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Systematic identification of conserved bacterial c-di-AMP receptor proteins

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Nucleotide signaling molecules are important messengers in key pathways that allow cellular responses to changing environments. Canonical secondary signaling molecules act through specific receptor proteins by direct binding to alter their activity. Cyclic diadenosine monophosphate (c-di-AMP) is an essential signaling molecule in bacteria that has only recently been discovered. Here, we report on the identification of four Staphylococcus aureus c-di-AMP receptor proteins that are also widely distributed among other bacteria. Using an affinity pull-down assay, we identified the potassium transporter-gating component KtrA as c-di-AMP receptor protein and it was further shown that this protein, together with c-di-AMP, enables S. aureus to grow in low potassium conditions. We defined the c-di-AMP binding activity within KtrA to the RCK (regulator of conductance of K⁺) domain. This domain is also found in a second S. aureus protein, CpaA, which as we show here also directly binds c-di-AMP. Since RCK domains are found in proteinaceous channels, transporters and antiporers from all kingdoms of life, these findings have broad implications for the regulation of different pathways through nucleotide-dependent signaling. Using a genome-wide nucleotide protein interaction screen, we further identified the histidine kinase protein KdpD that in many bacteria is also involved in the regulation of potassium transport and a PII-like signal transduction protein, which we renamed PstA, as c-di-AMP binding proteins. With the identification of these widely distributed c-di-AMP receptor proteins we link the c-di-AMP signaling network to a central metabolic process in bacteria.

c-di-AMP | receptor protein | Staphylococcus

Introduction. Nucleotide signaling molecules control fundamental processes in all forms of life. There is now a large body of evidence linking nucleotides such as cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP) and guanosine tetra-(pppGpp) and pentaphosphate (pppppGpp) to the control of fundamental metabolic pathways and stress response processes in eukaryotic and prokaryotic cells (1-3). Cyclic dinucleotides in particular have recently gained increased attention with the identification of novel nucleotides such as cyclic diadenosine monophosphate (c-di-AMP) and the hybrid cAMP-GMP molecule in bacterial cells (4-6), as well as the discovery that cyclic dinucleotides are also produced by eukaryotic cells (7-9). The dinucleotide cyclic diadenosine monophosphate (c-di-GMP) and the molecular mechanisms by which it controls cellular pathways has been well characterized and it is now recognized as a central regulator in bacterial cells that controls the switch from free-living planktonic to a sessile biofilm-associated lifestyles. In pathogenic organisms this is often linked to colonization of the human host and virulence (10). On the other hand, the function and the pathways controlled by the novel signaling nucleotide c-di-AMP are less clear, largely due to a gap in our knowledge of specific receptor proteins.

Many Gram-positive bacteria, including the important human pathogens Staphylococcus aureus (11), Streptococcus pyogenes (12), Listeria monocytogenes (5) and Mycobacterium tuberculosis (13) produce c-di-AMP and it is likely that c-di-AMP is also synthesized by several Gram-negative bacteria and a subset of archaea (14). c-di-AMP is synthesized by DisA_N domain-containing proteins DucA, DisA and YojJ and degraded by the DHH/DHHA1 domain-containing phosphodiesterase enzyme GdpP (4, 5, 11, 15-18). A variety of different phenotypes have been linked to altered c-di-AMP levels; an increase in c-di-AMP levels correlates with increased acid resistance (16, 19) and altered antibiosis resistance including an increase in methicillin resistance in S. aureus (11, 18, 20). Most notable, however, are the findings that L. monocytogenes (5) and Bacillus subtilis (18) cannot grow in the absence of c-di-AMP. Showing that in contrast to other signaling nucleotides, c-di-AMP controls essential cellular pathways. The molecular basis for this is currently not known, though it is assumed that, similar to other signalling molecules, c-di-AMP interacts with a specific set of target proteins and upon binding alters their activity or function. Currently only one bacterial c-di-AMP receptor protein, the TetR-type transcription factor DarR, has been identified in Mycobacterium smegmatis (21). However, the absence of close DarR homologs in many organisms that likely produce c-di-AMP implies that additional c-di-AMP target proteins must exist.

