays of amidotransferase activity.

Amidotransferase activity was assayed in 50 μl reaction volumes containing 6 mM ATP, 1 mM DTT, 1 mM $MgCl_2$, 1 μM purified LplA1 or LplA2, 20 μM substrate (GcvH or GcvH-L) and 35 μl of *lipA lipM lipL lplA1 lplA2* mutant whole cell lysates, prepared as described above. The reactions were incubated with or without DK^LA (2.4 mM) for 2 hours at 37°C with shaking. After incubation, proteins were resolved on parallel SDS-PAGE gels for staining with GelCode Blue (Thermo Scientific) and immunoblot with rabbit α-lipoic acid antibody as described above.

Reproducibility and experimental rigor

All growth curves were conducted three times in triplicate. Data shown are of a single representative growth curve conducted in triplicate with error bars reflecting the standard deviation between samples. When no error bars are shown, this reflects a data point where the error was smaller than the size of the symbol used to plot that point. All biochemical assays were conducted at least four times with two independent preparations of recombinant proteins to ensure reproducibility and rule out effects of any potential impurities. DKA, DK^LA, DKT and DK^LT tri-peptides were synthesized by Anaspec and purified by HPLC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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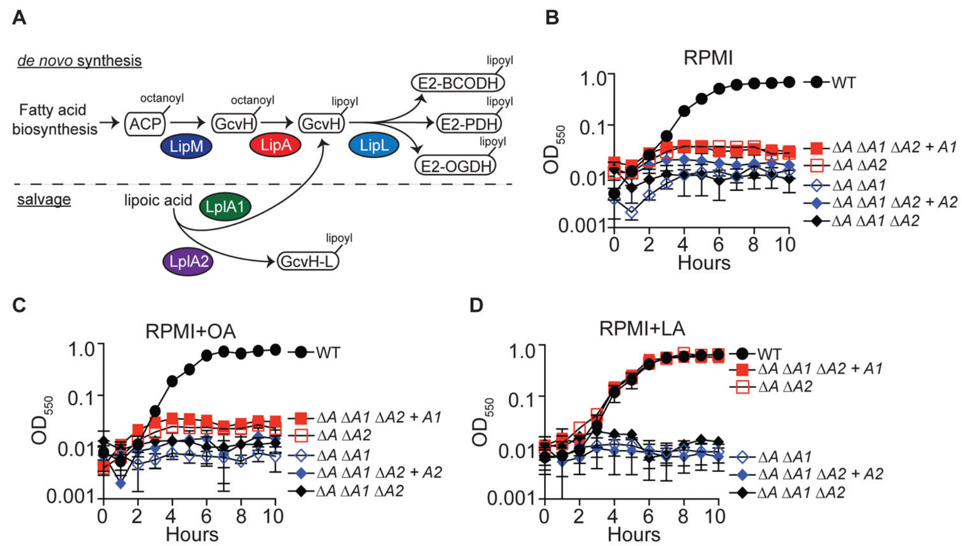

Figure 1. LplA1, but not LplA2, restores growth to lipoic acid auxotrophs *in vitro*

(**A**) Model of the lipoic acid biosynthesis and salvage pathways in *S. aureus. S. aureus* synthesizes lipoic acid via *de novo* biosynthesis using the enzymes LipM- octanoyl transferase; LipA – lipoyl synthethase; and LipL – amidotransferase. During lipoic acid salvage, *S. aureus* is thought to use two lipoic acid ligases, LplA1 and LplA2. Current evidence suggests LplA1 lipoylates GcvH, while LplA2 lipoylates the GcvH-Like protein, GcvH-L. (**B–D**) Growth (OD550) of the indicated lipoic acid auxotrophs in (**B**) RPMI, (**C**) RPMI + 25 μM octanoic acid (OA), and (**D**) RPMI + 5 μM lipoic acid (LA). Strain designations are as follows: Wildtype (WT), *lipA lplA1* (*A A1*), *lipA lplA2* (*A A2*), *lipA lplA1 lplA2* (*A A1 A2*), *lipA lplA1 lplA2* + *lplA1* (*A A1 A2* + *A1*), and *lipA lplA1 lplA2* + *lplA2* (*A A1 A2* + *A2*).

