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

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Keywords: *Staphylococcus aureus*, c-di-AMP, glutamine transporter, AlsT, glutamate transporter, GltS, amino acid transport, OpuD, osmolyte transport

Running title: Regulation of c-di-AMP by glutamine transport

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, S*taphylococcus aureus* can infect various niches, including, but not limited to, blood, kidneys, bones, heart, soft tissue and lungs (Kluytmans *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., 2015, Whiteley *et al.*, 2017). 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

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

113 The counterion of potassium in the cell is glutamate, which at the same time also
114 serves as main nitrogen donor for cellular metabolites and macromolecules. A correlation
115 between cellular levels of c-di-AMP, glutamate and glutamine has been reported for several
116 Firmicutes, including *B. subtilis* and *L. monocytogenes* (Whiteley *et al.*, 2017, Gundlach *et*
117 *al.*, 2015b, Gundlach *et al.*, 2017a, Sureka *et al.*, 2014). A two-fold increase in cellular c-di-
118 AMP levels was observed in *B. subtilis* when bacteria where grown in Spizizen minimal
119 medium with glutamate (Glu) as compared to growth in the same medium with glutamine
120 (Gln) as nitrogen source (Gundlach *et al.*, 2015b). In *L. monocytogenes,* c-di-AMP is a
121 negative regulator of the key TCA cycle enzyme pyruvate carboxylase (Sureka *et al.*, 2014).
122 The depletion of c-di-AMP in the cell resulted in an increased flux of glucose into the
123 production of glutamine and glutamate, likely due to increased pyruvate carboxylase activity
124 and increased flux into the TCA cycle at reduced c-di-AMP levels (Sureka *et al.*, 2014).
125 Interestingly, an *L. monocytogenes dacA* mutant that also lacks *citZ*, which codes for the key
126 TCA cycle enzyme citrate synthase, is viable in rich medium (Sureka *et al.*, 2014, Whiteley
127 *et al.*, 2017). In a *citZ* mutant the TCA cycle is blocked and it was shown that depletion of c-
128 di-AMP in this mutant no longer results in the accumulation of glutamate and glutamine in
129 the cell (Sureka *et al.*, 2014). Therefore, the amino acids glutamine and glutamate not only
130 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*$dacA_{G206S}$) 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**dacA/opuD* and LAC**dacA/alsT* suppressor strain bacteria was compared to that of WT LAC* and the low-level c-di-AMP LAC**dacA*$_{G206S}$ 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*$_{G206S}$ 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*$_{G206S}$ 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

187 constructed by phage transducing the *alsT::tn* region from the Nebraska Transposon Mutant
188 Library (NMTL) strain NE142 (Fey *et al.*, 2013) into the *S. aureus* LAC* strain background.
189 WT LAC* and the *alsT* mutant strain LAC**alsT::tn* showed similar growth rates when grown
190 in TSB medium (Fig. 2A). Next, their ability to take up different amino acids was assessed by
191 determining the levels of the individual amino acids in the culture supernatant at the start of
192 the experiment (T = 0 h) as compared to 6, 10 and 12 h following their growth in TSB
193 medium. While no significant differences were observed for most amino acids (Fig. S1), a
194 slight increase in the utilization of aspartate and a slight decrease in the uptake of serine
195 was observed (Fig. 2B and 2C), suggesting that AlsT could potentially be a serine
196 transporter. To test this, uptake assays were performed with radiolabelled serine using the
197 WT LAC* strain, the *alsT* mutant strain LAC**alsT::tn* piTET as well as the complementation
198 strain LAC**alsT::tn* piTET-*alsT*. However, no significant differences in the uptake rate of
199 serine were observed between the strains (Fig. 3A), indicating that AlsT is not the main
200 serine transporter in *S. aureus*. While slight differences in the amino acid uptake profile were
201 observed between the WT and *alsT* mutant strain, this analysis did not allow us to identify
202 the main substrate for AlsT. However, it is of note that using this method one cannot
203 distinguish between glutamine and glutamate or asparagine and aspartate utilization.
204 Additionally, tryptophan was not measured due to the limitations of the method used.
205
206 **AlsT is the main glutamine transporter in *S. aureus***
207 Next, a more detailed bioinformatics analysis was performed to identify potential AlsT
208 substrates. A BlastP search against the *B. subtilis* 168 genome led to the identification of
209 four close homologs of AlsT (SAUSA300_1252), namely AlsT (e-value: e-166), GlnT (e-
210 value: e-149), YrbD (e-value: e-117) and YflA (e-value: 2e-72). Of note, an AlsT homologue
211 SAUSA300_0914 is also present in the *S. aureus* FPR3757 genome (Fig. S2). While *S.*
212 *aureus* AlsT (SAUSA300_1252) shows the highest similarity to the *B. subtilis* AlsT and GlnT
213 proteins, SAUSA300_0914 has the highest similarity with the *B. subtilis* YrbD protein. AlsT is
214 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 *alsT* 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 *alsT* mutant LAC**alsT::tn* piTET and the complementation strain LAC**alsT::tn* piTET-*alsT*. Uptake of glutamine, but not of glutamate, was severely reduced in the *alsT* mutant when compared to the WT (Fig. 3B). This defect was restored upon expression of *alsT* in the complementation strain (Fig. 3C). To confirm that *alsT* also functions as main glutamine transporter in the LAC**dacA/alsT* suppressor strain, uptake assays were also performed with strain LAC**dacA/alsT* and compared to that of the WT LAC* and LAC**dacA::kan* control strains (Fig. 3D-F). Similar to what was observed for the *alsT* mutant, glutamine uptake was severely attenuated in strain LAC**dacA/alsT* 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

243 genomic region from the NMTL strain NE1463 (Fey *et al.*, 2013) containing a transposon
244 insertion in *SAUSA300_0914* into the *S. aureus* LAC* background. Subsequently uptake of
245 radiolabelled glutamine and glutamate was assessed (Fig. 4A-B). No significant differences
246 in the uptake of these amino acids was observed between WT LAC* and strain
247 LAC**0914::tn*, showing that SAUSA300_0914 does not function as glutamine or glutamate
248 transporter under our assay conditions.
249 AlsT and SAUSA300_0914 are members of the amino acid-sodium symporter family of
250 transporters, which are composed of a single multimembrane spanning protein. Besides this
251 type of transporter, GlnPQ-type ABC transporters play a major role in glutamine and
252 glutamate transport in other bacteria (Schuurman-Wolters & Poolman, 2005). *S. aureus*
253 contains a *glnPQ (SAUSA300_1808 - SAUSA300_1807)* operon with *glnP* coding for a
254 substrate binding domain-permease fusion protein and *glnQ* coding for the cytoplasmic
255 nucleotide-binding ATPase domain. The results from a previous study suggested that this
256 transporter functions as glutamine transporter in *S. aureus*, as a *glnP* mutant was more
257 resistant to the toxic glutamine analogue gamma-L-glutamyl hydrazide (Zhu *et al.*, 2009). To
258 assess the contribution of the GlnPQ transporter to glutamine and glutamate transport in *S.*
259 *aureus* under our assay conditions, strain LAC**glnQ::tn* was generated by transducing the
260 *glnQ::tn* region from the NMTL strain NE153 (Fey *et al.*, 2013) into the LAC* background.
261 The resulting LAC**glnQ::tn* mutant strain displayed no difference in glutamine or glutamate
262 uptake compared to WT LAC* (Fig. 4C-D), indicating that the ABC transporter GlnPQ does
263 not function as a main glutamate or glutamine transporter under our assay conditions.
264
265 **GltS (SAUSA300_2291) is the main glutamate transporter in *S. aureus***
266 Cellular glutamine levels are key in signalling nitrogen availability in bacterial cells (Fisher,
267 1999, Gunka & Commichau, 2012). In addition, differences in cellular c-di-AMP levels were
268 reported in *B. subtilis* depending on the presence of glutamine or glutamate as available
269 nitrogen source. More specifically, an increase in cellular c-di-AMP levels was observed in
270 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/amino 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*.