In this study we have identified KtrA as c-di-AMP target protein by using an affinity pull down assay. KtrA is a member of the widely distributed RCK (regulator of conductance of K⁺) protein family, known to be involved in the gating of ion channels. Here we show that KtrA is required for the growth of S. aureus under potassium limiting conditions. Through subsequent binding studies we show that c-di-AMP specifically interacts with the C-terminal RCK_C domain of KtrA. A second S. aureus RCK_C domain-containing protein CpaA, a predicted cation/proton antiporter, was subsequently identified bioinformatically and its interaction with c-di-AMP confirmed experimentally. Lastly, using a genome wide interaction screen, we identified the PII-like signal transduction protein PstA and the histidine kinase KdpD as additional c-di-AMP binding proteins. With the identification of these four novel and widely distributed c-di-AMP binding proteins we provide a link between c-di-AMP and a fundamental cellular process in bacteria, namely ion transport.

Results

Identification of the c-di-AMP target protein KtrA. To identify c-di-AMP receptor proteins, we performed an affinity pull down

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component. (MthK of an RCK B. subtilis S1) (23). part of a nucleotide-binding site and with aspartic acid residues assume the same fold with the conserved GxGxxG motif forming a structural model, it is likely that KtrA high similarity to the subtilis NAD trometry as di-AMP-coupled beads (Fig. 1A) and identified by mass spec-
trate derived from the S. aureus (Fig. S1) (25, 26). The cytoplasmic N domain and an RCK domain (amino acids 4-126) indicated in blue and RCK domain (amino acids 135-219) shown in orange. The RCK_N domain of the B. subtilis KtrA homolog is known to bind to nucleotides including ATP, ADP, NAD\(^+\) and NADH.

Fig. 1. Identification of S. aureus KtrA as a potential c-di-AMP binding protein. (A) Silver stained polyacrylamide gel of cytoplasmic S. aureus proteins retained on c-di-AMP-coupled (+) or uncoupled (-) beads. The protein band enriched in the c-di-AMP lane (indicated with an asterisk) was identified by mass spectrometry as S. aureus protein SAUSA300_0988 (KtrAS). (B) Illustration of Ktr-type potassium transport systems, which are composed of a KtrB-type membrane component and a cytoplasmic KtrA-type gating component. (C)Schematic representation of the KtrAS\(_{\text{SA}}\) domain structure with the RCK_N domain (amino acids 4-126) indicated in blue and RCK_C domain (amino acids 135-219) shown in orange. The RCK_N domain of the B. subtilis KtrA homolog is known to bind to nucleotides including ATP, ADP, NAD\(^+\) and NADH.

One protein band was enriched in samples obtained from c-di-AMP-coupled beads (Fig. 1A) and identified by mass spectrometry as S. aureus protein SAUSA300_0988. This protein has high similarity to the B. subtilis protein KtrAS\(_{\text{SA}}\) (51% identity) and KtrCB\(_{\text{BS}}\) (63% identity) that together with their respective membrane components KtrB and KtrD, form potassium transporters (Fig. 1B) (22). SAUSA300_0988 is the only KtrA/C-type protein in S. aureus and was renamed KtrAS\(_{\text{SA}}\). The cytoplasmic components of Ktr-systems are part of the RCK (regulator of conductance of K\(^+\)) protein family and play an important role in transporter gating (23, 24). KtrAS\(_{\text{SA}}\) is a typical RCK protein with an RCK_N domain and an RCK_C domain (Fig. 1C). Based on a structural model, it is likely that KtrAS\(_{\text{SA}}\) assumes a similar two-folded lobed fold as the RCK domain in the potassium channel protein MthK of Methanobacterium thermoautotrophicus (Fig. S1) (25, 26).