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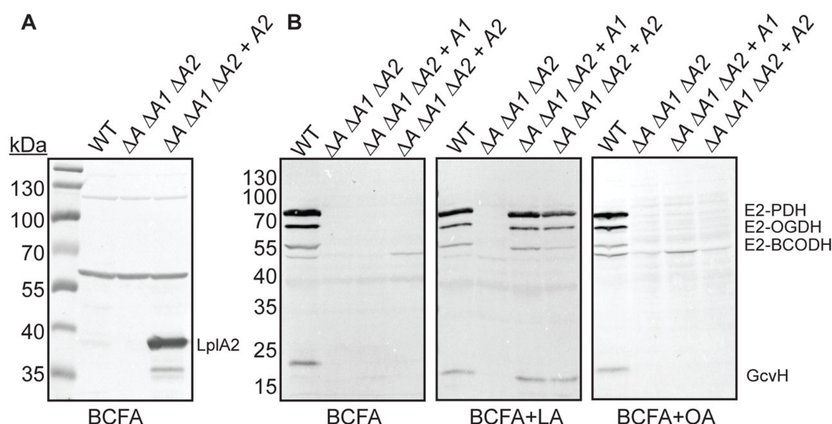

Figure 2. Constitutively expressed LplA1 and LplA2 promote lipoylation in medium containing free lipoic acid

(**A**) Immunoblot with α-LplA2 antibody of whole cell lysates derived from the indicated strains grown in RPMI supplemented with branched chain carboxylic acids (10 mM isobutyric acid, 9 mM 2-methylbutyric acid, 9 mM isovaleric acid, and 10 mM sodium acetate - BCFA) in order to bypass the requirement of lipoic acid for replication. (**B**) Immunoblot with α-lipoic acid antibody of whole cell lysates derived from the indicated strains grown in the same medium as (**A**) and supplemented with 5 μM lipoic acid (LA) or 150 μM octanoic acid (OA). The positions of the four lipoyl proteins in *S. aureus* (E2-PDH, E2-OGDH, E2-BCODH, and GcvH) are indicated. Strain designations are as follows: wildtype (WT), *lipA lplA1 lplA2* (*A A1 A2*), *lipA lplA1 lplA2* + *lplA1* (*A A1 A2* + *A1*), and *lipA lplA1 lplA2* + *lplA2* (*A A1 A2* + *A2*).

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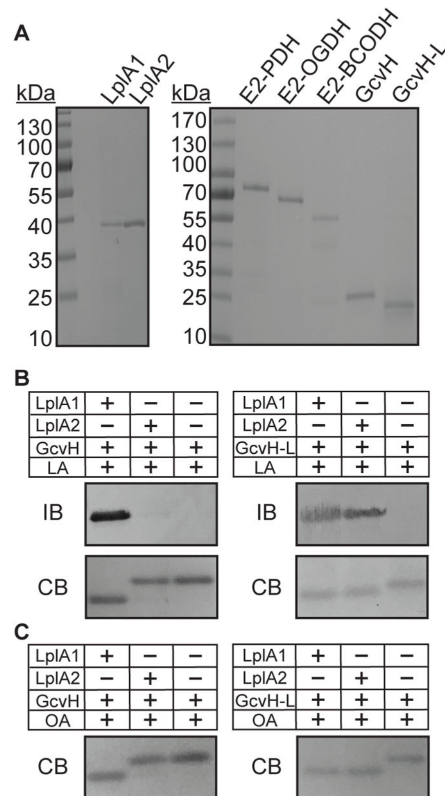

Figure 3. Targeting of GcvH and GcvH-L by LplA1 and LplA2

(**A**) GelCode Blue stained SDS-PAGE gels containing 2 μg of the salvage enzymes, LplA1 and LplA2, and lipoyl domain containing subunits E2-PDH, E2-OGDH, E2-BCODH, GcvH, and GcvH-L. (**B–C**) LplA1 and LplA2 attachment of (**B**) lipoic acid (2.4 mM) and (**C**) octanoic acid (2.4 mM) to GcvH and GcvH-L. Lipoylation was assessed by conducting an immunoblot (IB) with rabbit α-lipoic acid antibody. Parallel 12% SDS PAGE gels were stained with GelCode Blue (CB). Octanoylation was visualized as a shift in apparent molecular weight after resolving proteins on a 12% SDS-PAGE gel and staining with GelCode Blue (CB).

