Identification of the main glutamine and glutamate transporters in *Staphylococcus aureus* and their impact on c-di-AMP production

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Summary (200 words)

A *Staphylococcus aureus* strain deleted for the c-di-AMP cyclase gene *dacA* is unable to survive in rich medium unless it acquires compensatory mutations. Previously identified mutations were in *opuD*, encoding the main glycine-betaine transporter, and *alsT*, encoding a predicted amino acid transporter. Here, we show that inactivation of OpuD restores the cell size of a *dacA* mutant to near wild-type size, while inactivation of AlsT does not, suggesting two different mechanisms for the growth rescue. AlsT was identified as an efficient glutamine transporter, indicating that preventing glutamine uptake in rich medium rescues the growth of the *S. aureus dacA* mutant. In addition, GltS was identified as a glutamine transporter. By performing growth curves with WT, *alsT* and *gltS* mutant strains in defined medium supplemented with ammonium, glutamine or glutamate, we revealed that ammonium and glutamine, but not glutamate promote the growth of *S. aureus*. This suggests that besides ammonium also glutamine can serve as a nitrogen source under these conditions. Ammonium and uptake of glutamine via AlsT inhibited c-di-AMP production, while glutamate uptake had no effect. These findings provide, besides the previously reported link between potassium and osmolyte uptake, a connection between nitrogen metabolism and c-di-AMP signalling in *S. aureus*.

42 **Introduction**

43 In the human host, S*taphylococcus aureus* can grow in virous tissues such as
44 kidneys, bones, heart, soft tissues and lungs (Kluytmans *et al.*, 1997, Fridkin *et al.*, 2005).
45 Sensitive regulatory mechanisms enable this organism to rapidly respond to external stimuli
46 and environmental changes. Amongst others, this allows bacteria to adapt their metabolism
47 and utilize different carbon and nitrogen sources available in each specific niche (Fridkin *et*
48 *al.*, 2005, Spahich *et al.*, 2016, Vitko *et al.*, 2015, Crooke *et al.*, 2013, Fuller *et al.*, 2011,
49 Richardson *et al.*, 2008, Halsey *et al.*, 2017, Lehman *et al.*, 2019).

50 Glucose is the preferred carbon source for *S. aureus,* but it can be limiting during
51 infection due to the host immune response (Kelly & O'Neill, 2015, Spahich *et al.*, 2016,
52 Halsey *et al.*, 2017, Lehman *et al.*, 2019). In glucose-limiting conditions, *S. aureus* instead
53 catabolizes secondary carbon sources and amino acids, particularly glutamate and proline,
54 which serve as major carbon sources during growth in the absence of glucose (Halsey *et al.*,
55 2017). However, not much is known about amino acid uptake and catabolism in *S. aureus*
56 and how the availability of certain nutrients can affect virulence factor expression and
57 invasion of the host. While a large number of amino acid transporters and oligopeptide
58 permeases can be identified bioinformatically, their actual substrate specificities and
59 functions in *S. aureus* have not yet been studied in detail. Predicting the substrates for
60 transporters bioinformatically remains challenging and hence such questions need to be
61 addressed experimentally.

62 Secondary messenger molecules are crucial in allowing bacteria to rapidly adapt to
63 different environmental and host cell niches (Römling, 2008, Hengge, 2009). There is now
64 considerable evidence that one of these messengers, cyclic di-adenosine monophosphate
65 (c-di-AMP) plays a key role in osmoregulation in bacteria (Pham *et al.*, 2018, Pham &
66 Turner, 2019, Quintana *et al.*, 2019, Zarrella *et al.*, 2018, Teh *et al.*, 2019, Fahmi *et al.*,
67 2019, Devaux *et al.*, 2018, Bai *et al.*, 2014, Zeden *et al.*, 2018, Corrigan *et al.*, 2011, Rocha
68 *et al.*, 2019, Gundlach *et al.*, 2017a, Gundlach *et al.*, 2018, Witte *et al.*, 2013, Whiteley *et al.*,
69 2015, Whiteley *et al.*, 2017). c-di-AMP binds to and negatively regulates a number of

70 different potassium and osmolyte importers (Rocha *et al.*, 2019, Quintana *et al.*, 2019, Kim
71 *et al.*, 2015, Corrigan *et al.*, 2013, Moscoso *et al.*, 2015, Chin *et al.*, 2015, Huynh *et al.*,
72 2016, Schuster *et al.*, 2016, Pham & Turner, 2019, Pham *et al.*, 2018, Devaux *et al.*, 2018,
73 Zarrella *et al.*, 2018, Gundlach *et al.*, 2017a, Gundlach *et al.*, 2018, Gundlach *et al.*, 2017b).
74 In many Firmicutes, c-di-AMP is essential for bacterial growth under standard rich medium
75 growth conditions, but it is also toxic at high levels. Hence, the cellular levels must be tightly
76 regulated (Gundlach *et al.*, 2015, Mehne *et al.*, 2013, Corrigan *et al.*, 2011, Corrigan *et al.*,
77 2015, Woodward *et al.*, 2010, Witte *et al.*, 2013). In *S. aureus, Streptococcus agalactiae*
78 and *Listeria monocytogenes,* deletion of *dacA* (also referred to as *cdaA* in many bacteria),
79 coding for the diadenylate cyclase enzyme and responsible for the synthesis of c-di-AMP,
80 was only possible in chemically defined medium (Whiteley *et al.*, 2015, Zeden *et al.*, 2018,
81 Devaux *et al.*, 2018), whereas in *Bacillus subtilis* all three c-di-AMP cyclases could only be
82 inactivated in minimal medium also containing low amounts of potassium (Gundlach *et al.*,
83 2017a).
84 Previously, we found that inactivation of the main glycine betaine transporter OpuD
85 (SAUSA300_1245) as well as the predicted amino acid transporter AlsT (SAUSA300_1252)
86 allows an *S. aureus dacA* mutant to grow in rich medium in the absence of c-di-AMP (Zeden
87 *et al.*, 2018). In several other Firmicutes, including *B. subtilis, Lactococcus lactis,*
88 *Streptococcus pneumoniae, S. agalactiae, Streptococcus pyogenes* and *L. monocytogenes*,
89 inactivating mutations have also been identified in osmolyte and potassium transport
90 systems that allow these bacteria to grow in the absence of c-di-AMP (Pham *et al.*, 2018,
91 Pham & Turner, 2019, Quintana *et al.*, 2019, Zarrella *et al.*, 2018, Teh *et al.*, 2019, Fahmi *et*
92 *al.*, 2019, Devaux *et al.*, 2018, Bai *et al.*, 2014, Zeden *et al.*, 2018, Corrigan *et al.*, 2011,
93 Rocha *et al.*, 2019, Gundlach *et al.*, 2017a, Gundlach *et al.*, 2018, Witte *et al.*, 2013,
94 Whiteley *et al.*, 2015, Whiteley *et al.*, 2017). This suggests that potassium and osmolyte
95 transporters are more active in the absence of c-di-AMP, resulting in the accumulation of
96 toxic levels of potassium and osmolytes in the cell. Consistent with a key function of c-di-
97 AMP in regulating the osmotic balance in the cell, we found that *S. aureus* cells show

98 significant differences in cell size depending on their intracellular c-di-AMP levels (Zeden *et*
99 *al.*, 2018, Corrigan *et al.*, 2011). Cells of strain LAC**gdpP,* which have high c-di-AMP levels,
100 show a decrease in cell size, while cells of strain LAC**dacA*$_{G206S}$, containing low levels of c-
101 di-AMP, show an increase in cell size (Zeden *et al.*, 2018, Corrigan *et al.*, 2011). As c-di-
102 AMP negatively regulates potassium and osmolyte uptake (Corrigan *et al.*, 2013, Moscoso
103 *et al.*, 2015, Schuster *et al.*, 2016), the increase in cell size is consistent with the hypothesis
104 that an increase in potassium and osmolyte uptake and retention of water at reduced c-di-
105 AMP levels leads to the observed increase in cell size. c-di-AMP levels affecting bacterial
106 cell size has also been observed for other bacteria such as *S. pneumonia* and *L.*
107 *monocytogenes* (Commichau *et al.*, 2019, Bai *et al.*, 2013).

108 AlsT is a predicted amino acid transporter and a correlation between cellular levels of
109 c-di-AMP and the amino acids glutamate and glutamine has been reported for *B. subtilis* and
110 *L. monocytogenes* (Whiteley *et al.*, 2017, Gundlach *et al.*, 2015, Gundlach *et al.*, 2018,
111 Sureka *et al.*, 2014). A two-fold increase in cellular c-di-AMP levels was observed in *B.*
112 *subtilis* when bacteria where grown in Spizizen minimal medium with glutamate (Glu) as
113 compared to growth in the same medium with glutamine (Gln) as a nitrogen source
114 (Gundlach *et al.*, 2015). In *L. monocytogenes*, c-di-AMP was identified as a negative
115 regulator of the key TCA cycle enzyme pyruvate carboxylase (Sureka *et al.*, 2014).
116 Depletion of c-di-AMP resulted in an increased flux into the TCA cycle and as a
117 consequence an increase in the cellular glutamine/glutamate pool (Sureka *et al.*, 2014). In a
118 *citZ* mutant, which lacks the TCA cycle enzyme citrate synthase and thus has an early block
119 in the TCA cycle, the depletion of c-di-AMP no longer resulted in the accumulation of
120 glutamate/glutamine in the cell (Sureka *et al.*, 2014). Interestingly, an *L. monocytogenes*
121 *dacA*/*citZ* double mutant was again viable in rich medium (Sureka *et al.*, 2014, Whiteley *et*
122 *al.*, 2017).

123 As part of this study, we further investigated why inactivation of the main glycine-
124 betaine transporter OpuD and the predicted amino acid transporter AlsT allows *S. aureus* to
125 grow in the absence of c-di-AMP in rich medium. We show that AlsT is a main glutamine

126 transporter in *S. aureus* and that AlsT-mediated glutamine uptake represses c-di-AMP
127 production. Similarly, growth in ammonium-containing defined medium but not in glutamate-
128 containing medium, repressed c-di-AMP production. The repression of c-di-AMP production
129 was independent of the activity of the c-di-AMP phosphodiesterase GdpP and the predicted
130 cyclase regulator YbbR. With this study, we not only provide a further link between the c-di-
131 AMP signalling network and osmotic regulation in bacterial cells but also with the uptake of
132 specific nitrogen sources and amino acids in *S. aureus.*
133
134 **Results**
135 **Inactivation of OpuD but not AlsT reduces the cell size of an *S. aureus dacA* mutant**
136 In previous work, we reported a correlation between the cell size and c-di-AMP levels in *S.*
137 *aureus*: bacteria with high c-di-AMP level are smaller, whereas bacteria with low c-di-AMP
138 levels (strain LAC**dacA*$_{G206S}$) are larger as compared to wild-type bacteria (Zeden *et al.*,
139 2018, Corrigan *et al.*, 2011). We also reported that inactivating mutations in *opuD*
140 (*SAUSA300_1245*) coding for the main glycine betaine osmolyte transporter and *alsT*
141 (*SAUSA300_1252*) coding for a predicted amino acid sodium symporter, rescue the growth
142 defect observed for the c-di-AMP negative *S. aureus* strain LAC**dacA::kan* in rich medium
143 TSB (Zeden *et al.*, 2018). Here, we investigated further the mechanism by which the growth
144 defect of the *dacA* mutant strain is rescued in the LAC**dacA/opuD* and LAC**dacA/alsT*
145 suppressor strains. Initially, we compared the cell size of bacteria from the suppressor
146 strains LAC**dacA/opuD* and LAC**dacA/alsT* to that of WT LAC* and the low c-di-AMP level
147 strain LAC**dacA*$_{G206S}$ after growth in the rich medium TSB. As expected, the bacteria with
148 low-levels of c-di-AMP showed an increase in cell size as compared to WT bacteria (Fig. 1A
149 and B). While a similar increase in cell size was still observed for bacteria of strain
150 LAC**dacA/alsT*, the cell size of LAC**dacA/opuD* bacteria, while still increased as compared
151 to the wild type, was significantly smaller as compared to the low-level LAC**dacA*$_{G206S}$ strain
152 (Fig. 1A and B). Because regular TSB medium is not suitable for the growth of the c-di-AMP
153 null strain LAC**dacA::kan,* bacterial cell sizes were also determined following growth in TSB