Interestingly, a nucleotide-binding site for ATP and other nucleotides has been identified previously in the RCK_N domain of the B. subtilis protein KtrA (23) and based on a structural model the RCK_N domain of the S. aureus protein is likely to assume the same fold with the conserved GxGxxG motif forming part of a nucleotide-binding site and with aspartic acid residues D32 and D52 acting as crucial nucleotide-binding residues (Fig. S1) (23).

c-di-AMP binds to the RCK_C domain of KtrA. To confirm the interaction between KtrAS\(_{\text{SA}}\) and c-di-AMP and to define more precisely the interaction domain, we adapted the differential radial capillary action of ligand assay (DRaCALA), which was previously used to study c-di-GMP-protein interactions (27). This assay is based on the principle that free nucleotides migrate outward when spotted on nitrocellulose membranes, whereas bound ligand is sequestered to the protein and immobilized in a tight spot on the membrane (Fig. 2A). The distribution of free and bound ligand can be readily visualized and quantified using radiolabeled nucleotides. To determine if c-di-AMP-protein interactions could be measured with this assay, we produced \(^{32}\)P-labeled c-di-AMP (Fig. S2) and tested its interaction with purified S. au-
reus GdpP and B. subtilis DisA proteins, c-di-AMP degrading and synthesizing enzymes, respectively. c-di-AMP-specific binding to these control proteins was observed (Fig. S3), thus validating DRaCALA as a method to study c-di-AMP-protein interactions.

Using this method, we next investigated the interaction between c-di-AMP and purified His-KtrAS\(_{\text{SA}}\) protein and determined an interaction with a K\(_d\) of 64.4 ± 3.4 nM (Fig. 2B). Only an excess of unlabeled c-di-AMP, but not the other nucleotides tested, including ATP, competed for binding with labeled c-di-AMP (Fig. 2C).

This also indicated that c-di-AMP does not bind to the previously described nucleotide-binding site in RCK_N. Furthermore, c-di-
AMP bound to the KtrAS\(_{\text{SA}}\)-D32A/D52A variant with alamine substitutions of the two key nucleotide-binding residues within RCK_N (Fig. 2D). To determine more specifically which portion of KtrAS\(_{\text{SA}}\) interacts with c-di-AMP, the RCK_N and RCK_C do-
main were produced and purified separately. While the RCK_N domain interacted, as expected, with ATP it did not bind c-di-
AMP (Fig. 2E). In contrast, the RCK_C domain bound c-di-AMP with a K\(_d\) of 369.0 ± 44.4 nM (Fig. 2E and 2F), thus showing that the RCK_C domain is the receptor domain of c-di-AMP. To further validate the DRaCALA binding results, an interaction between c-di-AMP and KtrAS\(_{\text{SA}}\) was further confirmed by equilibrium dialysis (Fig. S4). Of note, a specific interaction between c-di-AMP and the RCK_C domain of KtrA was also obtained when DRaCALAs were performed using Escherichia coli extracts prepared from strains producing different KtrAS\(_{\text{SA}}\) variants in place of purified proteins (Fig. S5).

Further, E. coli extracts containing the full-length B. subtilis KtrA protein, but not an N-terminal fragment lacking the RCK_C domain, interacted with c-di-AMP (Fig. S5). Taken together, these results show that KtrA is a bona fide bacterial c-di-AMP receptor protein and support a model where the two domains in Gram-positive KtrA-type proteins bind different nucleotides: ATP, ADP, NAD\(^+\) or NADH with the RCK_N and c-di-AMP within the RCK_C domain.

KtrA is important for the growth of S. aureus in low potassium. To investigate the involvement of KtrAS\(_{\text{SA}}\) and c-di-AMP in the growth of S. aureus in low potassium conditions, the growth of ktrA and gdpP mutant strains was compared to that of the wildtype LAC\(^*\) strain. The gdpP mutant strain has 15-fold higher levels of intracellular c-di-AMP (11) and therefore KtrA should be in the nucleotide-bound state under these conditions. Since potassium uptake is especially important during osmotic stress, the different S. aureus strains were grown on chemically defined medium (CM) plates containing 0.75 M NaCl. Under these stress condition, a two to three log growth defect was observed for both the ktrA and gdpP\(^-\) mutant strains, which could be complemented either by the addition of potassium or by the introduction of a functional copy of ktrA or gdpP respectively (Fig. 3A and 3B). The ktrA mutant was also hyper-susceptible to the potassium ionophore nigericin, which causes an exchange of potassium for extracellular H\(^+\) (Fig. 3C). The hypersensitivity to nigericin could again be rescued by the addition of 250 mM potassium or by genetic complementation (Fig. 3C and 3D). Similarly a ktrA mutant strain in the methicillin sensitive S. aureus strain background Newman was also more sensitive to nigericin and did not grow as well as the wild-type strain under the osmotic stress conditions unless potassium was added (Fig. 3E).

