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Cyclic di-AMP targets the cystathionine beta-synthase domain of the osmolyte transporter OpuC

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SUMMARY

Cellular turgor is of fundamental importance to bacterial growth and survival. Changes in external osmolarity as a consequence of fluctuating environmental conditions and colonization of diverse environments can significantly impact cytoplasmic water content, resulting in cellular lysis or plasmolysis. To ensure maintenance of appropriate cellular turgor, bacteria import ions and small organic osmolytes, deemed compatible solutes, to equilibrate cytoplasmic osmolarity with the extracellular environment. Here, we show that elevated levels of c-di-AMP, a ubiquitous second messenger among bacteria, result in significant susceptibility to elevated osmotic stress in the bacterial pathogen *Listeria monocytogenes*. We found that levels of import of the compatible solute carnitine show an inverse correlation with intracellular c-di-AMP content and that c-di-AMP directly binds to the CBS domain of the ATPase subunit of the carnitine importer OpuC. Biochemical and structural studies identify conserved residues required for this interaction and transport activity in bacterial cells. Overall, these studies reveal a role for c-di-AMP mediated regulation of compatible solute import and provide new insight into the molecular mechanisms by which this essential second messenger impacts bacterial physiology and adaptation to changing environmental conditions.

Graphical abstract

Osmoadaptation is mediated in part through the uptake of the compatible solutes carnitine and betaine. Osmotolerance is inversely correlated with the production of the nucleotide c-di-AMP in *Listeria monocytogenes*, which directly binds to the CBS domain of the ATPase component of the carnitine transporter OpuC. These findings expand the link between c-di-AMP production and osmoadaptation among bacteria that produce this second messenger.

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INTRODUCTION

Bacterial signal transduction employs various proteins and small molecule second messengers to regulate multiple cellular processes. Among these signals, the nucleotide cyclic di-3',5'-adenosine monophosphate (c-di-AMP) has recently emerged as a near ubiquitous second messenger among bacteria, with essential roles in both bacterial physiology and host-pathogen interactions. Studies in different bacterial species revealed that this nucleotide is involved in broad cellular processes, such as DNA damage responses, central metabolism, cell wall homeostasis, stress responses, potassium transport, and virulence (Corrigan & Grundling, 2013, Commichau *et al.*, 2015). Intriguingly, although mechanisms of c-di-AMP synthesis and hydrolysis are seemingly conserved in bacteria, many molecular targets appear unique to certain species. This suggests both the conservation and divergence of c-di-AMP signaling networks for different bacterial lifestyles.

Within bacterial cells, c-di-AMP is synthesized by the diadenylate cyclase (Dac) activity of DisA_N domain-containing proteins. Hydrolysis occurs through enzymatic activities of phosphodiesterase (Pde) proteins with a catalytic DHH-DHHA1 domain or HD domain. For bacteria that produce c-di-AMP, a defect in c-di-AMP synthesis confers aberrant physiology and even lethality in several species. Conversely, the accumulation of this nucleotide also diminishes growth and virulence (Huynh & Woodward, 2016).

The intracellular pathogen *Listeria monocytogenes* synthesizes and secretes c-di-AMP during growth in broth culture and in host cells. Deletion of *dacA*, which encodes a diadenylate cyclase for c-di-AMP synthesis, is detrimental to *Listeria* growth. For c-di-AMP degradation, *L. monocytogenes* encodes PdeA and PgpH, which act cooperatively to hydrolyze c-di-AMP. The *pdeA pgpH* mutant, which accumulates approximately four times more c-di-AMP than the wild-type strain, is defective for growth in broth and macrophages. As shown for several other pathogens, the *pdeA pgpH* mutant is also

severely attenuated in a murine infection model, again highlighting the toxicity of elevated c-di-AMP levels (Huynh *et al.*, 2015).

Phosphodiesterase mutants in various bacteria exhibit aberrant phenotypes, such as altered cell wall metabolism, cell division, impaired potassium transport, defective stress responses, and diminished virulence (Bai *et al.*, 2013, Cho & Kang, 2013, Mehne *et al.*, 2013, Yang *et al.*, 2014, Ye *et al.*, 2014, Dey *et al.*, 2015). Nevertheless, there has been no systematic examination of the physiological effects of c-di-AMP accumulation. In an attempt to investigate the mechanism of c-di-AMP toxicity, we performed a phenotypic microarray screen on the *pdeA pgpH* mutant. Among the observed phenotypes, this mutant was highly sensitive to osmotic stress. As such, we interrogated the interaction of c-di-AMP with several membrane transporters involved in osmotic stress responses. We found that c-di-AMP interacts specifically with the ATPase component of the OpuC carnitine transporter, likely inhibiting carnitine uptake upon osmotic upshift. Thus, our studies expand the known targets of c-di-AMP, and generalize the role of c-di-AMP in osmotic regulation, a universal aspect of bacterial physiology.