154 medium supplemented with 0.4 M NaCl, which is permissive for the growth of the *dacA*
155 mutant (Fig. 1C-F). Similar to what was observed for the low c-di-AMP level $dacA_{G206S}$
156 mutant strain, the size of bacteria from the c-di-AMP null strain LAC**dacA::kan* was
157 significantly increased compared to WT bacteria. As observed before, the cell size was not
158 rescued for bacteria of the LAC**dacA/alsT* suppressor strain (Fig. 1C-F). On the other hand,
159 the size of LAC**dacA/opuD* bacteria was similar to that of WT bacteria (Fig. 1C-F). Taken
160 together, the observed differences in cell size indicate that the underlying molecular
161 mechanisms enabling the *opuD* and *alsT* mutant strains to survive in the absence of c-di-
162 AMP in rich medium might be different.
163
164 **AlsT is a glutamine transporter in *S. aureus***
165 AlsT (SAUSA300_1252) is a predicted amino acid transporter and annotated in the InterPro
166 database (www.ebi.ac.uk/interpro) as alanine/sodium symporter. However, in a previous
167 study no difference in the uptake of radiolabelled alanine was detected between a WT and
168 the LAC**dacA/alsT* mutant strain (Zeden *et al.*, 2018), indicating that AlsT is not an alanine
169 transporter. To identify potential substrates for the *S. aureus* AlsT transporter, we initially
170 followed the depletion of different amino acids from the culture supernatant during the
171 growth of the WT LAC* strain and an isogenic *alsT* transposon mutant strain in TSB
172 medium, where both strains exhibit similar growth rates (Fig. S1A). Of note, using this
173 method, tryptophan uptake cannot be measured and it is also not possible to distinguish
174 between glutamine/glutamate or asparagine/aspartate utilization. While no significant
175 differences were observed for most amino acids (Fig. S1), a slight increase in the utilization
176 of aspartate/asparagine and a slight decrease in the uptake of serine was observed (Fig.
177 S1D and Fig. S1O), suggesting that AlsT could potentially be a serine transporter. To test
178 this, uptake assays were performed with radiolabelled serine using WT LAC*, the *alsT*
179 mutant strain LAC**alsT::tn* piTET as well as the complementation strain LAC**alsT::tn* piTET-
180 *alsT*. However, no significant differences in the uptake rate of serine were observed between
181 the strains (Fig. 2A), indicating that AlsT is not a main serine transporter in *S. aureus*. Next,

182 a more detailed bioinformatics analysis was performed to identify potential AlsT substrates.
183 A BlastP search against the *B. subtilis* 168 genome led to the identification of four proteins
184 showing significant homology to the *S. aureus* AlsT (SAUSA300_1252) protein, namely AlsT
185 (e-value: e-166), GlnT (e-value: e-149), YrbD (e-value: e-117) and YflA (e-value: 2e-72).
186 Also in *S. aureus* a second AlsT homologue, SAUSA300_0914 (e-value 9e-108), could be
187 identified (Fig. S2), which is encoded at a different chromosomal region. AlsT is annotated in
188 *B. subtilis* as a potential glutamine sodium symporter (Zhu & Stülke, 2018), but to the best of
189 our knowledge, this has not yet been experimentally verified. To test if *S. aureus* AlsT is a
190 potential glutamine or glutamate transporter, uptake assays were performed with
191 radiolabelled glutamine and glutamate using the WT *S. aureus* strain LAC*, the *alsT* mutant
192 LAC**alsT::tn* piTET and the complementation strain LAC**alsT::tn* piTET-*alsT*. Uptake of
193 glutamine, but not of glutamate, was severely reduced in the *alsT* mutant when compared to
194 the WT strain (Fig. 2B-C). This defect was restored upon expression of *alsT* in the
195 complementation strain (Fig. 2C). To confirm that *alsT* functions as main glutamine
196 transporter also in the LAC**dacA/alsT* suppressor strain, uptake assays were also
197 performed with strain LAC**dacA/alsT* along with the WT LAC* and LAC**dacA::kan* control
198 strains (Fig. 2D-F). Similar as observed for the *alsT* single mutant, serine and glutamate
199 uptake were only marginally affected in strain LAC**dacA/alsT* (Fig. 2D-E), whereas
200 glutamine uptake was severely reduced in strain LAC**dacA/alsT* when compared to the
201 control strains (Fig. 2F). These data suggest that under the uptake assay conditions tested,
202 AlsT functions as the main glutamine transporter in *S. aureus.* A slight reduction in glutamine
203 and serine uptake was seen in the absence of c-di-AMP (Fig. 2D and Fig. 2F), suggesting
204 that c-di-AMP levels can impact glutamine and serine uptake in *S. aureus.* Taken together,
205 our data suggest that *S. aureus* cells that are unable to produce c-di-AMP can survive in rich
206 medium such as TSB, when glutamine uptake is reduced or blocked. However, we cannot
207 formally exclude that AlsT is able to transport other amino acid or substrates present in rich
208 medium.
209

210 **Investigating the contribution of SAUSA300_0914 and GlnQ to glutamine and**
211 **glutamate transport in *S. aureus***
212 *S. aureus SAUSA300_0914* codes for a predicted amino acid symporter, which shows 41%
213 identity to the *S. aureus* AlsT protein. After assigning AlsT a function as glutamine
214 transporter, we wanted to test if SAUSA300_0914 might also play a role in glutamine or
215 glutamate transport. To this end, strain LAC**0914::tn* was constructed by transducing the
216 genomic region from the NMTL strain NE1463 (Fey *et al.*, 2013) containing a transposon
217 insertion in *SAUSA300_0914* into the *S. aureus* LAC* background. Subsequently the uptake
218 of radiolabelled glutamine and glutamate was assessed (Fig. 3A-B). No significant
219 differences in the uptake of these amino acids was observed between WT LAC* and strain
220 LAC**0914::tn*, showing that SAUSA300_0914 does not function as a major glutamine or
221 glutamate transporter under our assay conditions (Fig. 3A-B). AlsT and SAUSA300_0914
222 are members of the amino acid/sodium symporter family of transporters, which are
223 composed of a single multimembrane spanning protein. Besides this type of transporter,
224 GlnPQ-type ABC transporters play a major role in glutamine and glutamate transport in other
225 bacteria (Schuurman-Wolters & Poolman, 2005). *S. aureus* contains a *glnPQ*
226 *(SAUSA300_1808 - SAUSA300_1807)* operon with *glnP* coding for a substrate binding
227 domain-permease fusion protein and *glnQ* coding for the cytoplasmic nucleotide-binding
228 ATPase domain. The results from a previous study suggested that this transporter functions
229 as glutamine transporter in *S. aureus*, as a *glnP* mutant was more resistant to the toxic
230 glutamine analogue γ-L-glutamyl hydrazide (Zhu *et al.*, 2009). To assess the contribution of
231 the GlnPQ transporter to glutamine and glutamate transport in *S. aureus* LAC*, the strain
232 LAC**glnQ::tn* was generated by transducing the *glnQ::tn* region from the NMTL strain NE153
233 (Fey *et al.*, 2013) into the LAC* background. The resulting *glnQ* mutant strain LAC**glnQ::tn*
234 displayed no difference in glutamine or glutamate uptake compared to WT LAC* (Fig. 3C-D).
235 This indicates that the ABC transporter GlnPQ does not function under our assay conditions
236 and in the *S. aureus* LAC* strain background as a main glutamate or glutamine transporter.
237

238 **Inactivation of AlsT but not SAUSA300_0914 or GlnQ reduces the susceptibility of *S.***
239 ***aureus* to the toxic glutamine analogue γ-L-glutamyl hydrazide**
240 To further validate the findings from the uptake assays and verify that AlsT is the main
241 glutamine transporter, we performed growth curves in the presence of increasing
242 concentrations of the toxic glutamine analogue γ-L-glutamyl hydrazide with the WT and
243 LAC**alsT::tn* mutant strains. Strains LAC**0914::tn* and LAC**glnQ::tn* were also included in
244 these assays, to uncover a potential low level glutamine uptake activity for the
245 SAUSA300_0914 and GlnPQ transporters. Strains defective in taking up this glutamine
246 analogue are expected to show reduced susceptibility to this toxic compound. In the
247 absence of the compound, all strains grew similarly in the chemically defined medium used
248 for this assay (Fig. 4A). As expected, addition of γ-L-glutamyl hydrazide reduced the growth
249 of the WT LAC* strain, in a dose-deponent manner (Fig. 4B and Fig. S3). Similar growth
250 inhibition curves to that of the WT strain were obtained for strains LAC**0914::tn* and
251 LAC**glnQ::tn*, while strain LAC**alsT::tn* showed increased resistance to the compound (Fig.
252 4B and Fig. S3). These findings support our earlier conclusion that AlsT is the main
253 glutamine transporter in *S. aureus*, while GlnPQ and SAUSA300_0914 are either unable to
254 take up glutamine or play only a minor role in its uptake under our growth conditions.
255
256 **GltS (SAUSA300_2291) is a glutamate transporter in *S. aureus***
257 *S. aureus* does not only take up glutamine but also shows robust glutamate uptake (Fig. 2
258 and Fig. 3). However, none of the transporters (AlsT, SAUSA300_0914 and GlnPQ)
259 investigated so far plays a major role in glutamate uptake under our growth conditions. In *B.*
260 *subtilis* GltT, belonging to the dicarboxylate/amino acid cation symporter (DAACS) family of
261 proteins, is a major high-affinity Na^+-coupled glutamate/aspartate symporter and can also
262 mediate the uptake of glyphosate (Wicke *et al.*, 2019). Two paralogs, DctP and GltP, are
263 found in *B. subtilis* of which GltP has also been shown to be a glutamate transporter (Tolner
264 *et al.*, 1995). The *S. aureus* protein SAUSA300_2329 (from here on referred to as GltT)

265 shows a high degree of similarity (52% identity) to the *B. subtilis* GltT protein. In addition,
266 SAUSA300_2291 (from here on referred to as GltS) is annotated in UniProt
267 (www.uniprot.org) as a potential glutamate transporter in *S. aureus*. To experimentally test if
268 GltT or GltS impact glutamate transport in *S. aureus*, strains LAC**gltT::tn* and LAC**gltS::tn*
269 were constructed by moving the respective *gltT and gltS* transposon insertion regions from
270 the NMTL strains NE566 and NE560 (Fey *et al.*, 2013) into the LAC* strain background.
271 Next, the uptake of radiolabelled glutamine and glutamate was assessed for WT LAC* and
272 strains LAC* *gltT::tn* and LAC* *gltS::tn*. No difference in the uptake of glutamine was
273 observed between the strains (Fig. 5A) and in the case of LAC**gltT::tn*, also no difference in
274 the uptake of glutamate was observed (Fig. 5B). However, a significant reduction in
275 glutamate uptake was observed for strain LAC**gltS::tn* when compared to the WT strain
276 (Fig. 5B). The glutamate uptake defect could be restored in a complementation strain
277 harbouring plasmid piTET-*gltS* allowing for inducible *gltS* expression (Fig. 5C). Indeed,
278 increased glutamate uptake was observed in the complementation strain, indicating
279 increased *gltS* expression in the complementation strain as compared to the WT strain.
280 Taken together, these data indicate that under the growth conditions tested, GltS is the main
281 glutamate transporter in *S. aureus*.