These results suggest a function for KtrAS\(_{\text{SA}}\) in potassium uptake in S. aureus strains and that c-di-AMP binding to KtrAS\(_{\text{SA}}\) might inactivate
channel activity, as the gdpP mutant strain, which has greatly increased levels of c-di-AMP, displays a phenotype similar to the ktrA mutant.

c-di-AMP interacts with CpaA, a second S. aureus RCK_C domain-containing protein. The identification of the RCK_C domain as a c-di-AMP interacting domain allows the bioinformatic prediction of other receptor proteins based on the presence of an RCK_C domain. In this manner we discovered the protein SAUSA300 -1-140 (RCK

Fig. 2. Characterization of the c-di-AMP / KtrA_S interaction by DRAcALAs. (A) Schematic representation of the differential radial capillary action of ligand assay (DRAcALAs) to study c-di-AMP protein interactions. (B) Binding curve and Kd determination for c-di-AMP and purified His-KtrA_S. Kd values were determined from the curve as previously described (27). (C) DRAcALAs with purified His-KtrA_S protein and 32P-labeled c-di-AMP and an excess of cold competitor nucleotide as indicated above each spot. (D) DRAcALAs with purified His-KtrA_S, His-KtrA_S-D32A or His-KtrA_S-D52A and 32P-labeled c-di-AMP. (E) DRAcALAs with purified His-KtrA_S-1-140 (RCK_N) or His-KtrA_S-133-220 (RCK_C) and 32P-labeled c-di-AMP or 32P-labeled ATP as indicated below the spots. (F) Binding curves and Kd determination for c-di-AMP and purified His-KtrA_S-133-220 protein containing only the RCK_C domain. The data were plotted and the best-fit line was determined by non-linear regression incorporating the hill equation using GraphPad Prism software.