297 **Glutamine but not glutamate stimulates the growth of *S. aureus* in CDM lacking**
298 **ammonium as nitrogen source.**

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

316

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

318 In a previous study, it has been reported that the presence of glutamine or glutamate in the
319 growth medium can affect cellular c-di-AMP levels in *B. subtilis* and it was proposed that
320 glutamate uptake leads to an activation of c-di-AMP synthesis in this organism (Gundlach *et*
321 *al.*, 2015b). To assess if the presence of glutamine or glutamate would also affect c-di-AMP

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

435 (see Model Fig. 8). In a study on *L. monocytogenes,* an increased flux of pyruvate into the
436 TCA cycle has been described for bacteria unable to produce c-di-AMP (Sureka *et al.*,
437 2014). As a consequence of this increased TCA cycle activity, an accumulation of citrate and
438 increased carbon flux into glutamine and glutamate was observed (Sureka *et al.*, 2014). Of
439 note, the authors did not distinguish between glutamine and glutamate in this study (Sureka
440 *et al.*, 2014). This provided experimental evidence that a decrease in c-di-AMP levels leads
441 to increased TCA cycle activity and accumulation of cellular metabolites such as citrate and
442 glutamine/glutamate. The essentiality of c-di-AMP in *L. monocytogenes* could be reversed
443 by mutating *citZ,* coding for the citrate synthase, which prevented the accumulation of high
444 levels of citrate as well as glutamine and glutamate pool (again analyzed as a combined
445 pool) in bacterial cells (Sureka *et al.*, 2014). Perhaps similar to the observations in *L.*
446 *monocytogenes*, the absence of c-di-AMP could also boost the metabolism and potentially
447 TCA cycle activity in *S. aureus*. As part of this study, we provide evidence that in *S. aureus*
448 glutamine is preferred over glutamate for growth in CDM lacking ammonium as nitrogen
449 source. The growth of an *S. aureus* strain in this glucose-containing but ammonium free
450 medium was improved by the addition of glutamine but not glutamate and the uptake of
451 glutamine mediated by AlsT was required for this growth improvement (Fig. 6). Hence, the
452 lack of c-di-AMP combined with glutamine uptake could fuel the bacterial metabolism and
453 the resulting metabolic imbalance might become toxic to the cell, similar as observed for *L.*
454 *monocytogenes* (Sureka *et al.*, 2014, Whiteley *et al.*, 2017). This futile cycle might be
455 blocked by preventing glutamine uptake and reducing the metabolic activity of cells.

456 The actual stimuli and underlying molecular mechanisms that regulate c-di-AMP
457 production in bacterial cells are at the moment poorly understood. As part of this study, we
458 show that glutamine uptake negatively impacts c-di-AMP production in *S. aureus* and
459 bacteria grown in medium lacking glutamine or inactivated for the main glutamine transporter
460 AlsT have significantly increased cellular c-di-AMP levels (Fig. 7B and 7C). The increase in
461 c-di-AMP production in the absence of glutamine uptake is likely achieved by activation of
462 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 anhydrotetracyline (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*

519 (*SAUSA300_0914::tn*; ANG5141), LAC**glnQ::tn* (*SAUSA300_0914::tn;* ANG5070),
520 LAC**gltT::tn* (*SAUSA300_1252::tn*; ANG5366) and LAC**gltS::tn* (*SAUSA300_2291::tn*;
521 ANG5367). The transposon insertion in the respective gene was again confirmed by PCR
522 and sequencing. For complementation analysis, the Atet inducible single copy integration
523 plasmids piTET-*alsT* and piTET-*gltS* were constructed. To this end, *alsT* (*SAUSA300_1252*)
524 and *gltS* (*SAUSA300_2291*) were amplified using LAC* chromosomal DNA and primers
525 ANG2250/ANG2251 and ANG3209/ANG3210, respectively. The products as well as piTET
526 were digested with AvrII and SacII and then ligated. Plasmid piTET-*alsT* was recovered in *E.*
527 *coli* strain XL1-Blue (yielding strain ANG3937), shuttled through *E. coli* strain IM08B (strain
528 ANG3955) and then introduced into LAC**alsT::tn* (ANG4803), yielding strain LAC**alsT::tn*
529 piTET-*alsT* (ANG4854). As a control, plasmid piTET was also introduced into LAC**alsT::tn*
530 (ANG4803) yielding strain LAC**alsT::tn* piTET (ANG4853). Plasmid piTET-*gltS* was
531 transformed into *E. coli* XL1-Blue (yielding strain ANG5494), shuttled through *E. coli* IM08B
532 (yielding strain ANG5495) and transformed into *LAC* gltS::tn*, yielding the complement strain
533 LAC* *gltS::tn* piTET-*gltS* (ANG5493). As a control, the piTET plasmid was transformed into
534 LAC* *gltS*:tn strain, yielding the strain LAC* *gltS*:tn piTET (ANG5492). Correct plasmid
535 integration into the *geh* locus was confirmed by PCR and the sequences of all plasmid
536 inserts were confirmed by fluorescent automated sequencing.
537
538 **Bacterial growth curves and amino acid analysis in culture supernatants**
539 *S. aureus* strains LAC* and LAC**alsT::tn* were grown overnight in TSB supplemented with
540 10 µg/ml erythromycin where appropriate. Overnight cultures were then diluted to an OD_{600}
541 of 0.01 into 50 ml of fresh TSB. Cultures were incubated at 37°C with aeration, and OD_{600}
542 values determined every hour. The experiment was performed with three biological
543 replicates and the average OD_{600} values and standard deviations were plotted. Using the
544 same cultures, supernatant samples were prepared at time 0, 6, 10 and 12 h after growth
545 and amino acid levels determined as previously described using an amino acid analyser
546 (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**dacA*$_{G206S}$, 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_2O 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.

575 **Uptake assays using ^{14}C-labelled amino acids**

576 Uptake assays were conducted as previously described with some minor modifications
577 (Zeden *et al.*, 2018). Briefly, *S. aureus* strains were streaked on TSA or TSA 0. 4M NaCl
578 plates with appropriate antibiotics and the plates incubated overnight at 37°C. Bacteria were
579 subsequently scraped off from the plates and suspended in 1 ml PBS pH 7.4 buffer and the
580 OD_{600} determined. Fifty ml of CDM (where indicated with 200 ng/ml of the inducer Atet
581 added) was inoculated with the appropriate bacterial suspensions to an OD_{600} of 0.05. The
582 cultures were grown at 37°C to an OD_{600} between 0.4 and 0.9 and then bacteria from an
583 OD_{600} equivalent of 8 were harvested by centrifugation for 10 min at 19,000 x g at RT.
584 Supernatants were discarded and the bacterial pellets were suspended in 2 ml of CDM
585 without glutamate (for glutamine and glutamate uptake assays), CDM without serine (for
586 serine uptake assays). The OD_{600} of the cell suspensions were measured and the cells
587 diluted to an OD_{600} of approximately 1. The OD_{600} was re-measured and this measurement
588 used for normalization purposes. Five hundred and fifty µl of these cell suspensions were
589 aliquoted into 50 ml conical tubes and 100 µl used to measure the background radiation, by
590 filtering the cells onto a nitrocellulose membrane filter, followed by a wash step with 16 ml
591 PBS. Then, 6.2 µl of Glutamine, L-[14C(U)] (Hartmann Analytic, MC1124), Glutamic acid, L-
592 [14C(U)] (Hartmann Analytic, MC156), or serine L-[14C(U)] (Hartmann Analytic, MC265)
593 was added to the remaining 450 µl sample. Hundred µl aliquots were filtered at 0, 3, 6 and 9
594 minutes and the filters were then washed with 2 x 16 ml of PBS pH 7.4. The filters were
595 subsequently dissolved in 9 ml of scintillation cocktail Filter Count (Perkin Elmer) and the
596 radioactivity measured in counts per minute (CPM) using a Wallac 1409 DSA liquid
597 scintillation counter. CPM for each sample were then normalized by the OD_{600} of the final cell
598 suspension and the means and standard deviations of the CPM/ml OD_{600} = 1 of three or four
599 (as indicated in the figure legends) independent experiments were plotted.
600
601
602

Determination of cellular c-di-AMP levels by competitive ELISA.

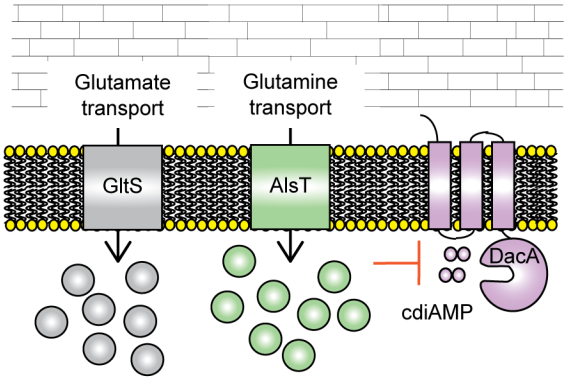
Intracellular c-di-AMP levels in WT LAC* and the indicated *S. aureus* mutant strains were determined using a previously described competitive ELISA method (Underwood *et al.*, 2014) and a slightly modified method for the preparation of *S. aureus* samples (Bowman *et al.*, 2016). Briefly, a single colony 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 Sientific, 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 Na_2C0_3, 50 mM $NaHCO_3$, 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 Sientific, 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 Na_2HPO_4 , 1.8 mM KH_2PO_4 137 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 $NaHPO_4$, 0.0485 M citric acid, 500 mg/l o-phenylenediamine dihydrochloride, 0.03% H_2O_2) 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 H_2SO_4 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.