282 **Ammonium and glutamine but not glutamate stimulate the growth of *S. aureus* in**
283 **defined medium containing glucose as carbon source**

284 Glutamine and glutamate are important amino acids that can serve, together with
285 ammonium, as nitrogen sources for the synthesis of many other cellular metabolites. Since
286 *S. aureus* is phenotypically auxotroph for many amino acids and at the same time can use
287 several amino acids as carbon and nitrogen sources, it is not possible to grow this organism
288 in any of the typical minimal media that are used to assess the ability of bacteria to
289 specifically use ammonium, glutamine or glutamate as nitrogen sources. However, to begin
290 to examine the effect of these compounds on the growth of *S. aureus*, growth curves were

291 performed with the WT LAC* strain in glucose containing defined medium (GDM), containing
292 essential vitamins, metals and 17 amino acids but lacking ammonium, glutamine and
293 glutamate as potential nitrogen/amino acid sources (see Table S1 for medium composition).
294 In addition, the WT LAC* was also grown in GDM containing glutamine (GDM+Gln),
295 glutamate (GDM+Glu), ammonium (GDM+NH_3), glutamine and ammonium (GDM+Gln+NH_3)
296 or glutamate and ammonium (GDM+Glu+NH_3). The addition of glutamine or ammonium
297 alone or in combinations stimulated the growth of the WT LAC* strain as compared to its
298 growth in GDM (Fig. 6A). On the other hand, no growth improvement was seen in the
299 presence of glutamate (GDM+Glu) (Fig. 6A). To examine the contribution of the glutamine
300 and glutamate transporters AlsT and GltS, additional growth curves were performed in the
301 different media with WT LAC* as well as strains LAC**alsT::tn* and LAC**gltS::tn* (Fig. S4).
302 Similar growth profiles were observed for all strains in the different media (Fig. S4), except in
303 GDM+Gln, in which the *alsT* mutant strain exhibited reduced growth compared to the WT
304 and *gltS* mutant strains (Fig. S4B). The growth defect could be restored in the *alsT*
305 complementation strain harbouring plasmid piTET-*alsT* (Fig. 6B). Taken together, these data
306 indicate that ammonium and glutamine are preferred over glutamate for the growth of *S.*
307 *aureus*. The observation that the addition of ammonium improves the growth of *S. aureus*
308 indicates that our base medium is likely nitrogen limiting and suggests that glutamine but not
309 glutamate can likely also serve as nitrogen source under these growth conditions. Finally,
310 these data further confirm the importance of AlsT for glutamine uptake in *S. aureus.*

311 **Ammonium and glutamine uptake lead to a reduction in c-di-AMP levels in *S. aureus***

312 For *B. subtilis,* it has been reported that the addition of glutamine, glutamate or ammonium
313 to a defined growth medium can affect cellular c-di-AMP levels (Gundlach *et al.*, 2015). It
314 was further proposed that glutamate uptake and to some extent also ammonium uptake
315 leads to an activation of c-di-AMP synthesis in this organism (Gundlach *et al.*, 2015). To
316 assess if the presence of glutamine, glutamate or ammonium would also affect c-di-AMP

317 levels in *S. aureus*, the intracellular c-di-AMP concentrations were determined for the WT *S.*
318 *aureus* strain LAC* following growth in GDM, GDM+Gln, GDM+Glu, GDM+NH_3,
319 GDM+Gln+NH_3 and GDM+Glu+NH_3. Using a competitive ELISA assay, c-di-AMP could be
320 readily detected in bacteria grown in GDM, our base medium (Fig. 7A). Similar amounts of c-
321 di-AMP were detected in bacteria grown in the glutamate-containing medium (GDM+Glu),
322 however the c-di-AMP levels were significantly lower in bacteria grown in medium containing
323 either ammonium or glutamine (GDM+Gln, GDM+NH_3, GDM+Gln+NH_3, GDM+Glu+NH_3)
324 (Fig. 7A). To verify that the addition of glutamine reduces c-di-AMP production and to
325 investigate the contribution of the glutamine transporter AlsT to this inhibition, c-di-AMP
326 levels were determined for WT piTET, the *alsT* mutant LAC**alsT::tn* piTET and the
327 complementation strain LAC**alsT::tn* piTET-*alsT* following growth in the glutamine
328 containing medium GDM+Gln. While again low c-di-AMP levels were detected for the WT
329 strain, the c-di-AMP levels increased significantly in the *alsT* mutant and were restored back
330 to wild-type levels in the complementation strain (Fig. 7B). A similar experiment was
331 performed with the *gltS* mutant and complementation strain in the glutamate containing
332 medium GDM+Glu. High and similar c-di-AMP levels were detected for all strains (Fig. 7C),
333 indicating that neither the addition of glutamate to the medium nor its uptake impacts c-di-
334 AMP production in *S. aureus* under our test conditions. Taken together, these data highlight
335 that ammonium as well as AlsT-mediated glutamine uptake represses c-di-AMP production
336 in *S. aureus*.

337 **The inhibition of the c-di-AMP production by glutamine and ammonium is not**
338 **mediated by GdpP or YbbR**

339 The observed reduction of c-di-AMP levels in the presence of glutamine or ammonium could
340 potentially be achieved through an increase in the activity of the c-di-AMP specific
341 phosphodiesterase GdpP. To investigate this, cellular c-di-AMP levels were compared
342 between the WT LAC* and the isogenic *gdpP* mutant strain LAC**gdpP::kan*. As previously

343 reported for strain LAC**gdpP::kan* following growth in TSB medium (Corrigan *et al.*, 2011),
344 the c-di-AMP levels were also increased in the *gdpP* mutant compared to the WT strain
345 following growth in GDM, the glucose containing defined medium used as part of this study
346 (Fig. 8A). However, a significant reduction in the cellular c-di-AMP levels was also seen for
347 the *gdpP* mutant following the addition of glutamine or ammonium to the medium (Fig. 8A).
348 This indicates that the reduction in c-di-AMP levels upon addition of glutamine or ammonium
349 is likely due to decreased synthesis by DacA and not increased degradation by GdpP. We
350 next tested the involvement of YbbR, a proposed c-di-AMP cyclase regulator, by comparing
351 the cellular c-di-AMP levels produced by WT LAC* and strain LAC*Δ*ybbR*. Similar c-di-AMP
352 levels were detected in the WT and *ybbR* mutant in GDM medium (Fig. 8B). The addition of
353 glutamine or ammonium to the medium led also to a large reduction in the cellular c-di-AMP
354 in the *ybbR* mutant strain (Fig. 8B). These data suggest that the observed reduction of c-di-
355 AMP production in the presence of glutamine and ammonium is neither mediated by GdpP
356 nor YbbR, and hence involves a different regulator protein, or that the cellular glutamine and
357 nitrogen levels are directly sensed by the cyclase DacA.

358 **Discussion**

359 Over the last decade, considerable evidence has emerged that c-di-AMP plays a
360 major role in osmotic regulation in bacteria, primarily by positively regulating potassium
361 export or negatively regulating potassium and osmolyte uptake (Rocha *et al.*, 2019,
362 Quintana *et al.*, 2019, Kim *et al.*, 2015, Corrigan *et al.*, 2013, Moscoso *et al.*, 2015, Chin *et*
363 *al.*, 2015, Huynh *et al.*, 2016, Schuster *et al.*, 2016, Pham & Turner, 2019, Pham *et al.*,
364 2018, Devaux *et al.*, 2018, Zarrella *et al.*, 2018, Gundlach *et al.*, 2017a, Gundlach *et al.*,
365 2018, Gundlach *et al.*, 2017b, Gundlach *et al.*, 2019). However, individual c-di-AMP target
366 proteins identified thus far are themselves not essential. Therefore, the essentiality of c-di-
367 AMP is likely due to its ability to regulate multiple target proteins simultaneously.
368 Furthermore, in the absence of this molecule, many transporters are activated rather than
369 inactivated, likely leading to accumulation of toxic levels of metabolites, such as potassium

370 and osmolytes. Consistent with this idea, inactivating mutations in potassium uptake
371 systems, oligopeptide and osmolyte transporters have been reported to rescue the growth
372 defect of bacteria unable to produce c-di-AMP (Whiteley *et al.*, 2015, Whiteley *et al.*, 2017,
373 Gundlach *et al.*, 2017a, Gundlach *et al.*, 2017b, Pham *et al.*, 2018, Devaux *et al.*, 2018,
374 Zeden *et al.*, 2018). We have previously shown that inactivation of the main glycine betaine
375 transporter OpuD bypasses the requirement of c-di-AMP for the growth of *S. aureus* in rich
376 medium (Zeden *et al.*, 2018). We hypothesize that inactivation of OpuD might help a c-di-
377 AMP null strain survive by allowing bacteria to re-establish their osmotic balance. Bacteria of
378 the *dacA/opuD* mutant strain, which cannot produce c-di-AMP but are also defective in
379 glycine betaine transport, are similar in size to WT bacteria (Fig. 1). At this point it is not
380 known if c-di-AMP can directly bind to and regulate the activity of the *S. aureus* OpuD
381 protein. We attempted to address this question; however, despite using multiple different
382 approaches, we were unable to produce sufficient amounts of the full-length OpuD
383 membrane protein to perform protein/nucleotide interaction studies. On the other hand, a
384 direct role for c-di-AMP in the control of glycine betaine or betaine transporters has been
385 reported in *S. agalactiae* and *L. lactis*. In these organisms, c-di-AMP binds to the
386 transcriptional regulator BusR, which controls the expression of the predicted glycine betaine
387 or betaine transporter BusAB (Devaux *et al.*, 2018, Pham *et al.*, 2018).

388 Bacteria of the *dacA/alsT* suppressor strain, which survive in the absence of c-di-
389 AMP, remained enlarged, indicating that the essentiality of c-di-AMP is bypassed in this
390 strain potentially through a different mechanism. Here, we show that AlsT is an efficient
391 glutamine transporter in *S. aureus* (Fig. 2). These findings indicate that eliminating or
392 reducing the ability of *S. aureus* to take up glutamine from rich growth medium rescues the
393 growth of an *S. aureus* unable to produce c-di-AMP. There are several (not mutually
394 exclusive) possibilities how preventing glutamine uptake could rescue the growth of a c-di-
395 AMP null strain in rich medium. Glutamine as well as proline have been shown to
396 accumulate in *S. aureus* under NaCl stress conditions (Anderson & Witter, 1982). While it
397 has been suggested that the glutamine accumulation is due to synthesis rather than uptake

398 (Anderson & Witter, 1982), these data highlight that glutamine likely plays an important role
399 in osmotic regulation in *S. aureus.* Despite the cell size not being restored in the *dacA/alsT*
400 suppressor strain, blocking glutamine uptake could potentially still help bacteria to better
401 balance their cellular osmolality during growth in rich medium in the absence of c-di-AMP.
402 Another possible explanation how eliminating glutamine uptake could allow *S. aureus* to
403 grow in the absence of c-di-AMP could be related to changes in metabolism and TCA cycle
404 activity. In *L. monocytogenes,* an increased flux of pyruvate into the TCA cycle has been
405 described for a strain unable to produce c-di-AMP (Sureka *et al.*, 2014). As a consequence,
406 an accumulation of citrate and increased carbon flux into glutamine/glutamate was
407 observed, which resulted in a metabolic imbalance and growth defect (Sureka *et al.*, 2014).
408 Perhaps similar to the observations in *L. monocytogenes*, the absence of c-di-AMP could
409 also boost TCA cycle activity in *S. aureus*, thus leading to glutamine accumulation and a
410 metabolic imbalance. Hence, the lack of c-di-AMP combined with active glutamine uptake
411 could fuel the bacterial metabolism and the resulting metabolic imbalance might become
412 toxic to the cell, similar as observed for *L. monocytogenes* (Sureka *et al.*, 2014, Whiteley *et*
413 *al.*, 2017).

414 In a recent study investigating genetic determinants required for eDNA release
415 during biofilm formation, it was found that inactivation of GdpP as well as of AlsT, resulted in
416 a significant decrease in eDNA release and in an increase in resistance to Congo red
417 (DeFrancesco *et al.*, 2017). Therefore, inactivation of AlsT and preventing/reducing
418 glutamine uptake might lead to alterations in the bacterial cell wall that make bacteria more
419 resistant to cell lysis. Such changes could also be an advantage during osmotic stress or c-
420 di-AMP deficiency. Indeed, we have recently shown a correlation between specific changes
421 in the peptidoglycan structure and the NaCl stress resistance in *S. aureus* (Schuster *et al.*,
422 2019). In addition, since the cellular c-di-AMP levels are significantly higher in the *gdpP* as
423 well as the *alsT* mutant strains compared to WT (Fig. 7B and Fig. 8A), the underlying
424 mechanistic bases for the decrease in eDNA release observed for the *gdpP* and *alsT* mutant
425 strains might be related.

