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

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Submitted to Proceedings of the National Academy of Sciences of the United States of America

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_C (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_C domains are found in proteinaceous channels, transporters and antiporters 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-(ppGpp) and pentaphosphate (pppGpp) 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 c-AMP-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 diguanosine 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 DacA, 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 antibiotic 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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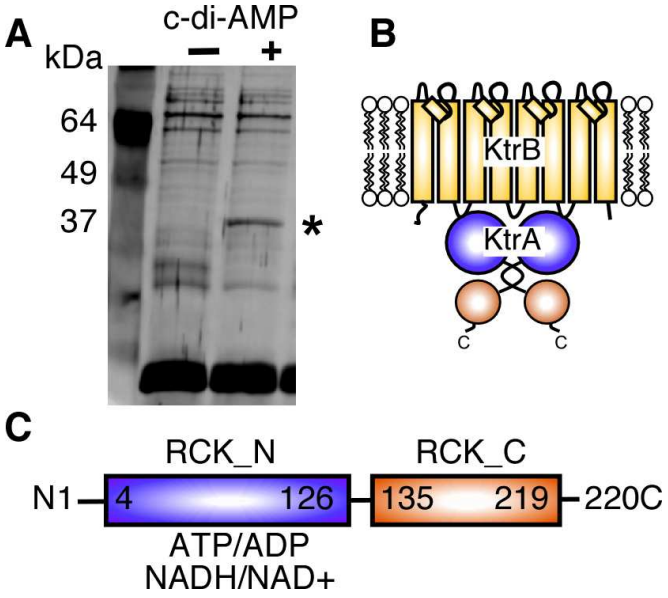

Fig. 1. Identification of *S. aureus* $KtrA_{SA}$ as a potential c-di-AMP binding protein.(*A*)Silver stained polyacrylamide gel of cytoplasmic *S. aureus* proteins retained on c-di-AMP-coupled (+) or uncoupled (-) beads. The protein band enriched in the c-di-AMP lane (indicated with an asterisk) was identified by mass spectrometry as *S. aureus* protein SAUSA300_0988 ($KtrA_{SA}$). (*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 $KtrA_{SA}$ 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.

assay using c-di-AMP-coupled magnetic beads and protein extracts derived from the *S. aureus* strain LAC*. This strain is an erythromycin sensitive derivative of the clinically relevant community-acquired methicillin resistant USA300 strain LAC. 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* proteins $KtrA_{BS}$ (51% identity) and $KtrC_{BS}$ (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 $KtrA_{SA}$. 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). $KtrA_{SA}$ 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 $KtrA_{SA}$ assumes a similar two-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 $KtrA_{SA}$ 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. aureus* 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-$KtrA_{SA}$ 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 $KtrA_{SA}$-D32A/D52A variant with alanine substitutions of the two key nucleotide-binding residues within RCK_N (Fig. 2D). To determine more specifically which portion of $KtrA_{SA}$ interacts with c-di-AMP, the RCK_N and RCK_C domains 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 KtrA or the RCK_C domain in the nM range 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 $KtrA_{SA}$ variants in place of purified proteins (Fig. S5). Furthermore, *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 $KtrA_{SA}$ 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 (CDM) 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 intracellular K^+ 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. S6). These results suggest a function for $KtrA_{SA}$ in potassium uptake in *S. aureus* strains and that c-di-AMP binding to $KtrA_{SA}$ might inactivate