Acknowledgments: This work was funded by the Wellcome Trust grants 100289/Z/12/Z and 210671/Z/18/Z to AG and the NIH/NIAID grants P01AI083211 and R01AI125588 to P.D.F. MSZ was supported by a Medical Research Council Centre for Molecular Bacteriology and Infection (MRC CMBI) studentship. The funders had no role in the study design, data collection and interpretation, or the decision to submit the work for publication.

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.

651 **Graphical abstract:**
652



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656 **Abbreviated Summary:**

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

662

663 **Table 1:** Bacterial strains used in this study

Unique ID	Strain name and resistance	Source
	***Escherichia coli* strains**	
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	
AH1263	LAC* Erm sensitive CA-MRSA USA300 strain (ANG1575)	(Boles *et al.*, 2010)
ANG1961	LAC**gdpP::kan;* KanR	(Corrigan *et al.*, 2011)
ANG3664	LAC**dacA*$_{G206S}$; 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
ANG4853	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

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665

666 **Table 2:** Cloning primers used in this study

Primer ID	Name	Sequence
ANG2250	5-AvrII-*alsT*	AGTCCCTAGGCGGTCTAATTTTATAGAAGG
ANG2251	3-SacII-*alsT*	TCCCCGCGGGGTTTATTTGATTTTTATATAATGAATCG
ANG3209	5-AvrII-*gltS*	ATACCTAGGAGGGAGAGGGATATTCAACAAGGGGGATTTG
ANG3210	3-SacII-*gltS*	GCCCGCGGTTTAACTAAACCATTGTATGAATCCCATAATG

667

Figures and Legends

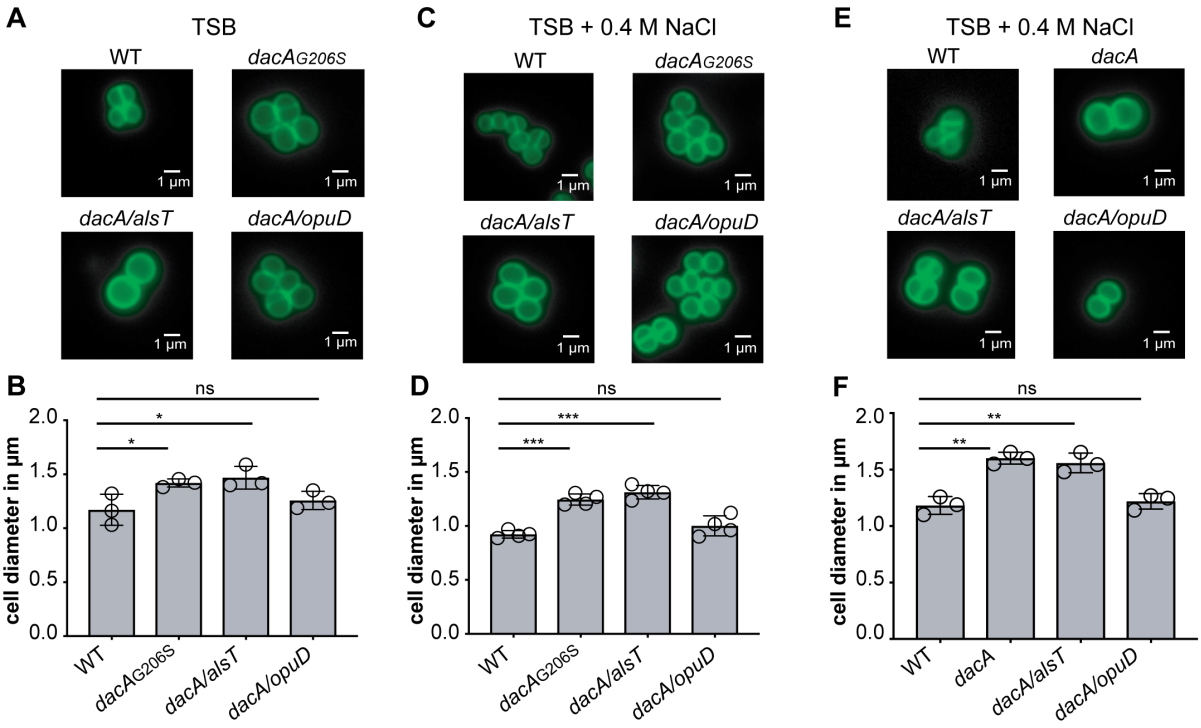


FIG 1: Inactivation of the glycine betaine transporter OpuD rescues the cell size of *S. aureus dacA* mutant bacteria. (A, C, E) Microscopy images of *S. aureus* cells stained with fluorescently labelled vancomycin. Cultures of *S. aureus* LAC* (WT), LAC**dacA*$_{G206S}$ (*dacA*$_{G206S}$) (panels A and C only), LAC**dacA::kan* (*dacA*) (panel E only) and the suppressor strains LAC**dacA/alsT* (*dacA/alsT*) and LAC**dacA/opuD* (*dacA/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).

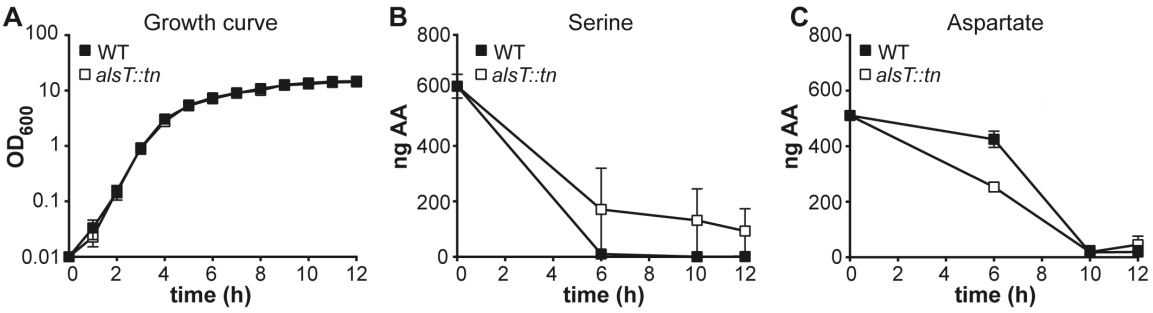


FIG 2: Amino acid uptake analysis to determine the function of AlsT. (A) Bacterial growth curves. *S. aureus* strains LAC* (WT) and LAC**alsT::tn* (*alsT::tn*) were grown in TSB medium and OD_{600} readings determined at hourly intervals and the average and standard deviations from three biological replicates 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.

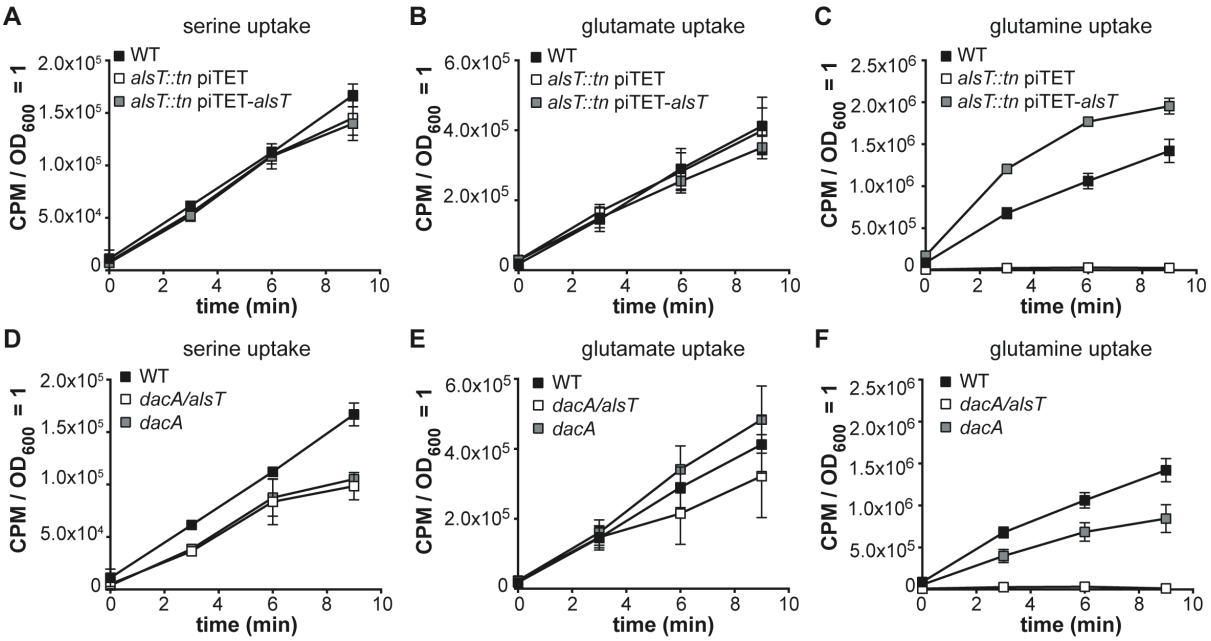


FIG 3: AlsT is a main glutamine transporter in *S. aureus*. Amino acid uptake assays. (A-C) *S. aureus* strain LAC* (WT), the *alsT* mutant LAC**alsT::tn* piTET (*alsT::tn* piTET) and the complementation strain LAC**alsT::tn* piTET-*alsT* (*alsT::tn* piTET-*alsT*) were grown to mid-log phase in CDM supplemented 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.