RESULTS

C-di-AMP accumulation diminishes compatible solute uptake

The Biolog phenotype microarray (Biolog Inc.) provides a platform for high-throughput screening of bacterial responses to >1000 distinct growth conditions. In this technology, bacterial strains are cultivated in 96-well microplates, with each well presenting a different culture condition. Metabolic activity is measured through cellular respiration, which reduces a tetrazolium dye and produces a purple color. The color intensity reflects altered levels of metabolism, and can be easily monitored and quantitated (Bochner *et al.*, 2001). We subjected the *L. monocytogenes* WT and *pdeA pgpH* strains to plates PM 1–10, which test metabolic activity under various carbon, phosphorous and sulfur sources, amino acid and vitamin supplements, peptide nitrogen sources, and pH and osmotic stresses. These two strains showed consistent metabolic differences in PM 9, with increasing osmolyte concentrations in the presence or absence of compatible solutes (Fig. 1A, for all plate images see Fig. S1).

To validate this observation, we examined *L. monocytogenes* strains for growth under osmotic stress. Since *dacA* is an essential gene, it was deleted in the presence of an IPTG-inducible allele, creating a conditionally null mutant (c *dacA*). In BHI broth with increasing concentrations of NaCl, the c *dacA* strain, which has the lowest level of c-di-AMP, was the most resistant to osmotic stress (Fig. 1B). By contrast, the *pdeA pgpH* strain, which has the highest level of c-di-AMP, was the most sensitive. Similar observations were made with the non-ionic osmolyte sorbitol (Fig. 1C). Additionally, whereas the WT and c *dacA* strains also exhibited growth defects in defined minimal medium supplemented with 2% NaCl (MMS), the *pdeA pgpH* strain was completely inhibited (Fig. 1D).

L. monocytogenes is well adapted to high-salt environments and accumulates various solutes to cope with osmotic stress, with the best studied examples of potassium, betaine, and carnitine (Sleator *et al.*, 2003). In the presence of salt stress, the WT and c *dacA* growth

defects were rescued by betaine and carnitine, but not by KCl, suggesting that potassium alone is insufficient for long-term adaptation to osmotic stress. Additionally, the *pdeA* *pgpH* growth defect was not rescued by any solute, indicating a defect for solute transport (Fig. 1D).

We selected carnitine as a representative compatible solute to test for transport activities by the WT and mutant strains. In these transport assays, exponential phase cultures in BHI broth were subjected to BHI + 4% NaCl + ¹⁴C-labeled carnitine and radioactivity was measured to evaluate intracellular carnitine accumulation. Compared to the WT strain, the *c dacA* mutant exhibited slightly higher transport activity (Fig. 2A). Among the phosphodiesterase mutants, the *pdeA* strain has wild-type c-di-AMP levels, whereas the *pgpH* mutant exhibits a two-fold increase, and the *pdeA* *pgpH* mutant has four-fold more c-di-AMP than WT (Fig. 2B). For carnitine transport, *pdeA* was indistinguishable from the WT strain, and *pgpH* exhibited a small but consistent decrease in transport activity. Finally, the *pdeA* *pgpH* mutant was highly impaired for carnitine transport (Fig. 2A). When the carnitine transported by these strains was compared to their relative intracellular c-di-AMP levels, a clear inverse correlation was observed (Fig. 2B). Together with the hyper-sensitivity of the *pdeA* *pgpH* strain to osmotic stress, its defect for compatible solute uptake indicates that c-di-AMP has an inhibitory effect on transport activity.

C-di-AMP interacts specifically with the carnitine transporter component OpuCA

Given the assumption that c-di-AMP antagonizes compatible solute transport, we wished to examine specific transporters for interactions with c-di-AMP. *L. monocytogenes* encodes various transport systems for solute uptake and efflux, including KdpABC and Ktr-type transporters for potassium uptake, BetL and GbuABC for betaine uptake, OpuC for carnitine uptake, various peptide transporters, aquaporins and mechanosensitive channels (Sleator et al., 2003).

Consistent with previous studies in *Staphylococcus aureus*, we observed that the histidine kinase sensor KdpD bound c-di-AMP via the USP domain (data not shown) (Moscoso et al., 2016). However, as potassium did not rescue long-term growth by *L. monocytogenes* under osmotic stress, we also investigated the carnitine and betaine transporters.