Fig. 3. Effect of potassium on growth of wildtype (WT), ktrA and gdpP S. aureus strains. (A) and (B), The indicated S. aureus strains were grown overnight in chemically defined medium (CDM) containing 2.5 mM KCl. Next day serial dilutions of washed cells were spotted onto CDM agar plates containing 0.75M NaCl and containing either 0 mM or 2.5 mM potassium. (C) and (D), Nigericin sensitivity curves of WT, ktrA mutant and complemented S. aureus strains. The different strains were grown in 96-well plates in CDM medium supplemented with 2.5 mM or 250 mM potassium and nigericin at the indicated concentration. OD600 readings were determined after 24 h growth and plotted as % growth compared to the growth in the absence of nigericin. Experiments were repeated a minimum of 5 times. When grown in 2.5 mM KCl the ktrA mutant consistently showed a two-fold reduced MIC for each experiment. The MIC for all the strains varied between experiments from 0.1-0.8 µM for the wildtype and complemented strain and 0.05-0.4 µM for the mutant strains.
COL ORFeome Gateway library is available and we reasoned that this library together with the DRaCALA method should allow us to identify additional *S. aureus* c-di-AMP binding proteins. The library contains 2,343 *S. aureus* ORFs (86% of all *S. aureus* COL genes) within the Gateway entry vector pDONR221. These ORFs were recombined into the pDEST17 protein expression vector, placing each ORF under the control of the IPTG-inducible T7 promoter. With the exception of eight reactions that failed, all other resulting plasmids were recovered in the *E. coli* protein expression strain T7IQ. Four percent of the library strains were subsequently analysed by PCR and all found to contain an insert of the expected size. Next, protein expression was induced and whole cell *E. coli* extracts prepared. Eight percent of these extracts were analyzed by SDS-PAGE and coomassie staining and visible protein overproduction was observed for approximately 70% of the lysates. Finally, these extracts, arrayed in twenty five 96-well plates, were used in DRaCALAs and the fraction of bound radiolabeled c-di-AMP determined for each spot. An average fraction bound value was determined for each plate and the cut off value for positive interactions was set at 1.4 times this average fraction bound background value. Extracts derived from strains expressing four different proteins gave c-di-AMP fraction bound values above background using these criteria, one of which was KtrA, thereby validating the DRaCALA ORFeome screen. The other positive clones, SACOL0525, SACOL2070 and SACOL2218, were confirmed by sequencing and renamed PstA (Pi-like signal transduction protein A), KdpD (a sensor histidine kinase and annotated as KdpD in other *S. aureus* strains) and Adk (adenylate kinase), respectively. To determine if these proteins are indeed bona fide c-di-AMP binding proteins, the corresponding genes were reamplified from *S. aureus* LAC*"* chromosomal DNA and cloned into the *E. coli* expression vector pET28b for overproduction as His-tag fusion proteins. Subsequently, extracts were prepared and used in DRaCALAs (Fig. S7). Of note, while the fraction bound values for PstA and KdpD were twice as high as the background value in the initial whole genome screen, the c-di-AMP fraction bound value obtained for Adk was only 1.45 times above background and so only just made the cut off (Fig. 5A and S7). When no interaction was observed with Adk after recloning, this protein was no longer regarded as a c-di-AMP receptor protein (Fig S7). On the other hand, c-di-AMP binding to PstA and KdpD was confirmed after recloning (Fig. S7) and both proteins interacted specifically with c-di-AMP as the only addition of an excess of cold c-di-AMP and not other cold nucleotides prevented the binding of radiolabeled c-di-AMP (Fig. 5B and 5C). CpaA was not identified in this screen as the gene encoding for this protein is not present in the *S. aureus* COL genome. Taken together, the genome-wide DRaCALA screen identified two additional *S. aureus* proteins, PstA and KdpD, as novel c-di-AMP receptor proteins.

### Discussion

Since the discovery of c-di-AMP, it has been speculated that this nucleotide binds to proteins to regulate their function. In this study we identified four c-di-AMP receptor proteins, namely KtrA, CpaA, KdpD and PstA by using an affinity pull down assay, bioinformatics analysis and a genome-wide protein nucleotide interaction screen (Figs. 1, 4 and 5). With the identification of three proteins (KtrA, CpaA, and KdpD) that have been implicated in potassium transport in other bacteria, we have linked c-di-AMP signaling to potassium transport in *S. aureus*. Interestingly, this distinguishes c-di-AMP from c-di-GMP, which regulates multiple cellular processes that help bacteria to transition between different lifestyles, such as extracellular carbohydrate and adhesion production, motility and biofilm formation. The link between c-di-AMP and the ion transport may explain why c-di-AMP, in contrast to other related signaling nucleotides, is essential for growth in bacterial species. Individually *ktrA*, *cpaA*, *pstA* and *kdpD* are not essential (28–30) however it is plausible that combined mutations may be lethal. Alternatively the existence of an as yet unidentified essential c-di-AMP receptor is also entirely possible.

The c-di-AMP binding region in *S. aureus* KtrA and CpaA was narrowed down to the RCK_C domain (Figs. 2 and 4). This domain is present in a large number of bacterial and archaeal proteins and there is a good correlation between the distribution of the c-di-AMP cyclase domain *DisA_N* and the presence of RCK_C domains. Most bacteria and archaea that potentially synthesize c-di-AMP also contain one or more proteins with an RCK_C domain. This raises the possibility that c-di-AMP may contribute to the regulation of ion transport in a large number of bacteria and archaea. The number of RCK_C domains per organism usually exceeds the number of cyclases, perhaps suggesting that c-di-AMP regulates the function of multiple proteins, which is similar to what we found in *S. aureus*. However, the RCK_C domain is phylogenetically more widely distributed than the c-di-AMP cyclase domain and is also found in some eukaryotes such as green algae, in additional archaeal species and most notably in a large number of Gram-negative proteobacteria where the c-di-AMP cyclase domain is absent. We would predict that in those organisms other small molecules interact with this domain to regulate transport processes. The RCK_C domain is associated...
as a soluble domain with potassium transporters, or in some cases directly linked to ion antiporers, such as in CpaA. However, this domain is also associated with predicted amino acid antiporers, citrate transporters and voltage-gated channels. This suggests that c-di-AMP or other small molecules might regulate a range of different transport processes, which have not been previously associated with signaling networks.