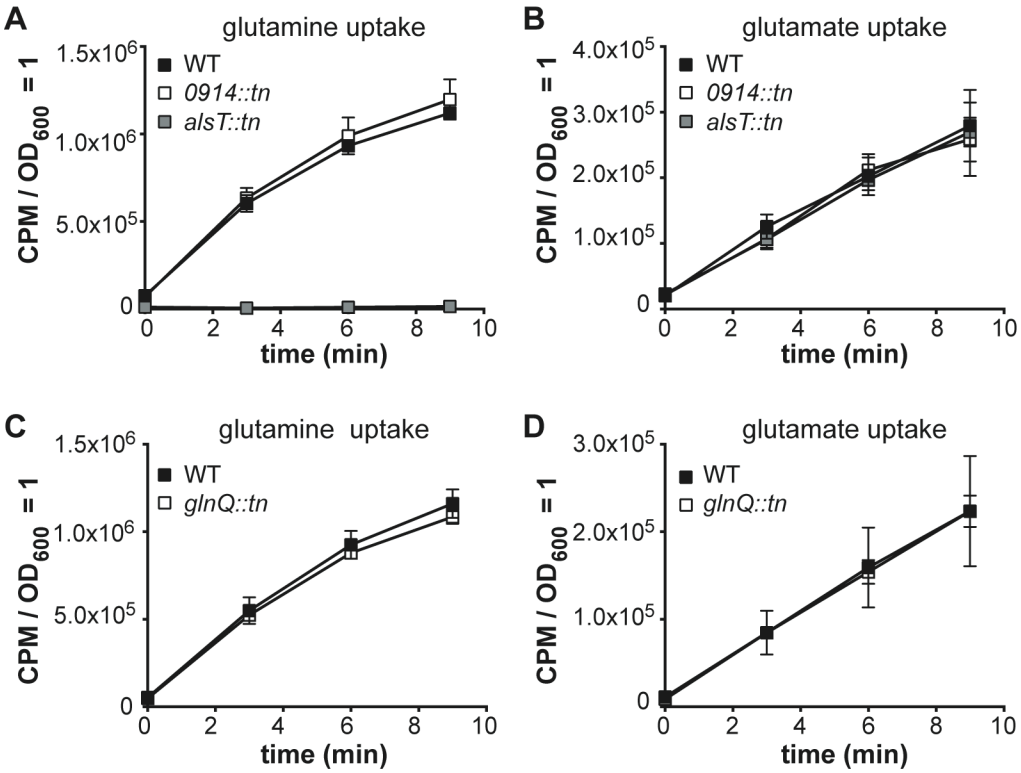


FIG 4: LAC*_0914::tn_ and LAC*_glnQ::tn_ strains do not show a defect in glutamine or glutamate uptake. (A and B) Amino acid uptake assays. *S. aureus* strains LAC* (WT) and LAC*_0914::tn_ were grown to mid-log phase in CDM. Subsequently radiolabelled (A) glutamine and (B) glutamate was added to culture 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*).

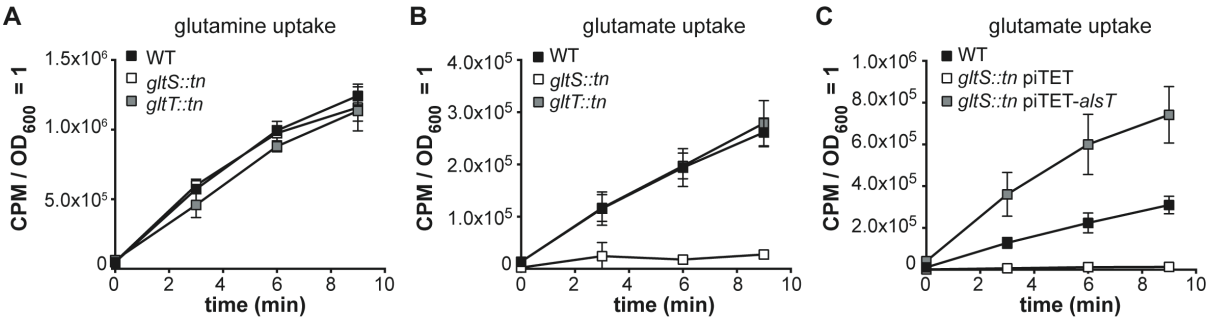


FIG 5: GltS is a main glutamate transporter in *S. aureus*. Amino acid uptake assays. (A and B) *S. aureus* strains LAC* (WT), LAC**gltT::tn* and LAC**gltS::tn* were grown to mid-log phase in CDM. Subsequently radiolabelled (A) glutamine or (B) glutamate was added to culture aliquots, samples removed 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**gltS::tn* piTET and the complementation strain LAC**gltS::tn* piTET-*gltS* and supplementing the CDM medium with 200 ng/µl Atet. The average values and standard deviations from three experiments were plotted.

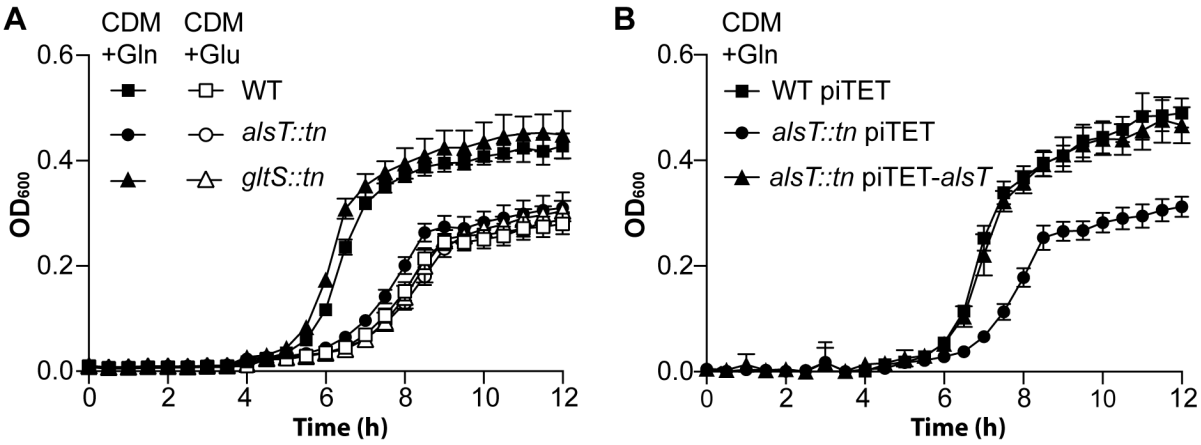
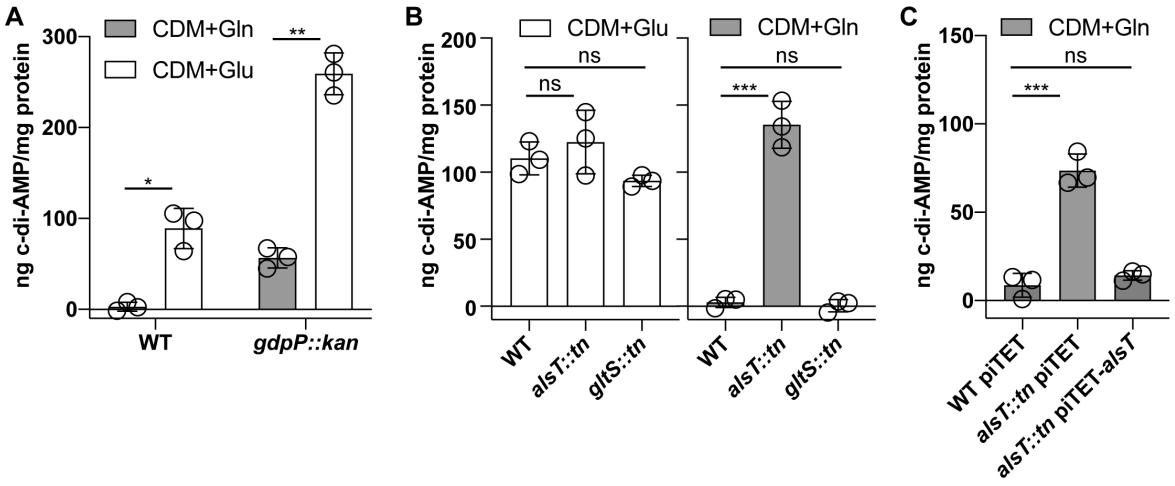


Figure 6. Addition of glutamine but not glutamate can stimulate the growth of *S. aureus* in CDM lacking ammonium as nitrogen source. (A) Growth curves of *S. aureus* strains LAC* (WT), LAC**gltS::tn* (*gltS::tn*) and LAC**alsT::tn* (*alsT::tn*). The strains were grown in CDM medium lacking ammonium and containing either glutamine (CDM+Gln) or glutamate (CDM+Glu). OD_{600} 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.