OpuC is a high affinity uptake system for carnitine and comprised of four proteins: OpuCA, OpuCB, OpuCC, and OpuCD. OpuCC is an extracellular substrate binding protein that is tethered to the cell membrane via a lipid modification. OpuCB and OpuCD form transmembrane permease subunits. OpuCA is a cytoplasmic protein with an ATP-binding cassette (ABC) and tandem cystathionine-β-synthase (CBS) domains (Fig. 3A). The ATPase activity of OpuCA is required for transport activity of the OpuC system. It has been shown that the uptake rate is stimulated by increased osmolality, and might be inhibited by intracellular metabolites (Fraser et al., 2000). The glycine betaine transporter GbuABC has a similar architecture to OpuC (Fig. 3A), in which GbuA is a cytoplasmic ATPase protein with tandem CBS domains. Interestingly, mutations in GbuABC transporter were recently reported in strains of *L. monocytogenes* suppressed for c-di-AMP essentiality (Whiteley et al., 2015). The Bile transport system is very closely related to OpuC, but has been implicated in bile acid resistance rather than osmolyte import (Sleator et al., 2005).

Furthermore, the ATPase subunit of Bile and OpuCA share ~60% amino acid identity but differs markedly in the C-terminus, where the CBS domain of the ATPase subunit of the Bile transporter is significantly truncated (Fig. 3A). As previous work in *L. monocytogenes* reported the interaction of c-di-AMP with two CBS domain-containing proteins, Lmo0553 and Lmo1009 (Sureka *et al.*, 2014), we hypothesized that c-di-AMP may also bind the CBS domains of osmolyte importers as well. Thus, we examined GbuA, OpuCA, and Bile, and observed c-di-AMP binding only for OpuCA (Fig. 3B). Furthermore, the CBS domain alone was able to directly interact with c-di-AMP but not the isolated ATP-binding domain (Fig. 3B). Together these observations suggested that c-di-AMP interacts with the CBS domain of the ATPase subunit of the solute transporter OpuC.

To further characterize OpuCA, we purified both the full-length protein fusion with maltose binding protein (MBP-OpuCA) and the 6xHis-tagged CBS domain (CBS). The full-length protein bound c-di-AMP at a high affinity (Fig. 3C), while the isolated CBS domain showed decreased affinity comparable to the full-length protein (Fig. 3D). CBS domains associated with several proteins have been shown to be regulated by adenosine nucleotides, such as ATP and AMP. In a competition-binding assay, we tested these and other related nucleotides in excessive concentrations (at least 1000-fold) relative to radio-labeled c-di-AMP, and found that CBS protein interacted with c-di-AMP specifically (Fig. 3E). To determine if this observation was unique to *L. monocytogenes*, we also tested the binding of an OpuCA homolog from *Enterococcus faecalis* and found a similar low micromolar affinity for the EfOpuCA protein (Fig. 3F). Together, these findings show that the CBS domain within the ATPase subunit of the osmolyte transporter OpuC specifically binds to c-di-AMP at physiologically relevant concentrations, and that this may be a widespread interaction among bacteria.

Structural studies reveal the mode of c-di-AMP binding

We solved the structure of *L. monocytogenes* OpuCA (LmOpuCA) tandem CBS domains (residues 247–371, Fig. 3A) at 2.9 Å resolution using the molecular replacement method with the CBSX1 protein structure from *Arabidopsis thaliana* as the search model (Jeong *et al.*, 2013) (PDB entry code: 4GQV, sequence identity 27%). There is a dimer of LmOpuCA in the asymmetric unit. The crystallographic statistics are summarized in Table 1. The crystal was found to have significant pseudo-translational symmetry (with a peak height of 64% relative to the Patterson origin peak), which is likely part of the reason for the somewhat higher *R* values for this structure. The data set used for refinement was the best one among the many that we collected on these crystals.

The structure of each CBS domain of LmOpuCA has a three-stranded anti-parallel β -sheet packed against two α -helices (Fig. 4A). The β -sheets of the two tandem CBS domains within the monomer are packed against each other, forming a Bateman domain. Two LmOpuCA Bateman domains then dimerize head-to-tail, mediated primarily by interactions between the α -helices, forming a disk-like assembly with a central cavity (Fig. 4A). Both head-to-head and head-to-tail dimeric associations have been commonly observed for Bateman domains (Baykov *et al.*, 2011).

The diffraction quality of the free OpuC CBS domain crystals was very poor, and we were not able to determine the structure of the free protein. However, soaking of these crystals with c-di-AMP overnight greatly improved their diffraction, which allowed us to determine the structure of the complex. This could indicate that c-di-AMP might induce some conformational rearrangement in the OpuC CBS domain.

