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Cyclice-di-AMP synthesis by the diadenylate cyclase CdaA is modulated by the peptidoglycan biosynthesis enzyme GlmM in *Lactococcus lactis*.

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Running Title: GlmM modulates c-di-AMP synthesis in *Lactococcus.*

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Keywords: c-di-AMP; osmotic stress, *Lactococcus*, peptidoglycan, GlmM

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SUMMARY

The second messenger cyclic-di-AMP plays important roles in cell growth, virulence, cell wall homeostasis, potassium transport and ~~is known to~~ affects resistance to antibiotics, heat and osmotic stress. Most Firmicutes contain only one c-di-AMP synthesizing diadenylate cyclase (CdaA) ~~and apart from the positively regulating CdaR protein~~however~~,~~ little is known about signals and effectors controlling CdaA activity. In this study, a genetic screening method was used to identify components which affect the c-di-AMP level in *Lactococcus*. We characterised suppressor mutations that restored osmoresistance to spontaneous c-di-AMP phosphodiesterase *gdpP* mutants which contain high c-di-AMP levels. Functionally ~~D~~destructive and restorative mutations were identified in the *cdaA* and *gdpP* genes, respectively, which le~~a~~d to lower c-di-AMP levels. A mutation was also identified in the phosphoglucosamine mutase ~~encoding~~ gene *glmM* which is commonly ~~commonly~~ located within~~in~~ the *cdaA* operon in bacteria. The *glmM* I154F mutation resulted in a lowering of the c-di-AMP level and a reduction in the key peptidoglycan precursor UDP-N-acetylglucosamine in *L. lactis*. C-di-AMP synthesis by CdaA was shown to be inhibited by GlmM and more by GlmM^{I154F}, while the GlmM^{I154F} variant was found to bind more strongly to CdaA than GlmM~~Using *Escherichia coli* as a heterologous expression host, c-di-AMP synthesis by CdaA was shown to be reduced by GlmM and furthermore by the GlmM^{I154F} variant~~. These findings identify GlmM as a c-di-AMP level modulating protein and provide a direct connection between c-di-AMP synthesis and peptidoglycan biosynthesis.

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INTRODUCTION

Ubiquitous nucleotide second messengers including cAMP, cGMP, (p)ppGpp, c-di-GMP and c-di-AMP have been shown to control a wide range of bacterial processes (Kalia *et al.*, 2013). The recently discovered ~~essential~~ nucleotide c-di-AMP has been found in some Gram-negative and many Gram-positive bacteria and has been shown to control bacterial growth, cell wall homeostasis, potassium transport, antibiotic resistance, osmotolerance, cell lysis, metabolism and immunomodulation (Woodward *et al.*, 2010; Corrigan *et al.*, 2011; Luo and Helmann, 2012a; Pozzi *et al.*, 2012; Smith *et al.*, 2012; Bai *et al.*, 2013; Mehne *et al.*, 2013; Witte *et al.*, 2013; Bai

64 *et al.*, 2014; Sureka *et al.*, 2014; Ye *et al.*, 2014). Up until recently c-di-AMP was considered
65 essential for cell viability, however work in *Listeria monocytogenes* has shown that c-di-AMP is
66 not essential for growth in minimal media (Whiteley *et al.*, 2015). For optimal growth on
67 nutrient rich media, however, ~~L~~levels of c-di-AMP need to be strictly controlled in the cell by
68 modulation of expression and/or activity of c-di-AMP synthesis and degradation enzymes
69 (Corrigan and Grundling, 2013). Most bacteria possess one diadenylate cyclase (DAC) enzyme
70 (CdaA [also called YbbP and DacA]) (Romling, 2008; Corrigan and Grundling, 2013) while
71 *Bacillus subtilis* has two additional DACs (CdaS [also called YojJ] and DisA) (Romling, 2008).
72 DHH/DHHA1 domain containing phosphodiesterases (PDEs) such as membrane bound GdpP-
73 type (also called YybT or Pde1) and standalone PDEs carry out c-di-AMP hydrolysis (Bai *et al.*,
74 2013; Corrigan and Grundling, 2013). Recently another c-di-AMP phosphodiesterase (PgpH) of
75 the HD-domain family has been identified in *L.~~isteria~~ monocytogenes* (Huynh *et al.*, 2015).
76 Diadenylate cyclase activity of the DNA integrity scanning protein DisA has been shown to be
77 negatively regulated by another DNA associated protein RadA (Zhang and He, 2013) while
78 CdaS is regulated by its own N-terminal inhibitory domain (Mehne *et al.*, 2014). The membrane
79 bound CdaA protein has been shown to be ~~positively~~ regulated by the YbbR-domain containing
80 protein CdaR through direct protein-protein interaction (Mehne *et al.*, 2013). GdpP and PgpH
81 activity is inhibited by the stringent response compound (p)ppGpp and ~~it~~ GdpP contains a
82 possibly regulatory hemin binding PAS domain (Rao *et al.*, 2010; Tan *et al.*, 2013; Huynh *et al.*,
83 2015). Expression of *gdpP* in *B. subtilis* is also transcriptionally regulated by σ^D (Luo and
84 Helmann, 2012b). Recent work has ~~also~~ identified cross-talk between c-di-AMP and (p)ppGpp
85 signalling nucleotides with both high and low c-di-AMP levels triggering high (p)ppGpp (Liu *et
86 al.*, 2006; Corrigan *et al.*, 2015; Whiteley *et al.*, 2015). Apart from these studies, little is known
87 regarding stimuli which control the c-di-AMP level within a cell. In single DAC containing
88 bacteria, c-di-AMP has been shown to be involved in cell wall homeostasis and osmotolerance
89 and therefore stimuli related to these processes are likely to be involved.

90 Modulation of signal transduction can occur by c-di-AMP binding to effector proteins
91 and RNA molecules which then influence their activity. Effectors which bind c-di-AMP have
92 been identified including the transcription factor DarR from *Mycobacterium smegmatis* (Zhang
93 *et al.*, 2013); the K^+ transport gating protein KtrA, ion transporter CpaA and PII-like signal
94 transduction protein PstA from *Staphylococcus aureus* (Corrigan *et al.*, 2013); the *ydaO*
95 riboswitch class in *Actinobacteria*, *Bacillales*, *Clostridia* and *Cyanobacteria* (Nelson *et al.*,
96 2013); and pyruvate carboxylase, CbpA, CbpB, NrdR, PstA and several other proteins which

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may bind c-di-AMP indirectly in *L. monocytogenes* (Sureka *et al.*, 2014). Crystal structures of pyruvate carboxylase (Sureka *et al.*, 2014), PII-like signal transduction proteins (Campeotto *et al.*, 2015; Choi *et al.*, 2015; Gundlach *et al.*, 2015a; Muller *et al.*, 2015), RCK domains of KtrA and CpaA (Chin *et al.*, 2015; Kim *et al.*, 2015) and the *ydaO* riboswitch (Gao and Serganov, 2014; Jones and Ferre-D'Amare, 2014; Ren and Patel, 2014) have provided insights into c-di-AMP binding sites. The first structure of a CdaA-type DAC has also been recently reported (Rosenberg *et al.*, 2015). Many of these c-di-AMP targets however are yet to be thoroughly characterized and thus their roles in c-di-AMP controlled phenotypes are still to be elucidated.

Previous research has identified osmosensitive phenotypes for *gdpP* mutants of *L. lactis* and *S. aureus* (Smith *et al.*, 2012; Corrigan *et al.*, 2013). In this study we used a genetic screening approach to identify proteins involved in controlling the c-di-AMP level and thus osmotolerance in *L. lactis*. The roles of the c-di-AMP synthesis and hydrolysis enzymes (CdaA and GdpP) were confirmed, while the broadly conserved cell wall biosynthesis enzyme GlmM was identified as a new c-di-AMP level modulator. ~~The results presented identify a connection between c-di-AMP signalling and cell wall biosynthesis pathways.~~

RESULTS

*Mutations in the diadenylate cyclase encoding gene (***cdaA***) restore osmoresistance in* **gdpP** *defective mutants*

Previous studies in our laboratory identified a role of the c-di-AMP phosphodiesterase GdpP in osmotolerance in two independent strains of *L. lactis* (Smith *et al.*, 2012). During this work we noticed that upon plating of spontaneous osmosensitive *gdpP* mutants (OS strains) on high salt containing agar, the presence of healthy looking colonies at low frequency (~1 in 10,000 to 100,000, as seen in Fig.~~ure~~ 1, panels A and C in (Smith *et al.*, 2012)). The OS strains used here contain either ~~a~~ single nucleotide changes (OS1 and OS3 contain *gdpP*$_{A573D}$ and *gdpP*$_{E472Stop}$, respectively) or a single nucleotide insertion (OS2 contains *gdpP*$_{K122Stop}$) (Smith *et al.*, 2012). We hypothesised that the reason for osmosensitivity of *gdpP* mutant strains was due to a high intracellular c-di-AMP level and that osmoresistant suppressor mutants (OR strains) which could grow on high salt agar would contain mutations which would lower the c-di-AMP level. Two colonies derived from OS1 growing on high salt agar (named OR1 and OR2 [Table S1]) were picked and confirmed to be stably osmoresistant (Fig. 1A). In fact OR2 became dependent upon high osmotic conditions for efficient growth (Fig. 1A). To identify the mutation(s) in OR1 and