426 The actual stimuli and underlying molecular mechanisms that regulate c-di-AMP
427 production in bacterial cells are at the moment poorly understood. As part of this study, we
428 show that ammonium and AlsT-mediated glutamine uptake but not GltS-mediated glutamate
429 uptake negatively impacts c-di-AMP production (Fig. 7). Changes in cellular c-di-AMP levels
430 depending on the presence of ammonium, glutamine or glutamate have already been
431 reported for *B. subtilis* (Gundlach *et al.*, 2015). For *B. subtilis* it has been suggested that
432 glutamine and to some extent ammonium uptake stimulates c-di-AMP production (Gundlach
433 *et al.*, 2015). Here we show that in *S. aureus* ammonium and glutamine uptake leads to an
434 inhibition of c-di-AMP production rather than glutamate promoting its synthesis (Fig. 7). The
435 decrease in c-di-AMP production in the presence of ammonium or glutamine is likely
436 achieved by reducing the activity of the c-di-AMP cyclase DacA and not by activation of the
437 c-di-AMP specific phosphodiesterase GdpP. This conclusion is based on our observation
438 that the cellular c-di-AMP levels also decreased in a *gdpP* mutant strain upon addition of
439 glutamine or ammonium (Fig. 8A). Current evidence suggests that the activity of DacA can
440 be regulated through the interaction with two proteins: the membrane anchored and
441 proposed DacA regulator protein YbbR (also name CdaR in other bacteria) and the
442 phosphoglucomutase enzyme GlmM (Tosi *et al.*, 2019, Zhu *et al.*, 2016, Gundlach *et al.*,
443 2015, Pham *et al.*, 2016). We could exclude that the observed reduction in cellular c-di-AMP
444 levels in the presence of ammonium or glutamine is mediated by YbbR, as a *ybbR* mutant
445 showed a similar decrease in the c-di-AMP levels as observed for the WT strain (Fig. 8B).
446 GlmM has been shown to be a negative regulator of DacA activity both *in vivo* and *in vitro*
447 (Tosi *et al.*, 2019, Pham *et al.*, 2018). However, since GlmM is likely an essential enzyme in
448 *S. aureus*, we were unable to construct a *glmM* mutant and test its involvement in the
449 observed repression of c-di-AMP synthesis in the presence of ammonium or glutamine as
450 we did for the *gdpP* and *ybbR* mutant strains. Nevertheless, with this work, we not only
451 identified main glutamine and glutamate transporters in *S. aureus,* but we also linked the c-
452 di-AMP signalling network to central nitrogen metabolism in *S. aureus.* It will be interesting to
453 determine in future studies the mechanistic bases for the observed changes in cellular c-di-

454 AMP levels depending on ammonium and glutamine uptake and the involvement of GlmM or
455 other factors in this process.
456
457 **Experimental Procedures**
458 **Bacterial strains and culture conditions**
459 Bacterial strains used in this study are listed in Table 1. *S. aureus* strains were grown in
460 Tryptic Soy Broth (TSB), Tryptic Soy Agar (TSA) or Glucose Defined Medium (GDM). GDM
461 was prepared similar to the chemically defined medium (CDM) reported in an earlier study
462 (Zeden *et al.*, 2018), with some modifications. The detailed content of the GDM (which
463 contains essential vitamins, trace metals, amino acids and glucose as a carbon source but
464 lacks ammonium, glutamine or glutamate as a potential nitrogen or amino acid source) is
465 shown in Table S1. In addition to the GDM, GDM containing glutamine (GDM+Gln),
466 glutamate (GDM+Glu), ammonium (GDM+NH_3), glutamine and ammonium (GDM+Gln+NH_3)
467 or glutamate and ammonium (GDM+Glu+NH_3) were used as part of this study (for exact
468 composition see Table S1). Where indicated, certain amino acids were removed from the
469 GDM recipe during uptake assays and when needed the TSB was supplemented with 0.4 M
470 NaCl. *Escherichia coli* strains were grown in Lysogeny Broth (LB). Where appropriate,
471 antibiotics and/or inducers were added to the media at the following concentration: 200
472 ng/ml anhydrotetracycline (Atet), 90 µg/ml Kanamycin (Kan), 10 µg/ml Erythromycin (Erm),
473 7.5 or 10 µg/ml Chloramphenicol (Cam), Ampicillin (Amp) 100 µg/ml.
474
475 **Bacterial strain construction**
476 All strains used in this study are listed in Table 1 and primers used in this study are listed in
477 Table 2. The transposon insertion sites in the Nebraska transposon mutant library (NTML)
478 strains (Fey *et al.*, 2013) used as part of this study were confirmed by PCR and sequencing.
479 The transposon and surrounding regions were moved by phage transduction using phage 85
480 into the *S. aureus* LAC* strain background. This resulted in the generation of *S. aureus*
481 strains LAC**alsT::tn* (*SAUSA300_1252::tn*; ANG4803), LAC**0914::tn* (*SAUSA300_0914::tn*;

482 ANG5141), LAC**glnQ::tn* (*SAUSA300_1807::tn;* ANG5070), LAC**gltT::tn*
483 (*SAUSA300_2329::tn*; ANG5366) and LAC**gltS::tn* (*SAUSA300_2291::tn*; ANG5367). The
484 transposon insertion in the respective gene was again confirmed by PCR and sequencing.
485 For complementation analysis, the Atet inducible single copy integration plasmids piTET-
486 *alsT* and piTET-*gltS* were constructed. To this end, *alsT* (*SAUSA300_1252*) and *gltS*
487 (*SAUSA300_2291*) were amplified using LAC* chromosomal DNA and primers
488 ANG2250/ANG2251 and ANG3209/ANG3210, respectively. The products as well as piTET
489 were digested with AvrII and SacII and then ligated. Plasmid piTET-*alsT* was recovered in *E.*
490 *coli* strain XL1-Blue (yielding strain ANG3937), shuttled through *E. coli* strain IM08B (strain
491 ANG3955) and then introduced into LAC**alsT::tn* (ANG4803), yielding strain LAC**alsT::tn*
492 piTET-*alsT* (ANG4854). As a control, plasmid piTET was also introduced into LAC**alsT::tn*
493 (ANG4803) yielding strain LAC**alsT::tn* piTET (ANG4853). Plasmid piTET-*gltS* was
494 transformed into *E. coli* XL1-Blue (yielding strain ANG5494), shuttled through *E. coli* IM08B
495 (yielding strain ANG5495) and transformed into *LAC*gltS::tn*, yielding the complement strain
496 LAC**gltS::tn* piTET-*gltS* (ANG5493). As a control, the piTET plasmid was transformed into
497 LAC**gltS::tn* strain, yielding the strain LAC**gltS*:tn piTET (ANG5492). Correct plasmid
498 integration into the *geh* locus was confirmed by PCR and the sequences of all plasmid
499 inserts were confirmed by fluorescent automated sequencing.
500

501 **Bacterial growth curves and amino acid analysis in culture supernatants**

502 *S. aureus* strains LAC* and LAC**alsT::tn* were grown overnight in TSB supplemented with
503 10 µg/ml erythromycin where appropriate. Overnight cultures were then diluted to an OD_{600}
504 of 0.01 into 50 ml of fresh TSB. Cultures were incubated at 37°C with aeration, and OD_{600}
505 values determined every hour. The experiment was performed with three biological
506 replicates and the average OD_{600} values and standard deviations (SDs) were plotted. Using
507 the same cultures, supernatant samples were prepared at the 0, 6, 10 and 12 h time points
508 and amino acid levels determined as previously described using an amino acid analyser
509 (Halsey *et al.*, 2017). For measuring the growth of *S. aureus* strains LAC*, LAC* piTET,

510 LAC**alsT::tn*, LAC**alsT::tn* piTET, LAC**alsT::tn* piTET-*alsT* and LAC**gltS::tn* in GDM,
511 GDM+Gln, GDM+Glu, GDM+NH_3, GDM+Gln+NH_3 or GDM+Glu+NH_3, the bacteria were
512 grown overnight in TSB medium supplemented with chloramphenicol and erythromycin
513 where appropriate. Next day, bacteria from a 1 ml aliquot were washed twice with PBS and
514 diluted to OD_{600} of 0.005 in the indicated GDM. LAC* WT, LAC**alsT::tn* and LAC**gltS::tn*
515 were grown in GDM, GDM+Gln, GDM+Glu, GDM+NH_3, GDM+Gln+NH_3 and GDM+Glu+NH_3
516 while LAC* piTET, LAC**alsT::tn* piTET and LAC**alsT::tn* piTET-*alsT* were grown in
517 GDM+Gln supplemented with 200 ng/ml Atet. One hundred µl of the diluted cultures (six
518 technical replicates) were transferred into wells of a 96-well plate and the plate was then
519 incubated with shaking (500 rpm) in a plate reader and OD_{600} readings determined every 30
520 min. The average values of the technical replicates were determined for each strain. The
521 experiment was performed three times and the average readings and standard deviations
522 were plotted.
523
524 **γ-L-glutamyl hydrazide susceptibility assay**
525 The susceptibility of *S. aureus* LAC*, LAC**alsT::tn* (ANG4803), LAC**0914::tn* (ANG5141)
526 and LAC**glnQ::tn* (ANG5242) to the toxic glutamine analogue γ-L-glutamyl hydrazide (Alfa
527 Aesar, MA, USA) was determined using a similar method as previously reported (Zhu *et al.*,
528 2009). Briefly, the different strains were grown overnight at 37°C in 5 ml TSB medium,
529 supplemented with 10 µg/ml erythromycin where appropriate. Next day, the bacteria were
530 washed twice with PBS, diluted to an OD_{600} of 0.005 in GDM+NH_3. Next, 10 µl of water (0
531 mM control) or 10 µl of a γ-L-glutamyl hydrazide solution dissolved in water was added to
532 0.99 ml aliquots of these bacterial suspension to give a final concentration of 20, 40, 60 or
533 80 µg/ml, respectively. One hundred µl were subsequently transferred in four replicates into
534 wells of a 96 well plates and the plate incubated at 37°C with shaking (500 rpm) in a plate
535 reader and OD_{600} readings determined every 10 min for 12 h. The experiment was
536 performed three times and the average OD_{600} values of the three experiments presented as

537 growth curves. The average values and SDs of the OD_{600} values from the 7 h time point
538 were also plotted against the different γ-L-glutamyl hydrazide concentrations.
539
540 **Microscopic analysis and cell size measurements**
541 The microscopic analysis to determine bacterial cell sizes was performed essentially as
542 previously described (Zeden *et al.*, 2018). Briefly, *S. aureus* strains LAC*, LAC**dacA::kan*,
543 LAC**dacA*$_{G206S}$, LAC**dacA/opuD* (ANG3835) and LAC**dacA/alsT* (ANG3838) were grown
544 overnight at 37°C in TSB or TSB supplemented with 0.4 M NaCl where stated. Next day, the
545 cultures were diluted to an OD_{600} of 0.01 and grown for 3 h at 37°C to mid-exponential phase
546 (OD_{600} of 0.5-0.9). One hundred µl of these cultures were then stained for 20 min at 37°C
547 with Vancomycin-BODIPY FL at a final concentration of 2 µg/ml. One and a half µl of each
548 sample was spotted onto a thin 1.5% agarose gel patch prepared in H_2O or in 0.4 M NaCl
549 and the bacteria subsequently imaged at 1000 x magnification using an Axio Imager A2
550 Zeiss microscope equipped with a GFP filter set. Images were acquired using the ZEN 2012
551 (blue edition) software. The bacterial cell diameters were determined using the Fiji software.
552 Only non-dividing cells (cells without any obvious fluorescent dots or lines at the mid-cell),
553 were used for cell diameter measurements. The cell diameters of 50 cells were measured
554 and the average cell diameter determined. The experiment was conducted three or four
555 times (as indicated in the figure legend) and the averages and standard deviations of the
556 average cell diameters plotted.
557
558 **Uptake assays using ^{14}C-labelled amino acids**
559 Uptake assays were conducted as previously described with some minor modifications
560 (Zeden *et al.*, 2018). Briefly, *S. aureus* strains were streaked on TSA or TSA 0. 4M NaCl
561 plates with appropriate antibiotics and the plates incubated overnight at 37°C. Bacteria were
562 subsequently scraped off from the plates and suspended in 1 ml PBS pH 7.4 buffer and the
563 OD_{600} determined. Fifty ml of GDM+Glu+NH_3 (where indicated with 200 ng/ml of the inducer
564 Atet added) was inoculated with the appropriate bacterial suspensions to an OD_{600} of 0.05.