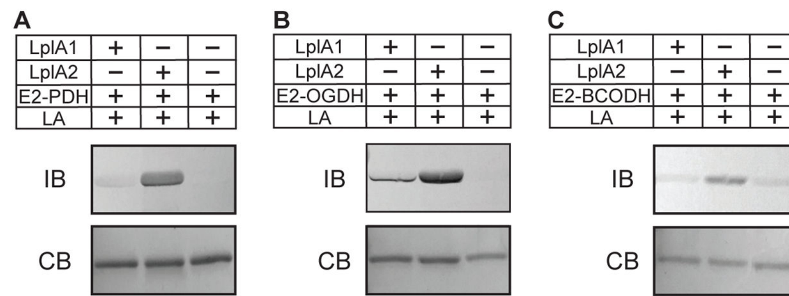

Figure 4. Targeting of E2 subunits by LplA1 and LplA2

(**A–C**) LplA1 and LplA2 attachment of lipoic acid (2.4 mM) to (**A**) E2-PDH, (**B**) E2-OGDH, and (**C**) E2-BCODH. Lipoyl attachment was assessed by conducting immunoblots (IB) with rabbit α-lipoic acid antibody. A parallel 12% SDS-PAGE gels was stained with GelCode Blue (CB).

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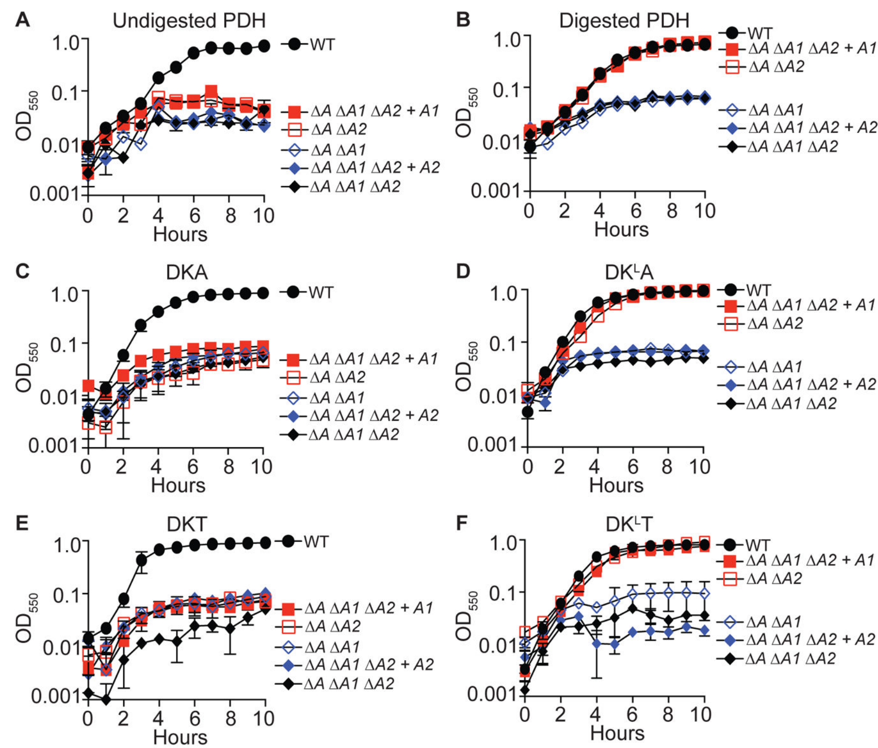

Figure 5. LplA1, but not LplA2, restores growth to lipoic acid auxotrophs in the presence of lipoyl peptides