129 OR2, whole genome sequencing was performed. Only one common gene was mutated in both
130 OR1 and OR2 which was *llmg_0448* (or herein named *cdaA*) (Table 1). This gene encodes a
131 diadenylate cyclase (DAC) which has homology to CdaA (also termed DacA and YbbP). The
132 mutations in *cdaA* are likely to cause ~~abnormal~~ abolished protein expression (CdaA^{E11frameshift} in
133 OR1) or reduced activity (CdaA^{M136I} in OR2) of ~~the enzyme~~CdaA resulting in a lowering of the
134 intracellular c-di-AMP level. Indeed intracellular c-di-AMP levels were significantly lower in the
135 OR1 and OR2 strains than in the parent OS1, which contains a *gdpP* mutation, though not as low
136 as for wild-type MG1363 (below detection limit) (Fig. 1B). CdaA in *L. lactis* is the only protein
137 containing a DAC domain. These results show that the c-di-AMP level influences salt resistance
138 in *L. lactis* and can be altered through mutation in the DAC~~c-di-AMP synthesis~~ (CdaA) ~~or~~ and
139 PDE~~degradation enzyme~~ (GdpP).

140 To identify more sites within CdaA which are important for c-di-AMP synthesis and also
141 other genes which may affect ~~the~~ c-di-AMP level and thus osmotolerance, 158 additional salt
142 resistant suppressor mutants from several different *gdpP* mutant backgrounds (OS1 [n=42], OS2
143 [n=112] and OS3 [n=4] [Table S1]) were obtained and characterized. The *cdaA* gene was
144 amplified by PCR and sequenced and 66 independent *cdaA* mutations were identified in total
145 (Fig. 2 and Fig. S1). These mutations resulted in amino acid changes, frameshift mutations, a
146 start codon change (from TTG to TTA) and the insertion of an *IS*905 element 12-bp upstream of
147 *cdaA*. Changes were found at the extracellular N-terminal region, both in and between the three
148 transmembrane spanning domains and also in the DAC enzymatic domain (Fig. 2), and therefore
149 may affect protein expression, signal sensing, oligomerization and/or enzymatic activity. A ~~vast~~
150 majority of the mutations ~~resulted in~~resulted in a ~~a~~ D123Y change in CdaA, suggesting a
151 significant proportion of sibling mutants were present in our culture stocks. CdaA mutations
152 were obtained in two independent *gdpP* mutants derived from both MG1363 (OS1 and OS2) and
153 an industrial *L. lactis* strain background (OS3). Salt resistant industrial strain derivatives OR3
154 and OR4 which contained CdaA^{T50K} and CdaA^{A195D} changes, respectively ~~which resulted in the~~
155 ~~same salt resistant phenotype and~~, had significantly reduced ~~reduction in~~ c-di-AMP levels
156 compared to their parent OS3 (Fig. 1C & 1D). Out of all the salt resistant suppressor mutants
157 obtained, only five mutants possessed no changes in the *cdaA* gene. Another way the cell might
158 reduce the c-di-AMP level is through restoration of PDE activity. Therefore the *gdpP* gene was
159 sequenced in the five mutants and in four (derived from OS1) new mutations were observed. The
160 OS1 parent contains an A573D change in GdpP from wild-type MG1363 (Smith *et al.*, 2012).
161 The four osmoresistant suppressor mutants either had reverted back to alanine at this position or

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162 a similar residue (glycine) thereby likely restoring c-di-AMP PDE activity. The final
163 characterized osmoresistant suppressor mutant (OR5) still possessed the same *gdpP* mutation
164 present in its parent OS2 (K122Stop) and therefore was predicted to still have no or low PDE
165 activity.
166

167 ***GlmM affects osmoresistance as well as peptidoglycan precursor and c-di-AMP levels***

168 The osmoresistant mutant (OR5) which contained unaltered *cdaA* and *gdpP* gene sequences as
169 its osmosensitive parent strain (OS2) was confirmed to be salt resistant (Fig. 3A). Interestingly
170 OR5 had a significantly reduced level~~s~~ of c-di-AMP (Fig. 3B), which ~~were similarly low as~~was
171 similar to that observed for *cdaA* mutants OR1 and OR2 (Fig. 1B). Whole genome sequencing
172 was carried out on this strain and its parent (OS2), and only one single nucleotide difference
173 (A→T) was identified. This change was in the *glmM* (*femD*) gene and was confirmed by Sanger
174 sequencing as causing an I154F amino acid change in the encoded protein. GlmM is the
175 phosphoglucosamine mutase enzyme responsible for interconversion of glucosamine-6-
176 phosphate to glucosamine-1-phosphate and forms an early part of the pathway leading to the
177 biosynthesis of cell wall peptidoglycan and other cell wall polymers including the cell wall
178 pellicle produced by *L. lactis* (Fig. 3C) (Chapot-Chartier *et al.*, 2010). The *glmM* gene is present
179 in the same operon as ~~the~~ *cdaA* ~~gene~~ in *L. lactis* (Fig. 3D), a genetic arrangement that is highly
180 conserved in almost all Firmicutes (Bai *et al.*, 2013; Corrigan and Grundling, 2013; Dengler *et*
181 *al.*, 2013; Mehne *et al.*, 2013). To determine if cell wall biosynthesis precursor levels are
182 affected by changes in the intracellular c-di-AMP level and by the GlmM I154F mutation, we
183 measured intracellular UDP-N-acetylglucosamine (UDP-NAG) levels. The level of UDP-NAG
184 was around 3-fold higher in the high c-di-AMP *gdpP* mutant strain (OS2) compared to wild-type
185 and was reduced to below wild-type levels as a result of the *glmM* mutation in OR5 (Fig. 3E).
186 This result demonstrates that c-di-AMP affects the level of cell wall biosynthesis precursor and
187 that the I154F GlmM mutation leads to reduced peptidoglycan precursor biosynthesis.
188 Interestingly the low c-di-AMP level observed in the *glmM* mutant strain also suggests that cell
189 wall precursor biosynthesis activity can also affect the c-di-AMP level in the cell.
190 Previous work has identified the involvement of c-di-AMP in cell lysis (Luo and
191 Helmann, 2012a; Witte *et al.*, 2013). We therefore investigated if the *glmM* mutation would also
192 alter autolysis activity. Like that found in *B. subtilis* and *L. monocytogenes*, ~~different~~ most OR
193 *cdaA* mutant strains lysed significantly faster than their parent (OS~~R~~2) (Fig. 4). The *glmM*
194 mutant (OR5) lysed similarly to the strains that contained *cdaA* mutations and faster than OS2

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195 (Fig. 4). The wild-type exhibited a ~~moderate~~ slower autolytic rate than OS2~~, in between the OS~~
196 ~~and OR derivatives~~. These results collectively provide support for the role of c-di-AMP and
197 *glmM* in cell wall stability.

198 The product of the gene located between the *cdaA* and *glmM* in *L. lactis* MG1363 (Fig.
199 3D) has significant homology to the CdaR protein ~~from *B. subtilis* which~~and has been identified
200 as a ~~positive~~ regulator of diadenylate cyclase activity of CdaA ~~(Fig. 3D)~~ (Mehne *et al.*, 2013). In
201 *B. subtilis*, *L. monocytogenes* and *S. aureus*, CdaR contains several YbbR domains (pfam07949)
202 and is also encoded by a single gene located between *cdaA* and *glmM*. In MG1363 there appears
203 to have been a single nucleotide deletion in a single ancestral *cdaR* gene resulting in a premature
204 stop codon and is annotated as a pseudogene in the MG1363 Genbank entry (NC_009004.1).
205 This ~~sequence~~ deletion was confirmed by Sanger sequencing in MG1363. It is possible that this
206 pseudogene could express two open reading frames (previously denoted in NC_009004.1 as
207 *llmg_0449* and *llmg_0450*), however the YbbR domain would not be linked to the N-terminal
208 transmembrane domain and thus not exposed on the extracellular side of the membrane like other
209 CdaR proteins. Investigation of other available *Lactococcus* spp. genomes (strains SK11,
210 IL1403, A76, TIFN3, TIFN6, UC509.9, A12, KF147, IO-1, CNCMI-1631 and CV56) and four
211 industrially used *Lactococcus* in our culture collection revealed a full length ~320 aa CdaR
212 encoding gene in all cases, therefore suggesting that the mutated CdaR in MG1363 is unusual for
213 *Lactococcus*.~~.~~ ~~Therefore in MG1363, the YbbR domains which are encoded by the downstream~~
214 ~~potential open reading frame within *cdaR'* (*llmg_0450*) are separated from the N-terminal~~
215 ~~transmembrane domains and therefore likely to not be anchored to the cytoplasmic membrane~~
216 ~~and possibly not even expressed due to the absence of an efficient ribosome binding site.~~
217

