Staphylococcus aureus can survive in the absence of c-di-AMP upon inactivation of
the main glutamine transporter

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Summary (250 words)

The nucleotide second messenger c-di-AMP negatively regulates potassium and osmolyte uptake in *Staphylococcus aureus* and many other bacteria. c-di-AMP is also important for growth and an *S. aureus* strain deleted for the c-di-AMP cyclase gene *dacA* is unable to survive in rich medium unless it acquires compensatory mutations. Previously, we have shown that an *S. aureus dacA* mutant can grow after the acquisition of inactivating mutants in *opuD*, encoding the main glycine-betaine osmolyte transporter, or mutations in *alsT*, encoding a predicted amino acid transporter. Using the size of bacterial cells as a proxy for their osmotic balance, we show that inactivation of OpuD helps bacteria to re-establish their osmotic balance, while inactivation of AlsT does not and bacteria remain enlarged, a characteristic of *S. aureus* cells unable to produce c-di-AMP. We show that AlsT is the main glutamine transporter in *S. aureus*, thus revealing that *S. aureus* can survive without c-di-AMP when glutamine uptake is prevented. Using a bioinformatics approach combined with uptake assays, we identified GltS as the main glutamate transporter in *S. aureus*. Using WT and mutant strain, we show that glutamine is preferred over glutamate for bacterial growth and that its uptake represses c-di-AMP production. Glutamine and glutamate are important players in osmotic regulation, but their cellular levels also serve as a key indicator of nitrogen availability in bacterial cells. Therefore, we not only provide a further connection between the c-di-AMP signalling network and osmotic regulation in *S. aureus* but also to central nitrogen metabolism.
Introduction

In the human host, *Staphylococcus aureus* can infect various niches, including, but not limited to, blood, kidneys, bones, heart, soft tissue and lungs (Kluymans *et al.*, 1997, Fridkin *et al.*, 2005). *S. aureus* can colonize these different tissues thanks to its refined regulatory mechanisms that allow it to rapidly respond to external stimuli. Amongst others, this allows the organism to adapt its metabolism and utilize different carbon and nitrogen sources available in each specific niche (Fridkin *et al.*, 2005, Spahich *et al.*, 2016, Vitko *et al.*, 2015, Crooke *et al.*, 2013, Fuller *et al.*, 2011, Richardson *et al.*, 2008, Halsey *et al.*, 2017, Lehman *et al.*, 2019).

Glucose is the preferred carbon source for *S. aureus*, but it can be limiting during infection due to the host immune response (Kelly & O'Neill, 2015, Spahich *et al.*, 2016, Halsey *et al.*, 2017, Lehman *et al.*, 2019). In glucose-limiting conditions, *S. aureus* instead catabolizes secondary carbon sources; amino acids, particularly glutamate and proline, serve as major carbon sources during growth in the absence of glucose (Halsey *et al.*, 2017). However, not much is known about amino acid uptake and catabolism in *S. aureus* and how the availability of certain nutrients can affect virulence factor expression and invasion of the host. While a large number of amino acid transporters and oligopeptide permeases can be identified bioinformatically, their actual substrate specificities and functions in *S. aureus* have not yet been studied in detail. Also, predicting the substrates for transporters bioinformatically remains difficult and hence such questions need to be addressed experimentally.

Secondary messenger molecules are crucial in allowing bacteria to rapidly adapt to different environmental and host cell niches (Römling, 2008, Hengge, 2009). There is now considerable evidence that one such messenger, cyclic di-adenosine monophosphate (c-di-AMP) plays a significant role in osmoregulation in bacteria (Pham *et al.*, 2018, Pham & Turner, 2019, Quintana *et al.*, 2019, Zarrella *et al.*, 2018, Teh *et al.*, 2019, Fahmi *et al.*, 2019, Devaux *et al.*, 2018, Bai *et al.*, 2014, Zeden *et al.*, 2018, Corrigan *et al.*, 2011, Rocha *et al.*, 2019, Gundlach *et al.*, 2017b, Gundlach *et al.*, 2017a, Witte *et al.*, 2013, Whiteley *et al.*...
c-di-AMP binds to and negatively regulates a number of different potassium and osmolyte importers (Rocha et al., 2019, Quintana et al., 2019, Kim et al., 2015, Corrigan et al., 2013, Moscoso et al., 2015, Chin et al., 2015, Huynh et al., 2016, Schuster et al., 2016, Pham & Turner, 2019, Pham et al., 2018, Devaux et al., 2018, Zarrella et al., 2018, Gundlach et al., 2017b, Gundlach et al., 2017a, Gundlach et al., 2017c). c-di-AMP is essential for bacterial growth under standard growth conditions but it is also toxic at high levels in many Firmicutes, hence its cellular levels must be tightly regulated (Gundlach et al., 2015b, Mehne et al., 2013, Corrigan et al., 2011, Corrigan et al., 2015, Woodward et al., 2010, Witte et al., 2013). In S. aureus and Listeria monocytogenes, deletion of the diadenylate cyclase gene dacA, the enzyme responsible for the synthesis of c-di-AMP, was only possible in chemically defined medium (Whiteley et al., 2015, Zeden et al., 2018, Devaux et al., 2018), whereas in Bacillus subtilis all three c-di-AMP cyclases could only be inactivated in minimal medium also containing low amounts of potassium (Gundlach et al., 2017b).

Previously, we found that inactivation of the main glycine betaine transporter OpuD as well as the predicted amino acid transporter AlsT (SAUSA300_1252) allows an S. aureus dacA mutant to grow in rich medium in the absence of c-di-AMP (Zeden et al., 2018). In several other Firmicutes, including B. subtilis, Lactococcus lactis, Streptococcus pneumoniae, Streptococcus pyogenes and L. monocytogenes, inactivating mutations have also been identified in osmolyte and potassium transport systems that allow these bacteria to grow in the absence of c-di-AMP (Pham et al., 2018, Pham & Turner, 2019, Quintana et al., 2019, Zarrella et al., 2018, Teh et al., 2019, Fahmi et al., 2019, Devaux et al., 2018, Bai et al., 2014, Zeden et al., 2018, Corrigan et al., 2011, Rocha et al., 2019, Gundlach et al., 2017b, Gundlach et al., 2017a, Witte et al., 2013, Whiteley et al., 2015, Whiteley et al., 2017). This is consistent with the idea that in the absence of c-di-AMP, potassium and osmolyte transporters are more active, resulting in the accumulation of toxic levels of potassium and osmolytes in the cell. Consistent with a key function of c-di-AMP in regulating the osmotic balance in the cell, we found that S. aureus cells show significant differences in cell size
depending on their intracellular c-di-AMP levels (Zeden et al., 2018, Corrigan et al., 2011).

Bacteria of the high c-di-AMP level *S. aureus* mutant strain LAC*gdpP* show a decrease in cell size, while cells of the low level c-di-AMP strain LAC*dacA_{G206S}* show an increase in cell size (Zeden et al., 2018, Corrigan et al., 2011). As c-di-AMP negatively regulates potassium and osmolyte uptake (Corrigan et al., 2013, Moscoso et al., 2015, Schuster et al., 2016), the increase in cell size is consistent with the hypothesis that an increase in potassium and osmolyte uptake and retention of water at reduced c-di-AMP levels leads to the observed increase in cell size. As part of this study, we further investigated the mechanisms by which inactivation of the main glycine betaine transporter OpuD and the predicted amino acid transporter AlsT allow *S. aureus* to survive in the absence of c-di-AMP.