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750 **Figure 7. Glutamine uptake inhibits c-di-AMP production in *S. aureus*. (A)** *S. aureus*
751 strains LAC* (WT) and LAC* *gdpP*::*kan* (*gdpP::kan*) were grown in CDM+Gln or CDM+Glu.
752 Cell extracts were prepared, and cellular c-di-AMP levels determined using a competitive
753 ELISA assay. The average values and standard deviation from three biological replicates
754 were determined and c-di-AMP levels plotted as ng c-di-AMP/ mg protein. For statistical
755 analysis f-tests and Student's t-tests were performed and c-di-AMP levels were found to be
756 statistically significantly different for both WT and the *gdpP* mutant, when grown in the
757 different media (* = p<0.01 and ** p<0.001). **(B)** *S. aureus* strains LAC* (WT), LAC**gltS::tn*
758 (*gltS::tn*) and LAC**alsT::tn* (*alsT::tn*) were grown in CDM+Glu (white columns) or CDM+Gln
759 (grey columns) and c-di-AMP concentrations determined and plotted as described in (A). **(C)**
760 *S. aureus* strains LAC* piTET, LAC**alsT::tn* piTET and the complementation strain
761 LAC**alsT::tn* piTET-*alsT* were grown in CDM+Gln supplemented with 200 ng/µl Atet and c-
762 di-AMP levels determined as described in (A). For panels B and C, one-way ANOVA
763 followed by Dunnett's multiple comparison tests were performed to identify statistically
764 significant differences in c-di-AMP levels between WT and the mutant strains (ns = not
765 significant, *** = p<0.0001).

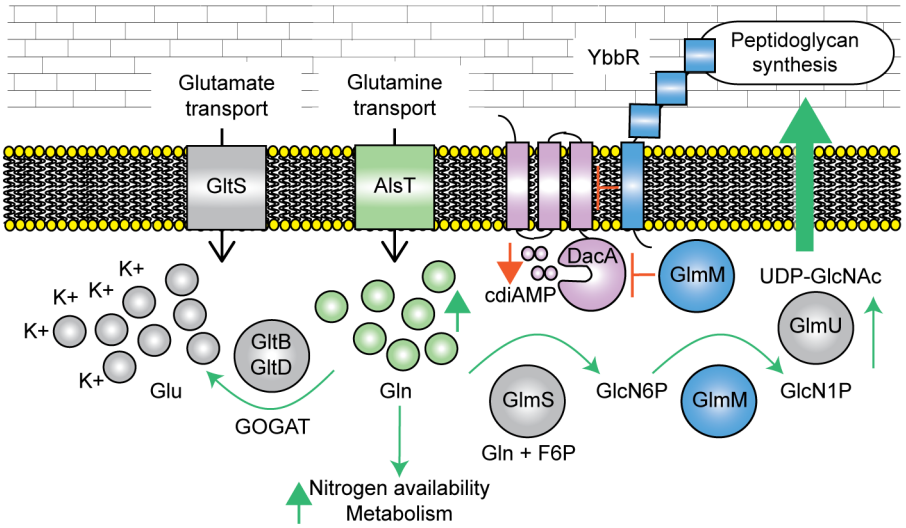

Figure 8. Model of how AlsT-mediated glutamine uptake impacts bacterial physiology and c-di-AMP production. As shown as part of this study, the proteins AlsT (SAUSA300_1252) and GltS (SAUSA300_2291) are the main glutamine (Gln) and glutamate (Glu) transporters in *S. aureus*, respectively. A high glutamine/glutamate 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.

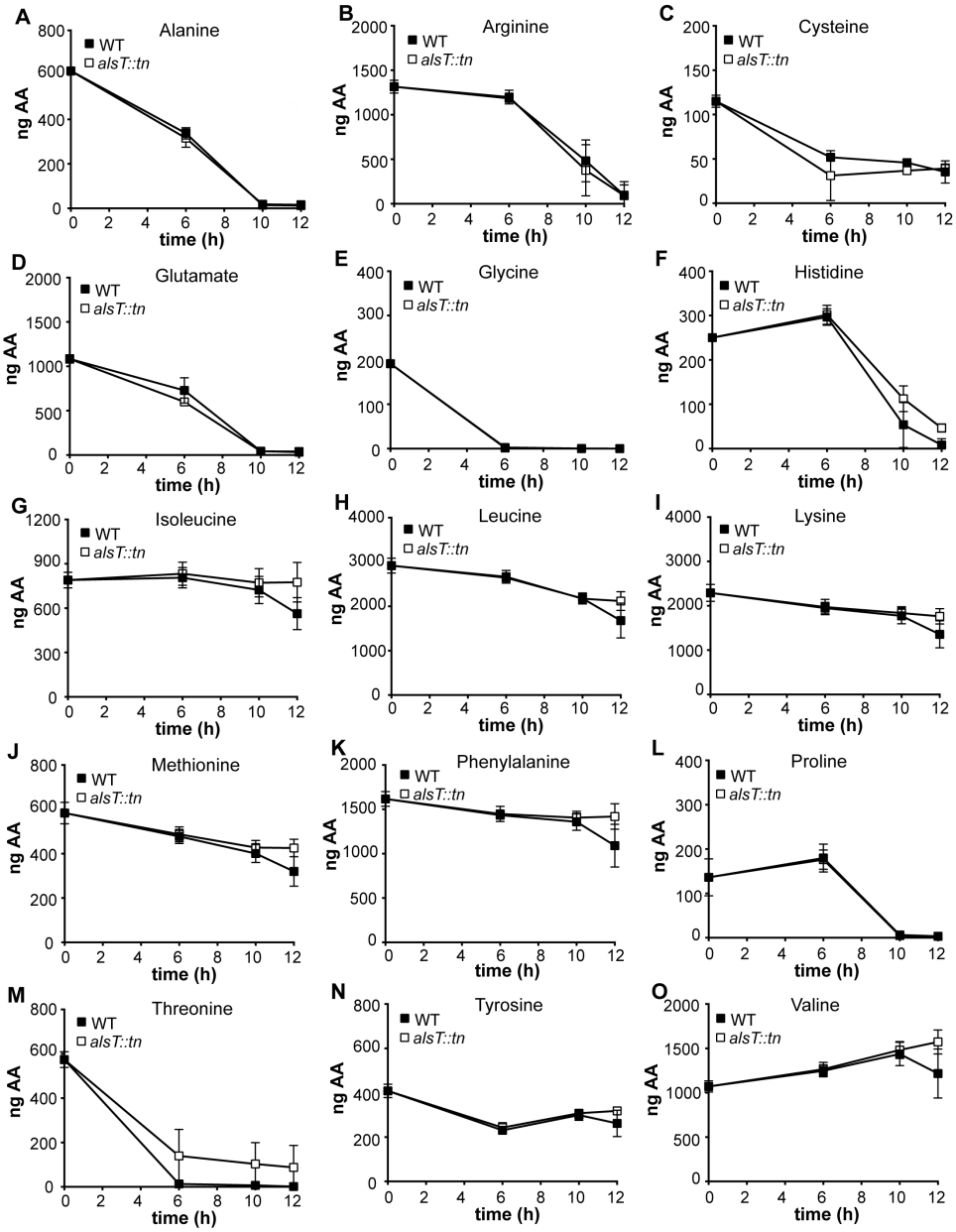

FIG S1: Amino acid uptake analysis to determine the function of AlsT. (A-O) Quantification of amino acid levels in culture supernatants. Spent medium samples from the cultures shown in Fig. 2 panel A were prepared at the 0, 6, 10 and 12 h time points and amino acid levels as indicated above each panel 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.