565 The cultures were grown at 37°C to an OD_{600} between 0.4 and 0.9 and bacteria from an
566 OD_{600} equivalent of 8 were harvested by centrifugation for 10 min at 19,000 x g at RT.
567 Supernatants were discarded and the bacterial pellets were suspended in 2 ml of GDM+NH_3
568 (for glutamine and glutamate uptake assays) or GDM+Glu+NH_3 without serine (for serine
569 uptake assays). The OD_{600} of the cell suspensions were measured and the cells diluted to
570 an OD_{600} of approximately 1. The OD_{600} was re-measured and this measurement used for
571 normalization purposes. Five hundred and fifty µl of these cell suspensions were aliquoted
572 into 50 ml conical tubes and 100 µl used to measure the background radiation, by filtering
573 the cells onto a nitrocellulose membrane filter, followed by a wash step with 16 ml PBS.
574 Then, 6.2 µl of glutamine, L-[14C(U)] (Hartmann Analytic, MC1124), glutamic acid, L-
575 [14C(U)] (Hartmann Analytic, MC156), or serine L-[14C(U)] (Hartmann Analytic, MC265)
576 was added to the remaining 450 µl sample. One hundred µl aliquots were filtered 0, 3, 6 and
577 9 min after addition of the radiolabelled amino acid and the filters were then washed two
578 times with 16 ml of PBS pH 7.4. The filters were subsequently dissolved in 9 ml of
579 scintillation cocktail Filter Count (Perkin Elmer) and the radioactivity measured in counts per
580 minute (CPM) using a Wallac 1409 DSA liquid scintillation counter. The CPMs were then
581 normalized to the OD_{600} reading of the final cell suspension and the means and standard
582 deviations of the CPM/ml OD_{600} = 1 of three or four (as indicated in the figure legends)
583 independent experiments were plotted.
584
585 **Determination of cellular c-di-AMP levels by competitive ELISA**
586 Intracellular c-di-AMP levels in WT LAC* and the indicated *S. aureus* mutant strains were
587 determined using a previously described competitive ELISA method (Underwood *et al.*,
588 2014) and a slightly modified method for the preparation of *S. aureus* samples (Bowman *et*
589 *al.*, 2016). Briefly, single colonies of the WT LAC* strain were picked from TSA plates and
590 used to inoculate 5 ml of GDM, GDM+Gln, GDM+Glu, GDM+NH_3, GDM+Gln+NH_3 and
591 GDM+Glu+NH_3. Colonies of the strains LAC**gdpP::kan* and LAC*Δ*ybbR* were inoculated
592 into 5 ml of GDM, GDM+Glu and GDM+Gln. Colonies of strains LAC* piTET, LAC**alsT::tn*

593 piTET and LAC**alsT::tn* piTET-*alsT* were inoculated into GDM+Gln containing 200 ng/ml
594 Atet and colonies of strains LAC* piTET, LAC**gltS::tn* piTET and LAC**gltS::tn* piTET-*gltS*
595 were inoculated into GDM+Glu supplemented with 200 ng/ml Atet. All cultures were
596 incubated for 18 h at 37°C with shaking. Next, bacteria from 4.5 ml culture were collected by
597 centrifugation, washed three times with PBS and subsequently suspended in 0.75 to 1 ml 50
598 mM Tris pH 8 buffer supplemented with 20 ng/ml lysostaphin and the cells were lysed by
599 bead beating. The lysates were cleared by centrifugation for 5 min at 17,000 x g and the
600 supernatant transferred to a new tube. A small sample aliquot was removed, and the protein
601 concentration determined for normalization purposes using a Pierce BCA protein assay kit
602 (Thermo Scientific, Waltham, MA, USA). The remainder of the sample was heated to 95°C
603 for 10 min. For the competitive ELISA assay, the samples were diluted to a protein
604 concentration of 100, 200, 400 or 500 µg/ml as, appropriate. ELISA plates were prepared by
605 adding 100 µl of coating buffer (50 mM Na_2C0_3, 50 mM $NaHCO_3$, pH 9.6) containing 10
606 µg/ml of the c-di-AMP binding protein $CpaA_{SP}$ to each well of a 96 well NUNC MaxiSorp
607 plate (Thermo Scientific, Waltham, MA, USA) and the plate was incubated for approximately
608 18 h at 4°C. Next, the plate was washed three times with 200 µl PBST pH 7.4 (10 mM
609 Na_2HPO_4 , 1.8 mM KH_2PO_4 137 mM NaCl , 2.7 mM KCl , 0.05% (v/v) Tween 20), blocked for
610 1 h at 18°C with 150 µl blocking solution (1% BSA in PBS pH 7.4) and washed three times
611 with 200 µl PBST. Fifty µl of the samples (three biological replicates and three technical
612 replicates) or standards (two technical replicates) were mixed with 50 µl of a 50 nM
613 biotinylated c-di-AMP solution prepared in 50 mM Tris pH 8 buffer. For the standard curve,
614 c-di-AMP standards were prepared in 50 mM Tris pH 8 buffer at concentrations of 0, 12.5,
615 25, 37.5, 50, 75, 100, 200 nM. Following the addition of the samples and the standards, the
616 plate was incubated for 2 h at 18°C and then washed three times with PBST. Next, 100 µl of
617 a high sensitivity streptavidin-HRP solution (Thermo Scientific, Waltham, MA, USA) diluted
618 1:500 in PBS was added to each well and the plate was incubated for 1 h at 18°C. The plate
619 was washed again 3 x with 200 µl PBST and 100 µl of a developing solution (0.103 M
620 $NaHPO_4$, 0.0485 M citric acid, 500 mg/l o-phenylenediamine dihydrochloride, 0.03% H_2O_2)

621 was added to each well and the plate incubated for 15 min at 18°C. The reaction was then
622 stopped by adding 100 µl of 2 M H_2SO_4 solution. The absorbance was measured in a plate
623 reader at a wavelength of 490 nm and c-di-AMP concentrations were calculated as ng c-di-
624 AMP / mg protein.
625
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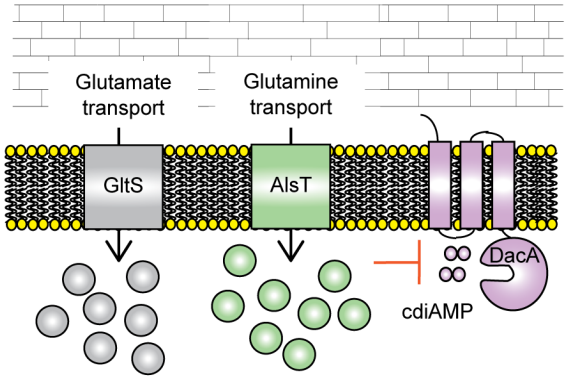
632 findings of this study will be openly available.
633
634 **Author contribution:** MSZ, IK and AG designed the study, MSZ, IK and CFS acquired the
635 data, MSZ, IK, CFS, VCT, PDF and AG, designed experiments, analyzed and interpreted
636 the data, MSZ, IK and AG prepared the figures and wrote the original draft of the
637 manuscript. All authors approved the final version of the manuscript.

638 **Graphical abstract:**
639



640
641
642

643 **Abbreviated Summary:**

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

649

650 **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)
ANG3301	LAC*Δ*ybbR*	(Bowman *et al.*, 2016)
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

651

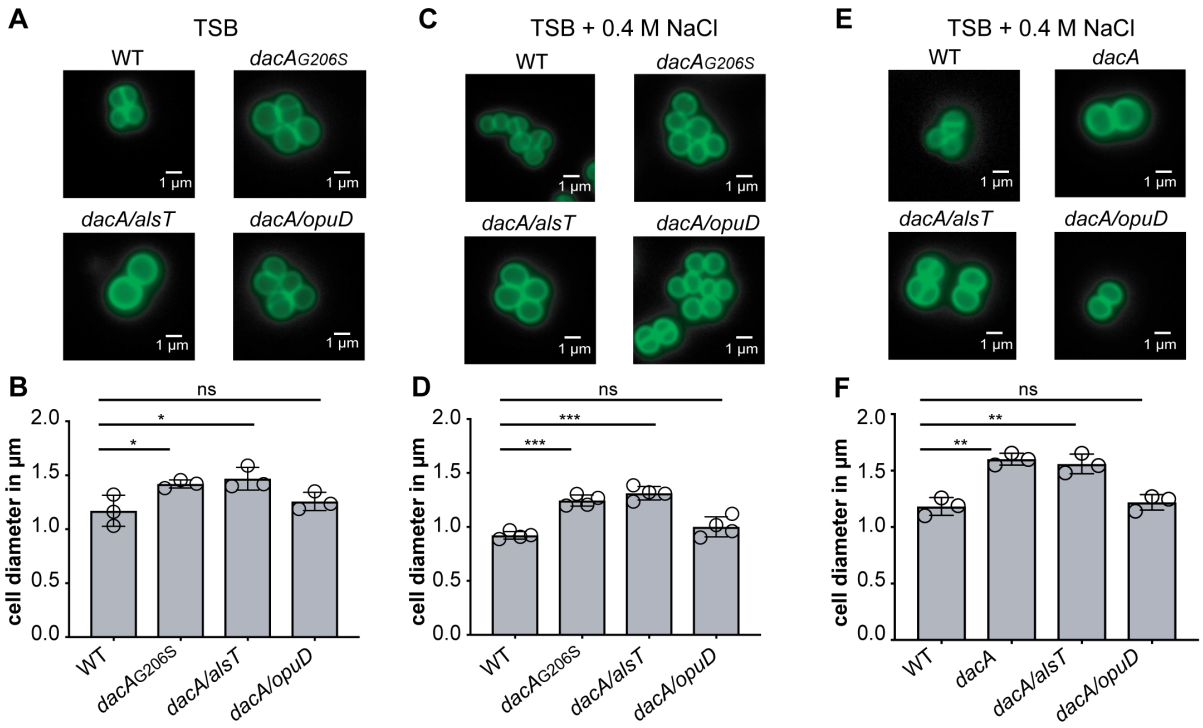
652

653 **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

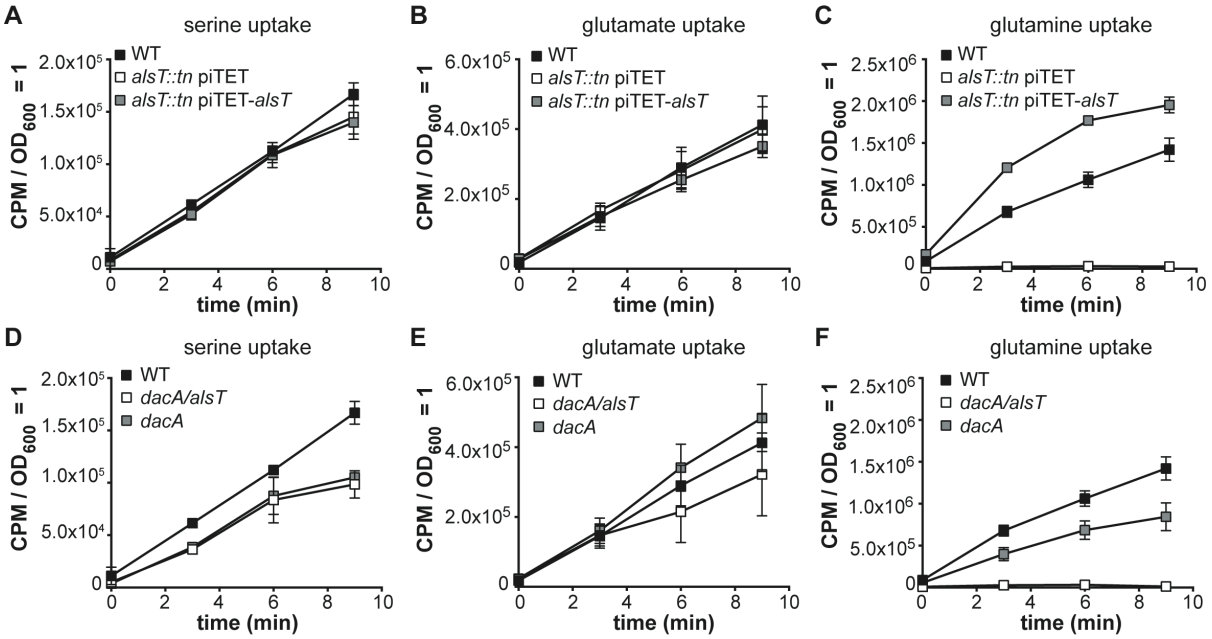
654

655 **Figures and Legends**



656

657 **Fig. 1. Inactivation of the glycine betaine transporter OpuD rescues the cell size of *S.***
658 ***aureus dacA* mutant bacteria.** (A, C, E) Microscopy images of *S. aureus* cells stained with
659 BODIPY-labelled vancomycin. Cultures of *S. aureus* LAC* (WT), LAC**dacA*$_{G206S}$ (*dacA*$_{G206S}$)
660 (panels A and C only), LAC**dacA::kan* (*dacA*) (panel E only) and the suppressor strains
661 LAC**dacA/alsT* (*dacA/alsT*) and LAC**dacA/opuD* (*dacA/opuD*) were grown in (A) TSB or (C
662 and E) TSB 0.4 M NaCl medium and subsequently stained with fluorescently labelled
663 vancomycin. The bacteria were then viewed using a fluorescent microscope and
664 representative images are shown. Scale bars are 1 µm. (B, D, F) Bacterial cell diameter
665 measurements. The diameter of non-dividing bacterial cells was measured as described in
666 the Materials and Method section for *S. aureus* strains grown in (B) TSB or grown in (D and
667 F) TSB 0.4 M NaCl medium. The diameters of 50 cells were determined and the average
668 diameter calculated. The experiment was performed in triplicate (B and F) or quadruplicate
669 (D) and the averages and SDs of the average cell diameters plotted. For statistical analysis,
670 one-way ANOVAs followed by Dunnett's multiple comparison tests were performed (ns = not
671 significant, * = $p<0.01$, ** = $p<0.001$, *** = $p<0.0001$).



