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***Staphylococcus aureus* SufT: an essential iron-sulfur cluster assembly factor in cells experiencing a high-demand for lipoic acid**

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Summary

S. aureus SufT is composed solely of the domain of unknown function 59 (DUF59) and has a role in the maturation of iron-sulfur (Fe-S) proteins. We report that SufT is essential for *S. aureus* when growth is heavily reliant upon lipoamide-utilizing enzymes, but dispensable when this reliance is decreased. LipA requires Fe-S clusters for lipoic acid (LA) synthesis and a *sufT* strain had phenotypes suggestive of decreased LA production and decreased activities of lipoamide-requiring enzymes. Fermentative growth, a null *clpC* allele, or decreased flux through the TCA cycle diminished the demand for LA and rendered SufT non-essential. Abundance of the Fe-S cluster carrier Nfu was increased in a *clpC* strain and a null *clpC* allele was unable to suppress the LA requirement of a *sufT nfu* strain. Over-expression of *nfu* suppressed the LA requirement of the *sufT* strain. We propose a model wherein SufT, and by extension the DUF59, is essential for the maturation of holo-LipA in *S. aureus* cells experiencing a high demand for lipoamide-dependent enzymes. The findings presented suggest that the demand for products of Fe-S enzymes is a factor governing the usage of one Fe-S cluster assembly factor over another in the maturation of apo-proteins.

Abbreviated Summary

Cells contain iron-sulfur (Fe-S) cluster utilizing proteins and proteins that facilitate cluster assembly. Lipoic acid (LA) is a product of the Fe-S cluster dependent enzyme LipA. The SufT Fe-S cluster assembly factor is essential in cells experiencing a high demand for lipoamide-dependent enzymes. We propose that the demand for products of Fe-S enzymes is a factor governing the usage of one Fe-S assembly factor over another in the maturation of apo-proteins.

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Author Contributions

AAM and JMB contributed to the conception and design of the study. AAM, CAR, SB, RKC, LNS, KLM and JMB contributed to the acquisition, analysis, and interpretation of the data. AAM and JMB wrote the original draft of the manuscript. AAM, CAR, SB, RKC, LNS, KLM and JMB proofread the manuscript revisions.

Keywords

Iron; sulfur; DUF59; SufT; lipoic acid; ClpC

Introduction

Iron (Fe) is necessary for the viability of most organisms. Fe is not readily available in environments and bacteria utilize multiple means to secure Fe. Once ferric iron passes across the cellular membrane, it is reduced to ferrous iron and is used to metallate proteins that require the Fe atom for function or to build Fe-containing prosthetic groups (reviewed here (Hammer & Skaar, 2011)). Much of the intracellular Fe is used to assemble inorganic prosthetic groups called iron-sulfur (Fe-S) clusters. Fe-S cluster assembly occurs in three steps (reviewed here (Py & Barras, 2010)). First, iron, sulfur, and electrons are combined on a molecular scaffolding protein to produce an Fe-S cluster. The Fe-S cluster is then trafficked to a target apo-protein by carrier molecules and subsequently inserted to produce the holo-protein.

Iron-sulfur cluster synthesis in *S. aureus* occurs upon the SufBCD molecular scaffold and it is an essential process ((Mashruwala *et al.*, 2015) and Roberts *et al.*, in preparation). The sulfur for Fe-S cluster synthesis is provided by the SufS cysteine desulfurase and the SufU sulfur carrier (Selbach *et al.*, 2014, Selbach *et al.*, 2010). The Fe and electron donors are unknown. SufA and Nfu are Fe-S cluster carriers that are responsible for shuttling the preformed Fe-S clusters to target apo-proteins and facilitating their maturation to holo-proteins (Rosario-Cruz *et al.*, 2015, Mashruwala *et al.*, 2015). The genes encoding for SufA and Nfu are dispensable for *S. aureus* viability. A strain lacking the low-molecular weight thiol bacillithiol has phenotypes that mimic strains lacking Fe-S cluster carrier molecules and these phenotypes are suppressed by overexpression of *sufA*, suggesting a role for bacillithiol in the maturation of Fe-S proteins (Rosario-Cruz & Boyd, 2015). *S. aureus*

strains defective in Fe-S cluster assembly have decreased virulence and fitness in *in vivo* and *in vitro* models of infection (Mashruwala *et al.*, 2015, Valentino *et al.*, 2014, Mashruwala *et al.*, 2016). Many of the factors that *S. aureus* uses to mature Fe-S proteins are not present in higher eukaryotes suggesting that Fe-S cluster assembly is a viable antimicrobial target.

S. aureus SufA and Nfu can both transfer Fe-S clusters to apo-aconitase (apo-AcnA) at comparable rates *in vitro* (Mashruwala *et al.*, 2015, Rosario-Cruz *et al.*, 2015). However, *in vivo* Nfu is utilized for the maturation of aconitase during aerobic growth while SufA is dispensable (Mashruwala *et al.*, 2016). Further experimentation found that *sufA* is negatively epistatic to *nfu* suggesting that the cell can utilize SufA for AcnA maturation *in vivo*. Therefore, *S. aureus* preferentially utilizes Nfu instead of SufA to mature AcnA. The forces governing the usage of one Fe-S maturation factor over another remain to be fully defined and are an area of active research (Vinella *et al.*, 2009).

We have recently identified an additional protein (SufT) that has a role in Fe-S cluster assembly in *S. aureus* (Mashruwala *et al.*, 2016). The *S. aureus* SufT is composed entirely of a domain of unknown function (DUF) 59 (Luo *et al.*, 2012, Weerapana *et al.*, 2010, Hausmann *et al.*, 2005). DUF59 domains are ~90 amino acids in length and contain one highly conserved cysteine (Mashruwala *et al.*, 2016). In *S. aureus*, SufT is an auxiliary factor involved in the maturation of proteins that utilize Fe-S cluster cofactors (AcnA, LeuCD and IlvD) and its role increases under conditions of high-demand for Fe-S clusters.

Eukaryotic proteins containing DUF59 domains also have roles in Fe-S cluster assembly. *Arabidopsis thaliana* HCF101 contains a N-terminal DUF59 domain, is localized in the chloroplast and is required for photosystem I function (Lezhneva *et al.*, 2004). HCF101 is also capable of binding a [Fe₄-S₄] cluster and transferring the cluster to an apo-protein resulting in the hypothesis that HCF101 is an Fe-S cluster carrier (Schwenkert *et al.*, 2010). Human Fam96a and Fam96b (CIA2A and CIA2B in *Saccharomyces cerevisiae*) also contain DUF59 domains and are involved in the assembly of Fe-S clusters upon cytosolic and nuclear proteins (Stehling *et al.*, 2013, Chen *et al.*, 2012). The *A. thaliana* AE7 protein, which is a CIA2 homologue, is also necessary for the maturation of nuclear and cytosolic Fe-S proteins (Luo *et al.*, 2012). Phylogenetic analyses found that nearly all eukaryotes possess a homologue of CIA2 (Tsaousis *et al.*, 2014). The role(s) of the DUF59 domains in these proteins is unknown.

Lipoic acid synthase (LipA) is a s-adenosylmethionine-radical enzyme that generates lipoic acid using s-adenosylmethionine and octanoic acid as substrates (Busby *et al.*, 1999, Ollagnier-de Choudens & Fontecave, 1999). LipA binds two [Fe₄-S₄] clusters (Harmer *et al.*, 2014). One of the LipA Fe-S clusters act as a substrate for lipoic acid synthesis by providing the two sulfur atoms (Cicchillo & Booker, 2005, Lanz *et al.*, 2015, McLaughlin *et al.*, 2016). *S. aureus* has four known enzymes that require lipoamide to function: pyruvate dehydrogenase (Pdh), 2-oxoglutarate dehydrogenase (Suc), branched chain keto-acid dehydrogenase (Bck), and the glycine cleavage system (Gcv).

This study was initiated to enhance our understanding of the roles of SufT, and by extension the DUF59 domain, in Fe-S cluster assembly and cellular physiology. We found that SufT

was essential for growth under conditions that impose a high demand for lipoamide-utilizing enzymes, but dispensable under low demand for these enzymes. A *sufT* strain had decreased Pdh activity and displayed phenotypes consistent with decreased activities of LipA, Bck and Gcv. We propose that SufT is essential for the maturation of holo-LipA during conditions of high demand for lipoic acid. These findings have led to a model wherein the demand for the product(s) of the Fe-S cofactor dependent protein target(s) is a factor governing the usage of one Fe-S cluster assembly factor over another for the maturation of the apo-protein.

Results

SufT is essential for growth in cells experiencing a high demand for lipoamide-requiring enzymes

A *S. aureus* *sufT* strain is capable of growth in liquid chemically defined medium containing the 20 canonical amino acids (AA) and glucose as a carbon source (hereafter 20AA medium) (Mashruwala *et al.*, 2016). In contrast, the *sufT* strain was unable to grow upon the same medium in solid form (Figure 1A). Supplementation of the solid 20 AA medium with lipoic acid fully corrected the growth of the *sufT* strain (Figure 1A). Returning the *sufT* gene *in trans* to the *sufT* strain also corrected growth in the absence of lipoic acid (Figure 1A). The initial experiments were conducted using strains cultured to stationary phase (18 hours; optical density ~9.5 (A_{600})). The *sufT* strain pre-cultured to post-exponential growth (6 hours; optical density ~5 (A_{600})) also required lipoic acid for growth on solid 20AA medium verifying that the phenotypes of the *sufT* strain did not arise as an artifact of stationary phase growth (data not shown).