218 ***GlmM down-regulates c-di-AMP synthesis by CdaA and the $GlmM^{I154F}$ variant has greater***
219 ***inhibitory activity.***

220 To ~~determine~~ evaluate the effect of GlmM and $GlmM^{I154F}$ in controlling CdaA activity more
221 directly, we expressed different combinations of *L. lactis* *cdaA* operon genes in *E. coli* which is
222 known to not produce c-di-AMP (Corrigan *et al.*, 2011; Mehne *et al.*, 2013) (Fig. 5A). ~~By~~
223 ~~comparing c-di-AMP levels in *E. coli* containing the complete *cdaAR'-glmM* operon, i~~It was
224 found that *E. coli* expressing the ~~the~~ *cdaAR'*-$glmM_{I154F}$ variant operon ~~led~~ had ~~to~~ a 5-fold lower
225 c-di-AMP concentration compared to that found ~~with the~~for *E. coli* expressing the wild-type
226 *cdaAR'-glmM* operon (Fig. 5A~~ & 5B~~). This suggests that the I154F mutation in GlmM results in
227 inhibition of CdaA activity and is in agreement with that found in *L. lactis* where the $glmM_{I154F}$

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mutation caused a lowering of the c-di-AMP level (Fig. 3B). To determine the effect of wild-type GlmM on CdaA activity, we compared c-di-AMP levels in *E. coli* containing the *cdaAR'-glmM* and *cdaAR'* only operons and found that the presence of GlmM resulted in a 19-fold reduction in c-di-AMP level (Fig. 5A~~ & 5B~~). Combined these results~~This~~ demonstrate~~s~~ a general inhibitory effect of GlmM on CdaA-mediated c-di-AMP synthesis and that the I154F mutation in GlmM enhances CdaA inhibition.

To extend these findings further we expressed combinations of *cdaA*, *cdaR* and *glmM* genes from another Gram-positive bacterium (*S. aureus* NCTC 8325) in *E. coli* and measured c-di-AMP levels (Fig. 5B). In agreement with that above, *S. aureus* GlmM was found to reduce c-di-AMP levels (Fig. 5B). Comparison of c-di-AMP levels in *E. coli* expressing *S. aureus* CdaA and CdaAR also suggests that CdaR inhibits CdaA activity. These results suggest that GlmM and CdaR are likely regulators of CdaA activity in a broad range of bacteria.

~~Global protein analysis reveals c-di-AMP affects the expression of the glycine-betaine transporter and a penicillin binding protein~~The GlmM^{I154F} variant binds more strongly to CdaA than GlmM.

~~C-di-AMP is expected to mediate its effects on cells by affecting gene expression or enzyme activity through binding of RNA (Nelson *et al.*, 2013) or effector proteins (Corrigan *et al.*, 2013). To determine the effects of elevated c-di-AMP on protein expression and to further explore how c-di-AMP controls cellular processes we compared the proteomes of wild-type MG1363 and OS2 using SWATH-MS (Gillet *et al.*, 2012). Whole genome sequencing of the OS2 mutant in this study revealed only one single nucleotide polymorphism when compared to wild-type. This was a thymine insertion which causes a K122Stop change in the GdpP previously reported (Smith *et al.*, 2012), thus making it a suitable *gdpP* mutant for this experiment. Using a 2-fold cut-off, 14 proteins were up-regulated and 16 proteins were down-regulated in the *gdpP* mutant compared to the wild-type (Table 2).~~ We hypothesized that the reason GlmM^{I154F} inhibits c-di-AMP synthesis by CdaA more than wild type GlmM is due to a stronger interaction between the two proteins. We examined the binding between CdaA and GlmM or GlmM^{I154F} using a bacterial two-hybrid system. Following measurement of β-galactosidase activities, a direct interaction was found between CdaA and GlmM while a significantly stronger interaction between GlmM^{I154F} and CdaA was observed (Fig. 6). The higher binding affinity of GlmM^{I154F} for CdaA provides a possible explanation for why this variant protein has greater CdaA inhibitory activity and why OS5 contains lower c-di-AMP levels.

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261 Of particular relevance to osmotolerance, the glycine betaine transporter BusAB was identified
262 in the proteome analysis as being downregulated 2.9 fold (Table 2). This ATP binding cassette
263 (ABC) type compatible solute transporter (BusAA-AB) has been identified in *L. lactis* as being a
264 major contributor to osmotolerance and is regulated by the transcriptional repressor BusR
265 (Romeo *et al.*, 2003). Previous work in our lab has identified that the RNA transcript level of
266 *busAA* is downregulated ~5 fold in a *gdpP* mutant strain Δ1816 (Smith *et al.*, 2012) and we
267 determine here that OS2 has a ~10-fold lower *busAA* transcript than wild-type (data not shown).
268 This suggests that c-di-AMP levels affect the expression of the BusAA-AB genes at a
269 transcriptional level.

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270 The most highly upregulated protein (18-fold) in OS2 was DacA which encodes a cell wall
271 peptidoglycan D-alanyl-D-alanine carboxypeptidase (note: DacA from *L. lactis* should not be
272 confused with DacA in other bacteria which is a diadenylate cyclase, othologous to CdaA). This
273 low molecular weight penicillin binding protein (PBP) has been shown to cleave the last alanine
274 from the interpeptide crossbridge to provide a substrate for peptide crosslinking and has also
275 been shown to affect resistance to the cell wall acting antimicrobial peptide Lcn972 (Roces *et al.*,
276 2012). An isogenic Δ*dacA* mutant was constructed in a wild-type MG1363 background and was
277 found to be more resistant to penicillin G (zone of inhibition size was 37.1±0.1 mm for wild-type
278 MG1363 and 29.8±0.4 mm for Δ*dacA*) confirming its role in peptidoglycan
279 biosynthesis/maturation. This is in contrast to what was expected however, as Δ*gdpP* mutants
280 are resistant to β-lactams (Corrigan *et al.*, 2011; Luo and Helmann, 2012; Smith *et al.*, 2012;
281 Witte *et al.*, 2013), therefore suggesting that other aspects of c-di-AMP signalling play a role in
282 antibiotic resistance in *L. lactis*.
283
284
285 **DISCUSSION**
286 Here we identify GlmM as a modulator of c-di-AMP synthesis through its regulation of
287 CdaA activity.Using a suppressor screen to isolate mutants with reduced c-di-AMP levels in
288 *gdpP* mutant backgrounds and subsequent genetic and biochemical studies, we identify GlmM as
289 an important modulator of CdaA-mediated c-di-AMP synthesis (Fig. 6). In turn, it is In
290 additionfound that, it was found that the c-di-AMP level affects peptidoglycan precursor
291 synthesis, thereby demonstrating a close interconnection between c-di-AMP and peptidoglycan
292 synthesis pathways. The broadly conserved clustering of *glmM* in the diadenylate cyclase *cdaA*
293 gene operon in Firmicutes hasd drawn speculation of a role of c-di-AMP in peptidoglycan

294 biosynthesis (Luo and Helmann, 2012a; Corrigan and Grundling, 2013; Mehne *et al.*, 2013;
295 Witte *et al.*, 2013; Commichau *et al.*, 2015). How GlmM regulates CdaA activity is uncertain at
296 present, however it appears likely to be through direct protein-protein interaction as
297 demonstrated here. In agreement with this, recent work by Gundlach et al (2015b) has
298 demonstrated binding between *B. subtilis* CdaA and GlmM. Coordinated activity of CdaA and
299 GlmM is likely to occur and therefore play a role in various phenotypic changes observed in cells
300 with altered c-di-AMP levels, such as cell lysis. A model likely to be broadly conserved based on
301 our results and others involving CdaA and GlmM is shown (Fig. 7).