We observed clear electron density for one c-di-AMP molecule bound to the OpuCA dimer (Fig. 4B), with the two-fold symmetry axis of the compound aligned with that of the dimer (Fig. 4A). This is similar to the binding mode of c-di-AMP to the CT dimer of LmPC (pyruvate carboxylase), in which the ligand is also aligned with the two-fold symmetry axis of that dimer (Sureka et al., 2014). In LmOpuCA, the c-di-AMP is located in a solvent-exposed, electropositive pocket in the center of the dimer (Fig. 4C). The c-di-AMP adopts a flat, nearly fully extended conformation, which appears to be unique among known c-di-AMP complexes. For example, c-di-AMP assumes a mostly folded conformation in the complex with LmPC.

The c-di-AMP compound bridges the two OpuCA monomers, and makes identical interactions with each (Fig. 4D). The adenine bases are buried deep into the binding pocket, packed between the side chains of Tyr342 and Ile355 on one face, and Val260 and Val280 on the other. The adenines are specifically recognized by the protein through hydrogen bonds between its N1 and N6 atoms to the main chain amide and carbonyl of Val280. In addition, the N7 atom of adenine makes a hydrogen bond with the side chain hydroxyl of Thr282. The phosphate group of c-di-AMP makes two hydrogen bonds to the main chain amide of Arg358 and Ala359, which are located at the N-terminal end of the last helix of the CBS domain, suggesting that the phosphate also has favorable interactions with the dipole of this helix. The residues in the c-di-AMP binding pocket are well conserved among OpuCA homologues (Fig. 4E), suggesting that c-di-AMP binding to OpuCA is of functional importance in many bacteria.

C-di-AMP binding mutants exhibit altered carnitine transport activities

Since c-di-AMP appeared to inhibit carnitine transport in bacterial cultures, we attempted to characterize this inhibition on the ATPase activity of OpuCA. However, for both the *L. monocytogenes* and *E. faecalis* homologs, the protein activity was rapidly lost, likely due to the requirement to associate with other components of the transport complex to perform repeated turnover. Thus, we performed a mutational analysis of OpuCA to examine the relationship between c-di-AMP binding and carnitine transport.

Based on the crystal structure of the OpuCA CBS domains, we generated several amino acid mutations in sites highly conserved among OpuCA homologs. Mutations of the conserved R279, V280 and D281 residues resulted in very weak to no observable binding to c-di-AMP (Fig. 5A). By contrast, mutations of the K341 and Y342 residues resulted in a slight increase and decrease in c-di-AMP binding affinity, respectively. Together, these data confirmed that conserved amino acids in the c-di-AMP binding pocket of OpuCA affected the affinity of the protein for this nucleotide.

To interrogate the effects of these amino acids on protein activity, we next performed allelic exchange to replace the *opuCA*⁺ allele with these point mutant alleles in the WT, *pgpH*, and *pgpH pde::Tn917* backgrounds (please see Experimental Procedures for an explanation). We reasoned that if c-di-AMP is an allosteric regulator of the ATPase activity of OpuCA, then c-di-AMP-binding site mutants could alter the transport of this osmolyte in *L. monocytogenes*. The V280 and K341 mutations completely abolished carnitine transport activity in all strains, irrespective of c-di-AMP levels (Fig. 5B and Fig. S2). In the wild-type and *pgpH* backgrounds, which have normal and intermediate c-di-AMP levels, no significant change in transport was observed for the other point mutations (Fig. S2). In the *pgpH pde::Tn917* background with elevated c-di-AMP activity, we found that relative to WT, the R279A mutant exhibited increased transport activity and the D281A and Y342A mutations resulted in lower transport activity (Fig. 5B). Together, these studies show that mutation of the conserved RVD and KY amino acids lining the c-di-AMP binding pocket drastically affect transport activity of OpuC, although only the RVD sequence is indispensable for high-affinity binding to c-di-AMP by OpuCA. No obvious correlation between c-di-AMP affinity and transport activity was revealed by these studies (Fig. 5C), suggesting a complex interplay between the role of these conserved amino acids in mediating c-di-AMP nucleotide recognition and osmolyte transport.

DISCUSSION

Regulation of CBS domains by c-di-AMP

CBS domains (Pfam PF00571) are widespread in all three domains of life, currently identifiable in more than 35,000 protein sequences, and assumed to regulate the catalytic activity of associated enzymatic or signal transduction domains. The regulatory function of many CBS domains has been shown to be dependent on adenine nucleotide ligands, such as S-adenosyl methionine, AMP, ATP, and diadenosine polyphosphates (Baykov et al., 2011, Anashkin et al., 2015). Through biochemical, structural, and genetic analyses, we examined the OpuCA CBS domain as another molecular target of c-di-AMP in bacteria.