Potassium is a major and essential intracellular ion and therefore bacteria have evolved several different types of uptake systems. The third c-di-AMP binding protein identified in this study was KdpD, which is a widely distributed membrane embedded sensor histidine kinase that in many bacteria controls, together with c-di-AMP, the production of secondary messengers of the second type of potassium uptake system. This ATP-dependent potassium uptake system has been best characterized in E. coli and consists of four membrane components KdpABC and KdpD, which are required for KdpABC expression at a very low potassium concentration when the other uptake systems are no longer sufficient to allow the cell to acquire the necessary amount of ion (31). However, a recent study on the S. aureus KdpDE system suggested that this two-component system has a different function in this organism (32). The S. aureus KdpDE two-component system, which still responds to the extracellular potassium concentration, was found to be no longer required for bacterial survival under low potassium conditions, but instead linked to ion homeostasis and cell division (33). The study of this new type of potassium uptake system raises future research to determine the molecular mechanisms underlying c-di-AMP dependent processes in Firmicutes.

The least characterized c-di-AMP receptor protein identified in this study is the DUF970 domain-containing PII-like signal transduction protein, which belongs to the GlnB superfamily of proteins and was renamed PstA. PII-type proteins are one of the most widely distributed signal transduction proteins in nature that are present in bacteria as well as archaea and plants. DUF970 domain-containing PII-like proteins are not only present in Staphylococcus species but widely distributed among Firmicutes. Characterized proteins belonging to this GlnB superfamily are the cation tolerance protein CutA (33) and the ATP phosphoribosyltransferase HisG, the first enzyme of the histidine pathway (34). However the best characterized proteins belonging to the GlnB superfamily are KdpDE. The discovery of the four protein expression library by DRaCALA provides a powerful platform for identifying and purifying new target proteins whose activity is modulated by c-di-AMP.

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protein extracts. 40 μl streptavidin dynabeads (Invitrogen) coupled with 2.4 μM biotinylated c-di-AMP (BioMol) were incubated with 1.2 mg cytoplasmic proteins in 1.5 ml 10% glycerol, 1 mM MgCl₂, 5 mM Tris pH 7.5, 230 mM NaCl, 0.5 mM DTT, 4 mM EDTA containing 50 μg/ml BSA for 30 min at room temperature. Samples were washed 4 times with the same buffer lacking BSA and resuspended in 50 μl of binding buffer. Samples were boiled for 5 min, beads removed and 18 μl run on 12% SDS-PAGE gels. Gels were stained using the SilverQuest kit (Invitrogen). Mass spectrometry was performed at the Taplin Mass Spectrometry Facility (Harvard Medical School).

**Protein purifications.** Proteins were purified from 0.5-4 L E. coli cultures. Cultures were grown to an OD₆₀₀ 0.5-0.7, protein expression induced at 16°C. Protein purifications were performed by affinity and size exclusion chromatography as previously described (11, 39). Protein concentrations were determined by A₂₈₀ readings.

**Minimum Inhibitory Concentrations.** Overnight cultures of S. aureus strains in CDM containing 2.5 mM KCl were adjusted to 5 x 10⁶ bacteria/ml in gene-involvement procedure of c-di-AMP. Nickel affinity was incubated for 30 min at room temperature. Samples were washed 4 times with the same buffer lacking MgCl₂ and resuspended in 50 μl of binding buffer. Samples were boiled for 5 min, beads removed and 18 μl run on 12% SDS-PAGE gels. Gels were stained using the SilverQuest kit (Invitrogen). Mass spectrometry was performed at the Taplin Mass Spectrometry Facility (Harvard Medical School).

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