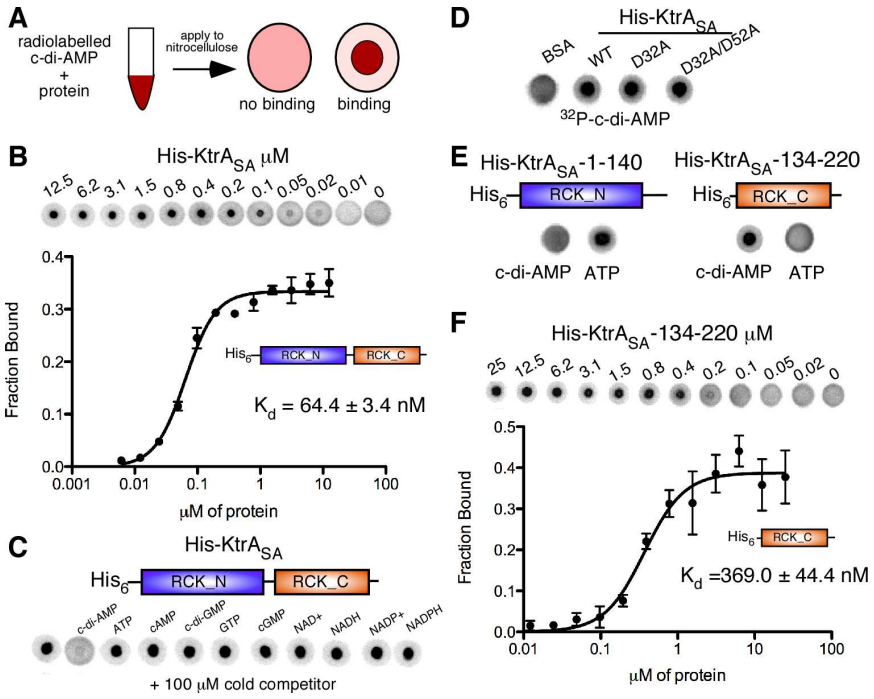

Fig. 2. Characterization of the c-di-AMP / KtrA$_{SA}$ interaction by DRaCALA. (*A*) Schematic representation of the differential radial capillary action of ligand assay (DRaCALA) to study c-di-AMP protein interactions. (*B*)Binding curve and K_d determination for c-di-AMP and purified His-KtrA$_{SA}$. K_d values were determined from the curve as previously described (27). (*C*)DRaCALAs with purified His-KtrA$_{SA}$ protein and ^{32}P-labeled c-di-AMP and an excess of cold competitor nucleotide as indicated above each spot. (*D*) DRaCALAs with purified His-KtrA$_{SA}$, His-KtrA$_{SA}$-D32A or His-KtrA$_{SA}$-D32A/D52A and ^{32}P-labeled c-di-AMP.(*E*)DRaCALAs with purified His-KtrA$_{SA}$-1-140 (RCK_N) or His-KtrA$_{SA}$-134-220 (RCK_C) and ^{32}P-labeled c-di-AMP or ^{32}P-labeled ATP as indicated below the spots. (*F*)Binding curves and K_d determination for c-di-AMP and purified His-KtrA$_{SA}$-134-220 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.