A previous study in *L. monocytogenes* revealed that c-di-AMP binds two CBS-domain containing proteins, Lmo1009 and Lmo0553 (Sureka et al., 2014). Additionally, as documented for *S. aureus* and *L. lactis* (Smith et al., 2012, Corrigan et al., 2013), we observed a negative regulatory effect of c-di-AMP in osmotic stress response. These findings prompted us to examine three ABC-type solute transporters, OpuC, GbuABC, and BilE, which all harbor CBS domains. Among these proteins, we found that c-di-AMP binds to the CBS domain of OpuCA, the ATPase subunit of OpuC but did not interact with GbuA, although it is highly similar to OpuCA in architecture and function. CBS domain sequences are highly divergent, despite their structural conservation (Baykov et al., 2011). Consistent with this notion, we observed low sequence conservation among these five bacterial CBS domains. Among c-di-AMP binding proteins, OpuCA exhibits the conserved RVD residues that are required for interaction, but the corresponding amino acids are different (GYS/R) for Lmo1009 and Lmo0553. Interestingly, GbuA exhibits the GTS residues at these positions, reminiscent of Lmo1009, although it did not interact with c-di-AMP (Fig. S3). Thus,

systematic analyses of more CBS domains are required to elucidate conserved structural elements of c-di-AMP interaction.

CBS domains are widespread among bacterial ABC transporters involved in compatible solute uptake, which presumably are important in regulating transport activity under osmotic stress. For instance, in *Pseudomonas* sp., *opuC* mutants with CBS-domain deletions were defective for growth under hyperosmotic conditions, reflecting the requirement of this element in OpuC function (Chen & Beattie, 2007). For the well-studied glycine and betaine transporter OpuA of *Bacillus subtilis* and *Lactococcus lactis*, the CBS domain has also been extensively characterized with a role for sensing ionic strength within the cytoplasm, and possibly in a conformational change that is required for catalytic activity (Horn *et al.*, 2003, Biemans-Oldehinkel *et al.*, 2006, Mahmood *et al.*, 2006, Horn *et al.*, 2008). However, no allosteric regulators have been reported for compatible solute transporters.

Our analyses indicated that c-di-AMP inhibits OpuC function, as c-di-AMP levels were inversely correlated to carnitine transport in *L. monocytogenes*. Since the OpuCA CBS domain interacted specifically with c-di-AMP at high affinity, we hypothesize that c-di-AMP is an allosteric regulator of OpuC transport function. Several mutations of the c-di-AMP binding site altered transport activity. In the *pdeA pgpH* background with a high c-di-AMP level, the weak-binding mutant R279A recovered carnitine transport, whereas the strong-binding mutant K341A abolished transport. These mutant phenotypes are consistent with a negative regulation of OpuCA by c-di-AMP, but further biochemical studies are required to establish a direct link between ligand binding and protein function. Indeed, the V280A and V280W mutations abolished both c-di-AMP binding and transport activity. Interestingly, the D281A mutation severely impaired c-di-AMP binding but inhibited activity only in the *pdeA pgpH* background. It is possible that this strain is altered for ionic contents which also affect OpuCA activity as discussed below. Together, the mutant phenotypes reflect the complexity in OpuCA regulation and c-di-AMP is perhaps another allosteric factor though the mechanism for this regulation is not yet elucidated in this study.

Interaction of c-di-AMP with the OpuCA CBS domains may cause conformational changes that affect protein function. In transport cycles of bacterial ABC transporters, including OpuA, the nucleotide-binding ATPase domain typically undergoes a dynamic interconversion between the dimer and monomer conformations, and the ATP-dependent closed dimer conformation allows ATP hydrolysis (Higgins & Linton, 2004). It is conceivable that c-di-AMP binding traps the nucleotide-binding domain in the open conformation, thereby inhibiting transport. Indeed, substrate binding to the CBS domains has been shown to induce large conformational changes in the associated domains, as shown for the magnesium transporter MgtE (Takeda *et al.*, 2014). As the c-di-AMP binding pocket is formed at the dimer interface of OpuCA CBS domain, it is likely that binding alters protein dimerization. Additionally, the CBS domain of OpuAA interacts with the negatively charged membrane surface and blocks transport function. Above a threshold ionic strength, Na⁺ and K⁺ ions relieve this inhibition and activate the transporter (Biemans-Oldehinkel *et al.*, 2006, Mahmood *et al.*, 2006, Mahmood *et al.*, 2009, Karasawa *et al.*, 2011). If this phenomenon also occurs for OpuCA, c-di-AMP binding may result in a conformational

change within the CBS domain that reduces ion sensing, for instance, by preventing the exposure of charged residues that interact with Na⁺ and K⁺ ions.