672

673 **Fig. 2. AlsT is a glutamine transporter in *S. aureus*.** (A-F) Amino acid uptake assays. (A-
674 C) *S. aureus* strain LAC* (WT), the *alsT* mutant LAC**alsT::tn* piTET (*alsT::tn* piTET) and the
675 complementation strain LAC**alsT::tn* piTET-*alsT* (*alsT::tn* piTET-*alsT*) were grown to mid-log
676 phase in glucose defined media as indicated in the method section and supplemented with
677 200 ng/ml Atet for the strains containing plasmids. Subsequently radiolabelled (A) serine, (B)
678 glutamate or (C) glutamine was added to culture aliquots, samples removed and filtered at
679 the indicated time points and the radioactivity accumulated in the cells measured. The
680 average values and SDs from three (A, C, D, F) or four (B, E) experiments were plotted. (D-
681 F) The same uptake assay experiment was performed as described in (A-C) but using *S.*
682 *aureus* strains LAC**dacA::kan* (*dacA*) and LAC**dacA/alsT* (*dacA/alsT*). The amino acid
683 uptake curve for the LAC* (WT) strain is the same as shown in panels A-C, as all strains
684 were grown and processed at the same time.

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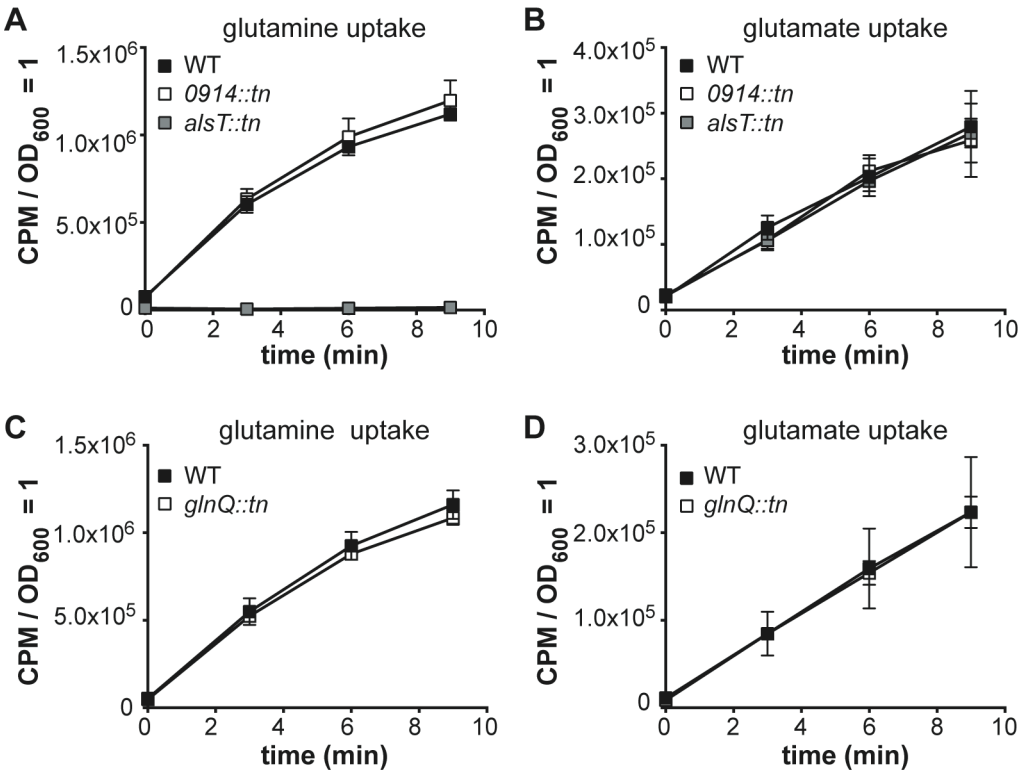

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688

689 **Fig. 3. LAC****0914::tn* **and LAC****glnQ::tn* **strains do not show a defect in glutamine or**
690 **glutamate uptake.** Amino acid uptake assays. (A and B) *S. aureus* strains LAC* (WT),
691 LAC**0914::tn* and LAC**alsT::tn* were grown to mid-log phase in GDM+Glu+NH_3.
692 Subsequently radiolabelled (A) glutamine or (B) glutamate was added to culture aliquots,
693 samples removed and filtered at the indicated time points and the radioactivity accumulated
694 in the cells measured. The average values and SDs from three experiments were plotted. (C
695 and D) Amino acid uptake assays were performed and the data plotted as described in
696 panels A and B, but using *S. aureus* strains LAC* (WT) and LAC**glnQ::tn* (*glnQ::tn*).

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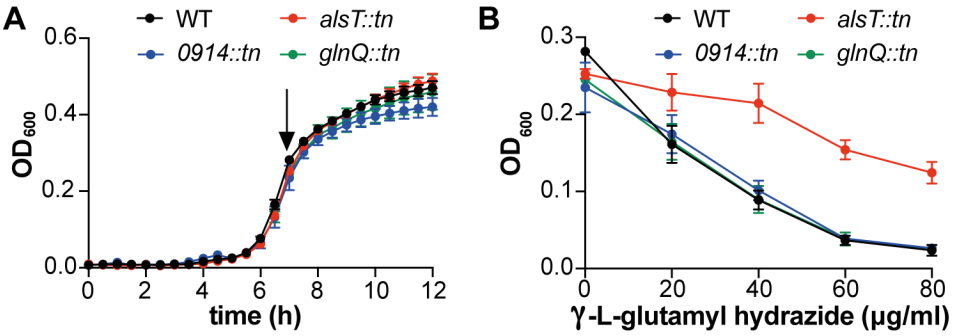
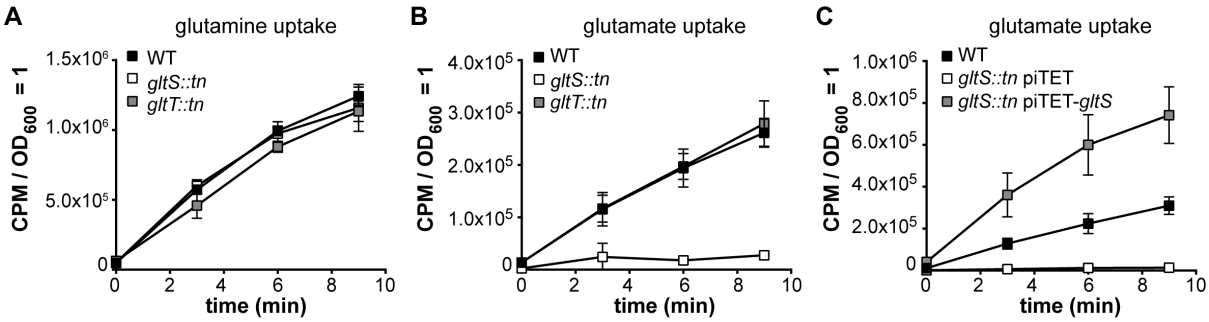


Fig. 4. LAC**alsT::tn*** **shows increased resistance to the toxic glutamine analogue γ-L-glutamyl hydrazide.** (A) Bacterial growth curves. *S. aureus* strains LAC* (WT), LAC**alsT::tn*, LAC**0914::tn* and LAC**glnQ::tn* were grown in GDM+NH_3 medium in 96-well plates and OD_{600} reading determined over 12 h. The average OD_{600} values and SDs from three independent biological replicates were plotted. (B) γ-L-glutamyl hydrazide susceptibility assay. The same *S. aureus* strains as in (A) were grown in GDM+NH_3 medium in the absence or presence of γ-L-glutamyl hydrazide at a final concentration of 20, 40, 60 or 80 µg/ml. OD_{600} reading were determined over 12 h and the complete growth curves are shown in Figure S3. In this graph, the average OD_{600} values from the 7 h time point (marked with an arrow in (A)) and SDs from three biological replicates were plotted against the γ-L-glutamyl hydrazide concentration in the growth medium.

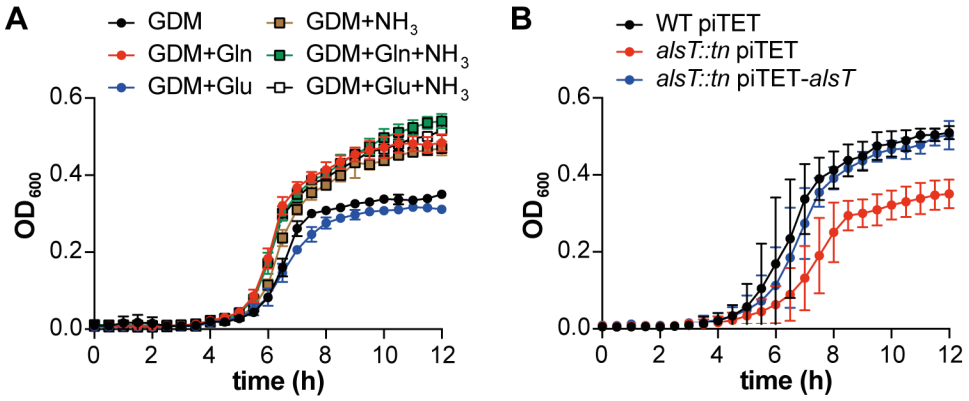
710



711

712 **Fig. 5: GltS is a glutamate transporter in *S. aureus*.** Amino acid uptake assays. (A and B)
713 *S. aureus* strains LAC* (WT), LAC**gltT::tn* and LAC**gltS::tn* were grown to mid-log phase in
714 GDM+Glu+NH_3. Subsequently radiolabelled (A) glutamine or (B) glutamate was added to
715 culture aliquots, samples removed and filtered at the indicated time points and the
716 radioactivity accumulated in the cells measured. (C) Same as (B) but using *S. aureus* strains
717 LAC* (WT), LAC**gltS::tn* piTET and the complementation strain LAC**gltS::tn* piTET-*gltS* and
718 supplementing the GDM+Glu+NH_3 medium with 200 ng/µl Atet. The average values and
719 SDs from three experiments were plotted.

720



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722

723 **Fig. 6. Addition of glutamine and ammonium but not glutamate stimulates the growth**

724 **of *S. aureus* in glucose-containing defined medium.** (A) Bacterial growth curves. The WT

725 *S. aureus* strain LAC* was grown in 96-well plates in glucose-containing defined medium

726 (GDM) or in GDM supplemented with glutamine (Gln), glutamate (Glu), ammonium (NH_3) or

727 combinations thereof as specified in the legend. OD_{600} readings were determined every 30

728 min and the average and SDs of three biological replicates plotted. (B) Bacterial growth

729 curves. *S. aureus* strains LAC* piTET (WT piTET), the *alsT* mutant strain LAC**alsT::tn*

730 piTET and the complementation strain LAC**alsT::tn* piTET-*alsT* were grown in GDM+Gln

731 medium supplemented with 200 ng/µl Atet. OD_{600} readings were determined every 30 min

732 and the average and SDS of three biological replicates plotted.