One explanation underlying the ability of the *sufT* strain to grow in liquid defined medium in the absence of lipoic acid supplementation was that there was a decreased reliance upon lipoamide-requiring enzymes. The growth of the WT, *sufT*, *bck::Tn*, *pdh::Tn*, *suc::Tn* and *gcv::Tn* strains was assessed upon solid and liquid 20 AA media. The *suc::Tn* and *gcv::Tn* mutants grew similar to the WT on solid and liquid defined media (Figure 1B and data not shown). There was no visible growth noted for the *sufT*, *bck::Tn* and *pdh::Tn* mutants upon solid medium (Figure 1B), while all three strains were capable of growth in liquid defined medium (Figure 1C). However, the *pdh::Tn* and *bck::Tn* strains displayed growth defects during the latter phases of growth in liquid medium (Figure 1C). These findings verified that growth of *S. aureus* cells upon solid defined medium has an increased reliance upon Pdh and Bck.

A strain lacking SufT displays phenotypes consistent with decreased activities of multiple lipoamide-dependent enzymes

WT *S. aureus* permitted growth of a *lipA::Tn* mutant when the two strains were cultured in close proximity on solid medium, suggesting that *S. aureus* releases lipoic acid or an alternate metabolite into the extracellular milieu that bypasses the need for LipA (data not shown). To quantify this effect, the *sufT* and WT strains were cultured in liquid 20AA medium (lacking lipoic acid), the spent medium supernatants were isolated, sterilized and used to support the growth of a *lipA::Tn* mutant. The spent medium from the *sufT* strain

supported growth to a lower final optical density than the spent medium from the WT strain (Figures 2A). The *lipA::Tn* strain was incapable of growth in the absence of exogenously supplied lipoic acid (Figures 2A and S1). Assuming that lipoic acid was the only metabolite present in the supernatants permitting growth of the *lipA::Tn* mutant, the concentration of lipoic acid in the WT and *sufT* supernatants was ~80 and ~46 pg/mL, respectively.

Bck is required for the synthesis of branch-chain fatty acids (BCFAs) and defects in Bck activity result in decreased growth in media lacking BCFAs (Keeney *et al.*, 2009). The *sufT*, *bck::Tn* and *pdh::Tn* strains were incapable of growth upon solid 20AA medium. Supplementing the medium with BCFAs fully restored growth of the *bck::Tn* mutant and partially restored growth of the *sufT* strain (Figure 2B). The growth of the *pdh::Tn* mutant was not altered by BCFAs.

Serine can be synthesized from glycine using the glycine cleavage system (Gcv) (Stauffer *et al.*, 1989) and decreased Gcv function result in a growth deficiency in media lacking serine (Plamann *et al.*, 1983). The *sufT* strain was deficient for growth in a defined liquid glucose medium lacking serine (hereafter 19AA-serine or serine dropout medium) and growth was restored by lipoic acid supplementation (Figure 2C). The *gcv::Tn* mutant grew poorly without serine and this growth defect was not alleviated by lipoic acid supplementation.

The *sufT* strain has decreased activities of the Fe-S cluster dependent enzymes AcnA, LeuCD and IlvD (Mashruwala *et al.*, 2016). Growth in defined media lacking leucine and isoleucine and containing glutamate as a carbon source (hereafter 18AA glutamate medium) is reliant upon AcnA, LeuCD and IlvD (Mashruwala *et al.*, 2016). As previously noted, the *sufT* strain was highly deficient for growth in liquid 18AA glutamate medium (Figure S2) (Mashruwala *et al.*, 2016). Supplementing the 18AA glutamate medium with lipoic acid did not rescue the growth defect of the *sufT* strain. This suggested that the phenotypic correction imparted by lipoic acid is specific to growth conditions imposing a requirement for lipoamide-dependent enzymes, and does not provide a general correction of SufT dependent phenotypes.

Pyruvate dehydrogenase is a lipoamide-dependent enzyme. Growth in liquid 20AA medium and liquid serine dropout medium imposes a low and an increased demand for lipoic acid, respectively. Pdh activity in the *sufT* strain was decreased by ~15% in cells cultured in 20AA media and ~40% when cells were cultured in serine dropout media (Figure 2D).

Catalase (Kat) activity was measured in the same lysates as those used to measure Pdh activity shown in Figure 2D. Kat activity was comparable in the WT and *sufT* strains (Figure S4). These data allowed us to rule out a general defect in enzyme activity in the *sufT* strain cultured in liquid serine dropout medium.

LipA requires Fe-S clusters for function (Lanz *et al.*, 2015). Therefore, conditions that impose an increased demand for lipoic acid should impose an increased demand for Fe-S cluster synthesis and assembly. Consistent with this prediction, the transcriptional activities of *sufT* and *sufC* were increased during culture in liquid serine dropout medium relative to liquid 20AA medium (Figure 2E).

The data in Figure 2 suggested that the *sufT* strain has decreased LipA, Pdh, Bck and Gcv activities in a manner that is dependent upon the cellular demand for lipoic acid. Further, a mechanism exists that increases the transcription of *sufT* and *sufC* upon increased demand for LipA and lipoic acid.

Methionine concentration influences the reliance upon lipoamide-requiring enzymes for growth

The initial phenotypic analyses presented, as well as the suppressor analyses discussed below, were conducted upon solid media that was prepared and stored at 4 °C prior to use. We noted that if the solid defined media was used on the same day as it was prepared (hereafter, freshly prepared media) the *sufT* strain was capable of growth in the absence of lipoic acid supplementation (Figure 3A). We hypothesized that a component of the solid defined medium decreased in bioavailability over time.

Individual amino acids were spread upon solid 20AA medium that had been previously prepared and stored at 4 °C. Supplementing the solid media with methionine (Met) permitted growth of the *sufT* strain (data not shown).

Growth of the *sufT* strain was assessed upon freshly prepared solid 20AA media or media lacking Met (hereafter 19AA-Met or Met dropout medium) and/or lipoic acid supplementation. The *sufT* strain was incapable of growth upon Met dropout medium unless it was supplemented with lipoic acid (Figure 3A).

The effect of Met supplementation upon the ability of the *sufT* strain to permit growth of the *lipA::Tn* strain was examined. The *lipA::Tn* mutant was cross-struck upon freshly prepared solid 20AA medium lacking lipoic acid. The *sufT* and WT strains were individually and perpendicularly cross-struck, bisecting the *lipA::Tn* streak. The WT strain permitted growth of the *lipA::Tn* strain when the cells were proximal to the WT streak, but not when the cells were distal (Figure 3B). The growth of the *lipA::Tn* mutant was visibly decreased when cross-struck with the *sufT* strain. These data suggested that although Met supplementation allowed growth of the *sufT* strain it did not correct the deficiency this strain has in producing the extracellular metabolite (presumably lipoic acid) that permits growth of the *lipA::Tn* mutant.

The effect of Met supplementation upon the requirement for Pdh or Bck for growth was assessed. The *pdh::Tn* mutant was capable of growth upon freshly prepared solid 20AA medium, but not upon Met dropout medium (Figure 3C). Methionine supplementation had no effect on the growth of the *bck::Tn* mutant. These findings suggested that solid media lacking Met had an increased requirement for Pdh, and therefore, an increased demand for lipoic acid.

The minimum concentration of Met necessary to limit growth of the *sufT* strain was determined. Supplementing freshly prepared solid growth media with concentrations greater than 1.65 pM Met supported growth of the *sufT* strain (data not shown). Unless specifically stated, for the remainder of this manuscript solid media were supplemented with

1.65 pM Met (defined as 20AA limiting Met (LM) medium) and were used on the same day as they were prepared.

The lipoic acid requirement of a *sufT* mutant is suppressed by mutations in either *clpC* or upstream of *fur*

Suppressor analyses were conducted to further dissect the role of SufT in lipoic acid dependent growth. Approximately $\sim 1 \times 10^9$ cells of the *sufT* (JMB2512) strain were plated on solid tryptic soy broth (TSB) medium containing rifampicin or solid chemically defined medium lacking lipoic acid. Colonies arose at a frequency of $\sim 1 \times 10^{-8}$ upon TSB rifampicin medium and $\sim 1 \times 10^{-6}$ upon the defined medium. One colony from each defined medium plate was retained for further analysis. Further experimentation verified that seven of the ten strains did not require lipoic acid for growth.

Whole genome sequencing was conducted to identify putative single nucleotide polymorphisms (SNP) that result in suppression. SNPs were verified in four of the seven genomes sequenced and were located in one of two locations. One strain had a mutation upstream of the *fur* gene at -90 base pairs from the translational start site (USA300_FPR3757 genome) (SAUSA300_1448*; *fur**) (Table 2). Three strains had individual SNPs within the *clpC* gene (SAUSA300_0510*; hereafter a general descriptor for these alleles is *clpC**) (Table 2). Preliminary analyses found that the suppression by the *fur** and *clpC** mutations are likely to be imparted through different mechanisms, and therefore, in the current report we elected to focus on the suppression imparted by the *clpC** mutations.