The global regulatory role of c-di-AMP in osmotic stress response is a conserved aspect of c-di-AMP signaling

The ability to cope with osmotic stress is a universal aspect of bacterial physiology, and is a major determining factor for growth and virulence (Poolman & Glaasker, 1998). The majority of bacterial species encode various strategies to cope with osmotic stress. The initial response is typically potassium uptake via Trk/Ktr and Kdp systems. C-di-AMP has been shown to interact with protein components of both systems, and possibly with riboswitches preceding certain genes (Corrigan et al., 2013, Nelson *et al.*, 2013, Bai *et al.*, 2014, Kim *et al.*, 2015, Moscoso et al., 2016). Similar to findings in *S. aureus*, we also observed an interaction of c-di-AMP with the KdpD USP domain in *L. monocytogenes*, confirming a role for c-di-AMP in regulating potassium transport. However, potassium supplementation did not rescue *L. monocytogenes* growth under long-term osmotic stress. Indeed, the role of the *kdp* system in *L. monocytogenes* osmotic tolerance has been shown to be strain dependent, as the EGD *kdpE* mutant exhibited no defect for growth under high salt (Brondsted *et al.*, 2003).

The regulatory effect of c-di-AMP in carnitine transport may partially explain the pleiotropic phenotypes conferred by elevated c-di-AMP levels. For *L. monocytogenes*, in addition to transporting carnitine under salt stress, OpuC is also important for bile resistance, and required for survival in the GI tract as well as systemic invasion (Wemekamp-Kamphuis *et al.*, 2002, Watson *et al.*, 2009). The human small intestine has an osmolarity equivalent to 0.3 M NaCl, requiring gastrointestinal pathogens to be highly tolerant to salt stress in order to persist in this environment and to further invade other tissues (Sleator *et al.*, 2009). Exposure to elevated osmolarity induces the expression of the σ^B regulon for general stress responses and GI epithelial cell invasion (Abram *et al.*, 2008, Bae *et al.*, 2012, Walecka-Zacharska *et al.*, 2013). Furthermore, σ^B also controls the transcription of PrfA, the master virulence regulator, and the σ^B and PrfA regulons significantly overlap, further emphasizing the role of osmotic stress as a primary stress and virulence signal (Ollinger *et al.*, 2009, Chaturongakul *et al.*, 2011). Thus, an impaired OpuC transport activity in the *pdeA* *pgpH* mutant may have adverse consequences in physiology and virulence. Indeed, this strain is also highly sensitive to bile stress (unpublished data) and future studies to interrogate the effects of c-di-AMP and OpuCA on virulence are warranted.

In summary, we report c-di-AMP as the first second messenger that targets the CBS domain of a bacterial membrane transporter and provide an atomic level description of this protein-nucleotide interaction. Allosteric regulation of solute transporters is a rapid strategy in coping with osmolarity fluctuations encountered by bacteria. The widespread presence of CBS domains among osmolyte transporters in bacteria suggests that this likely represents the first example of a conserved regulatory target of c-di-AMP. Despite the potentially conserved role of c-di-AMP in this context, why c-di-AMP is integrated in the pathway of osmolyte accumulation remains unresolved. Interestingly, CBS domain containing membrane transporters are broadly conserved among living organisms, many of which have

not been described to produce c-di-AMP, opening the possibility that other second messenger mediators of osmolyte import may await discovery.

EXPERIMENTAL PROCEDURES

Bacterial strains and culture conditions

L. monocytogenes strains used in this study are listed in Table S1. Deletion of *opuCA* (amino acid residues 4–395) was achieved by allelic exchange as previously described (Camilli *et al.*, 1993). Briefly, a truncated *opuCA* DNA fragment (deleted for amino acids 4–395), with upstream and downstream flanking regions, was cloned into a pKSV7 vector, to create a pKSV7-*opuCA* plasmid. This plasmid was transformed into *E. coli* SM10 strain, and conjugated into *L. monocytogenes*. Since pKSV7 is temperature sensitive, trans-conjugants were grown at 42°C to allow chromosomal integration of the plasmid, and plasmid curing was obtained by several passages at 30°C in the absence of selective antibiotics. Site-specific mutagenesis of *opuCA* was first obtained by Quick Change PCR on a pET20b-*opuCA* plasmid, and the mutated *opuCA* fragments were cloned into pKSV7 with ~500 base pairs of upstream and downstream flanking fragments. Mutant alleles of *opuCA* were then introduced into *opuCA* or *opuCA pgpH* by the same allelic exchange method. We found that if allelic exchange was performed in the *pdeA pgpH* background, growth at 42°C selected for suppressor mutations that grew equivalent to WT *L. monocytogenes* and had diminished levels of c-di-AMP (data not shown). As such, the knock-in strains in the *pgpH* background were subsequently transduced with U153 phage lysate generated from a *pdeA::Tr917* strain, to create the knock-in point mutants in the double phosphodiesterase mutant background. Broth growth curves were assessed in Brain Heart Infusion (BHI) broth at 37°C.