(**A–F**) Growth (OD_{550}) of the indicated lipoic acid auxotrophs in RPMI supplemented with (**A**) 2.5 mg ml^{-1} undigested pyruvate dehydrogenase (PDH), (**B**) 2.5 mg ml^{-1} proteinase K digested PDH, (**C**) 100 μM DKA tripeptide, (**D**) 100 μM $DK^{L}A$ tripeptide, (**E**) 100 μM DKT tripeptide, and (**F**) 100 μM $DK^{L}T$ tripeptide. Strain designations are as follows: Wildtype (WT), *lipA lplA1* (*A A1*), *lipA lplA2* (*A A2*), *lipA lplA1 lplA2* (*A A1 A2*), *lipA lplA1 lplA2* + *lplA1* (*A A1 A2* + *A1*), and *lipA lplA1 lplA2* + *lplA2* (*A A1 A2* + *A2*).

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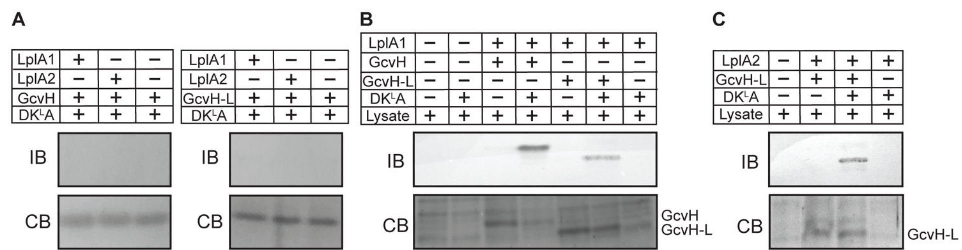

Figure 6. *S. aureus* crude lysates harbor lipoamidase activity that permits the use of DK^{L}A as a source of lipoic acid for attachment by LplA1 and LplA2

(**A**) LplA1 and LplA2 attachment of DK^{L}A tripeptide-derived lipoic acid (2.4 mM) to GcvH and GcvH-L. Lipoylation was assessed by conducting immunoblots (IB) with rabbit α-lipoic acid antibody. A parallel 12% SDS-PAGE gels was stained with GelCode Blue (CB). (**B**) LplA1-dependent attachment of DK^{L}A tripeptide-derived lipoic acid (2.4 mM) to GcvH, GcvH-L and (**C**) LplA2 attachment of DK^{L}A tripeptide-derived lipoic acid (2.4 mM) to GcvH-L in the presence of *S. aureus* whole cell lysates derived from a *lipA lipM lipL lplA1 lplA2* mutant. Lipoylation was assessed by conducting immunoblots (IB) with rabbit α-lipoic acid antibody. A parallel 12% SDS-PAGE gel was stained with GelCode Blue (CB). The positions of GcvH and GcvH-L are indicated.