Altered ClpC functionality suppresses the lipoic acid requirement of the *sufT* strain

Two of the *clpC** mutations were nonsense mutations whereas the third was a missense mutation (Table 2) leading to the hypothesis that these alleles result in altered ClpC function. Puromycin causes premature chain termination during translation resulting in an increased demand for the proteolytic machinery (Nathans, 1964). *S. aureus* strains lacking ClpC are sensitive to puromycin (Frees & Ingmer, 1999). The *sufT clpC** strains and a *sufT clpC* strain displayed increased sensitivity to puromycin (Figure 4A). All strains displayed similar growth upon solid TSB medium lacking puromycin (data not shown).

The ability of a *clpC* null allele to suppress the lipoic acid requirement of the *sufT* mutant was assessed. The *sufT clpC* strain was capable of growth upon solid 20AA LM medium whereas the *sufT* mutant was not (Figure 4B). These data verified that decreased ClpC activity suppressed the lipoic acid dependent growth defect of the *sufT* mutant.

The *clpC*_{G672R} allele is dominant negative

We sought to determine whether the effects of the *clpC** alleles were recessive or dominant. A wild type *clpC* allele was returned to the *sufT clpC** strains, as well as the *sufT* and WT strains via a plasmid (*pclpC*) and growth upon solid 20AA LM medium was monitored. The presence of *pclpC* abolished suppression in the *sufT clpC*_{E362→stop} strain whereas it had an intermediate effect upon the *sufT clpC*_{Y611SVIL→YFCDFNstop} strain (Figure 5A).

Notably, the *sufT clpC_{G672R}* strain containing *pclpC* remained incapable of growth in the absence of lipoic acid.

The *clpC_{G672R}* allele was introduced into the *sufT* strain via plasmid (*pclpC_{G672R}*) and growth upon solid 20AA LM medium was monitored. The presence of *pclpC_{G672R}*, but not *pclpC* allowed growth of the *sufT* strain (Figure 5B). We next assessed the effect of the *clpC_{G672R}* allele upon protein processing. The growth of the WT strain carrying empty vector, *pclpC* or *pclpC_{G672R}* upon solid TSB media with or without puromycin was monitored. The WT strain carrying *pclpC_{G672R}* displayed increased sensitivity towards puromycin relative to the WT strain carrying empty vector or *pclpC* (Figure 5C). As expected, the *clpC* strain carrying empty vector was sensitive to puromycin.

Strains lacking ClpP or TrfA mimic the suppression imparted by strains with decreased ClpC function

The Gly at position 672 of ClpC is located in an exposed disordered loop annotated as the IGF-loop. The IGF-loop facilitates interaction between ClpC and ClpP, which is the core peptidase of the ClpCP proteolytic complex (Singh *et al.*, 2001, Kim *et al.*, 2001). The IGF-loop is conserved in Clp AAA+ ATPases (ClpC and ClpX) that interact with ClpP. ClpX variants containing residue changes in the IGF-loop are active chaperones, but are defective in proteolysis because of altered interactions with ClpP (Singh *et al.*, 2001, Kim *et al.*, 2001).

We examined whether the absence of ClpP would suppress the lipoic acid requirement of the *sufT* strain. The *sufT clpP::Tn* strain was capable of growth on solid 20AA LM medium in the absence of lipoic acid (Figure 6). The strain displayed a slow growth phenotype, but this growth defect was similar to the growth defect of the isogenic *clpP::Tn* mutant and occurred irrespective of lipoic acid supplementation (data not shown). *S. aureus* contains an alternate peptidase (ClpQ) that interacts with AAA+ ATPases to process proteins (Frees *et al.*, 2005). Introduction of a *clpQ::Tn* mutation did not suppress the lipoic acid dependent growth of the *sufT* strain (Figure 6).

Small adaptor proteins facilitate regulated proteolysis by aiding recognition of target peptides. The TrfA adaptor is proposed to interact with ClpCP (Donegan *et al.*, 2014, Jousselin *et al.*, 2013). Introduction of a *trfA::Tn* mutation allowed for growth of the *sufT* strain in the absence of lipoic acid supplementation (Figure 6). The data in Figure 6 allowed for the conclusion that decreased protein processing by ClpCP, as well as decreased protein targeting to ClpCP by TrfA, bypassed the lipoic acid requirement of the *sufT* strain.

Decreased ClpC function lowers the requirement for lipoic acid

ClpC has a regulatory role in directing metabolic flux in *S. aureus* (Chatterjee *et al.*, 2011, Chatterjee *et al.*, 2005, Chatterjee *et al.*, 2009). We examined the premise that the *clpC** alleles were suppressing the lipoic acid dependent growth defect of the *sufT* strain by redirecting metabolic flux, and thereby, decreasing cellular demand for lipoic acid.

Individual top agar overlays containing the *sufT lipA::Tn* and *sufT clpC_{E362→stop} lipA::Tn* strains were poured upon solid 20AA medium and 1.5 pmol lipoic acid was spotted

in the center of each plate. The amount of growth was determined after 48 hours of incubation. Growth was enhanced in the strain carrying the *clpC*_{E362→stop} allele (Figures 7A and 7B), suggesting that this allele allowed for growth with less lipoic acid.

The ability of a null *clpC* allele to lower the requirement for lipoic acid was examined. The absence of ClpC also lowered the requirement for lipoic acid (Figure 7B).

Decreased TCA cycle function or fermentative growth decreases the demand for lipoic acid and for SufT

Strains lacking ClpC have reduced AcnA activity and decreased carbon flux through the TCA cycle (Chatterjee *et al.*, 2005, Chatterjee *et al.*, 2009). The expression of genes encoding for Pdh, Bck and Suc are decreased upon fermentative culture (Fuchs *et al.*, 2007). We examined whether modulating carbon flux through the TCA cycle or fermentative growth decreased the requirement for lipoic acid and bypassed the need for SufT.

Aconitase (AcnA) is an enzymatic gatekeeper for flux through the TCA cycle in *S. aureus* (Somerville *et al.*, 2002). Top agar overlays containing the *lipA::Tn* or *acnA lipA::Tn* strains were poured upon solid 20AA medium and 1.5 pmol of lipoic acid was spotted. The growth of the *acnA lipA::Tn* strain was significantly greater than that of the *lipA::Tn* mutant (Figure 8A).

AcnA activity was monitored in the WT, *sufT*, and *sufT clpC*_{E362→stop} strains after culture in liquid 20AA LM medium. AcnA activity was decreased in the *sufT clpC*_{E362→stop} strain relative to the *sufT* strain (Figure 8B).

The effect of modulating carbon flux through the TCA cycle was analyzed. We utilized *acnA::Tn* and *sufT acnA::Tn* strains that carried *acnA* upon a plasmid under the transcriptional control of a xylose inducible promoter (*pacnA*) allowing for modulation of *acnA* transcription. The *acnA::Tn* and *sufT acnA::Tn* strains containing *pacnA* were pre-cultured in liquid TSB media containing 0, 0.5 or 1% xylose to induce transcription of *acnA* and subsequently spot plated upon solid 20AA LM solid media supplemented with and without 1% xylose. In the absence of induction, the *sufT acnA::Tn* strain containing *pacnA* grew on 20AA LM medium (Figure 8C). Induction of *acnA* transcription resulted in decreased growth on 20AA medium.

The WT and *lipA::Tn* strains were cultured aerobically, microaerobically, and fermentatively (inside an anaerobic chamber) in liquid 20AA medium lacking lipoic acid and final culture optical densities were measured. The requirement for LipA was maximal during respiratory growth and minimal during fermentative growth (Figure 9A).

The activity of Pdh was examined in WT cells cultured aerobically and fermentatively in liquid 20AA medium. Pdh activity was lower during fermentative growth (Figure S3). The change in Pdh activity upon fermentative growth was similar to that reported for *Escherichia coli* and *Enterococcus faecalis* (Kaiser & Sawers, 1994, Snoep *et al.*, 1990).

The WT and *sufT* strains were cultured in liquid serine dropout medium aerobically or fermentatively. The *sufT* strain was deficient for growth in the presence of dioxygen, but

not during fermentative growth (Figure 9B). Likewise, SufT was essential for growth on solid 20AA LM medium lacking lipoic acid in the presence of dioxygen, but dispensable during fermentative growth (Figure 9C).

Nfu can compensate for the absence of SufT for lipoic acid-dependent growth

The epistatic relationships between *sufT*, *nfu* and *sufA* were investigated by phenotypically examining mutant strains lacking one, two or all three maturation factors. Growth was examined in liquid 20AA and serine dropout media, which impose varying demands for lipoic acid. The *sufA* strain did not display a growth deficiency in either medium (Figures 10A and 10B). The *sufA sufT* double mutant phenocopied the *sufT* strain in 20AA medium, but displayed a phenotype that was more severe than the phenotypes of the parental strains in serine dropout medium. The *nfu sufA* double mutant phenocopied the *nfu* strain in both media. The phenotypes of the *nfu* and *sufT* mutations displayed synergism and the *nfu sufT* double mutant displayed a severe growth defect in both media (Figures 10A and 10B). The *nfu sufT sufA* triple mutant strain phenocopied the *nfu sufT* strain (Figures 10A and 10B).

We further examined the phenotypes of the *nfu sufT* strain relative to the single mutants. Supplementing liquid 20AA or liquid serine dropout media with lipoic acid largely rescued the growth of the *nfu sufT* strain (Figures S5A and S5B). The *nfu sufT* strain has widespread metabolic defects including multiple growth deficiencies apart from those shown in the current study (Mashruwala *et al.*, 2016). Growth of the *nfu sufT* strain in liquid 18AA glutamate medium was not rescued by lipoic acid supplementation (Figure S5C), confirming that the *nfu sufT* strain displays both lipoic acid dependent, as well as lipoic acid independent defects. Growth upon solid 20AA medium was also examined. The WT, *sufT* and *nfu* strains were capable of growth. However, the *nfu sufT* double mutant strain was incapable of growth. Supplementation of medium with lipoic acid rescued the growth of the *nfu sufT* strain (Figure 10C).