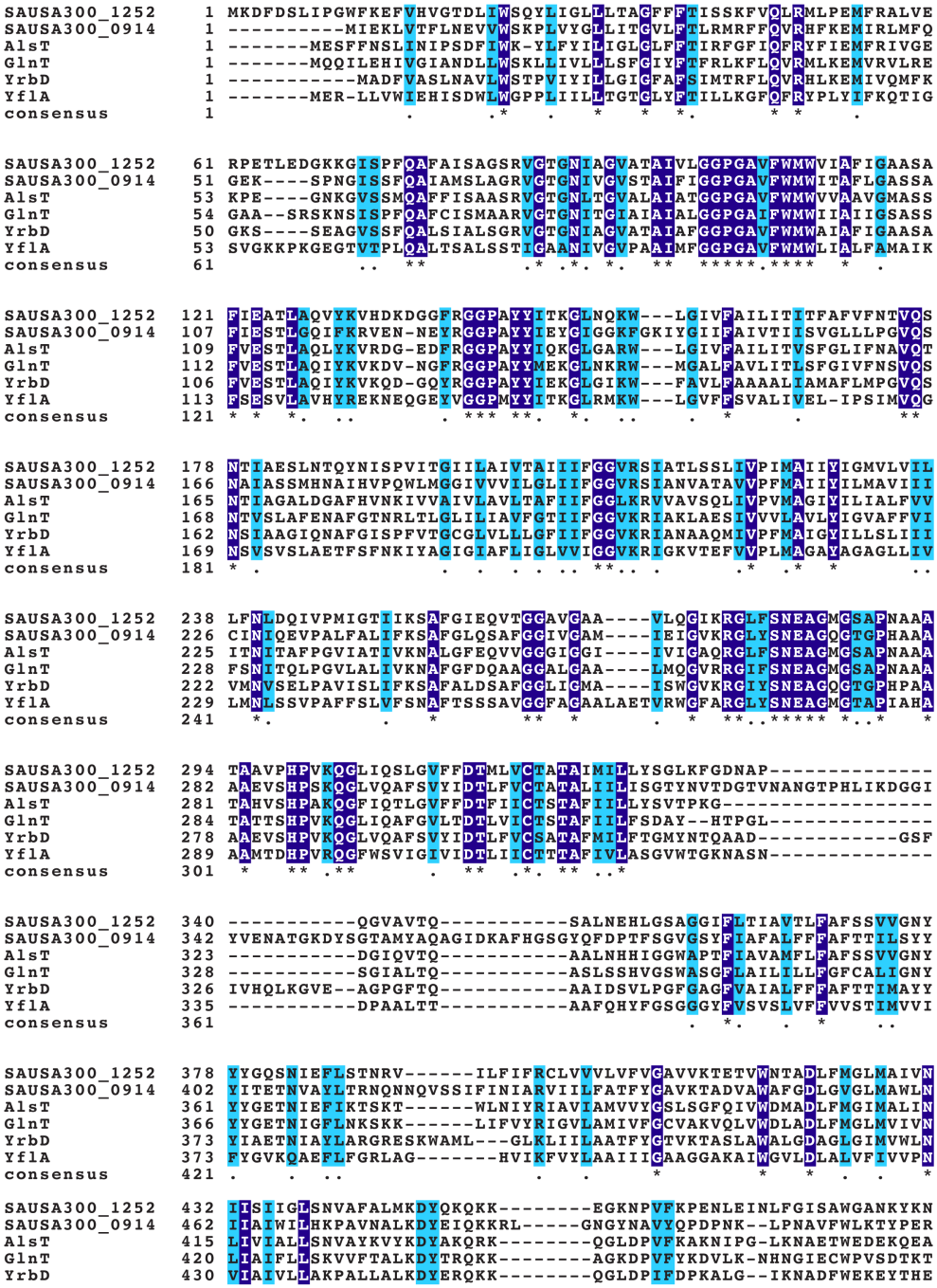

FIG S2: CLUSTAL Omega alignment of *B. subtilis* and *S. aureus* AlsT/GlnT homologs. The *S. aureus* AlsT (SAUSA300_1252) proteins was used as query sequence in a BLAST search to identity homologous proteins encoded in *S. aureus* FPR3757 and *B. subtilis* 168. One protein SAUSA300_0914 was identified in *S. aureus* and four close homologues $AlsT_{BS}$, $GlnT_{BS}$, $YrbD_{BS}$ (e-value: 353 e-117) and $YflA_{BS}$ (e-value: 237 2e-72) were found in *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.

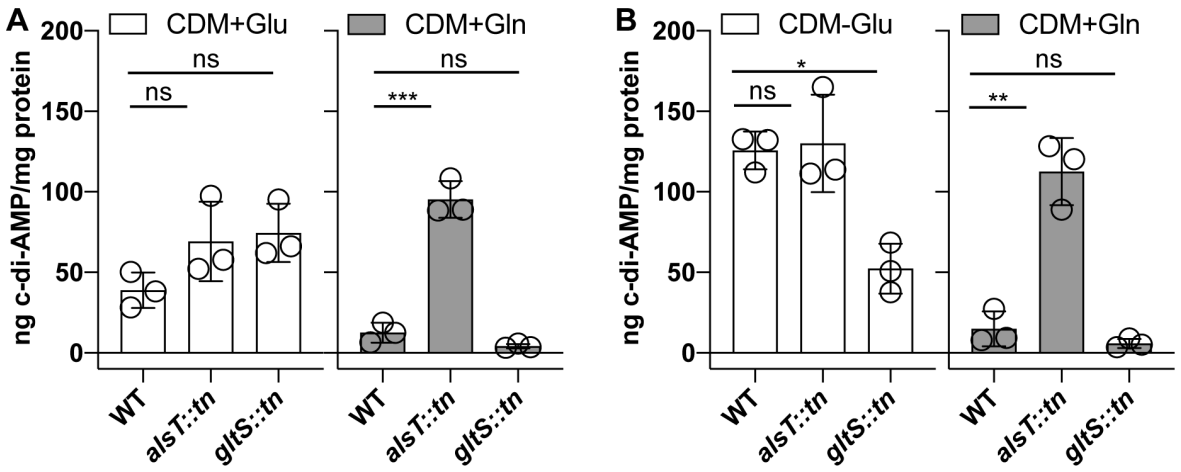

Figure S3. Glutamine uptake inhibits c-di-AMP production in *S. aureus,* but no clear differences are seen upon glutamate uptake. (A and B) *S. aureus* strains LAC* (WT), LAC**gltS::tn* (*gltS::tn*) and LAC**alsT::tn* (*alsT::tn*) were grown in CDM+Glu (white columns) or CDM+Gln (grey columns) and c-di-AMP 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; * = $p<0.01$; ** = $p<0.001$; *** = $p<0.0001$).

References

Anderson, C.B., and Witter, L.D. (1982) Glutamine and proline accumulation by *Staphylococcus aureus* with reduction in water activity. *Appl Environ Microbiol* **43**: 1501-1503.

Bai, Y., Yang, J., Zarrella, T.M., Zhang, Y., Metzger, D.W., and Bai, G. (2014) Cyclic di-AMP impairs potassium uptake mediated by a cyclic di-AMP binding protein in *Streptococcus pneumoniae*. *J Bacteriol* **196**: 614-623.

Boles, B.R., Thoendel, M., Roth, A.J., and Horswill, A.R. (2010) Identification of genes involved in polysaccharide-independent *Staphylococcus aureus* biofilm formation. *PLoS One* **5**: e10146.

Bowman, L., Zeden, M.S., Schuster, C.F., Kaever, V., and Gründling, A. (2016) New Insights into the Cyclic Di-adenosine Monophosphate (c-di-AMP) Degradation Pathway and the Requirement of the Cyclic Dinucleotide for Acid Stress Resistance in *Staphylococcus aureus*. *J Biol Chem* **291**: 26970-26986.

Chin, K.H., Liang, J.M., Yang, J.G., Shih, M.S., Tu, Z.L., Wang, Y.C., Sun, X.H., Hu, N.J., Liang, Z.X., Dow, J.M., Ryan, R.P., and Chou, S.H. (2015) Structural Insights into the Distinct Binding Mode of Cyclic Di-AMP with SaCpaA_RCK. *Biochemistry* **54**: 4936-4951.

Corrigan, R.M., Abbott, J.C., Burhenne, H., Kaever, V., and Gründling, A. (2011) c-di-AMP is a new second messenger in *Staphylococcus aureus* with a role in controlling cell size and envelope stress. *PLoS Pathog* **7**: e1002217.

Corrigan, R.M., Bowman, L., Willis, A.R., Kaever, V., and Gründling, A. (2015) Cross-talk between two nucleotide-signaling pathways in *Staphylococcus aureus*. *J Biol Chem* **290**: 5826-5839.

Corrigan, R.M., Campeotto, I., Jeganathan, T., Roelofs, K.G., Lee, V.T., and Gründling, A. (2013) Systematic identification of conserved bacterial c-di-AMP receptor proteins. *Proc Natl Acad Sci U S A* **110**: 9084-9089.

Crooke, A.K., Fuller, J.R., Obrist, M.W., Tomkovich, S.E., Vitko, N.P., and Richardson, A.R. (2013) CcpA-independent glucose regulation of lactate dehydrogenase 1 in *Staphylococcus aureus*. *PLoS One* **8**: e54293.