The counterion of potassium in the cell is glutamate, which at the same time also serves as main nitrogen donor for cellular metabolites and macromolecules. A correlation between cellular levels of c-di-AMP, glutamate and glutamine has been reported for several Firmicutes, including *B. subtilis* and *L. monocytogenes* (Whiteley et al., 2017, Gundlach et al., 2015b, Gundlach et al., 2017a, Sureka et al., 2014). A two-fold increase in cellular c-di-AMP levels was observed in *B. subtilis* when bacteria where grown in Spizizen minimal medium with glutamate (Glu) as compared to growth in the same medium with glutamine (Gln) as nitrogen source (Gundlach et al., 2015b). In *L. monocytogenes*, c-di-AMP is a negative regulator of the key TCA cycle enzyme pyruvate carboxylase (Sureka et al., 2014). The depletion of c-di-AMP in the cell resulted in an increased flux of glucose into the production of glutamine and glutamate, likely due to increased pyruvate carboxylase activity and increased flux into the TCA cycle at reduced c-di-AMP levels (Sureka et al., 2014). Interestingly, an *L. monocytogenes dacA* mutant that also lacks *citZ*, which codes for the key TCA cycle enzyme citrate synthase, is viable in rich medium (Sureka et al., 2014, Whiteley et al., 2017). In a *citZ* mutant the TCA cycle is blocked and it was shown that depletion of c-di-AMP in this mutant no longer results in the accumulation of glutamate and glutamine in the cell (Sureka et al., 2014). Therefore, the amino acids glutamine and glutamate not only play an important function in the osmotic regulation in bacterial cells, but also have a
particularly critical role in TCA cycle function and their cellular ratio has an important role in
signalling nitrogen-limiting (high glutamate levels and low glutamine levels) or nitrogen
excess (high glutamine and low glutamate levels) conditions (Halsey et al., 2017, Gundlach et al., 2017a).

As part of this study, we further investigated why inactivation of the main glycine-
betaine transporter OpuD and the predicted amino acid transporter AlsT allows S. aureus to
grow in the absence of c-di-AMP. Our results indicate that inactivation of OpuD helps
bacteria to re-establish their osmotic balance, while inactivation of AlsT, which we show here
is the main glutamine transporter in S. aureus, functions differently to bypass the essentiality
of c-di-AMP and possible mechanisms are discussed. We also identified the S. aureus GltS
protein as the main glutamate transporter in S. aureus and show that glutamine but not
 glutamate uptake represses the production of c-di-AMP. With this study, we not only provide
a further link between the c-di-AMP signalling network and osmotic regulation in bacterial
cells but also to the central nitrogen metabolism in S. aureus.

Results

Inactivation of OpuD but not AlsT reduces the cell size of an S. aureus dacA mutant

In previous work, we reported a correlation between the cell size and c-di-AMP levels in S.
aureus: bacteria with high c-di-AMP level are smaller, whereas bacteria with low c-di-AMP
levels (strain LAC∗dacA<sub>G206S</sub>) are larger as compared to wild-type bacteria (Zeden et al.,
2018, Corrigan et al., 2011). We also reported that inactivating mutations in opuD
(SAUSA300_1245) coding for the main glycine betaine osmolyte transporter and alsT
(SAUSA300_1252) coding for a predicted amino acid sodium symporter, rescue the growth
defect observed for the c-di-AMP negative S. aureus strain LAC∗dacA::kan in rich medium
(Zeden et al., 2018). To investigate the mechanism by which the growth defect of the dacA
mutant strain is rescued in the LAC∗dacA/opuD and LAC∗dacA/alsT suppressor strains and
to assess if restoring the osmotic imbalance could be a contributing factor, the cell size of
WT and different S. aureus mutants was determined. Initially, the cell size of
LAC* and the low-level c-di-AMP LAC* dacA<sub>G206S</sub> strain after growth in TSB medium. As expected, the low-level c-di-AMP bacteria showed an increase in cell size as compared to WT bacteria (Fig. 1A and B). While a similar increase in cell size was still observed for bacteria of strain LAC* dacA/alsT, the cell size of LAC* dacA/opuD bacteria, while still increased as compared WT, was significantly smaller as compared to the low-level LAC* dacA<sub>G206S</sub> strain (Fig. 1A and B). As TSB medium is not suitable for the growth of the c-di-AMP null strain LAC* dacA::kan, bacterial cell sizes were also determined for the WT and mutant S. aureus strains following growth in TSB supplemented with 0.4 M NaCl, which is permissive for the growth of the dacA mutant (Fig. 1C-F). Bacteria from all strains had a reduced cell size when grown in TSB supplemented with 0.4 M NaCl compared to bacteria grown in TSB (Fig. 1). Similar to what was observed for the low level c-di-AMP dacA<sub>G206S</sub> mutant strain, the size of bacteria from the c-di-AMP null strain LAC* dacA::kan was significantly increased compared to WT bacteria. As observed before, the cell size was not rescued for bacteria of the LAC* dacA/alsT suppressor strain (Fig. 1C-F). On the other hand, the size of LAC* dacA/opuD bacteria was similar to that of WT bacteria (Fig. 1C-F). Taken together, these data indicate that inactivation of OpuD, and hence reduced glycine betaine transport, likely helps bacteria to survive in the absence of c-di-AMP by re-establishing the osmotic balance in the mutant while a different mechanism is at play for the alsT mutant.

**Bacteria lacking alsT have an altered amino acid uptake profile**

AlsT (SAUSA300_1252) is a predicted amino acid transporter protein and annotated as an alanine/sodium symporter. However, no difference in the uptake of radiolabelled alanine was detected between a WT and the LAC* dacA/alsT mutant strain in our previous study (Zeden et al., 2018), indicating that AlsT is not an alanine transporter. To identify potential substrates for the S. aureus AlsT transporter, we followed the depletion of different amino acids from the culture supernatant during the growth of the WT and alsT mutant strains in TSB medium. To this end, strain LAC* alsT::tn containing a transposon insertion in alsT was
constructed by phage transducing the alsT::tn region from the Nebraska Transposon Mutant Library (NMTL) strain NE142 (Fey et al., 2013) into the S. aureus LAC* strain background. WT LAC* and the alsT mutant strain LAC*alsT::tn showed similar growth rates when grown in TSB medium (Fig. 2A). Next, their ability to take up different amino acids was assessed by determining the levels of the individual amino acids in the culture supernatant at the start of the experiment (T = 0 h) as compared to 6, 10 and 12 h following their growth in TSB medium. While no significant differences were observed for most amino acids (Fig. S1), a slight increase in the utilization of aspartate and a slight decrease in the uptake of serine was observed (Fig. 2B and 2C), suggesting that AlsT could potentially be a serine transporter. To test this, uptake assays were performed with radiolabelled serine using the WT LAC* strain, the alsT mutant strain LAC*alsT::tn piTET as well as the complementation strain LAC*alsT::tn piTET-alsT. However, no significant differences in the uptake rate of serine were observed between the strains (Fig. 3A), indicating that AlsT is not the main serine transporter in S. aureus. While slight differences in the amino acid uptake profile were observed between the WT and alsT mutant strain, this analysis did not allow us to identify the main substrate for AlsT. However, it is of note that using this method one cannot distinguish between glutamine and glutamate or asparagine and aspartate utilization. Additionally, tryptophan was not measured due to the limitations of the method used.

**AlsT is the main glutamine transporter in S. aureus**

Next, a more detailed bioinformatics analysis was performed to identify potential AlsT substrates. A BlastP search against the B. subtilis 168 genome led to the identification of four close homologs of AlsT (SAUSA300_1252), namely AlsT (e-value: e-166), GlnT (e-value: e-149), YrbD (e-value: e-117) and YflA (e-value: 2e-72). Of note, an AlsT homologue SAUSA300_0914 is also present in the S. aureus FPR3757 genome (Fig. S2). While S. aureus AlsT (SAUSA300_1252) shows the highest similarity to the B. subtilis AlsT and GlnT proteins, SAUSA300_0914 has the highest similarity with the B. subtilis YrbD protein. AlsT is annotated in B. subtilis as a potential glutamine sodium symporter, but to the best of our
knowledge, this has not yet been experimentally verified. Expression of adsT is controlled in
*B. subtilis* by GlnR and TnrA, the two main transcriptional regulators adjusting gene
expression in response to nitrogen availability (Randazzo *et al.*, 2017, Yoshida *et al.*, 2003,
Mirouze *et al.*, 2015). GlnT is a confirmed glutamine transporter in *B. subtilis* (Satomura *et al*.,
2005), whose production is induced in the presence of glutamine as nitrogen source by
the two-component system GlnKL. To test if *S. aureus* AlsT is a potential glutamine or
glutamate transporter, uptake assays were performed with radiolabelled glutamine and
glutamate using the WT *S. aureus* strain LAC*, the adsT mutant LAC*adsT::tn* piTET and the
complementation strain LAC*adsT::tn* piTET-adsT. Uptake of glutamine, but not of glutamate,
was severely reduced in the adsT mutant when compared to the WT (Fig. 3B). This defect
was restored upon expression of adsT in the complementation strain (Fig. 3C). To confirm
that adsT also functions as main glutamine transporter in the LAC*dacA/adsT* suppressor
strain, uptake assays were also performed with strain LAC*dacA/adsT* and compared to that
of the WT LAC* and LAC*dacA::kan* control strains (Fig. 3D-F). Similar to what was
observed for the adsT mutant, glutamine uptake was severely attenuated in strain
LAC*dacA/adsT* when compared to the control strains (Fig. 3D-F). These data highlight that
under the growth conditions tested, AlsT functions as the main glutamine transporter in *S.
aureus*. A slight reduction in glutamine uptake was seen in the absence of c-di-AMP,
suggesting that c-di-AMP levels can impact glutamine uptake in *S. aureus*. But perhaps
most importantly, our data suggest that bacteria lacking c-di-AMP can survive in rich
medium when glutamine uptake is blocked.