The *sufT* and *nfu* mutations display synergy with regard to AcnA activity (Mashruwala *et al.*, 2016). Dehydratase enzymes contain solvent-accessible Fe-S clusters that can be inactivated by stress such as reactive oxygen species insult resulting in Fe-S cluster turnover (Varghese *et al.*, 2003, Jang & Imlay, 2010, Mashruwala *et al.*, 2016). One of the Fe-S clusters upon LipA is a substrate for lipoic acid synthesis (Lanz *et al.*, 2015, Cicchillo & Booker, 2005). By inference, the maintenance of Fe-S clusters in dehydratases and LipA necessitate an increased demand for Fe-S cluster assembly. Succinate dehydrogenase also requires Fe-S clusters for function. However, the Sdh Fe-S clusters are protected by the polypeptide providing increased stability (Jang & Imlay, 2010, Yankovskaya *et al.*, 2003). Sdh activity was decreased by ~40% in the *nfu* strain, increased by ~200% in the *sufT* strain and decreased by ~80% in the *nfu sufT* strain, relative to the WT (Figure 10D).

Strains lacking genes encoding for proteins with functional overlap display synergistic (super-additive) phenotypic effects (Perez-Perez *et al.*, 2009). The synergistic and epistatic relationships amongst the Fe-S assembly factors led us to examine the effect of over-expression of *nfu* (*pnfu*) or *sufA* (*psufA*) on the lipoic acid requirement of the *sufT* strain. Growth was first examined upon solid 20AA LM medium, which imposes a high demand

for lipoic acid. The *sufT* strain carrying *pnf* was capable of growth whereas the *sufT* strain with empty vector was not. However, the presence of *pnf* did not rescue growth to the levels observed in the WT strain (Figure 11). The *sufT* strain carrying *psufA* did not grow without lipoic acid supplementation. Growth was also analyzed upon a medium that imposed a decreased demand for lipoic acid (containing 6.6 pM Met; 20AA intermediate Met (IM) medium). Solid 20AA IM medium allowed for intermediate growth of the *sufT* strain carrying the empty vector (Figure 11). The *sufT* strain carrying *pnf* grew similar to the WT strain containing empty vector. The presence of *pnf* did not have a notable effect on the growth of the WT strain.

Suppression imparted by the *clpC*_{E362→stop} allele requires Nfu

Nfu could compensate for SufT during decreased demand for lipoic acid. We tested the hypothesis that a lower demand for lipoic acid in strains with decreased ClpC function is accompanied by an increased abundance of Nfu and these two mechanisms simultaneously aid in phenotypic suppression of the *sufT* strain. We created a Nfu-Gfp translational fusion that was present upon an episome and complementation studies found that it functioned *in vivo* (Figure S6). GFP is highly resistant to proteolysis and it is stable in *S. aureus* and is not a substrate for ClpCP unless specifically manipulated to contain a SsrA degradation signal (Bokman & Ward, 1981, Donegan *et al.*, 2014). The fluorescence attributed to the Nfu-Gfp translational fusion was increased by ~10-fold in the *clpC* strain relative to the parental strain (Figure 12A).

We next examined whether the *clpC*_{E362→stop} allele could suppress the lipoic acid requirement of the *sufT* strain in the absence of Nfu. Growth of the *sufT clpC*_{E362→stop} *nfu::Tn* triple mutant strain and its parental strains was examined upon solid 20AA LM media with and without lipoic acid supplementation. The *sufT clpC*_{E362→stop} strain was capable of growth whereas the *sufT clpC*_{E362→stop} *nfu::Tn* strain was not (Figure 12B). Both strains grew in the presence of lipoic acid.

Discussion

The *S. aureus* SufT is composed entirely of the DUF59 domain. This study was initiated to further investigate the role of SufT, and by extension the DUF59 domain, in Fe-S cluster assembly and cellular physiology. SufT was essential for growth upon solid chemically defined medium (with limiting Met) in the absence of exogenous lipoic acid supplementation. Further experimentation found that *S. aureus* growth upon solid chemically defined media was highly reliant upon the lipoamide-requiring enzymes pyruvate dehydrogenase (Pdh) and branched chain keto-acid dehydrogenase (Bck). The reliance upon Pdh and Bck for growth was decreased upon culture in liquid defined media. The reliance on SufT for lipoic acid dependent growth was also decreased in liquid media and it was rendered non-essential.

Lipoic acid synthesis requires LipA, which utilizes two Fe-S clusters for catalysis (Ollagnier-de Choudens & Fontecave, 1999, Harmer *et al.*, 2014). The *sufT* strain displayed multiple phenotypes that could be attributed to decreased activities of enzymes requiring lipoate. A *sufT* strain also had decreased activity of the lipoamide-dependent

enzyme Pdh. In addition, supplementation of solid growth medium with branched-chain fatty acids, which are synthesized via the Bck enzyme, partially rescued growth of the *sufT* strain in the absence of lipoic acid supplementation. The finding that SufT was essential when cellular reliance upon Pdh and Bck was increased led to a working model wherein SufT is required for the maturation of LipA under growth conditions that impose a high demand for lipoic acid.

The bioavailability of methionine in the solid defined media used in this study decreased upon storage. This fact led to the finding that the lipoic acid dependent growth defect of the *sufT* strain was suppressed by excess methionine (Met). It is currently unclear why this occurs. Reports from numerous studies show that solvent accessible Fe-S clusters can be protected from damage by interactions with substrates (Varghese *et al.*, 2003, Gardner & Fridovich, 1991, Dougherty & Downs, 2006, Downs, 2006, Macomber & Imlay, 2009). Met is a precursor for s-adenosylmethionine (SAM) synthesis. It is possible that increasing intracellular Met results in an increased concentration of SAM, which provides protection to the Fe-S clusters of LipA, and thereby, decreases the need for SufT for the assembly of holo-LipA. Growth of *S. aureus* upon solid defined media containing excess Met had a decreased requirement for Pdh. Therefore, a more likely explanation is that Met supplementation decreased the demand for lipoic acid, and thereby, rendered SufT non-essential.

Strains deficient in ClpC activity were capable of suppressing the lipoic acid dependent growth defect of the *sufT* mutant. ClpC acts either independently as a molecular chaperone or in conjunction with the ClpP peptidase and the TrfA adaptor (Frees *et al.*, 2003, Jousselein *et al.*, 2013). The *sufT* mutant strains lacking either ClpP or TrfA were capable of growth in the absence of lipoic acid. These findings led to a model wherein altered protein targeting by TrfA and decreased protein processing by ClpCP resulted in the accumulation of proteins that facilitated suppression of the lipoic acid requirement of the *sufT* strain.

The manifest question arising from the suppressor analyses was how do the mutant *clpC** alleles alter the cellular requirement for lipoic acid. Strains lacking ClpC decreased the cellular demand for lipoic acid. ClpC positively influences the TCA cycle and decreased ClpC function results in reduced activity of the fulcrum TCA cycle enzyme aconitase (AcnA) (Chatterjee *et al.*, 2005, Chatterjee *et al.*, 2009). An *acnA* strain displayed a decreased demand for lipoic acid. A *sufT acnA* strain was capable of growth in the absence of lipoic acid while increased expression of *acnA* in this strain decreased growth. Fermentative growth also decreases flux through the TCA cycle (Ledala *et al.*, 2014) and the transcription of *pdh* and *bck* are decreased during fermentative growth (Fuchs *et al.*, 2007). Fermentative growth resulted in a decreased need for lipoic acid synthesis and it also rescued the lipoic acid dependent phenotypic abnormalities of the *sufT* strain.

Conditions that imposed a low demand for lipoic acid rendered SufT non-essential for growth. We inferred that an alternate cellular factor involved in Fe-S cluster assembly was compensating for the absence of SufT during these growth conditions. The phenotypes associated with the *nfu* and *sufT* mutations were synergistic with respect to their lipoic acid dependent growth phenotypes. When cultured on media where SufT and Nfu were individually dispensable for growth (20AA medium) a *nfu sufT* double mutant strain was

incapable of growth. Overexpression of *nfu* in the *sufT* strain also permitted growth in the absence of lipoic acid supplementation. Moreover, Nfu abundance was increased in a *clpC* strain and Nfu was required for the suppression imparted to the *sufT* strain by the *clpC* E362→stop allele. These data lead to a model wherein decreased ClpC function lowers the demand for lipoic acid and increases the abundance of Nfu, which act simultaneously to bypass the lipoic acid requirement of the *sufT* strain.

The phenotypes of *nfu* and *sufT* are synergistic and genes encoding for proteins with functional overlap often display synergistic phenotypic effects when the gene products are absent or non-functional (Perez-Perez *et al.*, 2009, Mashruwala *et al.*, 2016). Further, *sufA* was negatively epistatic to *sufT* for lipoic acid dependent growth in liquid serine dropout media. Since Nfu and SufA function as Fe-S cluster carriers, it is tempting to speculate that SufT also functions in this capacity. However, further biochemical evidence is necessary to draw this conclusion.