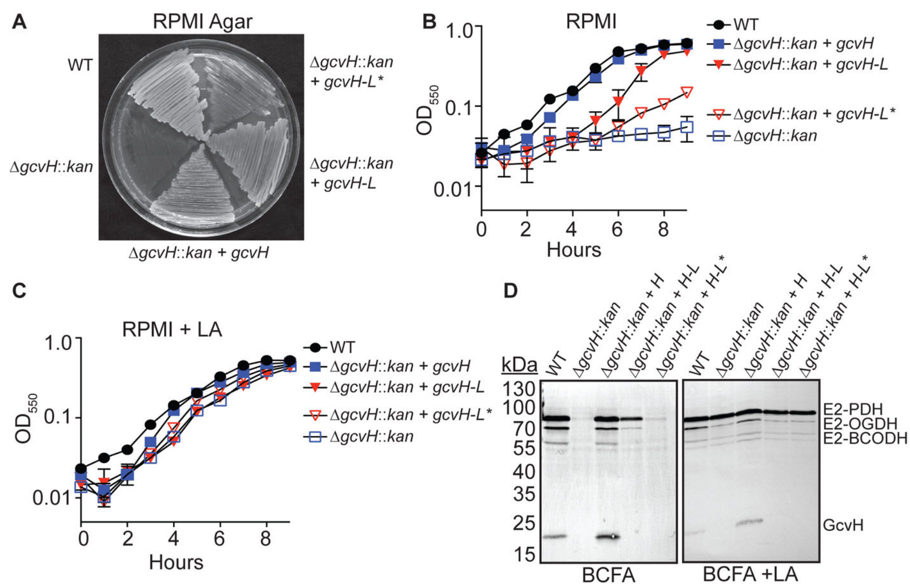

Figure 7. GcvH and GcvH-L both participate in lipoyl relay to E2 subunits

(**A**) Growth of the indicated strains on RPMI agar lacking free lipoic acid. (**B–C**) Growth (OD_{550}) of the indicated strains in (**B**) RPMI and (**C**) RPMI + 5 μM lipoic acid (LA). (**D**) α-lipoic acid immunoblot of whole cell lysates derived from the indicated strains grown in RPMI supplemented with branched chain carboxylic acids (10 mM isobutyric acid, 9 mM 2-methylbutyric acid, 9 mM isovaleric acid, and 10 mM sodium acetate - BCFA) in order to bypass the requirement of lipoic acid for replication with or without lipoic acid (LA). The positions of the four lipoyl proteins in *S. aureus* (E2-PDH, E2-OGDH, E2-BCODH, and GcvH) are indicated.

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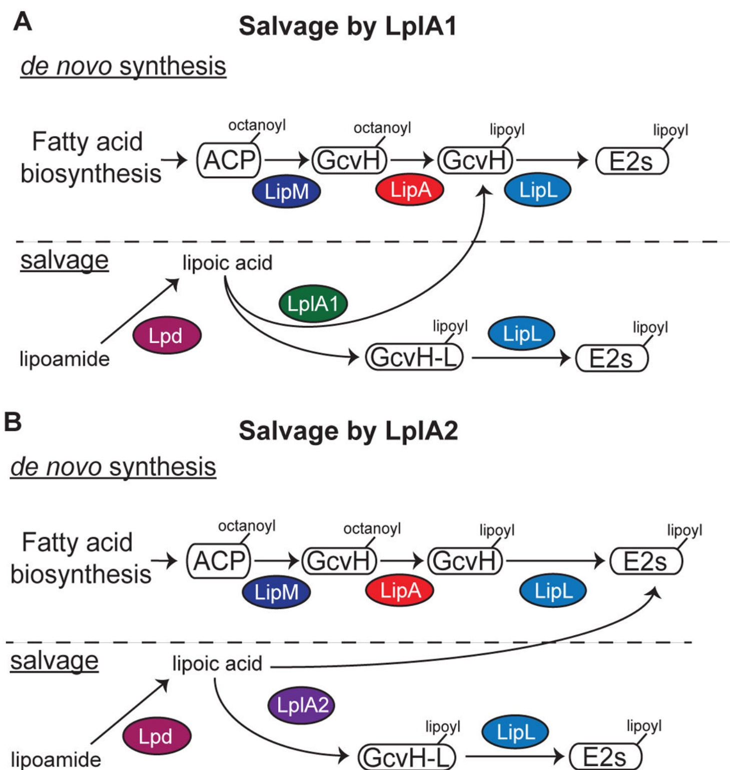

Figure 8. Model of lipoic acid salvage by LplA1 and LplA2

(**A–B**) *de novo* biosynthesis of lipoic acid occurs via an octanoic acid precursor attached to acyl carrier protein (ACP). The octanoyl group is transferred to the H subunit of GcvH by the octanoyl transferase LipM. The octanoyl moiety is converted to lipoic acid by LipA and transferred to the lipoyl domains of E2-PDH, E2-OGDH, or E2-BCODH by the amidotransferase LipL. (**A**) LplA1 scavenges free lipoic acid and primarily attaches the cofactor to the H subunits of GcvH and GcvH-L followed by transfer to E2 subunits by LipL. (**B**) LplA2 also scavenges free lipoic acid, but instead attaches the cofactor to the H subunit of GcvH-L as well as the E2 subunits of PDH, OGDH, and BCODH. The activity of both LplA1 and LplA2 requires free lipoic acid. However, *S. aureus* harbors lipoamidase (Lpd) activity that can hydrolyze the amide bond of lipoamide, providing an additional source of free lipoic acid for use by these ligases.