Protein expression, purification, and nucleotide binding

The DNA fragment encoding the OpuCA protein was PCR-amplified and cloned into the pMAL-c2x vector with the XbaI and HindIII restriction sites, generating an amino-terminal fusion of maltose binding protein (MBP-OpuCA). The CBS domain (aa 251–397) was cloned into a pET20b vector with the NdeI and XhoI restriction sites, generating a carboxy-terminal 6x Histidine tag (CBS protein). The resulting plasmids were transformed into *Escherichia coli* BL21 (DE3) strain for expression. Site directed mutagenesis was performed using QuikChange Site-Directed Mutagenesis (Agilent).

For MBP-OpuCA expression, an overnight *E. coli* culture were used to inoculate 1.5 L of LB medium containing 0.2% glucose and 100 µg mL⁻¹ Ampicillin, and grown at 37°C, 200 rpm until late-exponential phase (OD₆₀₀ ~0.8). At this stage, the culture was supplemented with 0.8 mM IPTG and shaken at 16°C, 200 rpm for approximately 14 hours. Cell pellets were collected by centrifugation and lysed by sonication in lysis buffer (20 mM Tris, pH 7.5, 500 mM NaCl, 5% glycerol, 1 mM phenylmethylsulfonyl fluoride, 0.1% β-mercaptoethanol). Cell lysate containing soluble proteins were run through 2.5 mL of amylose resin (New England Biolabs), washed with column buffer (20 mM Tris, pH 7.5, 500 mM NaCl, 5% glycerol) and protein was eluted with elution buffer (column buffer supplemented with 10 mM maltose monohydrate). For CBS protein, the *E. coli* expression

culture was induced with 0.5 mM IPTG for 3 hours at 37°C. Cell pellets were re-suspended in phosphate buffer (30 mM K₂HPO₄ pH 8.0, 300 mM NaCl) and purified with a HisPur NiNTA resin (ThermoFisher Scientific). After SDS-PAGE analysis, protein fractions were pooled, concentrated with 5 kDa MWCO spin column concentrators (GE Healthcare). The concentrated proteins were desalted with PD-10 columns (GE Healthcare) and exchanged into binding buffer (40 mM Tris pH 7.5, 100 mM NaCl, 20 mM MgCl₂). Nucleotide binding assays were performed using DRaCALA as previously described (Roelofs *et al.*, 2011).

Solute transport assay

Bacterial cultures were grown in BHI broth until mid-exponential phase (OD ~ 0.7) and diluted 1:1 (v/v) with BHI + 8% NaCl with ¹⁴C-labeled carnitine (final concentration 85 μM). At indicated time points, samples were taken, applied on a 0.45 μm filter, and washed with phosphate buffered saline containing 4.5% NaCl. The filters were dried and immersed in scintillation liquid for radioactivity count. Concurrently, a duplicate culture containing cold carnitine was assessed for bacterial growth density.

Protein expression and purification for crystallization—*L. monocytogenes* OpuCA (Imo1428) residues 247–371 were subcloned into a pET28a vector with an N-terminal His-tag. This construct was then transformed into BL21 Star (DE3) cells. The cells were cultured in LB (Luria–Bertani) medium with 35 mg L⁻¹ kanamycin and were induced for 14 h at 20°C with 1 mM isopropyl β-D-1-thiogalactopyranoside. The protein was purified through nickel-agarose affinity chromatography followed by gel filtration chromatography (S-300, GE Healthcare, Piscataway, New Jersey, USA). The purified protein was concentrated to 30 mg mL⁻¹ in a buffer containing 20 mM Tris (pH 8.0), 250 mM NaCl, 5% (v/v) glycerol, and 5 mM dithiothreitol, flash-frozen in liquid nitrogen and stored at –80°C. The N-terminal hexa-histidine tag was not removed for crystallization.

Crystallization—LmOpuCA (residues 247–371) crystals were grown by the sitting-drop vapor diffusion method at 20°C. The protein at 3 mg/mL was mixed with reservoir solution containing 0.1 M bicine (pH 9.0), 3% (w/v) PEG 20,000, and 2% (v/v) 1,4-dioxane. The crystals appeared within a few days. The crystals were then soaked overnight in the reservoir solution supplemented with 4 mM of c-di-AMP and 25% (v/v) ethylene glycol. The crystals were then flash-frozen in liquid nitrogen for data collection at 100 K.