302 It is possible that CdaA may be able to respond directly to GlmM activity through
303 protein-protein interaction, since other clustered genes in DAC encoding operons have been
304 shown to physically interact with the DAC and regulate c-di-AMP synthesis. These include
305 CdaR which positively regulates CdaA (Mehne *et al.*, 2013) and RadA which negatively
306 regulates DisA (Zhang and He, 2013). Alternatively, it may be an indirect effect with CdaA
307 sensing cell wall precursor levels or peptidoglycan changes. Regardless of this, the essential
308 nature of c-di-AMP may be due to its regulation of peptidoglycan precursor biosynthesisHere we
309 identify GlmM as a modulator of c-di-AMP through its regulation of CdaA activity. The variant
310 GlmM[I154F] identified in this study was found to inhibit CdaA activity and bind to CdaA more
311 strongly than to reduce c-di-AMP levels in *L. lactis* and in *E. coli* expressing CdaAR' more so
312 than wild-type GlmM. The structure of GlmM location of the I154 site can be examined based
313 on the known structure of the homologous GlmM from *Bacillus anthracis* (Mehra-Chaudhary *et*
314 *al.*, 2011). Using SWISS-MODEL, the 154 site in GlmM is predicted to be exposed on thea
315 surface region of opposite side of the protein away from the active site phosphate transferring
316 serine at residue 101. Interestingly the equivalent position of I154 in *L. lactis* GlmM is a
317 phenylalanine (F) in *S. aureus*, *B. subtilis* and *L. monocytogenes*. This suggests that the I154F
318 mutation that occurred in the *L. lactis* GlmM doesn't destroy activity of the enzyme. It is
319 possible that this region of the enzyme may be the point of contact between GlmM and CdaA,
320 however more work is needed to determine this., but may alter how it is regulated and how it
321 regulates CdaA.

322 The only other identified regulator of CdaA that has been identified to date is the
323 membrane bound extracellular exposed CdaR (Mehne *et al.*, 2013). In the heterologous host *E.*
324 *coli*, it was shown that *B. subtilis* CdaR positively regulated CdaA and binding between the two
325 proteins has been confirmed (Mehne *et al.*, 2013). In contrast, recent work in *L. monocytogenes*
326 has suggested that CdaR is a negative regulator of CdaA as a *cdaR* mutant contained a higher c-

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di-AMP level (Rismondo *et al.*, 2015). Like that found for *B. subtilis*, *L. monocytogenes* CdaR and CdaA directly interact (Rismondo *et al.*, 2015). Our findings comparing c-di-AMP levels in *E. coli* expressing various combinations of *cdaA* operon genes from *S. aureus* suggest that CdaR also negatively regulates CdaA activity in this system. Despite these differences, which may be due to the experimental conditions employed, it is clear that CdaR is an important regulator of CdaA. Although strain backgrounds and *gdpP* mutations are different, a ~3-fold higher level of c-di-AMP in OS1 compared to OS3 could perhaps be due to *L. lactis* MG1363 derivatives not carrying a functional negative regulator CdaR as mentioned above. In *B. subtilis* another DAC ~~(Mehne *et al.*, 2014)~~(DisA) is regulated by RadA (Zhang and He, 2013), suggesting that DAC enzymes likely operate as complexes with other proteins which regulate their activity and thus c-di-AMP levels.

The vast majority of mutations restoring osmoresistance in *gdpP* mutants were affected in the CdaA enzyme thus providing an excellent inverse correlation between c-di-AMP level and osmoresistance. Numerous single amino acid altering suppressor mutations were identified in regions in CdaA outside of the DAC domain including within and between the three N-terminal transmembrane domains. It is possible that the changes may affect CdaA activity by affecting dimerisation which has been shown recently for *L. monocytogenes* CdaA (Rosenberg *et al.*, 2015), interaction with the CdaR or GlmM regulatory proteins or sensing of cell envelope or external stimuli. Synthesis of c-di-AMP has been found to be essential in several Gram-positive bacteria (Luo and Helmann, 2012a; Mehne *et al.*, 2013) including *L. monocytogenes*, *S. aureus* and *Streptococcus pneumoniae*, where deletion of their single DAC gene is not possible when cultured on rich media (Song *et al.*, 2005; Chaudhuri *et al.*, 2009; Woodward *et al.*, 2010). Recent work has however identified that CdaA and thus c-di-AMP is not essential in *L. monocytogenes* when cells are cultured on minimal media or when suppressor mutations affecting (p)ppGpp levels or peptide transport exist (Whiteley *et al.*, 2015). Since we were using rich media for our suppressor selection experiment, ~~W~~we were surprised ~~therefore~~ to see frameshift mutations in the *cdaA* gene in osmoresistant suppressor mutants upstream of the DAC domain encoding region of *cdaA*, as this would be predicted to generate a cell devoid of DAC activity. However the location of an alternative ATG start codon (residue 136) just inside the start of the DAC domain could potentially allow expression of an N-terminally truncated enzyme (Fig. 2). In agreement with this, a low but detectable level of c-di-AMP was found in the OR1

mutant which has a frameshift mutation early in the gene (codon 11). Due to ~~destructive background~~ mutations in *gdpP* in the osmoresistant suppressor mutants, low levels of c-di-AMP are predicted to be more stable as the cell is likely devoid of significant c-di-AMP phosphodiesterase activity. It is also plausible that *cdaA* naturally encodes two variants, one of which is the full length membrane bound version and the other may begin with M136 and be devoid of the N-terminal transmembrane domains. Interestingly, this methionine is conserved in most CdaA proteins in other bacteria (Fig. S1).

Suppressor mutations in *cdaA* have recently been identified in a *B. subtilis* strain lacking both GdpP and PgpH phosphodiesterases that underwent lysis on agar plates (Gundlach *et al.*, 2015b). Overall c-di-AMP levels within the mutants however did not change, leading the authors to speculate that local pools of CdaA controlled c-di-AMP exist within the cell (Gundlach *et al.*, 2015b). Our findings of significantly lower c-di-AMP levels within *L. lactis cdaA* osmoresistant suppressor mutants perhaps reflect a more simple bacterium where only one DAC is present. In addition to this, the relatively high c-di-AMP levels we observed in the *L. lactis gdpP* mutant, which were for example 80-fold higher than the *cdaA* suppressor mutant OR1, is possibly reflective of *L. lactis* lacking a PgpH phosphodiesterase (Huynh *et al.*, 2015). Similarly the *gdpP* mutant of *S. aureus*, which also lacks PgpH~~(Huynh *et al.*, 2015b)~~, has ~15-fold higher c-di-AMP than wild-type (Corrigan *et al.*, 2011). Evidence is mounting showing that growth conditions can alter the importance of different enzymes in their regulation of c-di-AMP levels, including nitrogen source (Gundlach *et al.*, 2015b) and growth in media or host cells (Huynh *et al.*, 2015) and it is likely that additional yet to be discovered enzymes exist which can regulate c-di-AMP under certain environmental conditions. ~~(Huynh *et al.*, 2015)~~

The role of c-di-AMP in cell wall homeostasis has been established based on several findings from several laboratories. It has been found in different bacteria that there is a direct correlation between resistance to cell wall acting antibiotics and c-di-AMP level (Corrigan *et al.*, 2011; Luo and Helmann, 2012a; Smith *et al.*, 2012; Kaplan Zeevi *et al.*, 2013; Witte *et al.*, 2013). *S. aureus gdpP* mutants have increased peptidoglycan cross-linking (Corrigan *et al.*, 2011) and c~~C~~ells with low c-di-AMP have been found to exhibit a lysis phenotype which can be stabilized by ~~high osmotic~~NaCl ~~conditions~~ or Mg^{2+} (Luo and Helmann, 2012a; Witte *et al.*, 2013; Rismondo *et al.*, 2015). In agreement with this, ~~W~~we observed that many *cdaA* suppressor mutants exhibited growth defects on normal agar which ~~could~~ was ~~be~~ restored upon addition of salt, and also differences ~~and also changes~~ in autolysis rates ~~in~~ for strains~~cells~~ with different c-di-

393 AMP levels. Mutation in *gdpP* has been found to lead to increased peptidoglycan cross-linking
394 in *S. aureus* (Corrigan *et al.*, 2011) and in agreement with this we found that the PBP (DacA) is
395 highly upregulated in a *gdpP* mutant and its inactivation affects penicillin resistance. DacA in *L.*
396 *lactis* is a D-Ala-D-Ala carboxypeptidase which cleaves peptidoglycan pentapeptide side chains
397 to form tetrapeptide side chains (Roces *et al.*, 2012). Spontaneous *Lactococcus* mutants with
398 resistance to the bacteriocin Lcn972 were found to have lower levels of pentapeptide side chains
399 and were sensitive to penicillin and lysozyme further suggesting an important role for DacA in
400 peptidoglycan maturation and cell wall acting antimicrobial resistance (Roces *et al.*, 2012).
401 DacA is homologous to PBP3 from *S. pneumoniae*, DacA (PBP5) of *B. subtilis* and PBP4 of *S.*
402 *aureus* and its role in trimming the last residue in the peptide side chain likely reduces the
403 availability of donors for the transpeptidation reaction, thus regulating the degree of
404 peptidoglycan crosslinking (Morlot *et al.*, 2004). In a recent study of *S. aureus*, PBP4 was found
405 to be transcriptionally upregulated 2.7-fold in a *gdpP* mutant (Corrigan *et al.*, 2015). Further
406 work exploring how c-di-AMP regulates DacA/PBP4 levels and what role this PBP plays in β-
407 lactam resistance is of interest to allow understanding of how c-di-AMP level changes lead to
408 resistance to several different cell wall acting antibiotic classes (Luo and Helmann, 2012).