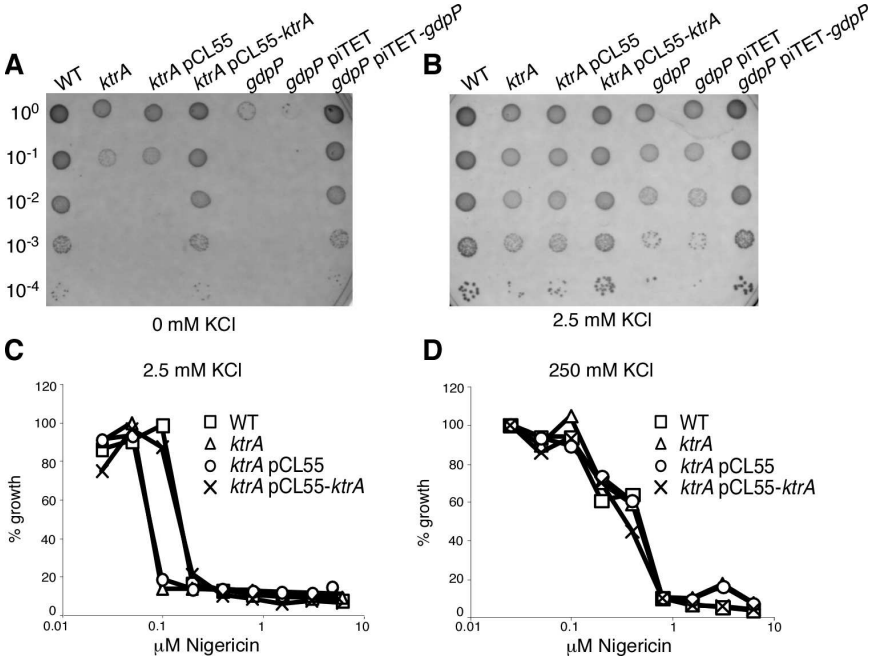

Fig. 3. Effect of potassium on growth of wildtype (WT), *ktrA* and *gdpP S. aureus* strains.(*A*) and (B), The indicated *S. aureus* strains were grown overnight in chemically defined medium (CDM) containing 2.5 mM KCl. Next day serial dilutions of washed cells were spotted onto CDM agar plates containing 0.75 M NaCl and containing either 0 mM or 2.5 mM potassium. (*C*) and (*D*), Nigericin sensitivity curves ofWT, *ktrA* mutantand 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. OD_{600} 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.

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_0911 in *S. aureus* strain LAC*, which we rename CpaA. This protein is a predicted cation/proton antiporter that is composed of an N-terminal transmembrane region followed by an RCK domain (Fig. 4A). An interaction between its RCK_C domain and c-di-AMP was tested by performing DRaCALAs with *E. coli* extracts prepared from strains either containing the empty vector as a control, or expressing the complete RCK or the RCK_C domain of CpaA. ^{32}P-labeled c-di-AMP interacted specifically with both the RCK and the RCK_C domain (Fig. 4B), thus showing that CpaA is a second c-di-AMP target protein.

Identification of PstA and KdpD as specific di-AMP binding proteins using a genome-wide open reading frame (ORFeome) DRaCALA screen. The DRaCALA method can be used to identify nucleotide/protein interactions using crude whole-cell *E. coli* lysates. This makes this assay ideally suited to perform a genome-wide protein/nucleotide interaction screen. An *S. aureus* strain