**Investigating the contribution of SAUSA300_0914 and GlnPQ to glutamine and

*glutamate transport in S. aureus***

*S. aureus* SAUSA300_0914 codes for a predicted amino acid symporter, which shows 41%
identity with the *S. aureus* AlsT protein. After assigning AlsT a function as glutamine
transporter, we wanted to test if SAUSA300_0914 might also play a role in glutamine or
glutamate transport. To this end, strain LAC*0914::tn* was constructed by transducing the
genomic region from the NMTL strain NE1463 (Fey et al., 2013) containing a transposon insertion in SAUSA300_0914 into the S. aureus LAC* background. Subsequently uptake of radiolabelled glutamine and glutamate was assessed (Fig. 4A-B). No significant differences in the uptake of these amino acids was observed between WT LAC* and strain LAC*0914::tn, showing that SAUSA300_0914 does not function as glutamine or glutamate transporter under our assay conditions.

AlsT and SAUSA300_0914 are members of the amino acid-sodium symporter family of transporters, which are composed of a single multimembrane spanning protein. Besides this type of transporter, GlnPQ-type ABC transporters play a major role in glutamine and glutamate transport in other bacteria (Schuurman-Wolters & Poolman, 2005). S. aureus contains a glnPQ (SAUSA300_1808 - SAUSA300_1807) operon with glnP coding for a substrate binding domain-permease fusion protein and glnQ coding for the cytoplasmic nucleotide-binding ATPase domain. The results from a previous study suggested that this transporter functions as glutamine transporter in S. aureus, as a glnP mutant was more resistant to the toxic glutamine analogue gamma-L-glutamyl hydrazide (Zhu et al., 2009). To assess the contribution of the GlnPQ transporter to glutamine and glutamate transport in S. aureus under our assay conditions, strain LAC*glnQ::tn was generated by transducing the glnQ::tn region from the NMTL strain NE153 (Fey et al., 2013) into the LAC* background. The resulting LAC*glnQ::tn mutant strain displayed no difference in glutamine or glutamate uptake compared to WT LAC* (Fig. 4C-D), indicating that the ABC transporter GlnPQ does not function as a main glutamate or glutamine transporter under our assay conditions.

**GltS (SAUSA300_2291) is the main glutamate transporter in S. aureus**

Cellular glutamine levels are key in signalling nitrogen availability in bacterial cells (Fisher, 1999, Gunka & Commichau, 2012). In addition, differences in cellular c-di-AMP levels were reported in B. subtilis depending on the presence of glutamine or glutamate as available nitrogen source. More specifically, an increase in cellular c-di-AMP levels was observed in the presence of glutamate as compared to glutamine (Gundlach et al., 2015b). S. aureus
does not only take up glutamine but also shows robust glutamate uptake (Figs 3 and 4).

However, none of the transporters (AlsT, SAUSA300_0914 and GlnPQ) investigated so far plays a major role in glutamate uptake under our growth conditions. In *B. subtilis* GltT, belonging to the dicarboxylate/amine acid cation symporter (DAACS) family of proteins, is a major high-affinity Na⁺-coupled glutamate/aspartate symporter and can also mediate the uptake of glyphosate (Wicke *et al.*, 2019). An additional two paralogs, DctP and GltP are found in *B. subtilis* of which GltP has also been shown to be a glutamate transporter (Tolner *et al.*, 1995). The *S. aureus* protein SAUSA300_2329 (from here on referred to as GltT) shows a high degree of similarity (52% identity) to the *B. subtilis* GltT protein. In addition, we identified SAUSA300_2330 (from here on referred to as GltS) as a potential glutamate transporter in *S. aureus* due to its similarity to the *E. coli* glutamate permease GltS (e-value: 6e-77; 38% identity) (Deguchi *et al.*, 1990). To test if *S. aureus* GltT or GltS are glutamate transporters or impact glutamine uptake in *S. aureus*, strains LAC* gltT::tn* and LAC* gltS::tn* were constructed by moving the respective *gltT* and *gltS* transposon insertion regions from the NMTL strains NE566 and NE560 (Fey *et al.*, 2013) into the LAC* strain background.

Next, the uptake of radiolabelled glutamine and glutamate was assessed for the WT LAC* strain and the LAC* gltT::tn* and LAC* gltS::tn* mutants. No difference in the uptake of glutamine was observed between the strains (Fig. 5A) and in the case of LAC* gltT::tn*, also no difference in the uptake of glutamate was observed. However, a significant reduction in glutamate uptake was observed for strain LAC* gltS::tn* when compared to the WT (Fig. 5B).

The glutamine uptake defect could be restored in a complementation strain harbouring plasmid piTET-*gltS* allowing for inducible *gltS* expression (Fig. 5C). Indeed, increased glutamate uptake was observed in the complementation strain, indicating increased *gltS* expression in the complementation strain as compared to a WT strain. Taken together, these data reveal that under the growth conditions tested, GltS is the main glutamate transporter in *S. aureus*. 
Glutamine but not glutamate stimulates the growth of *S. aureus* in CDM lacking ammonium as nitrogen source.

Glutamine and glutamate are important amino acids that can serve as nitrogen sources for the synthesis of many other cellular metabolites. To examine the effect of these amino acids on the growth of *S. aureus* as well as to evaluate the contribution of the glutamine (AlsT) and glutamate (GltS) transporters for growth, growth curves were performed with WT, *alsT::tn* and *gltS::tn* mutant strains in chemically defined medium (CDM) lacking ammonium and containing either glutamine (CDM+Gln) or glutamate (CDM+Glu). Reduced growth was seen when the *S. aureus* strains were grown in glutamate as compared to glutamine containing medium, suggesting that glutamine but not glutamate can stimulate the growth of *S. aureus* in CDM lacking ammonium as nitrogen source (Fig. 6A). Consistent with this, the *alsT::tn* mutant strain, which is deficient in glutamine uptake, showed a similar growth reduction even if grown in the glutamine-containing medium (CDM+Gln) (Fig. 6A). The observed growth defect for the *alsT* mutant in CDM+Gln could be restored in the complementation strain harbouring the plasmid piTET-alsT (Fig. 6B). On the other hand, the *gltS::tn* mutant, which is defective in glutamate uptake, grew similar to the WT strain under all conditions tested, suggesting that glutamate uptake does not impact the growth of *S. aureus* under the test conditions. Taken together, these data indicate that glutamine is preferred over glutamate for the growth of *S. aureus* in CDM lacking ammonium as nitrogen source.

Glutamine uptake leads to a reduction in the cellular c-di-AMP levels in *S. aureus*

In a previous study, it has been reported that the presence of glutamine or glutamate in the growth medium can affect cellular c-di-AMP levels in *B. subtilis* and it was proposed that glutamate uptake leads to an activation of c-di-AMP synthesis in this organism (Gundlach *et al.*, 2015b). To assess if the presence of glutamine or glutamate would also affect c-di-AMP
levels in *S. aureus*, the intracellular c-di-AMP concentrations were determined for the WT *S. aureus* strain LAC* following growth in CDM+Gln or CDM+Glu medium. c-di-AMP levels were significantly higher in the presence of glutamate as compared to glutamine (Fig. 7A).