S. aureus encodes for multiple factors involved in the maturation of Fe-S proteins including Nfu, SufA and SufT (Rosario-Cruz *et al.*, 2015, Mashruwala *et al.*, 2015). Thus far, it has been unclear what factors favor the use of one maturation factor over another. SufT is essential during conditions imposing a high demand for lipoic acid while Nfu appears to compensate for its absence during low demand for lipoic acid. By inference, the selective usage of SufT over alternate assembly factors for the maturation of LipA is a direct outcome of lipoic acid demand imposed by growth conditions. We propose a model wherein the utilization of an assembly factor for the maturation of a Fe-S protein is dictated by the cellular demand for the metabolite synthesized by the target apo-protein (increased need for lipoic acid, in the case of SufT).

It is currently unclear why SufT is required during growth conditions imposing a high demand for lipoic acid. However, the rate of apo-protein maturation, and thence, the rate of product formation is likely to be a critical factor in cells experiencing a high demand for the enzyme product. Moreover, it has been hypothesized that one of the two Fe-S clusters of LipA provides the two sulfur atoms for the synthesis for lipoic acid (Harmer *et al.*, 2014, Lanz *et al.*, 2015, McLaughlin *et al.*, 2016). Under such a scenario, the Fe-S cluster serving as the substrate must be either repaired or synthesized *de novo* prior to each round of catalysis. Both scenarios place an additional demand on the Fe-S protein maturation machinery. It is worth noting that results from our recent study suggest that SufT is not necessary for the repair of the Fe-S cluster in AcnA (Mashruwala *et al.*, 2016).

In summation, SufT is essential for *S. aureus* under growth conditions that impose a high demand for lipoic acid. SufT functions in Fe-S cluster assembly in *S. aureus* (Mashruwala *et al.*, 2016). The data presented are consistent with a model wherein SufT, and by extension the DUF59 domain, functions in the maturation of holo-LipA.

Experimental Procedures

Materials

Restriction enzymes, quick DNA ligase kit, deoxynucleoside triphosphates and Phusion DNA polymerase were purchased from New England Biolabs. The plasmid mini-prep and gel extraction kits were purchased from Qiagen. DNase I was purchased from Ambion. Lysostaphin was purchased from Ambi products. Oligonucleotides were purchased from Integrated DNA Technologies and sequences are listed in Table S1. Tryptic Soy broth (TSB) was purchased from MP biomedical. Difco BiTek agar was added (15 g L^{-1}) for solid medium. Unless specified, all chemicals were purchased from Sigma-Aldrich and were of the highest purity available.

Bacterial strains, media, and growth conditions

Unless specified, the *S. aureus* strains used in this study (Table 1) were isogenic and constructed in the community-associated *S. aureus* strain USA300_LAC that was cured of the native plasmid pUSA03 that confers erythromycin resistance (Boles *et al.*, 2010). *S. aureus* were cultured at 37°C with shaking at 200 rpm aerobically in 10 or 30 mL culture tubes containing either 1 mL or 5 mL of TSB media, respectively. Antibiotics were added to the following concentrations: $150 \mu\text{g mL}^{-1}$ ampicillin; $30 \mu\text{g mL}^{-1}$ chloramphenicol (Cm); $10 \mu\text{g mL}^{-1}$ erythromycin (Erm); $3 \mu\text{g mL}^{-1}$ tetracycline (Tet); rifampicin $1.25 \mu\text{g mL}^{-1}$; streptomycin 15 ng mL^{-1} ; puromycin $5\text{--}7 \mu\text{g mL}^{-1}$. To maintain plasmids, the media was supplemented with $15 \mu\text{g mL}^{-1}$ or $5 \mu\text{g mL}^{-1}$ of chloramphenicol or erythromycin, respectively.

A defined minimal medium was used for phenotypic analyses, which has been described previously (Mashruwala *et al.*, 2015). The three liquid growth medium formulations utilized for nutritional analyses were: 1) 20AA glucose medium, containing the 20 canonical amino acids and 14 mM glucose as a source of carbon; 2) 19AA glucose medium, lacking serine and 14 mM glucose as a source of carbon; and 3) 18AA glutamate medium, containing 18 lacking leucine and isoleucine and 44 mM glutamate as a source of carbon. Methionine was supplemented at 1.65 pM in limiting methionine (LM) media and 6.6 pM in intermediate methionine (IM) media. When provided at a non-limiting concentration, lipoic acid was supplemented at $0.5 \mu\text{g mL}^{-1}$. Methyl butyric acid, isobutyric acid, and isovaleric acid were each supplied at 5 μM .

Top-agar overlays were created using 0.5% agar prepared in water as follows. Cells were cultured overnight before pelleting by centrifugation. Cells were resuspended in an equal volume of phosphate buffered saline (PBS) and diluted 1:100 in PBS. One-hundred μL of cells were added to 3.5 mL of 0.5% molten agar before laying the soft-agar over the top of pre-warmed solid media.

Anaerobic growth was conducted in a COY anaerobic chamber. Culture media and plastic ware was allowed to reach anaerobiosis by incubating in the chamber overnight.

Growth analyses

Nutritional requirements were assessed in 200 μ L cultures in 96-well plates using a BioTek 808E Visible absorption spectrophotometer with medium shake speed at 37 °C. Culture optical density was monitored at 600 nm. Strains cultured overnight in TSB were used to inoculate staphylococcal chemically defined media to a final optical density of 0.025 (A_{600}) units. Before assessing nutritional requirements, cultures were harvested by centrifugation and the cell pellets were washed to prevent carryover of rich media components.

For culture on solid media, strains were cultured overnight in TSB and cells were harvested by centrifugation. Cell pellets were washed to prevent carryover of rich media components, serially diluted in PBS and 5 μ L aliquots were spotted upon solid media plates.

Suppressor analyses

Ten independent cultures of JMB2512 were propagated for 18 hours in 10 mL culture tubes containing 1 mL of TSB. The cells were pelleted and resuspended in an equal volume of phosphate buffered saline. One-hundred μ L of each replicate was plated onto both TSB-Rif plates and chemically defined media plates lacking lipoic acid containing amino acids (ARNDCQEGHLKMFSTWYV). Aliquots of each replicate were also serially diluted and plated on TSB agar plates to determine the total colony forming units plated. Mutational frequency was defined as the ratio of colonies present on selective media to total CFUs plated. One colony from each chemically defined media plate was retained for phenotypic analyses and genome sequencing.

Genome sequencing

The genomes of strains JMB2512, JMB4974, JMB4980, JMB4982 and JMB4986 were sequenced using an IonTorrent PGM as previously outlined (Mitra *et al.*, 2014). Briefly, genomic DNA (1 μ g) was sheared using the Ion Xpress™ Plus Fragment Library Kit to generate libraries for 200 bp sequencing. Following purification and size selection on an E-gel Size select 2% gel (Invitrogen), DNA was quantified using a Bioanalyzer High-sensitivity DNA chip (Agilent). Libraries were diluted and template-positive Ion Sphere Particles (ISPs) prepared using the Ion OneTouch 200 template kit (Life Technologies). ISPs were sequenced using the Ion PGM 200 Sequencing kit. Whole genome sequencing data was exported from the Ion Torrent Server and analyzed using the CLC Genomics Workbench software package (Qiagen). Reads were aligned to the *S. aureus* genome, using the USA300 FPR3757 genome sequence as a reference. Quality-based variant detection was then performed to identify polymorphisms in each strain. A minimum threshold detection frequency of 80% was employed. The lists of polymorphisms generated for each suppressor mutant strain (JMB4974, JMB4980, JMB4982 and JMB4986) were cross-referenced against the parental strain (JMB2512). Common polymorphisms were eliminated (as were polymorphisms in homopolymeric nucleotide tracts) resulting in the identification of specific genetic variations between the suppressor strains and parental strain.

SNP verification

The SNPs identified by whole chromosome sequencing were individually verified by sequencing of PCR fragments. The *clpC* alleles from strains JMB2512, JMB4974, JMB4980

and JMB4986 were amplified using the clpCup5EcoRI and clpCdown3saII primers. The *fur* gene and promoter region were amplified from strains JMB2512 and JMB4982 using the primers 1448upbamHI and 1448downPstI. The amplicons were gel purified and sequenced (Genewiz, South Plainfield, NJ) using the clpCabrseq or clpCglyseq primers.

Recombinant DNA and genetic techniques

Escherichia coli PX5 was used as a cloning host for plasmid constructions. All clones were passaged through *S. aureus* RN4220 (Kreiswirth *et al.*, 1983) and subsequently transduced into the appropriate *S. aureus* strains using bacteriophage 80α (Novick, 1991). All *S. aureus* mutant strains and plasmids were verified by phenotypic analysis, analyzing PCR products, or by sequencing PCR products or plasmids (Genewiz, South Plainfield, NJ).

Creation of plasmids and mutant strains

The *clpC* chromosomal deletion was constructed as previously described (Rosario-Cruz *et al.*, 2015). Briefly, the up and down-stream regions corresponding to the *clpC* gene were amplified using the clpCup5EcoRI/clpCup3fuse and clpCdown5fuse/clpCdown3saII primer pairs, respectively. The amplicons were joined using PCR using the clpCup5EcoRI and clpCdown3saII primers and the resulting amplicon was digested with EcoRI and SalI and ligated into similarly digested pJB38 creating pJB38_ *clpC*. The *tetM* gene with its native promoter was amplified from strain JMB1432 using the G+tet nheI and G+tet mluI primers. The G+tet amplicon was digested with NheI and MluI and ligated into similar digested pJB38_ *clpC* creating pJB38_ *clpC::tetM*. The pJB38_ *sufT::tetM* chromosomal deletion construct was constructed using pJB38_ *sufT* as outlined above. The procedure to create chromosomal deletions is described elsewhere (Mashruwala *et al.*, 2015). The *lipA::Tn* (*tetM*) strain was created using pTET as described elsewhere (Bose *et al.*, 2013).