Data collection and structure determination—The X-ray diffraction data set was collected using Saturn944HG CCD mounted on a Rigaku Micromax-003 X-ray generator. The diffraction images were processed using HKL-3000 (Minor *et al.*, 2006). Pseudo-translational symmetry (peak height 64% of Patterson origin) was identified in the crystal using the program Phenix.Xtriage (Adams *et al.*, 2010). The structure was solved using the molecular replacement method with the program Phaser (McCoy *et al.*, 2007), using the CBSX1 protein structure from *Arabidopsis thaliana* (PDB code 4GQV) as the search model. Manual rebuilding was carried out in Coot (Emsley & Cowtan, 2004) and refinement was done with the program Refmac (Murshudov *et al.*, 2011). Atomic coordinates and structure factors have been deposited in the Protein Data Bank with accession code xxxx.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Inverse correlation between c-di-AMP levels and osmotic stress tolerance. (A) Phenotype microarray results of WT vs. $\Delta pdeA \Delta pgpH$ on PM9 wells A1–A9 with increasing NaCl concentrations. (B) The *L. monocytogenes c dacA*, wild-type (WT), and $\Delta pdeA \Delta pgpH$ strains were tested for growth in BHI broth with increasing NaCl concentrations. One-hundred percent indicates growth rate for each strain in the absence of NaCl. (C) Similar to (B) but growth rates were examined in increasing sorbitol concentrations. (D) Growth yields

in minimal medium with no salt, or minimal medium + 2% NaCl (MMS) with or without betaine, carnitine, and potassium chloride at indicated concentrations.

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Figure 2.

Carnitine transport under salt stress in the presence of different c-di-AMP levels. (A) Mid-exponential phase cultures grown in BHI broth were shifted to BHI + 4% NaCl + 85 μM ^{14}C -carnitine. Imported carnitine was evaluated by radioactivity retained by bacterial cells, and normalized to optical density at each time point. (B) Plot comparing the intracellular c-di-AMP levels of various *L. monocytogenes* strains versus the amount of carnitine transported at 20 minutes in panel A. The WT c-di-AMP level is typically 4 μM . Data represents at least two independent experiments.

Figure 3.

C-di-AMP interacts specifically with the CBS domain of the carnitine transporter component OpuCA. (A) Domain organization of OpuCA (Lmo1428), GbuA (Lmo1014), and Bile (Lmo1421). (B) Binding of OpuCA, GbuA, and Bile to c-di-AMP as measured by DRaCALA to full-length (FL), ATP-binding (ATP), or CBS domains. (C) Binding titration for the OpuCA full-length OpuCA protein and (D) the isolated CBS domain. (E) Competition binding assay for ~1.5 nM radiolabeled c-di-AMP, in the presence of 200 μ M unlabeled nucleotides. (C) Binding titration for the *E. faecalis* full-length OpuCA homolog.

Figure 4.

Crystal structure of LmOpuCA tandem CBS domains in complex with c-di-AMP. (A) Schematic drawing of the dimer of LmOpuCA tandem CBS domains (monomer 1 in cyan, monomer 2 in yellow) in complex with c-di-AMP (labeled cdA, carbon atoms colored black). The two CBS domains in each monomer are labeled CBS1 and CBS2. (B) Omit $F_o - F_c$ electron density for c-di-AMP at 2.9 Å resolution, contoured at 2.5 σ . (C) Molecular surface of the LmOpuCA binding sites for c-di-AMP, colored according to the electrostatic potential (blue: positive, red: negative). (D) Detailed interactions between LmOpuCA and c-di-AMP. Hydrogen bonds are shown with dashed lines (red). (E) Sequence conservation of residues in the LmOpuCA c-di-AMP binding site, generated based on an alignment of 150 sequences by the program Consurf (Armon *et al.*, 2001). Purple indicates conserved

residues, cyan indicates variable residues, and white indicates average conservation. All structure figures were produced with PyMOL (www.pymol.org).

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Figure 5. C-di-AMP binding mutants exhibit altered carnitine transport activities. (A) Binding curves of OpuCA CBS point mutants with radiolabeled c-di-AMP (B) Effects of *opuCA* mutant alleles in the *pdeA pgpH* background on carnitine transport. (C) Plot comparing the amount of ^{14}C -carnitine transported in panel B at 30 minutes versus the observed binding constant for c-di-AMP. V280A and D281A K_d values were above the limit of detection of the binding assay. Data represents at least two independent experiments.