As previously reported (Corrigan *et al.*, 2011), in the absence of the c-di-AMP phosphodiesterase GdpP (strain LAC* gdpP::kan*) c-di-AMP levels were increased as compared to a WT strain (Fig. 7A). Interestingly and similar as observed for the WT strain, c-di-AMP levels were also higher in the *gdpP* mutant strain in the presence of glutamate as compared to glutamine. This indicates that the observed regulation of c-di-AMP synthesis depending on the presence of glutamine or glutamate is at the level of synthesis and not degradation. To test if glutamine uptake inhibits or glutamate uptake activates c-di-AMP production, c-di-AMP levels were also determined for the *alsT::tn* and *gltS::tn* mutants, which are defective in glutamine or glutamate uptake, respectively. Following growth in glutamate containing medium, all strains produced high and comparable levels of c-di-AMP (Fig. 7B white columns). However, some variation in the relative c-di-AMP levels produced by the *gltS* mutant compared to the WT strain was observed between experiments (Fig. 7B white columns and Fig. S3). The reason for this is currently not known. But taken together, our data suggest that the ability of *S. aureus* to take up glutamate does not drastically affect c-di-AMP production. On the other hand, clear differences in c-di-AMP levels were observed for strain *alsT::tn*, which is unable to take up glutamine. The WT and the *gltS::tn* mutant strains produced low c-di-AMP amounts following growth in CDM+Gln, while the c-di-AMP levels remained high in strain *alsT::tn*. (Fig. 7B grey columns and Fig. S3), suggesting that glutamine uptake inhibits c-di-AMP production. The c-di-AMP levels in the *alsT* mutant strain could be restored back to WT levels in the complementation strain harbouring plasmid piTET-*alsT* (Fig. 7C). Taken together, these results highlight that glutamine uptake blocks c-di-AMP production in *S. aureus* and that eliminating glutamine from the medium or preventing its uptake stimulates c-di-AMP production. Such an activation is likely achieved through stimulating the activity of the c-di-AMP cyclase DacA, rather than preventing its degradation by GdpP and possible mechanisms for this will be discussed.
Discussion

Under standard laboratory growth conditions, c-di-AMP is essential for growth in many bacteria (Mehne et al., 2013, Corrigan et al., 2015, Woodward et al., 2010, Gundlach et al., 2015a), but the exact molecular mechanisms behind this have remained unclear. Here we have shown that inactivation of OpuD likely helps a S. aureus c-di-AMP null strain survive by allowing bacteria to re-establish their osmotic balance. Bacteria unable to produce c-di-AMP are larger than WT cells but bacteria that are unable to produce c-di-AMP and also lack OpuD, the main transporter for the osmolyte glycine betaine in S. aureus, are similar in size to WT bacteria. This indicates that in these cells the osmotic balance has been restored.

Furthermore, we show that mutations in alsT, which we identify as part of this study to encode for the main glutamine transporter in S. aureus, suppress the essentiality of c-di-AMP in a different way and potential mechanisms for this are discussed here.

Over the last decade, considerable evidence has emerged that c-di-AMP plays a major role in osmotic regulation, primarily by positively regulating potassium export or negatively regulating potassium and osmolyte uptake (Rocha et al., 2019, Quintana et al., 2019, Kim et al., 2015, Corrigan et al., 2013, Moscoso et al., 2015, Chin et al., 2015, Huynh et al., 2016, Schuster et al., 2016, Pham & Turner, 2019, Pham et al., 2018, Devaux et al., 2018, Zardenella et al., 2018, Gundlach et al., 2017b, Gundlach et al., 2017a, Gundlach et al., 2017c, Gundlach et al., 2019). However, individual c-di-AMP target proteins identified thus far are themselves not essential. Therefore, the essentiality of c-di-AMP is likely due to its ability to regulate multiple target proteins simultaneously. Furthermore, in the absence of this molecule, many transporters are activated rather than inactivated, likely leading to accumulation of toxic levels of metabolites, such as potassium and osmolytes. Consistent with this idea, inactivating mutations in potassium uptake systems, oligopeptide and osmolyte transporters have been reported to rescue the growth defect of bacteria unable to produce c-di-AMP (Whiteley et al., 2015, Whiteley et al., 2017, Gundlach et al., 2017b, Gundlach et al., 2017c, Pham et al., 2018, Devaux et al., 2018, Zardenella et al., 2018). We have previously shown that in S. aureus inactivation of the main glycine betaine transporter
OpuD bypasses the requirement of c-di-AMP for the growth of *S. aureus* in rich medium (Zeden *et al.*, 2018). Using bacterial cell size as a proxy for the osmotic balance of cells, we show here that inactivation of OpuD likely helps an c-di-AMP null strain survive by allowing bacteria to re-establish their osmotic balance, as *dacA/opuD* mutant bacteria, which cannot produce c-di-AMP but are also lacking the main glycine betaine transport, are similar in size to WT bacteria (Fig. 1). Here it is interesting to note that while the carnitine osmolyte transporter OpuCA has been shown to be a direct target of c-di-AMP in both *S. aureus* and *L. monocytogenes* (Huynh *et al.*, 2016, Schuster *et al.*, 2016), no direct interaction between OpuD or any other glycine-betaine transporter and c-di-AMP has been reported for *S. aureus*. Hence, it remains unclear if glycine betaine osmolyte transport is directly regulated by c-di-AMP in *S. aureus* and hence the absence of c-di-AMP leads to an excess in glycine-betaine uptake and therefore inactivation of OpuD prevents such excess in uptake. Or alternatively, only potassium and carnitine uptake might be increased in the absence of c-di-AMP since transporters of these molecules are direct targets of c-di-AMP, and glycine betaine uptake remains unaffected but becomes toxic upon accumulation of potassium and carnitine. Therefore, it remains to be determined if the observed increase in cell size in the absence of c-di-AMP is solely caused by an increase in potassium transport by the Ktr, Kpd and potentially KimA systems and carnitine osmolyte transport by OpuCA or if also glycine betaine transport is directly affected by cellular c-di-AMP levels in *S. aureus*. A direct role for c-di-AMP in the control of glycine betaine or betaine transporters has been proposed for other bacteria where c-di-AMP binds to the transcriptional regulator BusR, which controls the expression of the predicted glycine betaine or betaine transporter BusAB (Devaux *et al.*, 2018, Pham *et al.*, 2018). However, such a system does not appear to be present in *S. aureus*.

Bacteria of the *dacA/alsT* suppressor strain, which survive in the absence of c-di-AMP, remained enlarged, indicating that the essentiality of c-di-AMP is bypassed in this strain through a different mechanism. Here, we show that AlsT is the main glutamine transporter in *S. aureus* (Fig. 3). Glutamine as well as proline accumulate under NaCl stress.
conditions in *S. aureus*, indicating that glutamine also plays an important role in osmotic regulation (Anderson & Witter, 1982). However, under the osmotic stress conditions tested in this previous study, glutamine accumulation was proposed to be due to synthesis rather than uptake (Anderson & Witter, 1982). In terms of other functions of the glutamine transporter AlsT in *S. aureus*; it is interesting to note that in a recent study investigating genetic determinants required for eDNA during biofilm formation, it was found that inactivation of GdpP as well as AlsT results in a significant decrease in eDNA release (DeFrancesco *et al.*, 2017). Since we show here that in an *alsT* mutant, which is unable to import glutamine, cellular c-di-AMP levels can be significantly higher as compared to a WT strain (Fig. 7), similar to a *gdpP* mutant, this could mean that the underlying mechanistic bases for the decrease in eDNA release observed for the *gdpP* and *alsT* mutant strains might be related.

There are several (not mutually exclusive) possibilities how preventing glutamine uptake could rescue the growth of a c-di-AMP null strain (Fig. 8). The cellular glutamine/glutamate ratio serves as a key indicator of nitrogen availability in bacterial cells with a high glutamine/glutamate ratio indicating nitrogen availability (Forchhammer, 2007). The cellular glutamate concentration is usually higher than the glutamine concentration and excess glutamine can be readily converted to glutamate via the GOGAT pathway by the glutamine oxoglutarate aminotransferase composed in *S. aureus* of the GltB and GltD proteins subunits (Gunka & Commichau, 2012). Hence, glutamine uptake and its availability in the cell will provide a flux towards glutamate synthesis and glutamate is the counterion of potassium in the cell. Therefore, glutamine uptake and its conversion to glutamate could indirectly facilitate further potassium uptake, which becomes toxic in a c-di-AMP null strain, in which potassium influx is already increased. As a result, reduction in glutamine uptake, as observed in the *alsT* mutant, and preventing its flux to glutamate, could also prevent the intoxication of cells with potassium.