The *pclpC* complementation vectors were created by amplifying the desired *clpC* alleles from *S. aureus* chromosomes using the ClpCRBSEcoRI and ClpC3BamHI primers. The amplicons were gel purified, digested with EcoRI and BamHI before ligating into similarly digested pEPSA5. Note that the *clpC* clones had an engineered *sodA* ribosomal binding site. The *psufT* complementation vector was created by amplifying the *sufT* gene and the native promoter using the primers sufT5BamHI and sufT3SalI before digesting the amplicon with BamHI and SalI and ligating into similarly digested pCM28 resulting in pCM28_ *sufT*.

The pLL39_ *native_nfu_FLAG* plasmid was constructed using yeast recombinational cloning (YRC) as previously described (Mashruwala & Boyd, 2016, Joska *et al.*, 2014). Amplicons were generated using the following primer pairs: pLLYCC5 and YCC_Nfu pro Rev; YCC_Nfu pro for and NfuCter_flag Rev; NfuCter_flag For and flag_pLLRev. The primers were designed to incorporate a NheI recognition sequence upstream of the FLAG sequence and PstI and HindIII recognition sequences downstream of the flag sequence and *nfu* was under the transcriptional control of its native promoter. The *nfu* allele and the upstream promoter region were amplified from the LAC chromosome and the pLL39 vector was linearized using SalI. The resultant pLL39_ *native_nfu_FLAG* plasmid contained additional NheI and HindIII recognition sequences that were native to the pLL39 vector backbone. We removed these native restriction sites using primers pairs pLL39_3_no NheI and

pLL39_5_no HindIII using YRC. The resultant plasmid, pLL39_*nfu*_FLAG was digested with NheI and PstI to remove the FLAG sequence. The *gfp* allele was amplified from the pCM11 plasmid (Malone *et al.*, 2009) using the sGFPnhe15 and sGFPHindIII3 primer pair. The amplicon was digested with NheI and HindIII and ligated into similarly digested pLL39_*nfu*_FLAG. The resultant pLL39_*nfu*_gfp plasmid was integrated as an episome into the chromosome of the *nfu* strain (JMB1165).

Transcriptional reporter fusion and translational fusion assays

WT containing pCM11_*nfu*, or pCM11_*sufT* were grown TSB-Erm and the *nfu* strain containing pLL39_*nfu*_gfp or pLL39 were grown in TSB overnight. The cultures were diluted to 0.1 A₆₀₀ in TSB and grown to exponential or stationary growth as described in the figure legend. Two-hundred μ L of cells were removed and assayed for culture optical density (A₆₀₀) and fluorescence using a Perkin Elmer Spectrometer HTS 7000 plus bioassay plate reader. GFP was excited at 485 nm and emission was read at 535 nm. For data analyses, fluorescence readings were normalized with respect to a strain not carrying a fluorescent plasmid and thereafter to culture optical density. Subsequent normalizations were as mentioned in the figures and/or figure legends.

Enzyme assays

Aconitase (AcnA) assays—AcnA assays were conducted as previously described (Mashruwala *et al.*, 2016). Briefly, *S. aureus* cells cultured overnight in TSB media were washed with PBS and used to inoculate 1 mL of liquid chemically 20AA medium. Strains were cultured in 10 mL culture tubes at 37 °C for 18 hours with shaking at 200 rpm. Cells were harvested by centrifugation and cell pellets were frozen and stored at –80 °C until assayed. Cell pellets were resuspended in anaerobic buffer within a COY anaerobic chamber and lysed by the addition of lysostaphin and DNase. AcnA activity was assessed as previously described (Mashruwala *et al.*, 2015).

Pyruvate dehydrogenase (Pdh) assays—*S. aureus* cells cultured overnight in TSB media were pelleted by centrifugation, washed with PBS, and subsequently used to inoculate 2 mL of liquid chemically defined 20AA or 19AA-serine media. Strains were cultured in 30 mL culture tubes for 8 hours (20AA medium) or 25 hours (19AA-serine medium) to ensure all strains were in similar growth phases. Where mentioned anaerobic growth was achieved as described earlier (Mashruwala *et al.*, 2016). Cell pellets were harvested and lysates prepared as for aconitase assays. Pdh activity was assessed using the assay kit from BMR services (University of Buffalo) and as per manufacturer's instructions.

Catalase assays—Cells were cultured, harvested, and cell pellets obtained as described above for Pdh assays. The cell lysate was further diluted 10-fold in lysis buffer and catalase activity was assayed by the addition of two μ L of the diluted extract to 985 μ L of assay buffer A (50 mM Tris, pH 7.5, 150mM NaCl, and 18 mM H₂O₂). The decomposition of H₂O₂ was monitored spectrophotometrically, as described elsewhere (Beers & Sizer, 1952).

Succinate dehydrogenase assays—Strains were cultured in TSB medium for 18 hours and cell lysates obtained as described above for AcnA assays. Sdh activity was

determined as described earlier, with minor changes (Spencer & Guest, 1973, Enos-Berlage & Downs, 1997). The reaction mixture contained PBS (pH 7.5), 2 mM KCN, 742 nM XTT, 19.5 nM phenazine methosulfate and 130 mM sodium succinate (pH 7.1). The reaction was initiated by the addition of cell-free extract and reduction of XTT was monitored at 490 nm.

Protein concentration determination—Protein concentrations was determined using a copper/bicinchoninic acid based colorimetric assay modified for a 96-well plate (Olson & Markwell, 2007).

Quantification of lipoic acid in spent culture media—*S. aureus* strains were cultured overnight in TSB and cells were harvested, washed twice with PBS and diluted into 20AA medium to a final optical density of 0.025 (A_{600}). Strains were cultured to stationary phase, optical densities were recorded and the cells were partitioned from the spent media supernatant using centrifugation. The spent media supernatants were filter sterilized by passage over a 0.22 μ m micron filter and standardized to culture optical density using sterile water. The *lipA::Tn* mutant was cultured overnight in TSB before the cells were harvested by centrifugation and cell pellets were washed using PBS. The *lipA::Tn* strain was subsequently cultured in fresh 20AA media that was supplemented with filter-sterilized spent media supernatant from the WT or *sufT* strains *in lieu* of lipoic acid supplementation. The *lipA::Tn* strain was cultured for 8 hours before optical densities (A_{600}) were recorded. The concentration of lipoic acid in spent media supernatants was estimated using a standard curve developed using the *lipA::Tn* strain. The *lipA::Tn* strain was prepared as described above before being inoculated into 20AA media that had been supplemented with varying concentrations of lipoic acid (0–250 pg/mL) (see Figure S1).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. SufT is essential for growth in cells experiencing a high demand for lipoamide-requiring enzymes

Panel A) A *S. aureus* Δ sufT strain is incapable of growth upon solid medium lacking liponic acid. Growth of the WT (JMB1100) carrying empty vector (pCM28) and the Δ sufT strain (JMB2512) carrying either empty vector or the complementing plasmid (pCM28_sufT) upon chemically defined solid media containing the 20 canonical amino acids (hereafter 20AA media) in the presence or absence of liponic acid. Panel B) Growth of *S. aureus* upon solid 20AA medium is reliant upon the lipoic acid requiring enzymes pyruvate dehydrogenase (Pdh) and branched chain keto-acid dehydrogenase (Bck). Growth of the WT

(JMB1100), *suc::Tn* (JMB2015), *gcv::Tn* (JMB7911), *bck::Tn* (JMB7888), *pdh::Tn* (JMB7891), and *sufT* (JMB2512) strains upon solid 20AA medium. Panel C) Reliance of *S. aureus* upon Pdh and Bck for growth is decreased during culture in liquid 20AA medium. Growth profiles for WT (JMB1100), *bck::Tn* (JMB7888), *pdh::Tn* (JMB7891) and *sufT* (JMB2512) strains are shown. Data in Panels A and B are representative of at least three independent experiments. Data in Panel C represent the average of biological duplicates and error bars represent standard deviations.