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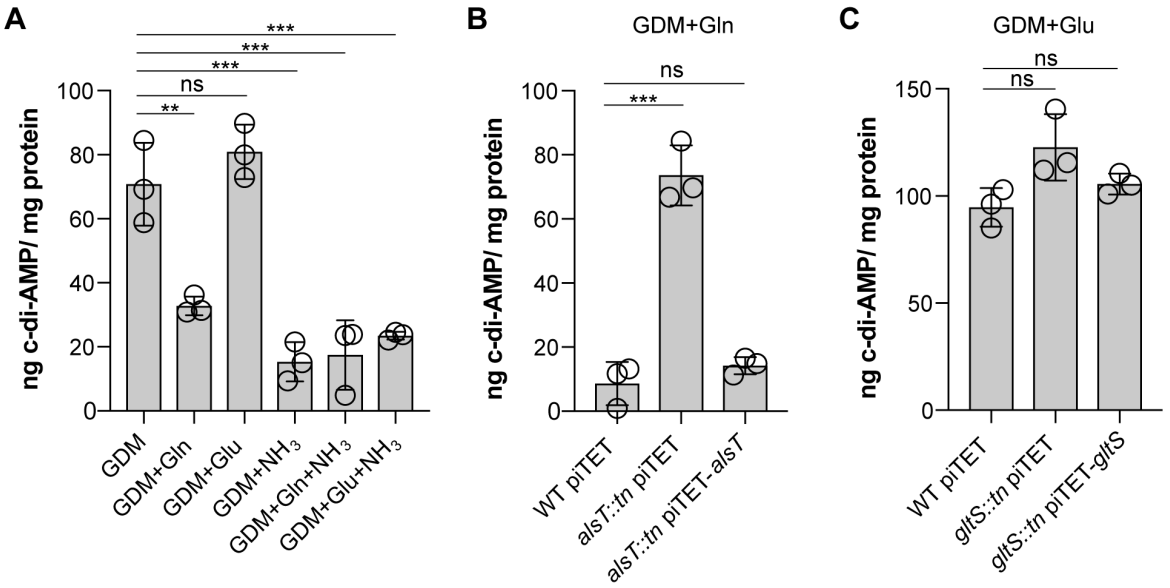
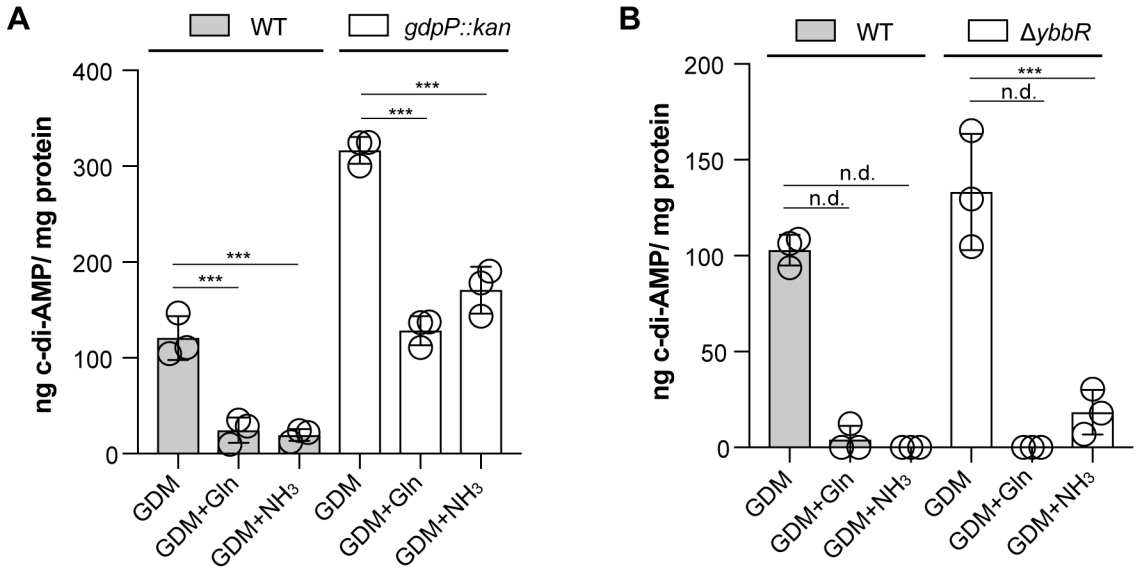


735

736 **Fig. 7. Ammonium and glutamine uptake inhibit c-di-AMP production in *S. aureus*.** (A-
737 C) Cellular c-di-AMP levels. (A) The WT *S. aureus* strain LAC* was grown in GDM or GDM
738 supplemented with glutamine (Gln), glutamate (Glu), ammonium (NH_3), or combinations
739 thereof as indicated on the X-axes. Cell extracts were prepared and c-di-AMP
740 concentrations measured using a competitive ELISA assay. The average values and SDs
741 from three biological replicates were plotted as ng c-di-AMP/mg protein. For statistical
742 analysis, one-way ANOVAs followed by Dunnett's multiple comparison tests were performed
743 to identify statistically significant differences between the different media as compared to
744 GDM (ns = not significant, ** = p<0.001, *** = p<0.0001). (B) *S. aureus* strains LAC* piTET
745 (WT piTET), LAC**alsT::tn* piTET and the complementation strain LAC**alsT::tn* piTET-*alsT*
746 were grown in GDM+Gln supplemented with 200 ng/µl Atet and c-di-AMP levels determined
747 and plotted as described in (A). (C) *S. aureus* strains LAC* piTET (WT piTET), LAC**gltS::tn*
748 piTET and the complementation strain LAC**gltS::tn* piTET-*gltS* were grown in GDM+Glu
749 supplemented with 200 ng/µl Atet and c-di-AMP levels determined and plotted as described
750 in (A).

751



752

753 **Fig. 8. The inhibition of c-di-AMP production by glutamine and ammonium is**
754 **independent of GdpP and YbbR.** (A and B) Cellular c-di-AMP levels. (A) *S. aureus* strains
755 LAC* (WT) and LAC**gdpP::kan* (*gdpP::kan)* were grown in GDM or in GDM containing
756 glutamine (GDM+Gln) or ammonium (GDM+NH_3). Cell extracts were prepared, and c-di-
757 AMP concentrations measured using a competitive ELISA assay. The average values and
758 SDs from three biological replicates were plotted as ng c-di-AMP/ mg protein. For statistical
759 analysis one-way ANOVAs followed by Dunnett's multiple comparison tests were performed
760 to identify statistically significant differences between the different media as compared to the
761 GDM medium (n.d. = not determined. Statistical analysis was not performed due to values
762 below our detection limit; these values were set to 0, ** = $p<0.001$, *** = $p<0.0001$). (B)
763 Same as in (A) but using *S. aureus* strains LAC* (WT) and LAC*Δ*ybbR* (Δ*ybbR*).

764

765

766 **References**

767

768 Anderson, C.B., and Witter, L.D. (1982) Glutamine and proline accumulation by
769 *Staphylococcus aureus* with reduction in water activity. *Appl Environ Microbiol* **43**:
770 1501-1503.
771 Bai, Y., Yang, J., Eisele, L.E., Underwood, A.J., Koestler, B.J., Waters, C.M., Metzger, D.W.,
772 and Bai, G. (2013) Two DHH subfamily 1 proteins in *Streptococcus pneumoniae*
773 possess cyclic di-AMP phosphodiesterase activity and affect bacterial growth and
774 virulence. *J Bacteriol* **195**: 5123-5132.
775 Bai, Y., Yang, J., Zarrella, T.M., Zhang, Y., Metzger, D.W., and Bai, G. (2014) Cyclic di-AMP
776 impairs potassium uptake mediated by a cyclic di-AMP binding protein in
777 *Streptococcus pneumoniae*. *J Bacteriol* **196**: 614-623.
778 Boles, B.R., Thoendel, M., Roth, A.J., and Horswill, A.R. (2010) Identification of genes
779 involved in polysaccharide-independent *Staphylococcus aureus* biofilm formation.
780 *PLoS One* **5**: e10146.
781 Bowman, L., Zeden, M.S., Schuster, C.F., Kaever, V., and Gründling, A. (2016) New Insights
782 into the Cyclic Di-adenosine Monophosphate (c-di-AMP) Degradation Pathway and
783 the Requirement of the Cyclic Dinucleotide for Acid Stress Resistance in
784 *Staphylococcus aureus*. *J Biol Chem* **291**: 26970-26986.
785 Chin, K.H., Liang, J.M., Yang, J.G., Shih, M.S., Tu, Z.L., Wang, Y.C., Sun, X.H., Hu, N.J.,
786 Liang, Z.X., Dow, J.M., Ryan, R.P., and Chou, S.H. (2015) Structural Insights into the
787 Distinct Binding Mode of Cyclic Di-AMP with SaCpaA_RCK. *Biochemistry* **54**: 4936-
788 4951.
789 Commichau, F.M., Heidemann, J.L., Ficner, R., and Stülke, J. (2019) Making and Breaking
790 of an Essential Poison: the Cyclases and Phosphodiesterases That Produce and
791 Degrade the Essential Second Messenger Cyclic di-AMP in Bacteria. *Journal of*
792 *Bacteriology* **201**: e00462-00418.
793 Corrigan, R.M., Abbott, J.C., Burhenne, H., Kaever, V., and Gründling, A. (2011) c-di-AMP is
794 a new second messenger in *Staphylococcus aureus* with a role in controlling cell size
795 and envelope stress. *PLoS Pathog* **7**: e1002217.
796 Corrigan, R.M., Bowman, L., Willis, A.R., Kaever, V., and Gründling, A. (2015) Cross-talk
797 between two nucleotide-signaling pathways in *Staphylococcus aureus*. *J Biol Chem*
798 **290**: 5826-5839.
799 Corrigan, R.M., Campeotto, I., Jeganathan, T., Roelofs, K.G., Lee, V.T., and Gründling, A.
800 (2013) Systematic identification of conserved bacterial c-di-AMP receptor proteins.
801 *Proc Natl Acad Sci U S A* **110**: 9084-9089.
802 Crooke, A.K., Fuller, J.R., Obrist, M.W., Tomkovich, S.E., Vitko, N.P., and Richardson, A.R.
803 (2013) CcpA-independent glucose regulation of lactate dehydrogenase 1 in
804 *Staphylococcus aureus*. *PLoS One* **8**: e54293.
805 DeFrancesco, A.S., Masloboeva, N., Syed, A.K., DeLoughery, A., Bradshaw, N., Li, G.W.,
806 Gilmore, M.S., Walker, S., and Losick, R. (2017) Genome-wide screen for genes
807 involved in eDNA release during biofilm formation by *Staphylococcus aureus*. *Proc*
808 *Natl Acad Sci U S A* **114**: E5969-E5978.
809 Devaux, L., Sleiman, D., Mazzuoli, M.V., Gominet, M., Lanotte, P., Trieu-Cuot, P., Kaminski,
810 P.A., and Firon, A. (2018) Cyclic di-AMP regulation of osmotic homeostasis is
811 essential in Group B *Streptococcus*. *PLoS Genet* **14**: e1007342.
812 Fahmi, T., Faozia, S., Port, G., and Cho, K.H. (2019) The Second Messenger c-di-AMP
813 Regulates Diverse Cellular Pathways Involved in Stress Response, Biofilm
814 Formation, Cell Wall Homeostasis, SpeB Expression and Virulence in *Streptococcus*
815 *pyogenes*. *Infect Immun* **87**: e00147-00119.

816 Fey, P.D., Endres, J.L., Yajjala, V.K., Widhelm, T.J., Boissy, R.J., Bose, J.L., and Bayles,
817 K.W. (2013) A genetic resource for rapid and comprehensive phenotype screening of
818 nonessential *Staphylococcus aureus* genes. *MBio* **4**: e00537-00512.
819 Fridkin, S.K., Hageman, J.C., Morrison, M., Sanza, L.T., Como-Sabetti, K., Jernigan, J.A.,
820 Harriman, K., Harrison, L.H., Lynfield, R., Farley, M.M., and Active Bacterial Core
821 Surveillance Program of the Emerging Infections Program, N. (2005) Methicillin-
822 resistant *Staphylococcus aureus* disease in three communities. *N Engl J Med* **352**:
823 1436-1444.
824 Fuller, J.R., Vitko, N.P., Perkowski, E.F., Scott, E., Khatri, D., Spontak, J.S., Thurlow, L.R.,
825 and Richardson, A.R. (2011) Identification of a lactate-quinone oxidoreductase in
826 *Staphylococcus aureus* that is essential for virulence. *Front Cell Infect Microbiol* **1**:
827 19.
828 Gründling, A., and Schneewind, O. (2007) Genes required for glycolipid synthesis and
829 lipoteichoic acid anchoring in *Staphylococcus aureus*. *J Bacteriol* **189**: 2521-2530.
830 Gundlach, J., Commichau, F.M., and Stülke, J. (2018) Perspective of ions and messengers:
831 an intricate link between potassium, glutamate, and cyclic di-AMP. *Curr Genet* **64**:
832 191-195.
833 Gundlach, J., Herzberg, C., Hertel, D., Thurmer, A., Daniel, R., Link, H., and Stülke, J.
834 (2017a) Adaptation of *Bacillus subtilis* to Life at Extreme Potassium Limitation. *MBio*
835 **8**: e00861-00817.
836 Gundlach, J., Herzberg, C., Kaever, V., Gunka, K., Hoffmann, T., Weiss, M., Gibhardt, J.,
837 Thurmer, A., Hertel, D., Daniel, R., Bremer, E., Commichau, F.M., and Stülke, J.
838 (2017b) Control of potassium homeostasis is an essential function of the second
839 messenger cyclic di-AMP in *Bacillus subtilis*. *Sci Signal* **10**: eaal3011.
840 Gundlach, J., Krüger, L., Herzberg, C., Turdiev, A., Poehlein, A., Tascon, I., Weiß, M.,
841 Hertel, D., Daniel, R., Hänelt, I., Lee, V.T., and Stülke, J. (2019) Sustained sensing in
842 potassium homeostasis: Cyclic di-AMP controls potassium uptake by KimA at the
843 levels of expression and activity. *J Biol Chem* **294**: 9605-9614.
844 Gundlach, J., Mehne, F.M., Herzberg, C., Kampf, J., Valerius, O., Kaever, V., and Stülke, J.
845 (2015) An Essential Poison: Synthesis and Degradation of Cyclic Di-AMP in *Bacillus*
846 *subtilis*. *J Bacteriol* **197**: 3265-3274.
847 Halsey, C.R., Lei, S., Wax, J.K., Lehman, M.K., Nuxoll, A.S., Steinke, L., Sadykov, M.,
848 Powers, R., and Fey, P.D. (2017) Amino Acid Catabolism in *Staphylococcus aureus*
849 and the Function of Carbon Catabolite Repression. *MBio* **8**: e01434-01416.
850 Hengge, R. (2009) Principles of c-di-GMP signalling in bacteria. *Nat Rev Microbiol* **7**: 263-
851 273.
852 Huynh, T.N., Choi, P.H., Sureka, K., Ledvina, H.E., Campillo, J., Tong, L., and Woodward,
853 J.J. (2016) Cyclic di-AMP targets the cystathionine beta-synthase domain of the
854 osmolyte transporter OpuC. *Mol Microbiol* **102**: 233-243.
855 Kelly, B., and O'Neill, L.A. (2015) Metabolic reprogramming in macrophages and dendritic
856 cells in innate immunity. *Cell Res* **25**: 771-784.
857 Kim, H., Youn, S.J., Kim, S.O., Ko, J., Lee, J.O., and Choi, B.S. (2015) Structural Studies of
858 Potassium Transport Protein KtrA Regulator of Conductance of K+ (RCK) C Domain
859 in Complex with Cyclic Diadenosine Monophosphate (c-di-AMP). *J Biol Chem* **290**:
860 16393-16402.
861 Kluytmans, J., van Belkum, A., and Verbrugh, H. (1997) Nasal carriage of *Staphylococcus*
862 *aureus*: epidemiology, underlying mechanisms, and associated risks. *Clin Microbiol*
863 *Rev* **10**: 505-520.
864 Lehman, M.K., Nuxoll, A.S., Yamada, K.J., Kielian, T., Carson, S.D., and Fey, P.D. (2019)
865 Protease-Mediated Growth of *Staphylococcus aureus* on Host Proteins Is *opp3*
866 Dependent. *MBio* **10**: e02553-02518.
867 Mehne, F.M., Gunka, K., Eilers, H., Herzberg, C., Kaever, V., and Stülke, J. (2013) Cyclic di-
868 AMP homeostasis in *Bacillus subtilis*: both lack and high level accumulation of the
869 nucleotide are detrimental for cell growth. *J Biol Chem* **288**: 2004-2017.