As stated above, a high glutamine/glutamate ratio indicates nitrogen availability and in the absence of other limitations, this will boost the general metabolism of bacterial cells...
In a study on *L. monocytogenes*, an increased flux of pyruvate into the TCA cycle has been described for bacteria unable to produce c-di-AMP (Sureka *et al.*, 2014). As a consequence of this increased TCA cycle activity, an accumulation of citrate and increased carbon flux into glutamine and glutamate was observed (Sureka *et al.*, 2014). Of note, the authors did not distinguish between glutamine and glutamate in this study (Sureka *et al.*, 2014). This provided experimental evidence that a decrease in c-di-AMP levels leads to increased TCA cycle activity and accumulation of cellular metabolites such as citrate and glutamine/glutamate. The essentiality of c-di-AMP in *L. monocytogenes* could be reversed by mutating *citZ*, coding for the citrate synthase, which prevented the accumulation of high levels of citrate as well as glutamine and glutamate pool (again analyzed as a combined pool) in bacterial cells (Sureka *et al.*, 2014). Perhaps similar to the observations in *L. monocytogenes*, the absence of c-di-AMP could also boost the metabolism and potentially TCA cycle activity in *S. aureus*. As part of this study, we provide evidence that in *S. aureus* glutamine is preferred over glutamate for growth in CDM lacking ammonium as nitrogen source. The growth of an *S. aureus* strain in this glucose-containing but ammonium free medium was improved by the addition of glutamine but not glutamate and the uptake of glutamine mediated by AlsT was required for this growth improvement (Fig. 6). Hence, the lack of c-di-AMP combined with glutamine uptake could fuel the bacterial metabolism and the resulting metabolic imbalance might become toxic to the cell, similar as observed for *L. monocytogenes* (Sureka *et al.*, 2014, Whiteley *et al.*, 2017). This futile cycle might be blocked by preventing glutamine uptake and reducing the metabolic activity of cells.

The actual stimuli and underlying molecular mechanisms that regulate c-di-AMP production in bacterial cells are at the moment poorly understood. As part of this study, we show that glutamine uptake negatively impacts c-di-AMP production in *S. aureus* and bacteria grown in medium lacking glutamine or inactivated for the main glutamine transporter AlsT have significantly increased cellular c-di-AMP levels (Fig. 7B and 7C). The increase in c-di-AMP production in the absence of glutamine uptake is likely achieved by activation of the c-di-AMP cyclase DacA and not inhibition of the c-di-AMP phosphodiesterase GdpP, as
an increase in cellular c-di-AMP levels was also detected in a gdpP mutant strain when grown in medium lacking glutamine (Fig. 7A). Current evidence suggests that the activity of DacA can be regulated through the interaction with two proteins: the membrane anchored regulator protein YbbR (also name CdaR in other bacteria) and the phosphoglucomutase enzyme GlmM (Tosi et al., 2019, Zhu et al., 2016, Gundlach et al., 2015b, Pham et al., 2016) (see Model Fig. 8). YbbR and GlmM are encoded in the same operon with DacA. GlmM converts glucosamine-6-P to glucosamine-1-P, an essential precursor for UDP-GlcNAc production and hence peptidoglycan synthesis. GlmM can block the c-di-AMP cyclase activity of DacA through a direct interaction (Tosi et al., 2019, Zhu et al., 2016).

Glutamine is a key precursor for the production of the GlmM substrate glucosamine-6-P since it and fructose-6-P are converted by GlmS to glutamate and glucosamine-6-P. Therefore, the cellular glutamine levels will impact GlmS activity and hence also the availability of the GlmM substrate, which could in turn impact the ability of GlmM to interact with DacA. Based on our findings that c-di-AMP levels are increased in the absence of glutamine in the medium or in the alsT mutant would suggest that low glutamine levels would prevent an interaction between GlmM and DacA, resulting in an increase in c-di-AMP production. Vice versa, high glutamine level could stimulate the interaction between GlmM and DacA and in this manner reduce c-di-AMP production and perhaps stimulate the bacterial metabolism (Fig. 8). In other bacteria it has been reported that YbbR can, depending on the growth conditions, either act as an activator or repressor of DacA (Mehne et al., 2013, Rismondo et al., 2016). It is thought that this regulation is achieved through a direct interaction between the membrane spanning helix of YbbR and the transmembrane domain in DacA (Gundlach et al., 2015b). YbbR has an extracellular sensor domain, which could respond to changes in the peptidoglycan structure (e.g. caused by changes in glutamine levels and flux of precursors towards peptidoglycan synthesis) or alternatively biophysical changes in the membrane bilayer depending on osmotic status and/or nitrogen availability. In this manner the availability of glutamine and resulting cellular changes could also be sensed by YbbR and transduced to DacA (Fig. 8).
Taken together, with this work, we provide a further connection between the c-di-AMP signalling network and osmotic regulation in *S. aureus* but also to central nitrogen metabolism. It will be interesting to determine in future studies the mechanistic bases for the observed changes in cellular c-di-AMP levels depending on glutamine uptake and the involvement of GlmM and YbbR in this process.

Experimental Procedures

Bacterial strains and culture conditions

Bacterial strains used in this study are listed in Table 1. *S. aureus* strains were grown in Tryptic Soy Broth (TSB), Tryptic Soy Agar (TSA) or Chemically Defined Medium (CDM). CDM was prepared as described previously (Zeden et al., 2018). Where indicated, certain amino acids were removed from the CDM recipe during uptake assays and when needed the TSB was supplemented with 0.4 M NaCl. An ammonium free chemically defined medium, CDM+Glu, was prepared the same way as CDM containing the standard glutamine concentration of 100 mg/L but lacking ammonium sulphate and CDM+Gln was prepared the same way as CDM+Glu but replacing the glutamate with 100 mg/L glutamine. *Escherichia coli* strains were grown in Lysogeny Broth (LB). Where appropriate, antibiotics and/or inducers were added to the media at the following concentration: 200 ng/ml anhydrotetracycline (Atet), 90 µg/ml Kanamycin (Kan), 10 µg/ml Erythromycin (Erm), 7.5 or 10 µg/ml Chloramphenicol (Cam), Ampicillin (Amp) 100 µg/ml.

Bacterial strain construction

All strains used in this study are listed in Table 1 and primers used in this study are listed in Table 2. The transposon insertion sites in the Nebraska transposon mutant library (NTML) strains (Fey et al., 2013) used as part of this study were confirmed by PCR and sequencing. The transposon and surrounding region were also moved by phage transduction using phage 85 into the *S. aureus* LAC* strain background. This resulted in the generation of *S. aureus* strains LAC*alsT::tn* (SAUSA300_1252::tn; ANG4803), LAC*0914::tn*
SAUSA300_0914::tn; ANG5141), LAC*glnQ::tn (SAUSA300_0914::tn; ANG5070), LAC*gltT::tn (SAUSA300_1252::tn; ANG5366) and LAC*gltS::tn (SAUSA300_2291::tn; ANG5367). The transposon insertion in the respective gene was again confirmed by PCR and sequencing. For complementation analysis, the Atet inducible single copy integration plasmids piTET-alsT and piTET-gltS were constructed. To this end, alsT (SAUSA300_1252) and gltS (SAUSA300_2291) were amplified using LAC* chromosomal DNA and primers ANG2250/ANG2251 and ANG3209/ANG3210, respectively. The products as well as piTET were digested with AvrII and SacII and then ligated. Plasmid piTET-alsT was recovered in E. coli strain XL1-Blue (yielding strain ANG3937), shuttled through E. coli strain IM08B (strain ANG3955) and then introduced into LAC*alsT::tn (ANG4803), yielding strain LAC*alsT::tn piTET-alsT (ANG4854). As a control, plasmid piTET was also introduced into LAC*alsT::tn (ANG4803) yielding strain LAC*alsT::tn piTET (ANG4853). Plasmid piTET-gltS was transformed into E. coli XL1-Blue (yielding strain ANG5494), shuttled through E. coli IM08B (yielding strain ANG5495) and transformed into LAC*gltS::tn, yielding the complement strain LAC* gltS::tn piTET-gltS (ANG5493). As a control, the piTET plasmid was transformed into LAC* gltS:tn strain, yielding the strain LAC* gltS:tn piTET (ANG5492). Correct plasmid integration into the geh locus was confirmed by PCR and the sequences of all plasmid inserts were confirmed by fluorescent automated sequencing.