DeFrancesco, A.S., Masloboeva, N., Syed, A.K., DeLoughery, A., Bradshaw, N., Li, G.W., Gilmore, M.S., Walker, S., and Losick, R. (2017) Genome-wide screen for genes involved in eDNA release during biofilm formation by *Staphylococcus aureus*. *Proc Natl Acad Sci U S A* **114**: E5969-E5978.

Deguchi, Y., Yamato, I., and Anraku, Y. (1990) Nucleotide sequence of *gltS*, the Na+/glutamate symport carrier gene of *Escherichia coli B*. *J Biol Chem* **265**: 21704-21708.

Devaux, L., Sleiman, D., Mazzuoli, M.V., Gominet, M., Lanotte, P., Trieu-Cuot, P., Kaminski, P.A., and Firon, A. (2018) Cyclic di-AMP regulation of osmotic homeostasis is essential in Group B *Streptococcus*. *PLoS Genet* **14**: e1007342.

Fahmi, T., Faozia, S., Port, G., and Cho, K.H. (2019) The Second Messenger c-di-AMP Regulates Diverse Cellular Pathways Involved in Stress Response, Biofilm Formation, Cell Wall Homeostasis, SpeB Expression and Virulence in *Streptococcus pyogenes*. *Infect Immun*.

Fey, P.D., Endres, J.L., Yajjala, V.K., Widhelm, T.J., Boissy, R.J., Bose, J.L., and Bayles, K.W. (2013) A genetic resource for rapid and comprehensive phenotype screening of nonessential *Staphylococcus aureus* genes. *MBio* **4**: e00537-00512.

Fisher, S.H. (1999) Regulation of nitrogen metabolism in *Bacillus subtilis*: vive la difference! *Mol Microbiol* **32**: 223-232.

Forchhammer, K. (2007) Glutamine signalling in bacteria. *Front Biosci-Landmrk* **12**: 358-370.

Fridkin, S.K., Hageman, J.C., Morrison, M., Sanza, L.T., Como-Sabetti, K., Jernigan, J.A., Harriman, K., Harrison, L.H., Lynfield, R., Farley, M.M., and Active Bacterial Core Surveillance Program of the Emerging Infections Program, N. (2005) Methicillin-

resistant *Staphylococcus aureus* disease in three communities. *N Engl J Med* **352**: 1436-1444.

Fuller, J.R., Vitko, N.P., Perkowski, E.F., Scott, E., Khatri, D., Spontak, J.S., Thurlow, L.R., and Richardson, A.R. (2011) Identification of a lactate-quinone oxidoreductase in *Staphylococcus aureus* that is essential for virulence. *Front Cell Infect Microbiol* **1**: 19.

Gründling, A., and Schneewind, O. (2007) Genes required for glycolipid synthesis and lipoteichoic acid anchoring in *Staphylococcus aureus*. *J Bacteriol* **189**: 2521-2530.

Gundlach, J., Commichau, F.M., and Stülke, J. (2017a) Perspective of ions and messengers: an intricate link between potassium, glutamate, and cyclic di-AMP. *Curr Genet*.

Gundlach, J., Dickmanns, A., Schröder-Tittmann, K., Neumann, P., Kaesler, J., Kampf, J., Herzberg, C., Hammer, E., Schwede, F., Kaever, V., Tittmann, K., Stülke, J., and Ficner, R. (2015a) Identification, characterization, and structure analysis of the cyclic di-AMP-binding PII-like signal transduction protein DarA. *J Biol Chem* **290**: 3069-3080.

Gundlach, J., Herzberg, C., Hertel, D., Thurmer, A., Daniel, R., Link, H., and Stülke, J. (2017b) Adaptation of *Bacillus subtilis* to Life at Extreme Potassium Limitation. *MBio* **8**.

Gundlach, J., Herzberg, C., Kaever, V., Gunka, K., Hoffmann, T., Weiss, M., Gibhardt, J., Thurmer, A., Hertel, D., Daniel, R., Bremer, E., Commichau, F.M., and Stülke, J. (2017c) Control of potassium homeostasis is an essential function of the second messenger cyclic di-AMP in *Bacillus subtilis*. *Sci Signal* **10**.

Gundlach, J., Krüger, L., Herzberg, C., Turdiev, A., Poehlein, A., Tascon, I., Weiß, M., Hertel, D., Daniel, R., Hänelt, I., Lee, V.T., and Stülke, J. (2019) Sustained sensing in potassium homeostasis: Cyclic di-AMP controls potassium uptake by KimA at the levels of expression and activity. *J Biol Chem* **294**: 9605-9614.

Gundlach, J., Mehne, F.M., Herzberg, C., Kampf, J., Valerius, O., Kaever, V., and Stülke, J. (2015b) An Essential Poison: Synthesis and Degradation of Cyclic Di-AMP in *Bacillus subtilis*. *J Bacteriol* **197**: 3265-3274.

Gunka, K., and Commichau, F.M. (2012) Control of glutamate homeostasis in *Bacillus subtilis*: a complex interplay between ammonium assimilation, glutamate biosynthesis and degradation. *Mol Microbiol* **85**: 213-224.

Halsey, C.R., Lei, S., Wax, J.K., Lehman, M.K., Nuxoll, A.S., Steinke, L., Sadykov, M., Powers, R., and Fey, P.D. (2017) Amino Acid Catabolism in *Staphylococcus aureus* and the Function of Carbon Catabolite Repression. *MBio* **8**.

Hengge, R. (2009) Principles of c-di-GMP signalling in bacteria. *Nat Rev Microbiol* **7**: 263-273.

Huynh, T.N., Choi, P.H., Sureka, K., Ledvina, H.E., Campillo, J., Tong, L., and Woodward, J.J. (2016) Cyclic di-AMP targets the cystathionine beta-synthase domain of the osmolyte transporter OpuC. *Mol Microbiol*.

Kelly, B., and O'Neill, L.A. (2015) Metabolic reprogramming in macrophages and dendritic cells in innate immunity. *Cell Res* **25**: 771-784.

Kim, H., Youn, S.J., Kim, S.O., Ko, J., Lee, J.O., and Choi, B.S. (2015) Structural Studies of Potassium Transport Protein KtrA Regulator of Conductance of K+ (RCK) C Domain in Complex with Cyclic Diadenosine Monophosphate (c-di-AMP). *J Biol Chem* **290**: 16393-16402.

Kluytmans, J., van Belkum, A., and Verbrugh, H. (1997) Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks. *Clin Microbiol Rev* **10**: 505-520.

Lehman, M.K., Nuxoll, A.S., Yamada, K.J., Kielian, T., Carson, S.D., and Fey, P.D. (2019) Protease-Mediated Growth of *Staphylococcus aureus* on Host Proteins Is *opp3* Dependent. *MBio* **10**.

Mehne, F.M., Gunka, K., Eilers, H., Herzberg, C., Kaever, V., and Stülke, J. (2013) Cyclic di-AMP homeostasis in *Bacillus subtilis*: both lack and high level accumulation of the nucleotide are detrimental for cell growth. *J Biol Chem* **288**: 2004-2017.

Mirouze, N., Bidnenko, E., Noirot, P., and Auger, S. (2015) Genome-wide mapping of TnrA-binding sites provides new insights into the TnrA regulon in *Bacillus subtilis*. *Microbiologyopen* **4**: 423-435.

Monk, I.R., Shah, I.M., Xu, M., Tan, M.W., and Foster, T.J. (2012) Transforming the untransformable: application of direct transformation to manipulate genetically *Staphylococcus aureus* and *Staphylococcus epidermidis*. *MBio* **3**.

Monk, I.R., Tree, J.J., Howden, B.P., Stinear, T.P., and Foster, T.J. (2015) Complete Bypass of Restriction Systems for Major *Staphylococcus aureus* Lineages. *MBio* **6**: e00308-00315.

Moscoso, J.A., Schramke, H., Zhang, Y., Tosi, T., Dehbi, A., Jung, K., and Gründling, A. (2015) Binding of Cyclic Di-AMP to the *Staphylococcus aureus* Sensor Kinase KdpD Occurs via the Universal Stress Protein Domain and Downregulates the Expression of the Kdp Potassium Transporter. *J Bacteriol* **198**: 98-110.

Pham, H.T., Nhiep, N.T.H., Vu, T.N.M., Huynh, T.N., Zhu, Y., Huynh, A.L.D., Chakrabortti, A., Marcellin, E., Lo, R., Howard, C.B., Bansal, N., Woodward, J.J., Liang, Z.X., and Turner, M.S. (2018) Enhanced uptake of potassium or glycine betaine or export of cyclic-di-AMP restores osmoresistance in a high cyclic-di-AMP *Lactococcus lactis* mutant. *PLoS Genet* **14**: e1007574.