Table 1

List of strains used in this study.

USA300 LAC	*S. aureus* USA300 Strain LAC (AH-1264). Plasmid cured.	AH-LAC (WT)	(Boles *et al.*, 2010)
DC10B	*E. coli* strain for recombinant pJC plasmids (*dcm-*)	DC10B	(Monk *et al.*, 2012)
lysY/I^q	*E. coli lysY/I*^{q} for expression of LplA1 and LplA2	*lysY/I*^{q}	NEB
FA-E1344	*E. coli lysY/I*^{q} *lipA::kan* for expression of H and E2 subunits	*lysY/I*^{q} *lipA::kan*	(Grayczyk *et al.*, 2017)
RN4220	Restriction deficient *S. aureus* for plasmid passage	RN4220	(Fairweather *et al.*, 1983)
RN9011	RN4220 + pRN7203 expressing SaPI integrase	RN9011	(Chen *et al.*, 2014)
FA-S1249	AH-LAC with in-frame deletions of *lipA* and *lplA1*	*lipA* *lplA1*	(Zorzoli *et al.*, 2016)
FA-S1180	AH-LAC with in-frame deletions of *lipA* and *lplA2*	*lipA* *lplA2*	(Zorzoli *et al.*, 2016)
FA-S1178	AH-LAC with in-frame deletions of *lipA, lplA1,* and *lplA2*	*lipA* *lplA1* *lplA2*	(Zorzoli *et al.*, 2016)
FA-S1200	FA-S1178 complemented with pJC1111-*lplA1*	*lipA* *lplA1* *lplA2+lplA1*	(Zorzoli *et al.*, 2016)
FA-S1212	FA-S1178 complemented with pJC1111-*lplA2*	*lipA* *lplA1* *lplA2+lplA2*	(Zorzoli *et al.*, 2016)
FA-S953	AH-LAC *lipL::kan*	*lipL::kan*	(Zorzoli *et al.*, 2016)
FA-S698	JE2 *lipM::erm* transposon insertion mutant (NE1334)	*lipM::erm*	(Zorzoli *et al.*, 2016)
FA-S1476	AH-LAC *lipA* *lplA1* *lplA2* *lipL::kan lipM::erm*	*lipA* *lipM* *lipL* *lplA1* *lplA2*	This work
FA-S1038	AH-LAC with gene replacement of *gcvH*	*gcvH::kan*	(Zorzoli *et al.*, 2016)
FA-S1637	AH-LAC *gcvH::kan* containing pJC1111-*gcvH*	*gcvH::kan+gcvH*	This work
FA-S1582	AH-LAC *gcvH::kan* containing pJC1111-*gcvH-L*	*gcvH::kan+gcvH-L*	This work
FA-S1636	AH-LAC *gcvH::kan* containing pJC1111-*gcvH-L (K56A)*	*gcvH::kan+gcvH-L**	This work
FA-S1435	AH-LAC with in-frame deletion of *gcvH-L*	*gcvH-L*	This work
FA-S1498	AH-LAC *gcvH-L* containing pJC1111-*gcvH-L*	*gcvH-L* + *gcvH-L*	This work
FA-S1645	AH-LAC with gene replacement of *gcvH* and in-frame deletion of *gcvH-L*	*gcvH::kan* *gcvH-L*	This work
FA-S1684	AH-LAC *gcvH::kan* *gcvH-L* containing pJC1111-*gcvH*	*gcvH::kan* *gcvH-L* + *gcvH*	This work
FA-S1686	AH-LAC *gcvH::kan* *gcvH-L* containing pJC1111-*gcvH-L*	*gcvH::kan* *gcvH-L* + *gcvH-L*	This work
FA-E1357	*E. coli lysY/I*^{q} *lipA::kan* + pET15b-*6x-His-gcvH*	6x-His-GcvH	This work
FA-E1383	*E. coli lysY/I*^{q} *lipA::kan* + pET15b-*6x-His-gcvH-L*	6x-His-GcvH-L	This work
FA-E1359	*E. coli lysY/I*^{q} *lipA::kan* + pET15b-*6x-His-e2-pdh*	6x-His-E2-PDH	This work
FA-E1363	*E. coli lysY/I*^{q} *lipA::kan* + pET15b-*6x-His-e2-ogdh*	6x-His-E2-OGDH	This work
FA-E1367	*E. coli lysY/I*^{q} *lipA::kan* + pET15b-*6x-His-e2-bcodh*	6x-His-E2-BCODH	This work
FA-E1284	*E. coli lysY/I*^{q} + pET15b-*6x-His-lplA1*	6x-His-LplA1	This work
FA-E1278	*E. coli lysY/I*^{q} + pET15b-*6x-His-lplA2*	6x-His-LplA2	This work