Bacterial growth curves and amino acid analysis in culture supernatants

S. aureus strains LAC* and LAC*alsT::tn were grown overnight in TSB supplemented with 10 µg/ml erythromycin where appropriate. Overnight cultures were then diluted to an OD$_{600}$ of 0.01 into 50 ml of fresh TSB. Cultures were incubated at 37°C with aeration, and OD$_{600}$ values determined every hour. The experiment was performed with three biological replicates and the average OD$_{600}$ values and standard deviations were plotted. Using the same cultures, supernatant samples were prepared at time 0, 6, 10 and 12 h after growth and amino acid levels determined as previously described using an amino acid analyser (Halsey et al., 2017). For measuring the growth of S. aureus strains LAC*, LAC* piTET,
LAC*alsT::tn, LAC*alsT::tn piTET, LAC*alsT::tn piTET-alsT, LAC*gltS::tn in CDM+Gln and CDM+Glu, the bacteria were grown overnight in TSB medium supplemented with chloramphenicol and erythromycin where appropriate. Next day, bacteria from a 1 ml aliquot were washed twice in PBS and diluted to OD$_{600}$ of 0.005 in either CDM-Glu or CDM+Gln supplemented with 200 ng/ml Atet were indicated. One hundred µl of the diluted cultures (6 technical replicates) were transferred into well of a 96-well plate and the plate was then incubated with shaking (500 rpm) in a plate reader and OD$_{600}$ measured every 30 min. The experiment was performed with three biological replicates and one representative graph is shown.

**Microscopic analysis and cell size measurements**

Microscopic analysis to determine bacterial cell sizes was performed essentially as previously described (Zeden et al., 2018). Briefly, *S. aureus* strains LAC*, LAC*dacA::kan*, LAC*dacAG206s*, LAC*dacA/opuD* (ANG3835) and LAC*dacA/alsT* (ANG3838) were grown overnight at 37°C in TSB or TSB supplemented with 0.4 M NaCl where stated. Next day, the cultures were diluted to an OD$_{600}$ of 0.01 and grown for 3 hours at 37°C. 100 µl of these cultures were then stained for 20 min at 37°C with Vancomycin-BODIPY FL at a final concentration of 2 µg/ml. 1.5 µl of each sample was spotted onto a thin 1.5% agarose gel patch prepared in H$_2$O or in 0.4 M NaCl and the bacteria subsequently imaged at 1000 x magnification using an Axio Imager A2 Zeiss microscope equipped with a GFP filter set. Images were acquired using the ZEN 2012 (blue edition) software. The bacterial cell diameters were determined using the Fiji software. Only non-dividing cells (cells without any obvious fluorescent dots or lines at the mid-cell), were used for cell diameter measurements. The cell diameters of 50 cells were measured and the average cell diameter determined. The experiment was conducted with three or four biological replicates (as indicated in the figure legend) and the averages and standard deviations of the average cell diameters plotted.
Uptake assays using $^{14}$C-labelled amino acids

Uptake assays were conducted as previously described with some minor modifications (Zeden et al., 2018). Briefly, *S. aureus* strains were streaked on TSA or TSA 0.4 M NaCl plates with appropriate antibiotics and the plates incubated overnight at 37°C. Bacteria were subsequently scraped off from the plates and suspended in 1 ml PBS pH 7.4 buffer and the OD$_{600}$ determined. Fifty ml of CDM (where indicated with 200 ng/ml of the inducer Atet added) was inoculated with the appropriate bacterial suspensions to an OD$_{600}$ of 0.05. The cultures were grown at 37°C to an OD$_{600}$ between 0.4 and 0.9 and then bacteria from an OD$_{600}$ equivalent of 8 were harvested by centrifugation for 10 min at 19,000 x g at RT. Supernatants were discarded and the bacterial pellets were suspended in 2 ml of CDM without glutamate (for glutamine and glutamate uptake assays), CDM without serine (for serine uptake assays). The OD$_{600}$ of the cell suspensions were measured and the cells diluted to an OD$_{600}$ of approximately 1. The OD$_{600}$ was re-measured and this measurement used for normalization purposes. Five hundred and fifty μl of these cell suspensions were aliquoted into 50 ml conical tubes and 100 μl used to measure the background radiation, by filtering the cells onto a nitrocellulose membrane filter, followed by a wash step with 16 ml PBS. Then, 6.2 μl of Glutamine, L-[14C(U)] (Hartmann Analytic, MC1124), Glutamic acid, L-[14C(U)] (Hartmann Analytic, MC156), or serine L-[14C(U)] (Hartmann Analytic, MC265) was added to the remaining 450 μl sample. Hundred μl aliquots were filtered at 0, 3, 6 and 9 minutes and the filters were then washed with 2 x 16 ml of PBS pH 7.4. The filters were subsequently dissolved in 9 ml of scintillation cocktail Filter Count (Perkin Elmer) and the radioactivity measured in counts per minute (CPM) using a Wallac 1409 DSA liquid scintillation counter. CPM for each sample were then normalized by the OD$_{600}$ of the final cell suspension and the means and standard deviations of the CPM/ml OD$_{600}$ = 1 of three or four (as indicated in the figure legends) independent experiments were plotted.
Determination of cellular c-di-AMP levels by competitive ELISA.

Intracellular c-di-AMP levels in WT LAC* and the indicated S. aureus mutant strains were determined using a previously described competitive ELISA method (Underwood et al., 2014) and a slightly modified method for the preparation of S. aureus samples (Bowman et al., 2016). Briefly, a single colony of the WT and different S. aureus mutant strains were picked from TSA plates and used to inoculate 5 ml of either CDM+Gln or CDM+Glu and the cultures were incubated for 18 h at 37°C with shaking. Next, bacteria from 4.5 ml culture were collected by centrifugation, washed three times with PBS and subsequently suspended in 0.75 to 1 ml 50 mM Tris pH 8 buffer supplemented with 20 ng/ml lysostaphin and the cells were lysed by bead beating. The lysates were cleared by centrifugation for 5 min at 17,000 x g and the supernatant transferred to a new tube. A small sample aliquot was removed, and the protein concentration determined using a Pierce BCA protein assay kit for normalization purposes (Thermo Scientific, Waltham, MA, USA). The remainder of the sample was heated to 95°C for 10 min. For the ELISA assay, the samples were diluted to a protein concentration of 100, 200, 400 or 500 μg/ml as, appropriate. ELISA plates were prepared by adding 100 μl of coating buffer (50 mM Na₂CO₃, 50 mM NaHCO₃, pH 9.6) containing 10 μg/ml of the c-di-AMP binding CpaA_SP protein to each well of a NUNC MaxiSorp 96 well plate (Thermo Scientific, Waltham, MA, USA) and the plate was incubated for approximately 18 h at 4°C. Next, the plate was washed three times with 200 μl PBST pH 7.4 (10 mM Na₂HPO₄, 1.8 mM KH₂PO₄ 137 mM NaCl, 2.7 mM KCl, 0.05% (v/v) Tween 20), blocked for 1 h at 18°C with 150 μl blocking solution (1% BSA in PBS pH 7.4) and washed three times with 200 μl PBST. Fifty μl of the samples (three biological replicates and three technical replicates) or standards (two technical replicates) were mixed with 50 μl of a 50 nM biotinylated c-di-AMP solution prepared in 50 mM Tris pH 8 buffer. For the standard curve, c-di-AMP standards were prepared in 50 mM Tris pH 8 buffer at concentrations of 0, 12.5, 25, 37.5, 50, 75, 100, 200 nM. Following the addition of the samples and the standards, the plate was incubated for 2 h at 18°C and then washed three times with PBST. Next, 100 μl of a high sensitivity streptavidin-HRP solution (Thermo Scientific, Waltham, MA, USA) diluted 1:500 in PBS was
added to each well and the plate was incubated for 1 h at 18°C. The plate was washed again 3 x with 200 μl PBST and 100 μl of a developing solution (0.103 M NaHPO₄, 0.0485 M citric acid, 500 mg/l o-phenylenediamine dihydrochloride, 0.03% H₂O₂) was added to each well and the plate incubated for 15 min at 18°C. The reaction was then stopped by adding 100 μl of 2 M H₂SO₄ solution. The absorbance was measured in a plate reader at a wavelength of 490 nm and c-di-AMP concentrations were calculated as ng c-di-AMP / mg protein.