Pham, H.T., and Turner, M.S. (2019) Onwards and [K(+)]upwards: a new potassium importer under the spell of cyclic-di-AMP. *J Bacteriol*.

Pham, T.H., Liang, Z.X., Marcellin, E., and Turner, M.S. (2016) Replenishing the cyclic-di-AMP pool: regulation of diadenylate cyclase activity in bacteria. *Curr Genet* **62**: 731-738.

Quintana, I.M., Gibhardt, J., Turdiev, A., Hammer, E., Commichau, F.M., Lee, V.T., Magni, C., and Stülke, J. (2019) The KupA and KupB proteins of *Lactococcus lactis* IL1403 are novel c-di-AMP receptor proteins responsible for potassium uptake. *J Bacteriol*.

Randazzo, P., Aucouturier, A., Delumeau, O., and Auger, S. (2017) Revisiting the in vivo GlnR-binding sites at the genome scale in *Bacillus subtilis*. *BMC Res Notes* **10**: 422.

Richardson, A.R., Libby, S.J., and Fang, F.C. (2008) A nitric oxide-inducible lactate dehydrogenase enables *Staphylococcus aureus* to resist innate immunity. *Science* **319**: 1672-1676.

Rismondo, J., Gibhardt, J., Rosenberg, J., Kaever, V., Halbedel, S., and Commichau, F.M. (2016) Phenotypes Associated with the Essential Diadenylate Cyclase CdaA and Its Potential Regulator CdaR in the Human Pathogen Listeria monocytogenes. *J Bacteriol* **198**: 416-426.

Rocha, R., Teixeira-Duarte, C.M., Jorge, J.M.P., and Morais-Cabral, J.H. (2019) Characterization of the molecular properties of KtrC, a second RCK domain that regulates a Ktr channel in *Bacillus subtilis*. *J Struct Biol* **205**: 34-43.

Römling, U. (2008) Great times for small molecules: c-di-AMP, a second messenger candidate in Bacteria and Archaea. *Sci Signal* **1**: pe39.

Satomura, T., Shimura, D., Asai, K., Sadaie, Y., Hirooka, K., and Fujita, Y. (2005) Enhancement of glutamine utilization in *Bacillus subtilis* through the GlnK-GlnL two-component regulatory system. *J Bacteriol* **187**: 4813-4821.

Schuster, C.F., Bellows, L.E., Tosi, T., Campeotto, I., Corrigan, R.M., Freemont, P., and Gründling, A. (2016) The second messenger c-di-AMP inhibits the osmolyte uptake system OpuC in *Staphylococcus aureus*. *Sci Signal* **9**: ra81.

Schuurman-Wolters, G.K., and Poolman, B. (2005) Substrate specificity and ionic regulation of GlnPQ from *Lactococcus lactis*. An ATP-binding cassette transporter with four extracytoplasmic substrate-binding domains. *J Biol Chem* **280**: 23785-23790.

Spahich, N.A., Vitko, N.P., Thurlow, L.R., Temple, B., and Richardson, A.R. (2016) *Staphylococcus aureus* lactate- and malate-quinone oxidoreductases contribute to nitric oxide resistance and virulence. *Mol Microbiol* **100**: 759-773.

976 Sureka, K., Choi, P.H., Precit, M., Delince, M., Pensinger, D., Huynh, T.N., Jurado, A.R.,
977 Goo, Y.A., Sadilek, M., Iavarone, A.T., Sauer, J.D., Tong, L., and Woodward, J.J.
978 (2014) The cyclic dinucleotide c-di-AMP is an allosteric regulator of metabolic
979 enzyme function. *Cell* **158**: 1389-1401.
980 Teh, W.K., Dramsi, S., Tolker-Nielsen, T., Yang, L., and Givskov, M. (2019) Increased
981 Intracellular Cyclic di-AMP Levels Sensitize *Streptococcus gallolyticus* subsp.
982 gallolyticus to Osmotic Stress and Reduce Biofilm Formation and Adherence on
983 Intestinal Cells. *J Bacteriol* **201**.
984 Tolner, B., Ubbink-Kok, T., Poolman, B., and Konings, W.N. (1995) Characterization of the
985 proton/glutamate symport protein of *Bacillus subtilis* and its functional expression in
986 *Escherichia coli*. *J Bacteriol* **177**: 2863-2869.
987 Tosi, T., Hoshiga, F., Millership, C., Singh, R., Eldrid, C., Patin, D., Mengin-Lecreulx, D.,
988 Thalassinos, K., Freemont, P., and Gründling, A. (2019) Inhibition of the
989 *Staphylococcus aureus* c-di-AMP cyclase DacA by direct interaction with the
990 phosphoglucosamine mutase GlmM. *PLoS Pathog* **15**: e1007537.
991 Underwood, A.J., Zhang, Y., Metzger, D.W., and Bai, G. (2014) Detection of cyclic di-AMP
992 using a competitive ELISA with a unique pneumococcal cyclic di-AMP binding
993 protein. *J Microbiol Methods* **107**: 58-62.
994 Vitko, N.P., Spahich, N.A., and Richardson, A.R. (2015) Glycolytic dependency of high-level
995 nitric oxide resistance and virulence in *Staphylococcus aureus*. *MBio* **6**.
996 Whiteley, A.T., Garelis, N.E., Peterson, B.N., Choi, P.H., Tong, L., Woodward, J.J., and
997 Portnoy, D.A. (2017) c-di-AMP modulates *Listeria monocytogenes* central
998 metabolism to regulate growth, antibiotic resistance and osmoregulation. *Mol*
999 *Microbiol* **104**: 212-233.
1000 Whiteley, A.T., Pollock, A.J., and Portnoy, D.A. (2015) The PAMP c-di-AMP Is Essential for
1001 *Listeria monocytogenes* Growth in Rich but Not Minimal Media due to a Toxic
1002 Increase in (p)ppGpp. *Cell Host Microbe* **17**: 788-798.
1003 Wicke, D., Schulz, L.M., Lentes, S., Scholz, P., Poehlein, A., Gibhardt, J., Daniel, R.,
1004 Ischebeck, T., and Commichau, F.M. (2019) Identification of the first glyphosate
1005 transporter by genomic adaptation. *Environ Microbiol* **21**: 1287-1305.
1006 Witte, C.E., Whiteley, A.T., Burke, T.P., Sauer, J.D., Portnoy, D.A., and Woodward, J.J.
1007 (2013) Cyclic di-AMP is critical for *Listeria monocytogenes* growth, cell wall
1008 homeostasis, and establishment of infection. *MBio* **4**: e00282-00213.
1009 Woodward, J.J., Iavarone, A.T., and Portnoy, D.A. (2010) c-di-AMP secreted by intracellular
1010 *Listeria monocytogenes* activates a host type I interferon response. *Science* **328**:
1011 1703-1705.
1012 Yoshida, K., Yamaguchi, H., Kinehara, M., Ohki, Y.H., Nakaura, Y., and Fujita, Y. (2003)
1013 Identification of additional TnrA-regulated genes of *Bacillus subtilis* associated with a
1014 TnrA box. *Mol Microbiol* **49**: 157-165.
1015 Zarrella, T.M., Metzger, D.W., and Bai, G. (2018) Stress Suppressor Screening Leads to
1016 Detection of Regulation of Cyclic di-AMP Homeostasis by a Trk Family Effector
1017 Protein in *Streptococcus pneumoniae*. *J Bacteriol* **200**.
1018 Zeden, M.S., Schuster, C.F., Bowman, L., Zhong, Q., Williams, H.D., and Gründling, A.
1019 (2018) Cyclic di-adenosine monophosphate (c-di-AMP) is required for osmotic
1020 regulation in *Staphylococcus aureus* but dispensable for viability in anaerobic
1021 conditions. *J Biol Chem* **293**: 3180-3200.
1022 Zhu, Y., Pham, T.H., Nhiep, T.H., Vu, N.M., Marcellin, E., Chakrabortti, A., Wang, Y.,
1023 Waanders, J., Lo, R., Huston, W.M., Bansal, N., Nielsen, L.K., Liang, Z.X., and
1024 Turner, M.S. (2016) Cyclic-di-AMP synthesis by the diadenylate cyclase CdaA is
1025 modulated by the peptidoglycan biosynthesis enzyme GlmM in *Lactococcus lactis*.
1026 *Mol Microbiol* **99**: 1015-1027.
1027 Zhu, Y., Xiong, Y.Q., Sadykov, M.R., Fey, P.D., Lei, M.G., Lee, C.Y., Bayer, A.S., and
1028 Somerville, G.A. (2009) Tricarboxylic acid cycle-dependent attenuation of
1029 *Staphylococcus aureus* in vivo virulence by selective inhibition of amino acid
1030 transport. *Infect Immun* **77**: 4256-4264.