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Table 2

List of oligonucleotides used in this study.

Name	Sequence
His-LplA1-F	ATAT-CATATG(NdeI)-AAATTCATTAGTAATAATAATATT
His-LplA1-R	ATAT-GGATCC(BamHI)-TTATGACATTAATCTAATTAATT
His-LplA2-F	ATAT-CATATG(NdeI)-TACTTAATAGAACCGATTAG
His-LplA2-R	ATAT-GGATCC(BamHI)-TTAACTTAAAATCATATCCAC
His-GcvH-F	ATAT-CATATG(NdeI)-GCAGTACCAAATGAATTGAA
His-GcvH-R	ATAT-GGATCC(BamHI)-TTATTCACCAATCATTTCTGA
His-GcvH-L-F	ATAT- CATATG (NdeI)-AAAAAGTTAGCCAATTATTTAT
His-GcvH-L-R	ATAT- GGATCC(BamHI)-TTAAGCCTCCGGTAATGC
His-E2PDH-F	ATAT-CATATG(NdeI)-GCATTTGAATTTAGATTACCC
His-E2PDH-R	ATAT-GGATCC(BamHI)-TTACCCCTCCATTAATAATAA
His-E2OGDH-F	ATAT-CATATG(NdeI)-CCAGAGGTTAAAGTTCCAG
His-E2OGDH-R	ATAT-GGATCC(BamHI)-TTAAGATTCTAATAATAAGTCTT
His-E2BCODH-F	ATAT-CATATG(NdeI)-GAAATAACAATGCCTAAGTTA
His-E2BCODH-R	ATAT-GGATCC(BamHI)-CTAATATATATTTGTATTTTCTAA
UniCompSOE1	ATAT-CTGCAG(PstI)-ATCCCATTATGCTTTGGCA
gcvH-L-CompSOE2	CCCATAAATAATTGGCTAACTTTTTCATGGGTTTCACTCTCCTTCTA
gcvH-L-CompSOE3	TAGAAGGAGAGTGAAACCCATGAAAAAGTTAGCCAATTATTTATGGG
gcvH-L-CompSOE4	ATAT-GAGCTC(SacI)-TGCCGGTCTGACATTTGC
gcvH-CompSOE2	TGGTACTGCCAAGGGTTTCACTCTCCTTCTA
gcvH-CompSOE3	TAGAAGGAGAGTGAAACCCTTGGCAGTACCA
gcvH-CompSOE4	ATAT-GAGCTC(SacI)-AATAGCTAAATTCATTATTTCCATCATTCTTGA
gcvH-L-K56AF	[Phos]CGAAGCATCG**GC**AACGGTCATT
gcvH-L-K56AR	[Phos]ATACTCACAATTTCATCATCCACTTTAACTTC
gcvH-L-SOE1	ATAT-GGTACC(KpnI)-GGTATAGGGCTTTCTGTTGGAACA
gcvH-L-SOE2	CTCGCTTTATTTGATTTTAACGTTTCCATCTTATCTTCATCCTTTCTCTTC
gcvH-L-SOE3	GAAGAGAAAGGATGAAGATAAGATGGAAACGTTAAAATCAAATAAAGCGAG
gcvH-L-SOE4	CAACA-GAGCTC(SacI)-CTTTGCTGACAAATTATATCCACGTG