409 Here we identify GlmM as a modulator of c-di-AMP through its regulation of CdaA
410 activity. The variant GlmM^{I154F} was found to reduce c-di-AMP levels in *L. lactis* and in *E. coli*
411 expressing CdaAR' more so than wild-type GlmM. The structure of GlmM can be examined
412 based on the known structure of the homologous GlmM from *Bacillus anthracis* (Mehra-
413 Chaudhary *et al.*, 2011). Using SWISS-MODEL, the 154 site in GlmM is predicted to be
414 exposed on the surface of opposite side of the protein away from the active site phosphate
415 transferring serine at residue 101. Interestingly the equivalent position of I154 in *L. lactis* is a
416 phenylalanine (F) in *S. aureus*, *B. subtilis* and *L. monocytogenes*. This suggests that the I154F
417 mutation that occurred in the *L. lactis* GlmM doesn't destroy activity of the enzyme, but may
418 alter how it is regulated and how it regulates CdaA.

419 Comparison of the proteome of wild-type *L. lactis* to the *gdpP* mutant OS2 revealed 30
420 proteins with a >2 fold difference in expression. The glycine betaine transporter BusAB was
421 downregulated in the *L. lactis gdpP* mutant. Another major function of c-di-AMP that has been
422 identified is in the control of osmotolerance. Appropriate turgor pressure needs to be strictly
423 controlled in bacteria by regulation of the accumulation of osmolytes including ions such as K^+
424 and compatible solutes such as glycine-betaine (Kempf and Bremer, 1998). Recent work has
425 identified the K^+ transport gating component KtrA as a c-di-AMP effector which affects

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osmoresistance by regulating K^+ transport in a c-di-AMP dependent manner (Corrigan *et al.*, 2013; Bai *et al.*, 2014). Additionally the riboswitch *ydaO* which regulates the expression of a broad range of genes, including osmoprotection genes such as those encoding K^+ and compatible solute transporters, has been shown to bind c-di-AMP (Nelson *et al.*, 2013). In *L. lactis* we have previously shown that the glycine-betaine transporter BusAA-AB is downregulated 5-fold in a *gdpP* mutant suggesting it may be a reason for the osmosensitive phenotype in this strain (Smith *et al.*, 2012). Interestingly numerous destructive mutations were identified in the GbuABC glycine-betaine transporter genes in c-di-AMP deficient suppressor mutants of *L. monocytogenes* (Whiteley *et al.*, 2015) suggesting that high levels of intracellular glycine-betaine is detrimental to growth. We hypothesise that K^+ and glycine-betaine transporters are closed or not expressed, respectively, in cells with high c-di-AMP and are open or overexpressed, respectively, in cells with low c-di-AMP. This would explain why high c-di-AMP cells are osmosensitive and low c-di-AMP cells are osmoresistant or even become dependent upon high osmolarity for normal growth, as we found in the current study. Further exploration of the role of c-di-AMP in osmoregulation is required to confirm this model and studies are needed to elucidate other important and possibly interrelated roles this signalling molecule plays within the cell. ~~In contrast to our findings, in a microarray experiment performed with a *gdpP* mutant of *L. monocytogenes*, expression of the homologous osmolyte transporter (OpuCA encoded by *lmo1428*) was found to be upregulated 2.2-fold (Witte *et al.*, 2013). Only slightly increased levels of c-di-AMP were observed in the supernatant of this mutant therefore it is possible that the c-di-AMP level is not high enough to trigger downregulation of this osmolyte transporter. Whether c-di-AMP affects BusAA-AB expression directly or indirectly through possible effects on ion homeostasis remains to be determined.~~

~~Several other differentially regulated proteins in our dataset have been reported in other studies examining c-di-AMP signalling. The acetaldehyde-CoA/alcohol dehydrogenase AdhE and the oligopeptide binding protein transporter OppA were downregulated 3.6- and 2.5-fold, respectively in the *L. lactis gdpP* mutant. AdhE was also transcriptionally downregulated 11-fold in a *gdpP* mutant of *S. aureus* (Corrigan *et al.*, 2015). Interestingly, both AdhE (Lmo1634) and OppA were also identified recently in a chemical proteomics screen to identify c-di-AMP interacting proteins in *L. monocytogenes* (Sureka *et al.*, 2014), indicating that this binding may affect protein levels. Further work is needed to identify how c-di-AMP levels alter the expression of these proteins and importantly how this binding may influence the cell's phenotype.~~

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EXPERIMENTAL PROCEDURES

Isolation of osmoresistant suppressor mutants from **gdpP** *mutant strains OS1, OS2 and OS3*

L. lactis subsp. *cremoris* strains used in this study are shown in Table S1. Strain MG1363 and derivatives were cultured as described previously (Smith *et al.*, 2012). Two independent MG1363 derivatives (OS1 and OS2) and an ASCC892185 derivative (OS3) contain mutations in the *gdpP* gene which were shown to be osmosensitive (Smith *et al.*, 2012). To isolate osmoresistant suppressor mutants, cells were taken from different growth phases in broth (lag, mid-log and stationary phase), from colonies of agar plates and from frozen glycerol (40% final v/v) stocks and plated onto GM17 or LM17 containing an additional 0.2, 0.25 or 0.3 M sodium chloride. Following incubation for 2-3 days at 30°C, colonies were then re-streaked onto agar containing the same level of salt again to ensure purity before being subcultured in broth without added salt and frozen in 40% glycerol at -80°C. To confirm stable osmoresistance, cells were tested for growth on salt containing agar similarly to that described previously (Smith *et al.*, 2012).

Whole-genome sequencing and mutation identification

Whole genome sequencing of strains OS2, OR1, OR2 and OR5 was carried out using an Illumina HiSeq2000 instrument (Macrogen, South Korea). Trimming and mapping of 100-bp raw reads to the reference *L. lactis* MG1363 genome was performed using Geneious Pro 6.1.6 as described previously (Smith *et al.*, 2012). Criteria for SNP identification were >100-fold coverage with >90% variant frequency as used previously (Linares *et al.*, 2010; Smith *et al.*, 2012). In a few cases, SNPs which were called in one strain but not another (due ~~to a slight greater than~~ to having slightly lower than 90% variation frequency), were confirmed using a slightly lower threshold cut-off and manual inspection. SNPs in OS1 have been identified before (Smith *et al.*, 2012) while SNPs in strains OS2, OR1, OR2 and OR5 are identified here from mapping using average read depths of 554-, 659-, 704- and 633-fold, respectively. SNPs in *cdaA, gdpP* and *glmM* were confirmed following PCR using GoTaq green (Qiagen) and Sanger sequencing (Macrogen, South Korea) using primers listed in Table S1.

491

492 ***Penicillin susceptibility test***

493 Susceptibility tests for penicillin were performed using a disk diffusion assay (Luo and Helmann,
494 2012a) with slight modifications. A volume of 0.1 ml of the strain harvested in mid-log phase
495 (OD_{600} ~ 0.6) was mixed with 5 ml of GM17 containing 0.75% agar and poured onto an agar
496 base consisting of GM17 containing 1.5% agar (15ml). Sterile 8 mm diameter filter paper disks
497 (Advantec, CA) were loaded with 20 µg penicillin G (Sigma-Aldrich, MO) and put on the agar.
498 The diameter of each growth inhibition zone was measured after overnight incubation at 30°C.
499 The difference between strains tested in triplicate was analyzed using the Student's *t*-test.

500

501 ***Extraction of c-di-AMP***

502 Overnight *L. lactis* cultures were diluted 1:100 into 30 ml GM17 or LM17 broth and incubated at
503 30°C till OD_{600}~0.7 (mid-log phase). Cells were pelleted by centrifugation at 3,500 × *g* (Awel
504 MF20, Awel International, France) for 5 min, and re-suspended in 1 ml ice-cold extraction buffer
505 (40% methanol, 40% acetonitrile and 20% ddH_2O in vol.). Fifty microliters of 2′-,2″-dideoxy-
506 cyclic diadenosine monophosphate (Biolog, Denmark) was added and used as an internal
507 standard. Samples were mixed with 0.5 ml equivalent of 0.1 mm zirconia/silica beads and
508 disrupted using a Mini-Beadbeater-16 (Biospec, USA) 3 times for 1 min each with 1 min cooling
509 on ice in between. Glass beads were separated by centrifugation at 16,873 × *g* (Eppendorf,
510 Germany) for 5 min. The supernatant was dried under liquid nitrogen before resuspended in 1ml
511 ddH_2O before being filtered (0.22 µm pore size). Levels of protein in a duplicate culture sample
512 taken were determined following equivalent cell homogenisation using a Qubit Protein Assay Kit
513 and Qubit 2.0 Fluorometer (Invitrogen, Life Technologies).