870 Monk, I.R., Shah, I.M., Xu, M., Tan, M.W., and Foster, T.J. (2012) Transforming the
871 untransformable: application of direct transformation to manipulate genetically
872 *Staphylococcus aureus* and *Staphylococcus epidermidis*. *MBio* **3**: e00277-00211.
873 Monk, I.R., Tree, J.J., Howden, B.P., Stinear, T.P., and Foster, T.J. (2015) Complete Bypass
874 of Restriction Systems for Major *Staphylococcus aureus* Lineages. *MBio* **6**: e00308-
875 00315.
876 Moscoso, J.A., Schramke, H., Zhang, Y., Tosi, T., Dehbi, A., Jung, K., and Gründling, A.
877 (2015) Binding of Cyclic Di-AMP to the *Staphylococcus aureus* Sensor Kinase KdpD
878 Occurs via the Universal Stress Protein Domain and Downregulates the Expression
879 of the Kdp Potassium Transporter. *J Bacteriol* **198**: 98-110.
880 Pham, H.T., Nhiep, N.T.H., Vu, T.N.M., Huynh, T.N., Zhu, Y., Huynh, A.L.D., Chakrabortti,
881 A., Marcellin, E., Lo, R., Howard, C.B., Bansal, N., Woodward, J.J., Liang, Z.X., and
882 Turner, M.S. (2018) Enhanced uptake of potassium or glycine betaine or export of
883 cyclic-di-AMP restores osmoresistance in a high cyclic-di-AMP *Lactococcus lactis*
884 mutant. *PLoS Genet* **14**: e1007574.
885 Pham, H.T., and Turner, M.S. (2019) Onwards and [K(+)]upwards: a new potassium
886 importer under the spell of cyclic-di-AMP. *J Bacteriol* **201**: e00150-00119.
887 Pham, T.H., Liang, Z.X., Marcellin, E., and Turner, M.S. (2016) Replenishing the cyclic-di-
888 AMP pool: regulation of diadenylate cyclase activity in bacteria. *Curr Genet* **62**: 731-
889 738.
890 Quintana, I.M., Gibhardt, J., Turdiev, A., Hammer, E., Commichau, F.M., Lee, V.T., Magni,
891 C., and Stülke, J. (2019) The KupA and KupB proteins of *Lactococcus lactis* IL1403
892 are novel c-di-AMP receptor proteins responsible for potassium uptake. *J Bacteriol*
893 **201**: e00028-00019.
894 Richardson, A.R., Libby, S.J., and Fang, F.C. (2008) A nitric oxide-inducible lactate
895 dehydrogenase enables *Staphylococcus aureus* to resist innate immunity. *Science*
896 **319**: 1672-1676.
897 Rocha, R., Teixeira-Duarte, C.M., Jorge, J.M.P., and Morais-Cabral, J.H. (2019)
898 Characterization of the molecular properties of KtrC, a second RCK domain that
899 regulates a Ktr channel in *Bacillus subtilis*. *J Struct Biol* **205**: 34-43.
900 Römling, U. (2008) Great times for small molecules: c-di-AMP, a second messenger
901 candidate in Bacteria and Archaea. *Sci Signal* **1**: pe39.
902 Schuster, C.F., Bellows, L.E., Tosi, T., Campeotto, I., Corrigan, R.M., Freemont, P., and
903 Gründling, A. (2016) The second messenger c-di-AMP inhibits the osmolyte uptake
904 system OpuC in *Staphylococcus aureus*. *Sci Signal* **9**: ra81.
905 Schuster, C.F., Wiedemann, D.M., Kirsebom, F.C.M., Santiago, M., Walker, S., and
906 Gründling, A. (2019) High-throughput transposon sequencing highlights the cell wall
907 as an important barrier for osmotic stress in methicillin resistant *Staphylococcus*
908 *aureus* and underlines a tailored response to different osmotic stressors. *Mol*
909 *Microbiol*: DOI: 10.1111/mmi.14433.
910 Schuurman-Wolters, G.K., and Poolman, B. (2005) Substrate specificity and ionic regulation
911 of GlnPQ from *Lactococcus lactis*. An ATP-binding cassette transporter with four
912 extracytoplasmic substrate-binding domains. *J Biol Chem* **280**: 23785-23790.
913 Spahich, N.A., Vitko, N.P., Thurlow, L.R., Temple, B., and Richardson, A.R. (2016)
914 *Staphylococcus aureus* lactate- and malate-quinone oxidoreductases contribute to
915 nitric oxide resistance and virulence. *Mol Microbiol* **100**: 759-773.
916 Sureka, K., Choi, P.H., Precit, M., Delince, M., Pensinger, D., Huynh, T.N., Jurado, A.R.,
917 Goo, Y.A., Sadilek, M., Iavarone, A.T., Sauer, J.D., Tong, L., and Woodward, J.J.
918 (2014) The cyclic dinucleotide c-di-AMP is an allosteric regulator of metabolic
919 enzyme function. *Cell* **158**: 1389-1401.
920 Teh, W.K., Dramsi, S., Tolker-Nielsen, T., Yang, L., and Givskov, M. (2019) Increased
921 Intracellular Cyclic di-AMP Levels Sensitize *Streptococcus gallolyticus* subsp.
922 gallolyticus to Osmotic Stress and Reduce Biofilm Formation and Adherence on
923 Intestinal Cells. *J Bacteriol* **201**: e00597-00518.

924 Tolner, B., Ubbink-Kok, T., Poolman, B., and Konings, W.N. (1995) Characterization of the
925 proton/glutamate symport protein of *Bacillus subtilis* and its functional expression in
926 *Escherichia coli*. *J Bacteriol* **177**: 2863-2869.
927 Tosi, T., Hoshiga, F., Millership, C., Singh, R., Eldrid, C., Patin, D., Mengin-Lecreulx, D.,
928 Thalassinos, K., Freemont, P., and Gründling, A. (2019) Inhibition of the
929 *Staphylococcus aureus* c-di-AMP cyclase DacA by direct interaction with the
930 phosphoglucosamine mutase GlmM. *PLoS Pathog* **15**: e1007537.
931 Underwood, A.J., Zhang, Y., Metzger, D.W., and Bai, G. (2014) Detection of cyclic di-AMP
932 using a competitive ELISA with a unique pneumococcal cyclic di-AMP binding
933 protein. *J Microbiol Methods* **107**: 58-62.
934 Vitko, N.P., Spahich, N.A., and Richardson, A.R. (2015) Glycolytic dependency of high-level
935 nitric oxide resistance and virulence in *Staphylococcus aureus*. *MBio* **6**: e00045-
936 00015.
937 Whiteley, A.T., Garelis, N.E., Peterson, B.N., Choi, P.H., Tong, L., Woodward, J.J., and
938 Portnoy, D.A. (2017) c-di-AMP modulates *Listeria monocytogenes* central
939 metabolism to regulate growth, antibiotic resistance and osmoregulation. *Mol*
940 *Microbiol* **104**: 212-233.
941 Whiteley, A.T., Pollock, A.J., and Portnoy, D.A. (2015) The PAMP c-di-AMP Is Essential for
942 *Listeria monocytogenes* Growth in Rich but Not Minimal Media due to a Toxic
943 Increase in (p)ppGpp. *Cell Host Microbe* **17**: 788-798.
944 Wicke, D., Schulz, L.M., Lentes, S., Scholz, P., Poehlein, A., Gibhardt, J., Daniel, R.,
945 Ischebeck, T., and Commichau, F.M. (2019) Identification of the first glyphosate
946 transporter by genomic adaptation. *Environ Microbiol* **21**: 1287-1305.
947 Witte, C.E., Whiteley, A.T., Burke, T.P., Sauer, J.D., Portnoy, D.A., and Woodward, J.J.
948 (2013) Cyclic di-AMP is critical for *Listeria monocytogenes* growth, cell wall
949 homeostasis, and establishment of infection. *MBio* **4**: e00282-00213.
950 Woodward, J.J., Iavarone, A.T., and Portnoy, D.A. (2010) c-di-AMP secreted by intracellular
951 *Listeria monocytogenes* activates a host type I interferon response. *Science* **328**:
952 1703-1705.
953 Zarrella, T.M., Metzger, D.W., and Bai, G. (2018) Stress Suppressor Screening Leads to
954 Detection of Regulation of Cyclic di-AMP Homeostasis by a Trk Family Effector
955 Protein in *Streptococcus pneumoniae*. *J Bacteriol* **200**: e00045-00018.
956 Zeden, M.S., Schuster, C.F., Bowman, L., Zhong, Q., Williams, H.D., and Gründling, A.
957 (2018) Cyclic di-adenosine monophosphate (c-di-AMP) is required for osmotic
958 regulation in *Staphylococcus aureus* but dispensable for viability in anaerobic
959 conditions. *J Biol Chem* **293**: 3180-3200.
960 Zhu, B., and Stülke, J. (2018) SubtiWiki in 2018: from genes and proteins to functional
961 network annotation of the model organism *Bacillus subtilis*. *Nucleic Acids Res* **46**:
962 D743-D748.
963 Zhu, Y., Pham, T.H., Nhiep, T.H., Vu, N.M., Marcellin, E., Chakrabortti, A., Wang, Y.,
964 Waanders, J., Lo, R., Huston, W.M., Bansal, N., Nielsen, L.K., Liang, Z.X., and
965 Turner, M.S. (2016) Cyclic-di-AMP synthesis by the diadenylate cyclase CdaA is
966 modulated by the peptidoglycan biosynthesis enzyme GlmM in *Lactococcus lactis*.
967 *Mol Microbiol* **99**: 1015-1027.
968 Zhu, Y., Xiong, Y.Q., Sadykov, M.R., Fey, P.D., Lei, M.G., Lee, C.Y., Bayer, A.S., and
969 Somerville, G.A. (2009) Tricarboxylic acid cycle-dependent attenuation of
970 *Staphylococcus aureus* in vivo virulence by selective inhibition of amino acid
971 transport. *Infect Immun* **77**: 4256-4264.
972
973