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Author contribution: MSZ, IK and AG design the study, MSZ and IK acquired the data, MSZ, IK, VTC, PF and AG, designed experiments, analyzed and interpreted the data, MSZ, IK and AG prepared the figures and writing of the manuscript. All authors approved the final version of the manuscript.
Abbreviated Summary:

A large number of amino acid transporters and oligopeptide permeases are encoded in bacterial genomes. However, their actual substrate specificity and functions are hard to predict bioinformatically. In this study, we report that GltS and AlsT are main glutamate and glutamine transporters in Staphylococcus aureus, respectively and show that glutamine uptake inhibits the production of the nucleotide signalling molecule c-di-AMP.
Table 1: Bacterial strains used in this study

| Unique ID | Strain name and resistance | Source |
|-----------|-----------------------------|--------|
| ANG284    | XL1-Blue piTET; AmpR        | (Gründling & Schneewind, 2007) |
| ANG2154   | DH10B pIMAY; CamR           | (Monk et al., 2012) |
| ANG3724   | IM08B                       | (Monk et al., 2015) |
| ANG3928   | IM08B piTET; AmpR           | (Zeden et al., 2018) |
| ANG3937   | XL1-Blue piTET-alsT; AmpR   | This study |
| ANG3955   | IM08B piTET-alsT; AmpR      | This study |
| ANG5494   | XL1-Blue piTET-gltS; AmpR   | This study |
| ANG5495   | IM08B piTET-gltS; AmpR      | This study |
| ANG2452   | xl1-Blue piTET-gltS; AmpR   | This study |
| ANG2453   | IM08B piTET-gltS; AmpR      | This study |

**Escherichia coli strains**

| Unique ID | Strain name and resistance | Source |
|-----------|-----------------------------|--------|
| ANG284    | XL1-Blue piTET; AmpR        | (Gründling & Schneewind, 2007) |
| ANG2154   | DH10B pIMAY; CamR           | (Monk et al., 2012) |
| ANG3724   | IM08B                       | (Monk et al., 2015) |
| ANG3928   | IM08B piTET; AmpR           | (Zeden et al., 2018) |
| ANG3937   | XL1-Blue piTET-alsT; AmpR   | This study |
| ANG3955   | IM08B piTET-alsT; AmpR      | This study |
| ANG5494   | XL1-Blue piTET-gltS; AmpR   | This study |
| ANG5495   | IM08B piTET-gltS; AmpR      | This study |

**Staphylococcus aureus strains**

| Unique ID | Strain name and resistance | Source |
|-----------|-----------------------------|--------|
| AH1263    | LAC* Erm sensitive CA-MRSA USA300 strain (ANG1575) | (Boles et al., 2010) |
| ANG1961   | LAC*gdpP::kan; KanR         | (Corrigan et al., 2011) |
| ANG3664   | LAC*dacA6206s; KanR         | (Bowman et al., 2016) |
| ANG3666   | LAC*dacA::kan (dacA) KanR   | (Zeden et al., 2018) |
| ANG3835   | LAC*dacA::kan-S7 (LAC*dacA/opuD); KanR | (Zeden et al., 2018) |
| ANG3838   | LAC*dacA::kan-S10 (LAC*dacA/alsT); KanR | (Zeden et al., 2018) |
| ANG3940   | NE142 (alsT::tn) – NMTN strain | (Fey et al., 2013) |
| ANG4054   | LAC* piTET; CamR            | (Zeden et al., 2018) |
| ANG4803   | LAC*alsT::tn; ErmR          | This study |
| ANG4854   | LAC*alsT::tn piTET-alsT ; ErmR CamR | This study |
| ANG4855   | LAC*alsT::tn piTE ; ErmR CamR | This study |
| ANG4968   | NE1463 (JE2 SAUSA300_0914::tn) – NMTN strain | (Fey et al., 2013) |
| ANG5070   | NE153 (JE2 glnQ::tn) – NMTN strain | (Fey et al., 2013) |
| ANG5141   | LAC*0914::tn; ErmR          | This study |
| ANG5242   | LAC*glnQ::tn; ErmR          | This study |
| ANG5309   | NE566 (JE2 gltT::tn) – NMTN strain | (Fey et al., 2013) |
| ANG5310   | NE560 (JE2 gltS::tn) – NMTN strain | (Fey et al., 2013) |
| ANG5366   | LAC*gltT::tn; ErmR          | This study |
| ANG5367   | LAC*gltS::tn; ErmR          | This study |
| ANG5492   | LAC*gltS::tn piTET; ErmR CamR | This study |
| ANG5493   | LAC*gltS::tn piTET-gltS; ErmR CamR | This study |
Table 2: Cloning primers used in this study