514

515 ***Quantification of c-di-AMP***

516 C-di-AMP was detected and quantified by UPLC-coupled tandem mass spectrometry (UPLC-
517 MS/MS; Waters Corporation, Milford City, MA). The chromatographic separation was
518 performed on an ACQUITY UPLC, using a C18-BEH 1.7µm x 2.1 x 100mm column. Eluent A
519 consisted of 10 mM ammonium acetate in water and eluent B was methanol. Sample volume of
520 10µl, with a flow rate of 0.3 ml/min. Eluent A (98%) was used from 0 to 1 min followed by a
521 linear gradient from 98% to 50% of eluent A until 10 min. The column was then washed with
522 100% B for 4 mins, and then re-equilibrated with 98% A for 6 mins prior to re-injection. The

internal standard of 2′-,2″-dideoxy-c-di-AMP and commercial c-di-AMP (Biolog, Denmark) were used. Analyte detection was performed on a Synapt G2-Si mass spectrometer equipped with an electrospray ionization source (Waters Corporation) using MRM transitions of the internal standard 627→216 and c-di-AMP 659→330 in positive ionization mode, using argon as collision gas. Detection of c-di-AMP was carried out with Mass Lynx software (Waters Corporation).

Quantification of UDP-N-acetylglucosamine

UDP-NAG was detected and quantified using a modified method (Marcellin *et al.*, 2009). Cultures in mid-exponential growth phase were spun at 6,000 × *g* (Allegra X14, Coulter Beckman, USA) for 5 min, and the cell pellet was resuspended in ice cold 50% acetonitrile containing 50 nmol of azidothymidine (Sigma-Aldrich) as internal standard. Samples were lysed using 0.1 mm glass beads (Biospec, USA) using a Precellys 24 homogenizer equipped with a cryolys unit (Precellys, France) to maintain the temperature bellow 4°C. The supernatant was freeze dried and resuspended in 200 µL of MilliQ water. UDP-NAG was detected and quantified using LCMS. The mass spectrometer, an ABSciex 4000 QTRAP (ABSciex, Canada), was attached to a Dionex Ultimate 3000 liquid chromatography system (Dionex, USA). Chromatographic separation was achieved on a Gemini-NX C18 150×2.0 mm, 3 µm 110 Å particle column (Phenomenex, Germany) maintained at 55°C in the column oven as previously described (McDonald *et al.*, 2014).

Autolysis of* L. lactis *strains

Overnight cultures of *L. lactis* MG1363, OS2, OR5 and several OR mutants with differing *cdaA* mutations were diluted 1/100 in 35 mL of fresh GM17 broth. OD_{600} measurements using a spectrophotometer (Lovibond, Germany) were taken using 2 mL of culture regularly during the growth phase and then less regularly during lysis. Before each sample was taken the culture was mixed by vortexing to ensure homogeneity of the cells.

***Expression of* cdaA *operon genes from* L. lactis *and* S. aureus *in* E. coli**

Genes from *L. lactis* MG1363 were synthesised and cloned into XhoI and PstI sites in pRSET-A downstream of the T7 promoter by Geneart (Life Technologies, Germany). Gene names in MG1363 are named *llmg_0448* (*cdaA*), *llmg_0449* and *llmg_0450* (*cdaR*') and *llmg_0451* (*glmM*). The start codon for CdaA was changed from a TTG to an ATG in all the constructs to

improve expression. For the *glmM*$_{I154F}$ mutant construct, a single nucleotide change was introduced which was identical to that found in *L. lactis* OR5. Genes from *S. aureus* NCTC 8325 were synthesised and cloned into XhoI and PstI sites in pRSET-A downstream of the T7 promoter by Geneart (Life Technologies, Germany). Gene names in NCTC 8325 are named *SAOUHSC_02407* [*cdaA*], *SAOUHSC_02406* [*cdaR*] and *SAOUHSC_02405* [*glmM*]). *L. lactis* gene ~~P~~plasmids were transformed into *E. coli* BL21 (DE3) (New England Biolabs, USA), while *S. aureus* gene plasmids were transformed in to *E. coli* T7 Express *lysY/I*^{q} (New England Biolabs, USA). ~~and G~~genes were expressed from OD$_{600}$ ~0.5 (mid-log phase) with 1 mM isopropyl-B-D-thiogalactopyranoside (IPTG) for 3 hours at 37°C with shaking at 220 rpm. Cells from 6 ml culture were harvested by centrifugation at 16,873 × *g* for 5 mins and c-di-AMP levels were determined as above for *L. lactis*. Levels of protein in a duplicate culture sample taken were determined following equivalent cell homogenisation using a Qubit Protein Assay Kit and Qubit 2.0 Fluorometer (Invitrogen, Life Technologies).

Bacterial two-hybrid system analysis of CdaA-GlmM interaction

The bacterial adenylate cyclase two-hybrid system (Euromedex, France) allows evaluation of protein-protein interactions following co-expression of test proteins fused to T25 and T18 subunits of the *Bordetella pertussis* adenylate cyclase. If the proteins interact, T25 and T18 are brought together leading to cAMP synthesis and activation of the catabolite activator protein CAP resulting in increased expression of the *lacZ* reporter gene and β-galactosidase activity. PCR primers used to cloned genes into plasmids are shown in Table S1. The *L. lactis cdaA* gene encoding a protein without the N-terminal transmembrane domains (αα 98-292) was cloned into PstI and KpnI digested pKNT25 in frame with the T25 *cya* gene fragment at the 3' end. The wild-type *glmM* and *glmM*$_{I154F}$ variant genes were cloned into PstI and KpnI digested pUT18C in frame with the T18 *cya* gene fragment at the 5' end. Plasmids, including empty pUT18C and pKNT25 and also positive control *zip* gene containing plasmids were transformed into *E. coli* DHP-1 and selection using kanamycin (50 μg/mL) and ampicillin (100 μg/mL) in Luria-Bertani (LB) agar. From this point onwards antibiotics were always included when growing these strains and incubation was always carried out at 29°C (Gloeckl *et al.*, 2012). β-galactosidase activity on agar was examined following spotting of mid-log phase (OD$_{600}$ ~0.5) *E. coli* cultures grown in LB broth onto LB agar with IPTG (0.5 mM) and X-gal (40 μg/mL) and incubation for 16.5 hr at 29°C. Following this the plates were stored for 4 d at 4°C to allow colour development. β-galactosidase activity in broth was quantified from mid-log phase (OD$_{600}$ ~0.5) *E. coli* cultures

589 grown in LB broth with IPTG (0.5 mM) and assayed according to the BACTH kit instructions
590 (Euromedex, France) except chloroform was used to permeabilize cells instead of toluene.
591 Enzymatic activity is presented as Miller units (Ref needed here XXXX).

592

593 ***Proteome comparison of MG1363 and OS2 using SWATH MS***

594 Proteins were extracted from cell pellets obtained from triplicate mid-exponential phase cultures.
595 Cells were lysed using glass beads for 5 minutes at 4,800 rpm using a Precellys 24 homogenizer
596 equipped with a cryolys unit (Precellys, France) at 4°C. RNaseA and DNaseI (Thermo Scientific,
597 USA) were added and incubated for 15 minutes at room temperature. The lysate was clarified at
598 16,000 × *g* for 10 min at 0°C and the protein concentration in the supernatant was measured
599 using the 2D Quant Kit (GE Healthcare, USA). Proteins (100 µg) were digested overnight with
600 Trypsin Gold (Promega, USA) using FASP (Wisniewski *et al.*, 2009) and analysed via Nano-LC
601 MS/MS. The mass spectrometer (ABSciex TripleTof 5600, Canada), was equipped with a nano-
602 spray ESI sources operated in positive ion mode coupled to a Nano-LC (Shimadzu Prominence,
603 Japan). Peptides were separated using a flow rate of 30 µl/min on a Vydac Everest C18 column
604 (300 A, 5 µm, 150 mm × 150 µm) at a flow rate of 1 µl/min and a gradient of 10-60% mobile
605 phase B over 90 min. Analyst Software was used for peak picking with a method searching for
606 masses of 300 to 1800 Da. A SWATH reference library was generated from information-
607 dependent acquisition on a pooled sample containing 30 µg of proteins selecting for +2 to +4
608 charges which exceeded 150 counts using Enhanced Resolution scans. The two most abundant
609 ions in each of these scans (or with unknown charge) were subjected to MS/MS. Protein Pilot
610 Software v 4.5 (ABSciex, Canada) and the Paragon Algorithm were used for peptide
611 identification. The theoretical ions and peaks were matched using the tolerance used by the
612 Paragon Algorithm search, based on information about the mass accuracy of the instrument
613 chosen in the Paragon Method dialog box. Search parameters used were the same as specified
614 previously (Marcellin *et al.*, 2013). SWATH MS was used for protein quantification (Gillet *et*
615 *al.*, 2012). SWATH analyses scanned across *m/z* 350-1800 for 0.05 sec followed by high
616 sensitivity DIA mode, using 26 *m/z* (1 *m/z* for window overlap) isolation windows for 0.1 sec,
617 across *m/z* 400-1250. Collision energy for SWATH samples was automatically assigned based
618 on *m/z* mass windows by Analyst. C_{18} ZipTips (Millipore, USA) were used to avoid overloading
619 the detector and to ensure that equal amount of peptides (1 µg) were loaded in SWATH MS
620 mode. Prior to MS analysis, samples were concentrated using a vacuum centrifuge to remove
621 residual acetonitrile and resuspended in 99.5 µl of 0.1% formic acid (Mobile phase A). Each