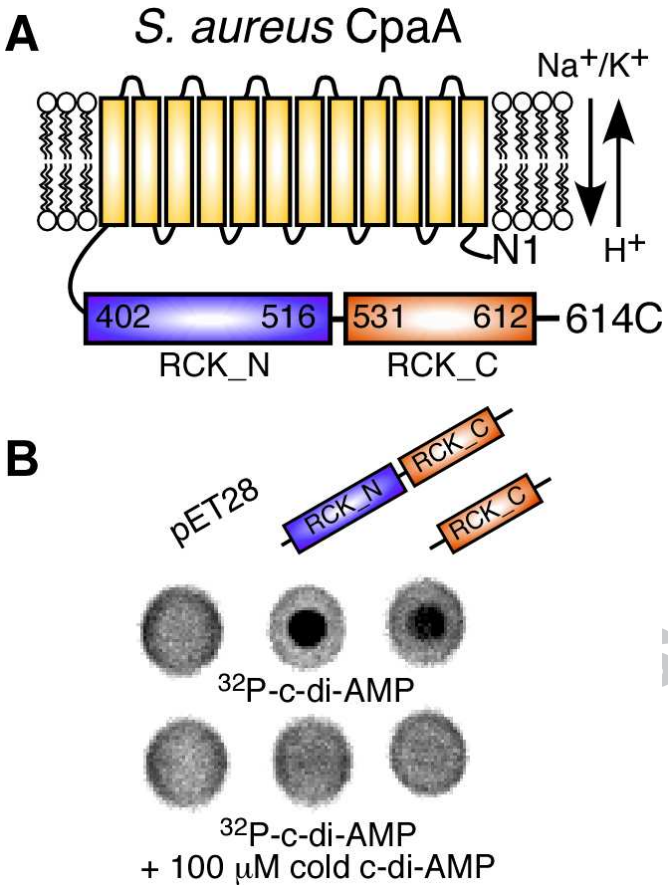

Fig. 4. Identification of CpaA as an additional c-di-AMP target protein (*A*)Schematic representation of the predicted K^+ or Na^+ antiporter CpaA (SAUSA300_0911), containing an N-terminal transmembrane (yellow) and cytoplasmically located RCK_N (blue) and RCK_C (orange) domains. (*B*) DRaCALAswith ^{32}P-labeled c-di-AMP and *E. coli* extracts prepared from the vector control strain (pET28b) or strains overproducing His-CpaA$_{SA}$-402-614 (RCK_N and RCK_C) or His-CpaA$_{SA}$-513-614 (RCK_C). Cold c-di-AMP was added as a competitor where indicated.

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 (PII-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 only the 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

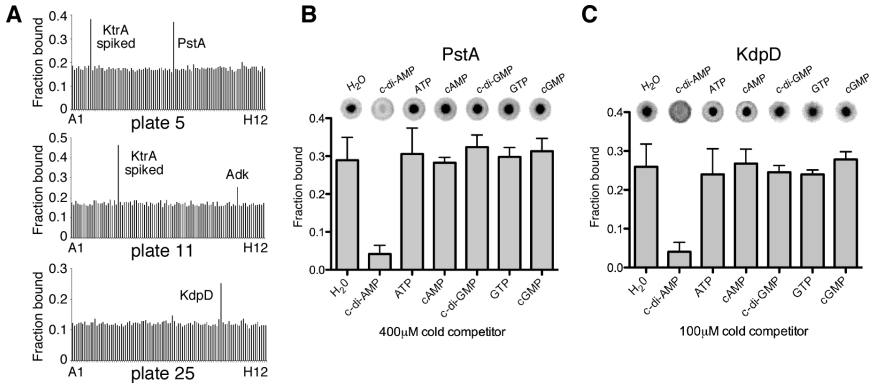

Fig. 5. Identification of PstA and KdpD as specific c-di-AMP target proteins. (*A*) For the whole genome DRaCALA screen, ^{32}P-labeled c-di-AMP was dispensed into 96-well plates containing *E. coli* lysates and aliquots were subsequently spotted in duplicate onto nitrocellulose membrane. The fraction of bound c-di-AMP was calculated for each well as described in Roelofs *et al.* (27) and the average values from the duplicate spots plotted. Plates 5, 11 and 25 with positive interactions are shown. The average fraction bound value for plate 5 was 0.178 ± 0.029. Well A10 was spiked with a KtrA lysate and well E3 contained the PstA lysate, which had a fraction bound value of 0.370 (2 x background). The average fraction bound value for plate 11 was 0.174 ± 0.032. Well B12 was spiked with a KtrA lysate and well G11 contained the Adk lysate, which had a fraction bound value of 0.253 (1.45 x background). The average fraction bound value for plate 25 was 0.122 ± 0.015. Well G2 contained the KdpD lysate, with a fraction bound value of 0.252 (2 x background). (*B* and *C*) DRaCALAs were preformed with *E. coli* extracts prepared from strains overproducing His-PstA (*B*) or KdpD-His (*C*) and ^{32}P-labeled c-di-AMP and an excess of cold competitor nucleotide as indicated above each spot. Fraction of bound nucleotide was determined as described in Roelofs *et al.* (27) and values from three independent experiments were plotted with standard deviations.

as a soluble domain with potassium transporters, or in some cases directly linked to ion antiporters, such as in CpaA. However, this domain is also associated with predicted amino acid antiporters, 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 its cognate response regulator KdpE, the expression of a 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 KdpABCF and the two-component system KdpDE, which is required for KdpABCF 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 to control the expression of several well-characterized *S. aureus* virulence factors (32). However, additional work is needed to fully understand the function of this two-component system in *S. aureus* and other Gram-positive bacteria and based on this study its interplay with cellular c-di-AMP levels.