Figure 2. The *sufT* strain has phenotypes consistent with decreased activities of multiple lipamide-dependent enzymes

Panel A) Spent medium metabolites isolated from the *sufT* strain are deficient in supporting growth of a *lipA::Tn* mutant in media that otherwise lacks liponic acid. The *lipA::Tn* mutant (JMB7156) was cultured in liquid 20 AA media supplemented with no spent media or spent media from the WT (JMB1100) or *sufT* (JMB2512) strains and the final optical densities are presented. Panel B) Supplementing solid 20AA medium with branch chain fatty acids (BCFAs) allows the *bck::Tn* and *sufT* strains to grow in the absence of liponic acid. Growth of the WT (JMB1100), *bck::Tn* (JMB7888), *pdh::Tn* (JMB7891) and *sufT* (JMB2512) strains upon solid 20AA media with and without 5 μ M each of methyl butyric acid, isobutyric acid, and isovaleric acid. Panel C) A strain lacking Suft is deficient for growth upon liquid 19AA medium lacking serine (hereafter 19AA-serine) and this deficiency is corrected by liponic acid. Growth profiles for the WT (JMB1100), *gcv::Tn* (JMB7911) and *sufT* (JMB2512) strains cultured in liquid 19AA-serine media in the presence or absence of liponic acid (LA). Panel D) Activity of the lipamide-dependent enzyme pyruvate dehydrogenase (Pdh) is decreased in a *sufT* strain. Pdh activity was assessed in cell-free lysates generated from the WT (JMB1100) and *sufT* (JMB2512) strains cultured in media imposing a low (20AA) or increased (19AA-serine)

demand for lipoic acid. Panel E) Transcriptional activities of *sufC* and *sufT* are increased upon culture in serine dropout medium. Transcriptional activities of *sufC* and *sufT* were assessed in the WT (JMB1100) strain after culture in 20AA or 19AA-serine media. Data represent the average value of biological triplicates (Panels A, C and E) or biological duplicates (Panel D) and error bars represent standard deviations. Data in panel B are representative of at least three independent experiments.

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Figure 3. Methionine concentration influences the reliance upon lipoamide-requiring enzymes for growth

Panel A) The lipoic acid requirement of the *sufT* strain is influenced by the presence of methionine (Met) in the growth medium. Growth of the WT (JMB1100) and *sufT* (JMB2512) strains upon freshly prepared 19AA media lacking Met (hereafter 19AA-Met) in the presence or absence of lipoic acid supplementation or solid 20AA medium. Panel B) Extracellular metabolites from the *sufT* strain are deficient in supporting growth of the *lipA::Tn* strain when cultured upon freshly prepared solid 20AA medium. The *lipA::Tn* mutant (JMB7156) was cross-struck on a freshly prepared solid 20AA medium plate lacking lipoic acid and the WT (JMB1100) or *sufT* (JMB2512) strains were perpendicularly struck, bisecting the *lipA::Tn* streak. Panel C) Methionine concentration alters the reliance upon Pdh for growth. Growth of the WT (JMB1100), *sufT* (JMB2512), *bck::Tn* (JMB7888) and *pdh::Tn* (JMB7891) strains on freshly prepared solid 20AA and 19AA-Met media. Data in panels A and C are representative of at least three independent experiments.

Figure 4. Altered ClpC function suppresses the lipoic acid requirement of a *sufT* mutant

Panel A) Strains with *clpC** alleles are sensitive to puromycin toxicity and phenocopy a *clpC* strain. Growth of the WT (JMB1100), *sufT* (JMB2512), *sufT clpC* (JMB7121), *sufT clpC*_{G672R} (JMB4974), *sufT clpC*_{E362→stop} (JMB4980), and *sufT clpC*_{Y611SVIL→YFCDFNstop} (JMB4986) strains upon solid TSB medium with 7 μ g mL⁻¹ puromycin is shown. Panel B) Introduction of a null *clpC* allele suppresses the lipoic acid requirement of the *sufT* strain. Growth of the WT (JMB1100), *sufT* (JMB2512), *clpC* (JMB5674), *sufT clpC* (JMB7121), and *sufT clpC*_{E362→stop} (JMB4980) strains upon

solid 20AA LM media with and without lipoic acid supplementation is shown. Data in both panels are representative of at least three independent experiments.

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Figure 5. The *clpC*_{G672R} allele is dominant negative

Panel A) Genetic complementation reveals the presence of both recessive, as well as non-recessive *clpC** alleles. Growth of the WT (JMB1100), *sufT* (JMB2512), *sufT clpC*_{G672R} (JMB4974), *sufT clpC*_{E362→stop} (JMB4980) and *sufT clpC*_{Y611SVIL→YFCDFNstop} (JMB4986) strains containing either empty vector (pEPSA5) or *pclpC* upon solid 20AA LM medium lacking lipoic acid is shown. Panel B) The *clpC*_{G672R} allele exerts a dominant effect upon the lipoic acid requirement of a *sufT* mutant strain. Growth of the WT (JMB1100) and *sufT* (JMB2512) strains containing either empty vector (pEPSA5), *pclpC* or *pclpC*_{G672R} upon solid 20AA LM medium containing 1% xylose to induce transcription. Panel C) The *clpC*_{G672R} allele exerts a dominant effect upon puromycin resistance in the WT strain. Growth of the WT (JMB1100) containing empty vector (pEPSA5), *pclpC* or *pclpC*_{G672R}, as well as the *clpC* mutant (JMB7384) containing empty vector (pEPSA5), upon TSB media with and without 6 μ g mL⁻¹ puromycin is shown. Data in all panels are representative of at least three independent experiments.

Figure 6. The *sufT* strains lacking ClpC, ClpP or TrfA do not require lipoic acid supplementation for growth

Growth of the WT (JMB1100), *sufT* (JMB2512), *sufT clpQ*::Tn (JMB7729), *sufT clpP*::Tn (JMB7709), *sufT clpC* (JMB7121) and *sufT trfA*::Tn (JMB7703) strains upon solid 20AA LM media with and without lipoic acid supplementation. Data are representative of at least three independent experiments.

Figure 7. Strains with decreased ClpC function have a decreased requirement for lipoic acid
 Panels A and B) Top-agers containing the $\textit{sufT } \textit{lipA}::\textit{Tn}$ (JMB7750), $\textit{sufT } \textit{clpC}_{\text{E362} \rightarrow \text{stop}} \textit{lipA}::\textit{Tn}$ (JMB7746), $\textit{lipA}::\textit{Tn}$ (JMB7156), or $\textit{clpC } \textit{lipA}::\textit{Tn}$ (JMB7385) were overlaid upon 20AA LM medium plates before 1.5 pmol of lipoic acid was spotted in the center. Representative photographs of zones of growth are displayed in Panel A. Quantification of the zones of growth are displayed in Panel B and the data represent the average of biological triplicates and error bars represent standard deviations.

Figure 8. Decreased TCA cycle function results in a lower demand for lipioic acid and renders SufT non-essential

Panel A) Strains lacking aconitase (AcnA) have a decreased requirement for lipioic acid. Top agar overlays containing the *lipA::Tn* (JMB7156) or *lipA::Tn acnA* (JMB7825) strains were overlaid upon 20AA LM medium and 1.5 pmol of lipioic acid was spotted in the center. Zones of growth were measured after incubation. Panel B) A strain with decreased ClpC function has decreased AcnA activity. The WT (JMB1100), *sufT* (JMB2512) and *sufT clpC*_{E362→stop} (JMB4980) strains were cultured in liquid 20AA medium and AcnA activity was determined in cell-free lysates. Panel C) A *sufT* mutant with decreased AcnA

expression is capable of growth upon solid 20AA LM medium and this phenotype is suppressed upon induction of *acnA* transcription. Growth of the *acnA::Tn* (JMB2475) and *acnA::Tn sufT* (JMB3539) strains carrying *pacnA*, which contains *acnA* under the transcriptional control of a xylose inducible promoter, were cultured overnight in the presence or absence of varying concentrations of xylose. The strains were subsequently serially diluted and spot plated upon solid 20AA LM media with and without 1% xylose. Data in Panel A and B represent the average values of biological triplicates and error bars represent standard deviations. Data in Panel C are representative of at least three independent experiments.

Figure 9. Fermentative growth imposes a decreased demand for lipoic acid and renders SufT non-essential

Panel A) Requirement of lipoic acid for growth is decreased upon fermentative culture. Growth of the WT (JMB1100) and *lipA::Tn* (JMB7156) strains grown in liquid 20AA medium lacking lipoic acid under aerobic, microaerobic or fermentative (growth inside an anaerobic chamber) conditions. Panel B) SufT is dispensable for growth in serine dropout medium during fermentative culture. Growth of the WT (JMB1100) and *sufT* (JMB2512) strains after growth in liquid 19AA-serine medium in the presence or absence (fermentative growth) of dioxygen. Panel C) SufT is dispensable for growth upon solid media lacking

lipoic acid during fermentative culture. Growth of the WT (JMB1100) and *sufT* (JMB2512) strains upon solid medium lacking lipoic acid and in the presence or absence of dioxygen is shown. Data in Panel A and B represent the average values of biological triplicates and error bars represent standard deviations. Data in Panel C are representative of at least three independent experiments. For data in Panels A and B final optical densities were assessed and to facilitate comparative analyses the data were normalized with respect to the WT strain under each growth condition.

Figure 10. *nfu* is synergistic with *sufT*, while *sufA* is epistatic to *sufT*

Panels A and B) *sufT* displays synergy with *nfu* and *sufA* is epistatic to *sufT*. Growth was assessed for the WT (JMB1100), *sufT* (JMB1146), *nfu* (JMB1165), *sufA* (JMB2223), *sufA sufT* (JMB2224), *nfu sufA* (JMB6834), *nfu sufT* (JMB2514), and the *nfu sufT sufA* (JMB6835) strains in 20AA (Panel A) or 19AA-serine (Panel B) media. Panel C) The phenotypes of the *nfu* and *sufT* mutations display synergism for lipoic acid dependent growth. The WT (JMB1100), *sufT* (JMB2512), *nfu* (JMB1165) and *sufT nfu* (JMB2514) strains were plated on solid 20AA media with and without lipoic acid. Panel D) *nfu* and *sufT* display synergy with respect to succinate dehydrogenase (Sdh) activity. Sdh activity was assessed in cell-free lysates generated from the same strains used

in Panel C. Data represent the average value of biological triplicates (Panel D) or biological duplicates (Panel A and B) and error bars represent standard deviations. Data in panel C are representative of at least three independent experiments.