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sample was spiked with 0.5 µl of synthetic peptides in order to correct for retention time shifts between samples (HRM calibration kit from Biognosys, Switzerland). All IDA and SWATH samples were injected twice. Peak View (ABSciex, Canada) was used for peak selection.

Contruction of a ΔdacA mutant

Flanking regions either side of *dacA* (*llmg_2560*) were amplified and fused by splicing by overlap extension PCR (SOE-PCR) using primers listed in Table S1. These fragments were then cloned into suicide plasmid pRV300 (Leloup *et al.*, 1997) to generate pRV300ΔdacA which was transformed into the wt MG1363 using a standard method (Wells *et al.*, 1993) and integrants were selected using 3 µg/ml erythromycin and confirmed by PCR. The vector was then excised from the chromosome by successive subculturing in fresh GM17 broth without erythromycin. Replica plating on GM17 agar with and without erythromycin allowed detection of the deletion mutant which was confirmed by PCR (Table S1).

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795

Table 1. Polymorphisms identified in osmoresistant suppressor mutants OR1 and OR2 compared with the parent OS1 by whole genome sequencing.

Strain	Reference position	Gene or region	Gene function	Nucleotide change	Protein effect
OR1	442839	*llmg_0448* (*cdaA*)	c-di-AMP synthase	A deletion	Frameshift from codon 11
	1010850	142-bp upstream of *busAA*	Glycine betaine transporter	G → T	Intergenic
OR2	443214	*llmg_0448* (*cdaA*)	c-di-AMP synthase	G → A	M136I
	824826	16-bp upstream of *uxuB*	Fructuronate reductase	A → G	Intergenic

816

817

818

819 **Table 2.** Proteome comparison of wild-type MG1363 and high c-di-AMP containing strain

820 (OS2). Only proteins which had a >2.0 fold change and P<0.05 are presented and up-regulated

821 proteins are shaded grey.

GI #	Protein^	Alternate protein name	Putative function	Fold change (OS2 vs wt)	P-value*
125625313	Llmg_2560	DacA	D-alanyl-D-alanine carboxypeptidase	18.2	0.01
125624549	Llmg_1759		NADH-flavin reductase	12.2	0.04
125625244	Llmg_2488	RuvA	Holliday junction resolvasome, DNA-binding subunit	6.7	0.04
125623715	Llmg_0871	Asd	Aspartate-semialdehyde dehydrogenase	6.3	0.04
125624459	Llmg_1660		TetR family transcriptional regulator	5.1	0.02
125623296	Llmg_0429	SodA	Superoxide dismutase	3.4	0.01
125623422	Llmg_0557	PrfA	Protein chain release factor A	3.3	0.03
125625088	Llmg_2327	GlpF3	Glycerol uptake facilitator and related permeases	3.2	0.02
125623323	Llmg_0456	PgmB	β-phosphoglucomutase	2.8	0.00003
125623774	Llmg_0933		RNA binding protein	2.7	0.001
125623372	Llmg_0505	NifZ	Cysteine sulfinate desulfinase/cysteine desulfurase and related enzymes	2.4	0.005
125624134	Llmg_1314		Uncharacterized FAD-dependent dehydrogenases	2.1	0.01
125624935	Llmg_2164		Uncharacterized conserved protein	2.1	0.0009
125623623	Llmg_0769		DivIVA cell division initiation protein	2.0	0.0005
125624150	Llmg_1330		hypothetical protein	0.48	0.002
125623375	Llmg_0508		Cysteine synthase	0.47	0.003
125623558	Llmg_0702	PepO	Predicted metalloendopeptidase	0.46	0.004
125623934	Llmg_1103		hypothetical protein	0.44	0.003
125623969	Llmg_1138	MtsA	Manganese transporter (substrate binding protein)	0.43	0.0003
125624260	Llmg_1452		Amino acid transporters	0.41	0.004
125623557	Llmg_0701	OppA	Peptide transport system (substrate binding protein)	0.40	0.002
125623967	Llmg_1136	MtsB	Manganese transporter (ATPase component)	0.40	0.002
125622903	Llmg_0022	MtlA	Phosphotransferase system, mannitol-specific IIBC component	0.40	0.0001
125625268	Llmg_2514		Universal stress protein family (UspA)	0.37	0.003
125623880	Llmg_1049	BusAB	Glycine betaine transporter (permease component)	0.35	0.00007
125623830	Llmg_0993	HprT	Hypoxanthine-guanine phosphoribosyltransferase	0.34	0.0003
125623241	Llmg_0372	AsnB	Asparagine synthase (glutamine-hydrolyzing)	0.29	0.0002
125625190	Llmg_2432	AdhE	bifunctional acetaldehyde-CoA/alcohol dehydrogenase	0.28	<0.00001

125625247	Llmg_2491	MutS	Mismatch repair ATPase (MutS family)	0.13	0.008
125624224	Llmg_1412	GuaC	Guanosine 5' monophosphate oxidoreductase	0.10	0.00004

^from Genbank entry AM406671.1 *Statistical analysis of these data (three biological replicates per strain) was performed by fitting the data for each protein to a linear model using the R package Limma. Limma's empirical Bayes method was used to calculate a moderated t-statistic test for the contrast and proteins were classified as differentially expressed if the adjusted p-value (Benjamini-Hochberg) was lower than 0.05.

FIGURE LEGENDS

Figure 1.

Osmoresistant suppressor mutants of *L. lactis* OS1 and OS3 have reduced c-di-AMP. (A) Comparison of growth of *L. lactis* MG1363 background strains on GM17 agar or GM17 agar + 0.25 M NaCl following spotting of serial dilutions. (B) Levels of c-di-AMP in wt, OS1, OR1 and OR2 mutants from three biological replicates. (C) Comparison of growth of industrial *L. lactis* ASCC892185 background strains on LM17 agar or LM17 agar + 0.2 M NaCl following spotting of serial dilutions. (D) Levels of c-di-AMP in wt, OS3, OR3 and OR4 mutants from three biological replicates. Significant $P<0.01$ (**) and $P<0.05$ (*) results were determined using Tukey's test. ND, none detected.

Figure 1.

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Osmoresistant suppressor mutants of OS1 have reduced c-di-AMP. (A) Comparison of growth of *L. lactis* MG1363 background strains on GM17 agar or GM17 agar + 0.25 M NaCl following spotting of serial dilutions. (B) Levels of c-di-AMP in wt, OS1, OR1 and OR2 mutants. (C) Comparison of growth of industrial *L. lactis* ASCC892185 background strains on LM17 agar or LM17 agar + 0.2 M NaCl following spotting of serial dilutions. (B) Levels of c-di-AMP in wt, OS3, OR3 and OR4 mutants. **, $P<0.01$ using Tukey's test based on results from duplicate cultures. ND, none detected.

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Figure 2.

Location of mutations in the CdaA protein in osmoresistant suppressor mutants. Red dots indicate amino acid changes and dark blue horizontal bars indicate frameshift mutations. The transmembrane domains are highlighted as thick yellow regions and the DAC domain is marked green. The DAC active site residues are highlighted in cyan.

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Figure 2.