| Primer ID | Name       | Sequence                                      |
|-----------|------------|-----------------------------------------------|
| ANG2250   | 5-AvrII-alsT | AGTCCCTAGGCGGTCTAATTTTATAGAAGG                |
| ANG2251   | 3-SacII-alsT | TCCCCGCGGGGTTTATTTTGATTTTTATATAATGAATCG       |
| ANG3209   | 5-AvrII-gltS | ATACCTAGGAGGGAGAGGGATATTCAACAAGGGGATT         |
| ANG3210   | 3-SacII-gltS | GCCCGCGGTTAACTAAACCATTGTATGAATCCCATAATG       |
**FIG 1: Inactivation of the glycine betaine transporter OpuD rescues the cell size of S. aureus dacA mutant bacteria.** (A, C, E) Microscopy images of S. aureus cells stained with fluorescently labelled vancomycin. Cultures of S. aureus LAC* (WT), LAC*\textit{dacA}\textsubscript{G206S} (\textit{dacA}\textsubscript{G206S}) (panels A and C only), LAC*\textit{dacA}::\textit{kan} (\textit{dacA}) (panel E only) and the suppressor strains LAC*\textit{dacA}/\textit{als} T (\textit{dacA}/\textit{als} T) and LAC*\textit{dacA}/\textit{opuD} (\textit{dacA}/\textit{opuD}) were grown in (A) TSB or (C and E) TSB 0.4 M NaCl medium and subsequently stained with fluorescently labelled vancomycin. The bacteria were then viewed using a fluorescent microscope and representative images are shown. Scale bars are 1 μm. (B, D, F) Bacterial cell diameter measurements. The diameter of non-dividing bacterial cells was measured as described in the Materials and Method section for S. aureus strains grown in (B) TSB or grown in (D and F) TSB 0.4 M NaCl medium. The diameters of 50 cells were determined and the average diameter calculated. The experiment was performed in triplicate (B and F) or quadruplicate (D) and the averages and SD of the average cell diameters plotted. For statistical analysis, one-way ANOVA followed by Dunnett’s multiple comparison tests were performed (ns = not significant, * = p<0.01, ** = p<0.001, *** = p<0.0001).
FIG 2: Amino acid uptake analysis to determine the function of AlsT. (A) Bacterial growth curves. *S. aureus* strains LAC* (WT) and LAC*alsT::tn (alsT::tn) were grown in TSB medium and OD$_{600}$ readings determined at hourly intervals and the average and standard deviations from three biological replicates plotted. (B and C) Quantification of amino acid levels in culture supernatants. Spent medium samples from the cultures shown in panel A were prepared at the 0, 6, 10 and 12 h time points and (B) aspartate and (C) serine levels determined as previously described using an amino acid analyzer (Halsey et al., 2017). The average values and standard deviations from 3 biological replicates were plotted. The plots for all other amino acids measured are shown in Figure S1.
FIG 3: AlsT is a main glutamine transporter in S. aureus. Amino acid uptake assays. (A-C) S. aureus strain LAC* (WT), the alsT mutant LAC*alsT::tn piTET (alsT::tn piTET) and the complementation strain LAC*alsT::tn piTET-alsT (alsT::tn piTET-alsT) were grown to mid-log phase in CDM supplemented with 200 ng/ml Atet for the strains containing the piTET plasmids. Subsequently radiolabelled (A) serine, (B) glutamate or (C) glutamine was added to culture aliquots, samples removed and filtered at the indicated time points and the radioactivity accumulated in the cells measured. The average values and standard deviations from three (A,C,D,F) or four (B,E) experiments were plotted. (D-F) The same uptake assay experiment was performed as described in (A-C) but using S. aureus strains LAC*dacA::kan (dacA) and LAC*dacA/alsT (dacA/alsT). The amino acid uptake curve for the LAC* (WT) strain is the same as shown in panels A-C, as all strains were grown and processed at the same time.
FIG 4: LAC*0914::tn and LAC*glnQ::tn strains do not show a defect in glutamine or glutamate uptake. (A and B) Amino acid uptake assays. S. aureus strains LAC* (WT) and LAC*0914::tn were grown to mid-log phase in CDM. Subsequently radiolabelled (A) glutamine and (B) glutamate was added to culture aliquots, samples removed and filtered at the indicated time points and the radioactivity accumulated in the cells measured. The average values and standard deviations from three experiments were plotted. (C and D) Amino acid uptake assays. Amino acid uptake assays were performed and the data plotted as described in panels A and B, using S. aureus LAC* (WT) and LAC*glnQ::tn (glnQ::tn).
FIG 5: GltS is a main glutamate transporter in *S. aureus*. Amino acid uptake assays. (A and B) *S. aureus* strains LAC* (WT), LAC*<i>gltT::tn</i> and LAC*<i>gltS::tn</i> were grown to mid-log phase in CDM. Subsequently radiolabelled (A) glutamine or (B) glutamate was added to culture aliquots, samples removed and filtered at the indicated time points and the radioactivity accumulated in the cells measured. (C) Same as (B) but using *S. aureus* strains LAC* (WT), LAC*<i>gltS::tn</i> piTET and the complementation strain LAC*<i>gltS::tn</i> piTET-<i>gltS</i> and supplementing the CDM medium with 200 ng/µl Atet. The average values and standard deviations from three experiments were plotted.
Figure 6. Addition of glutamine but not glutamate can stimulate the growth of *S. aureus* in CDM lacking ammonium as nitrogen source. (A) Growth curves of *S. aureus* strains LAC* (WT), LAC*gltS::tn* (*gltS::tn*) and LAC*alsT::tn* (*alsT::tn*). The strains were grown in CDM medium lacking ammonium and containing either glutamine (CDM+Gln) or glutamate (CDM+Glu). OD<sub>600</sub> readings were measured every 30 min. Average and standard deviations of six technical replicates were plotted. This experiment is a representative result of three independent experiments. (B) Same as (A) but using the *S. aureus* strains LAC* (WT), LAC*alsT::tn* piTET and the complementation strain LAC*alsT::tn* piTET-*alsT* grown in CDM+Gln medium supplemented with 200 ng/µl Atet.
Figure 7. Glutamine uptake inhibits c-di-AMP production in S. aureus. (A) S. aureus strains LAC* (WT) and LAC* gdpP::kan (gdpP::kan) were grown in CDM+Gln or CDM+Glu. Cell extracts were prepared, and cellular c-di-AMP levels determined using a competitive ELISA assay. The average values and standard deviation from three biological replicates were determined and c-di-AMP levels plotted as ng c-di-AMP/ mg protein. For statistical analysis f-tests and Student’s t-tests were performed and c-di-AMP levels were found to be statistically significantly different for both WT and the gdpP mutant, when grown in the different media (* = p<0.01 and ** p<0.001). (B) S. aureus strains LAC* (WT), LAC*gltS::tn (gltS::tn) and LAC*alsT::tn (alsT::tn) were grown in CDM+Glu (white columns) or CDM+Gln (grey columns) and c-di-AMP concentrations determined and plotted as described in (A). (C) S. aureus strains LAC* piTET, LAC*alsT::tn piTET and the complementation strain LAC*alsT::tn piTET-alsT were grown in CDM+Gln supplemented with 200 ng/µl Atet and c-di-AMP levels determined as described in (A). For panels B and C, one-way ANOVA followed by Dunnett’s multiple comparison tests were performed to identify statistically significant differences in c-di-AMP levels between WT and the mutant strains (ns = not significant, *** = p<0.0001).
Figure 8. Model of how AlsT-mediated glutamine uptake impacts bacterial physiology and c-di-AMP production. As shown as part of this study, the proteins AlsT (SAUSA300_1252) and GltS (SAUSA300_2291) are the main glutamine (Gln) and glutamate (Glu) transporters in S. aureus, respectively. A high glutamine/glutamate ratio signals nitrogen availability and hence glutamine uptake is thought to stimulate the bacterial metabolism. Glutamine is further converted by the GOGAT enzymes GltB and GltD to glutamate, the cellular counterion of potassium. Glutamine is also an important precursor for the synthesis of the essential peptidoglycan precursor UDP-GlcNAc. Glutamine and fructose-6-P (F6P) are converted by GlmS to glucosamine-6-P (GlcN6P), which is converted by GlmM to glycosamine-1-P (GlcN1P) and then utilized by GlmU for the production of UDP-GlcNAc. GlmM has been shown to directly interact and inhibit the activity of the c-di-AMP cyclase DacA. Based on the data presented in this study, glutamine uptake could potentially stimulate the GlmM/DacA interaction resulting in the observed reduction in c-di-AMP production. Alternatively, a potential increased flow of glutamine into peptidoglycan precursor and actual peptidoglycan synthesis could be sensed by the proposed regulator protein YbbR. Once such a signal is perceived by YbbR, it will be transduced to the c-di-AMP cyclase DacA leading to reduced c-di-AMP production. Reactions predicted to increase upon glutamine update are indicated by green arrows and the reduced c-di-AMP synthesis (potentially mediated by interaction with GlmM or YbbR) is indicated by a red arrow.
FIG S1: Amino acid uptake analysis to determine the function of AlsT. (A-O)

Quantification of amino acid levels in culture supernatants. Spent medium samples from the cultures shown in Fig. 2 panel A were prepared at the 0, 6, 10 and 12 h time points and amino acid levels as indicated above each panel determined as previously described using an amino acid analyzer (Halsey et al., 2017). The average values and standard deviations from 3 independent biological replicates were plotted.
FIG S2: CLUSTAL Omega alignment of \textit{B. subtilis} and \textit{S. aureus} AlsT/GlnT homologs.

The \textit{S. aureus} \textit{AlsT} (SAUSA300_1252) proteins was used as query sequence in a BLAST search to identity homologous proteins encoded in \textit{S. aureus} FPR3757 and \textit{B. subtilis} 168.

One protein SAUSA300_0914 was identified in \textit{S. aureus} and four close homologues AlsT\textit{BS}, GlnT\textit{BS}, YrbD\textit{BS} (e-value: 353 e-117) and \textit{YflA\textit{BS}} (e-value: 237 2e-72) were found in \textit{B. subtilis}. A Clustal-Omaga alignment was performed with the six proteins and is shown.

Using BOXSHADE, identical residues were boxed dark blue and similar residues light blue.
Figure S3. Glutamine uptake inhibits c-di-AMP production in *S. aureus*, but no clear differences are seen upon glutamate uptake. (A and B) *S. aureus* strains LAC* (WT), LAC*gltS::tn* (*gltS::tn*) and LAC*alsT::tn* (*alsT::tn*) were grown in CDM+Glu (white columns) or CDM+Gln (grey columns) and c-di-AMP concentrations determined and plotted as described in Figure 7. The data shown in panels A and B are two additional experimental replicates of the data shown in Fig. 7B. One-way ANOVA tests followed by a Dunnett’s multiple comparison tests were performed to identify statistically significant differences in c-di-AMP levels between WT and the mutant strains (ns = not significant; * = p<0.01; ** = p<0.001; *** = p<0.0001).
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