Figure 11. Nfu can compensate for the absence of SufT for lipoic acid dependent growth
Overexpression of *nfu*, but not *sufA*, permits growth of the *sufT* strain on solid media lacking lipoic acid. The *sufT* (JMB2512) and WT (JMB1100) strains carrying pEPSA5 (empty vector), *pnfu* or *psufA* were pre-cultured for 6 hours with 1% xylose, serial diluted and spot plated upon solid 20AA LM medium, 20AA LM medium with lipoic acid or 20AA intermediate methionine (IM) medium containing 1% xylose. Data are representative of at least three independent experiments.

Figure 12. Nfu-Gfp abundance is increased in a *clpC* mutant and Nfu is required for the suppression imparted by the *clpC*_{E362→stop} allele on the lipoic acid requirement of the *sufT* strain

Panel A) The abundance of Nfu-Gfp is increased in a *clpC* mutant strain. Abundance of Nfu-Gfp was assessed by monitoring fluorescence in the *nfu* (parent; JMB3403) and *clpC* *nfu* mutant (*clpC*; JMB8246) strains containing the pLL39_ *nfu_gfp* episome. Panel B) Nfu is required for the suppression imparted by the *clpC*_{E362→stop} allele on the lipoic acid requirement of the *sufT* strain. Growth of the WT (JMB1100), *sufT* (JMB2512), *nfu* (JMB1165), *sufT clpC*_{E362→stop} (JMB4980), *sufT clpC*_{E362→stop} *nfu::Tn* (JMB8121)

and *nfu sufT*(JMB2514) strains cultured on solid chemically defined 20AA LM media with and without lipoic acid supplementation is shown. Data in Panel A represent the average value of biological triplicates and error bars represent standard deviations. Data in panel B are representative of at least three independent experiments.

Table 1

Strains and plasmids used in this study.

Strains used in this study		
Strain name	genotype	Source/reference
JMB1100	USA300_LAC	A.R. Horswill (Boles <i>et al.</i> , 2010)
RN4220	Restriction minus	NARSA (Kreiwirth <i>et al.</i> , 1983)
JMB1146	<i>sufT</i> (SAUSA300_0875)	This study
JMB2512	<i>sufT::tetM</i>	This study
JMB1432	<i>fur::tetM</i>	(Torres <i>et al.</i> , 2010)
JMB2475	<i>acnA::TN(ermR)</i> (SAUSA300_1246)	NARSA (Fey <i>et al.</i> , 2013)
JMB3539	<i>acnA::TN(ermR) sufT</i>	This study
JMB5674	<i>clpC::TN(ermR)</i> (SAUSA300_0510)	NARSA (Fey <i>et al.</i> , 2013)
JMB7121	<i>sufT::tetM clpC::TN(ermR)</i>	This work
JMB4982	<i>sufT::tetM fur*</i>	This work
JMB4974	<i>sufT::tetM clpC*</i> _{G672→R}	This work
JMB4980	<i>sufT::tetM clpC*</i> _{E362→stop}	This work
JMB4986	<i>sufT::tetM clpC*</i> _{Y611SVIL→YFCDFNstop}	This work
JMB7384	<i>clpC::tetM</i>	This work
JMB7156	<i>lipA::TN(ermR)</i> (SAUSA300_0829)	NARSA (Fey <i>et al.</i> , 2013)
JMB7750	<i>lipA::TN(ermR) sufT::tetM</i>	This work
JMB7746	<i>lipA::TN(ermR) sufT::tetM clpC*</i> _{E362→stop}	This work
JMB7825	<i>lipA::TN(ermR) acnA::tet</i>	this study
JMB7385	<i>clpC::tetM lipA::TN(ermR)</i>	This work
JMB1165	<i>nfu</i> (SAUSA300_0839)	(Mashruwala <i>et al.</i> , 2015)
JMB2223	<i>sufA::tetM</i> (SAUSA300_0843)	(Rosario-Cruz <i>et al.</i> , 2015)
JMB2224	<i>sufA::tetM sufT</i>	(Mashruwala <i>et al.</i> , 2016)
JMB6834	<i>sufA::tetM nfu::kanR</i>	(Mashruwala <i>et al.</i> , 2016)
JMB6835	<i>sufA::tetM nfu::kanR sufT</i>	(Mashruwala <i>et al.</i> , 2016)
JMB3403	<i>nfu pLL39_gfp_nfu</i>	This work
JMB8246	<i>nfu clpC::TN(ermR) pLL39_gfp_nfu</i>	This work
JMB2015	<i>sucA::TN(ermR)</i> (SAUSA300_1306)	NARSA (Fey <i>et al.</i> , 2013)
JMB2916	<i>pdx::TN(ermR)</i> (SAUSA300_0955)	NARSA (Fey <i>et al.</i> , 2013)
JMB7505	<i>bckA::TN(ermR)</i> (SAUSA300_1465)	NARSA (Fey <i>et al.</i> , 2013)
JMB7714	<i>clpQ::TN(ermR)</i> (SAUSA300_1147)	NARSA (Fey <i>et al.</i> , 2013)
JMB7911	<i>gcv::TN(ermR)</i> (SAUSA300_1496)	NARSA (Fey <i>et al.</i> , 2013)
JMB7729	<i>sufT::tetM clpQ::TN(ermR)</i>	This work
JMB4898	<i>clpP::TN(ermR)</i> (SAUSA300_0752)	NARSA (Fey <i>et al.</i> , 2013)
JMB7709	<i>sufT::tetM clpP::TN(ermR)</i>	This work
JMB7702	<i>trfA::TN(ermR)</i> (SAUSA300_0899)	NARSA (Fey <i>et al.</i> , 2013)
JMB7703	<i>sufT::tetM trfA::TN(ermR)</i>	This work
JMB2514	<i>sufT::tetM nfu</i>	This work
JMB8121	<i>sufT::tetM clpC*</i> _{E362→stop} <i>nfu::TN(ermR)</i>	This work

Strains used in this study		
Strain name	genotype	Source/reference
JMB8123	<i>sufT::tetM clpC*</i> _{Y611SVIL→YFCDFNstop} <i>nfr::TN(ermR)</i>	This work
JMB8124	<i>sufT::tetM clpC*</i> _{G672→R} <i>nfr::TN(ermR)</i>	This work
<i>Escherichia coli</i> PX5	Used for gene cloning	Protein Express
Plasmids used in this study		
Plasmid name	Insert	Function and Reference
pEPSA5	None	Genetic complementation (Forsyth <i>et al.</i> , 2002)
pEPSA5_ <i>clpC</i>	SAUSA300_0510	<i>clpC</i> overexpression; this work
pEPSA5_ <i>clpC</i> _{G672→R}	SAUSA300_0510 _{G672→R}	<i>clpC</i> overexpression; this work
pEPSA5_ <i>acnA</i>	SAUSA300_1246	<i>acnA</i> overexpression (Mashruwala <i>et al.</i> , 2015)
pEPSA5_ <i>nfr</i>	SAUSA300_0839	<i>nfr</i> overexpression (Mashruwala <i>et al.</i> , 2015)
pEPSA5_ <i>sufA</i>	SAUSA300_0839	<i>sufA</i> overexpression (Rosario-Cruz <i>et al.</i> , 2015)
pCM28	None	Genetic complementation (Pang <i>et al.</i> , 2010)
pCM28_ <i>sufT</i>	SAUSA300_0875	<i>sufT</i> complementation; This work
pJB38	None	Mutant construction; (Bose <i>et al.</i> , 2013)
pJB38_ <i>sufT</i>	SAUSA300_0875	(Mashruwala <i>et al.</i> , 2016)
pJB38_ <i>sufT::tetM</i>	SAUSA300_0875	Mutant construction; This work
pJB38_ <i>clpC</i>	SAUSA300_0510	Mutant construction; This work
pJB38_ <i>clpC::tetM</i>	SAUSA300_0510	Mutant construction; This work
pCM11	None	Promoterless <i>gfp</i> (Malone <i>et al.</i> , 2009)
pCM11_ <i>sufC</i>	<i>sufC</i> promoter	Transcriptional activity (Mashruwala <i>et al.</i> , 2016)
pCM11_ <i>sufT</i>	<i>sufT</i> promoter	Transcriptional activity (Mashruwala <i>et al.</i> , 2016)
pLL39		(Luong & Lee, 2007)
pLL39_ <i>native_nfr_FLAG</i>	SAUSA300_0839	Translational fusion; this work
pLL39_ <i>nfr_gfp</i>	SAUSA300_0839 and Gfp	Translational fusion; this work
pTET	None	Replace <i>ermR</i> with <i>tetM</i> (Bose <i>et al.</i> , 2013)

Table 2 Properties of *sufT* suppressor mutations.

Strain	Location	Mutation type	Mutation	Effect
JMB4974	<i>clpC</i>	Missense	G ₆₇₂ →R	dominant
JMB4980	<i>clpC</i>	Amber	E ₃₆₂ →stop	recessive
JMB4982	<i>fur</i> promoter	Base-pair change	−90 promoter thy→cyt	Not tested
JMB4986	<i>clpC</i>	Frame shift	Y ₆₁₁ SVIL→YFCDFN _{stop}	recessive