852 Location of mutations in the CdaA protein in osmoresistant suppressor mutants. Red dots
853 indicate amino acid changes and dark blue horizontal bars indicate frameshift mutations. The
854 transmembrane domains are highlighted as thick yellow regions and the intracellular DAC
855 domain is marked green. The DAC active site residues are highlighted in cyan.
856
857 **Figure 3.**
858 Mutation of *glmM* affects salt resistance, c-di-AMP level and peptidoglycan precursor synthesis.
859 (A) Comparison of growth of strains on GM17 agar or GM17 agar + 0.25 M NaCl following
860 spotting of serial dilutions. (B) Levels of c-di-AMP in wt, OS2 and OR5 mutants from three
861 biological replicates. (C) Early steps in the bacterial cell wall peptidoglycan biosynthesis
862 pathway. (D) Location of the *glmM* (*femD)* gene in the same operon as *cdaA* and the frameshift
863 mutated *cdaR* pseudogene (denoted as *cdaR'*) in MG1363. The GlmM I154F mutation in OR5 is
864 shown with an arrow. (E) Levels of peptidoglycan precursor UDP-N-acetylglucosamine in
865 strains from three biological replicates. Significant $P<0.01$ (**) and $P<0.05$ (*) results were
866 determined using Tukey's test. ND, none detected.
867 Figure 3.
868 Mutation of the *glmM* gene affects salt resistance, the c-di-AMP level and peptidoglycan
869 precursor synthesis. (A) Comparison of growth strains on GM17 agar or GM17 agar + 0.25 M
870 NaCl following spotting of serial dilutions. (B) Levels of c-di-AMP in wt, OS2 and OR5
871 mutants. **, $P<0.01$ using Tukey's test based on duplicate cultures. ND, none detected. (C) Early
872 steps in the bacterial cell wall peptidoglycan biosynthesis pathway. (D) Location of the *glmM*
873 gene in the same operon as *cdaA* and the frameshift mutated *cdaR* pseudogene (denoted as
874 *cdaR'*) in MG1363. The GlmM I154F mutation in OR5 is shown with an arrow. (E) Levels of
875 peptidoglycan precursor UDP-N-acetylglucosamine in strains. *, $P<0.05$ and **, $P<0.01$ using
876 Tukey's test based on triplicate cultures.
877
878 **Figure 4.**
879 Autolysis is affected by the c-di-AMP level in cells. Wild-type MG1363 (solid grey line), a high
880 c-di-AMP containing osmosensitive *gdpP* mutant (OS2 [solid black line]), and several low c-di-
881 AMP osmoresistant suppressor mutants derived from OS2 containing *cdaA* (dashed grey lines)
882 or *glmM* (OR5 [dashed black line]) mutations were incubated in GM17 broth at 30°C and
883 monitored with regular OD_{600} measurements. Averages and standard deviation error bars are
884 shown based on three biological replicates.

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887 c-di-AMP containing osmosensitive *gdpP* mutant (OS2 [solid grey line]), and several low c-di-
888 AMP osmoresistant suppressor mutants containing *cdaA* (dashed grey lines) or *glmM* (OR5
889 [dashed black line]) mutations were incubated in GM17 broth at 30°C and monitored with
890 regular OD_{600} measurements. The experiment was carried out in duplicate (SD is shown as error
891 bars).
892
893 **Figure 5.**
894 GlmM reduces c-di-AMP synthesis by CdaA and the $GlmM^{I154F}$ variant shows greater inhibitory
895 action. (A) Three different combinations of CdaA operon genes from *L. lactis* MG1363 were
896 expressed in *E. coli* and c-di-AMP levels from three biological replicates were determined.
897 CdaR' indicates the product(s) from the frameshift mutated *cdaR*' pseudogene which has
898 homology to the YbbR domain containing protein CdaR in other bacteria. The star indicates the
899 I154F mutation in GlmM. (B) Three different combinations of CdaA operon genes from *S.*
900 *aureus* NCTC 8325 were expressed in *E. coli* and c-di-AMP levels from three biological
901 replicates were determined. Significant *P*<0.01 (**) results were determined using Tukey's test.
902 Figure 5.
903 $GlmM^{I154F}$ reduces c-di-AMP synthesis by CdaAR' in *E. coli* more than wild-type GlmM. (A)
904 Three different combinations of CdaA operon genes from *L. lactis* MG1363 were expressed in *E.*
905 *coli*. CdaR' indicates the product(s) from the frameshift mutated *cdaR*' pseudogene which has
906 homology to the YbbR domain containing protein CdaR in other bacteria. (B) Levels of c-di-
907 AMP in three *E. coli* strains expressing genes shown in (A) were determined **, *P*<0.01 using
908 Tukey's test based on triplicate cultures.
909
910 **Figure 6.**
911 *L. lactis* $GlmM^{I154F}$ binds more strongly to CdaA than GlmM. Bacterial two-hybrid analysis
912 showing β-galactosidase activity in *E. coli* strains containing different combinations of two
913 plasmids encoding domains of the *B. pertussis* adenylate cyclase. Different genes cloned into
914 pKNT25 or pKT25 and pUT18C are indicated. The leucine zipper GCN4 (Zip) proteins are
915 positive controls ([a]note that *zip* in cloned in pKT25 while *cdaA* was cloned in pKNT25), while
916 negative controls containing at least one empty plasmid (indicated as '—') are shown. The
917 negative control strain which had the highest β-galactosidase activity was used for comparison to

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918 the strain expressing both CdaA and GlmM. Significant $P<0.01$ (**) results were determined
919 using Tukey's test using three biological replicates.
920
921 **Figure 7.**
922 Model of the c-di-AMP signalling pathway involving CdaA from results obtained in this and
923 other studies. Solid black lines indicate enzymatic reactions, dashed black lines indicate a
924 translation product, red lines indicate a negative effect and green arrows indicate a positive
925 effect. Synthesis and degradation of c-di-AMP is carried out by diadenylate cyclase (CdaA as the
926 most common and is shown here) and phosphodiesterase (GdpP is the most common and is
927 shown here). CdaR binds to and regulates CdaA mediated c-di-AMP synthesis (note that in *L.*
928 *lactis* MG1363 *cdaR* is a pseudogene). The stringent response nucleotide (p)ppGpp inhibits
929 GdpP thereby reducing c-di-AMP hydrolysis while high or low c-di-AMP levels can result in an
930 increase in the (p)ppGpp level. GlmM binds directly to and inhibits CdaA mediated c-di-AMP
931 synthesis and c-di-AMP levels affect peptidoglycan precursor (UDP-NAG) biosynthesis, most
932 likely via GlmM.
933
934
935 ~~**Figure 6.**~~
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942 ~~that in *L. lactis* MG1363 *cdaR* is a pseudogene). The stringent response nucleotide (p)ppGpp~~
943 ~~inhibits GdpP thereby reducing c-di-AMP hydrolysis while high c-di-AMP levels result in an~~
944 ~~increase in the (p)ppGpp level. GlmM inhibits CdaA mediated c-di-AMP synthesis and c-di-~~
945 ~~AMP levels affect peptidoglycan precursor (UDP-NAG) biosynthesis, most likely via GlmM.~~
946
947 **Supplementary Figure 1.**
948 Alignment of CdaA homologs in Gram-positive bacteria. Darker red shading indicates highly
949 conserved residues while darker blue shading indicates less conserved residues. The location of
950 the mutations found in suppressor mutant strains here are indicated by spots above the amino

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951 acid. Bacteria listed are as follows: llm, *Lactococcus lactis* subsp. *cremoris* MG1363; bce,
952 *Bacillus cereus* ATCC 14579; bsu, *Bacillus subtilis* subsp. *subtilis* 168; btk, *Bacillus*
953 *thuringiensis* 97-27; cac, *Clostridium acetobutylicum* ATCC 824; cpe, *Clostridium perfringens*
954 13; efa, *Enterococcus faecalis* V583; gka, *Geobacillus kaustophilus*; hhd, *Halobacillus*
955 *halophilus*; lac, *Lactobacillus acidophilus* NCFM; lca, *Lactobacillus casei* ATCC 334; ljo,
956 *Lactobacillus johnsonii* NCC 533; lla, *Lactococcus lactis* subsp. *lactis* Il1403; llc, *Lactococcus*
957 *lactis* subsp. *cremoris* SK11; lme, *Leuconostoc mesenteroides* subsp. *mesenteroides* ATCC
958 8293; lmo, *Listeria monocytogenes* EGD-e; lpl, *Lactobacillus plantarum* WCFS1; lrh,
959 *Lactobacillus rhamnosus* GG; lsa, *Lactobacillus sakei*; ooe, *Oenococcus oeni*; ppe, *Pediococcus*
960 *pentosaceus* ATCC 25745; sav, *Staphylococcus aureus* subsp. *aureus* Mu50 (MRSA/VISA);
961 spm, *Streptococcus pyogenes* MGAS8232 (serotype M18); spn, *Streptococcus pneumoniae*
962 TIGR4 (virulent serotype 4); spy, *Streptococcus pyogenes* SF370 (serotype M1); stc,
963 *Streptococcus thermophilus* CNRZ1066.