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 archea 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 CutA1 (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 PII nitrogen regulatory proteins, which are key signal transduction protein that report on the nitrogen and carbon status of cells by sensing glutamine and 2-ketoglutarate levels (35). Since proteins belonging to this superfamily are known to bind diverse ligands and function by protein-protein interaction to control the activity of enzymes, transcription factor or transport proteins, we would assume that upon c-di-AMP binding or release the *S. aureus* PstA protein interacts with other cellular proteins. However these still need to be discovered.

This work demonstrates the feasibility of a DRaCALA-based ORFeome screen as a high-throughput platform for identifying c-di-AMP receptor proteins. While the DRaCALA ORFeome screen will identify receptors whose binding site does not require additional proteins, biochemical pull-down assays will only yield receptors that are expressed in the assayed growth conditions. Together the combination of biochemical pull-down assays, bioinformatic analysis and systematic screening of a whole genome protein expression library by DRaCALA provides a powerful synergistic approach for the systematic elucidation of protein-metabolite interaction networks (36). The discovery of the four different and widely distributed c-di-AMP receptor proteins allows future research to determine the molecular mechanisms underlying c-di-AMP dependent processes in prokaryotes.

Methods

Bacterial strains and culture conditions. *E. coli* strains were grown in LB or LB-M9 (37), *B. subtilis* strains in LB and *S. aureus* strains in TSB or chemically defined medium (CDM) at 37°C with aeration. CDM was prepared as referenced (38), with the following modifications: KH_2PO_4 was substituted with Na Phosphate buffer and KCl was added at concentrations stated in the text. In addition, Gly 50mg/L; L-Ser 30 mg/L; L-Asp 90 mg/L; L-Lys 50 mg/L; L-Ala 60 mg/L; L-Trp 10 mg/L; L-Met 10 mg/L; L-His 20 mg/L; L-Ile 30 mg/L; L-Tyr 50 mg/L and thymine 20 mg/L were added. Information on strain construction is provided in the SI section. Strains and primers used are listed in Tables S1 and S2 and the *S. aureus* (MRSA), Strain COL Gateway® Clone Set, Recombinant in *Escherichia coli*, Plates 1-25, NR-19277 were obtained through BEI Resources, NIAID, NIH.

Affinity-pull down assay. 20 ml of an *S. aureus* LAC* culture with an OD_{600} of 1 was harvested and suspended in 1 ml 10 mM Tris HCl pH 7.5, 50 mM NaCl buffer containing EDTA-free complete protease inhibitor (Roche). Cells were mixed with 0.1 mm glass beads and lysed in a Fast-Prep machine twice for 45 sec. at setting 6 (MP Biomedicals). Samples were centrifuged for 5 min at 17,000 x g and subsequently for 1 h at 100,000 x g to obtain cytoplasmic

protein extracts. 40 µl streptavidin dynabeads (Invitrogen) coupled with 2.4 µM biotinylated c-di-AMP (BioLog) were incubated with 1.2 mg cytoplasmic proteins in 1.5 ml 10% glycerol, 1 mM $MgCl_2$, 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 suspended in 50 µl protein sample 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 OD_{600} 0.5-0.7, protein expression induced with 0.5 mM IPTG and incubated overnight at 16°C. Protein purifications were performed by nickel affinity and size exclusion chromatography as previously described (11, 39). Protein concentrations were determined by A_{280} readings.

Minimum Inhibitory Concentrations. Overnight cultures of *S. aureus* strains in CDM containing 2.5 mM KCl were adjusted to 5 x 10^5 bacteria/ml in CDM supplemented with either 2.5 or 250 mM KCl. 100 µl of these suspensions were incubated in 96 well plates with 2-fold dilutions of nigericin starting at 6.25 µM. Plates were incubated at 37°C with shaking for 24 h. MICs were determined as the antimicrobial concentration at which growth was inhibited by >75% compared to growth without antimicrobial. Five independent experiments were performed and one representative graph is shown.

Bacterial Stress Testing. Overnight cultures of *S. aureus* strains in CDM containing 2.5 mM KCl were washed three times in CDM lacking K^+. Cultures were adjusted to an OD_{600} of 0.05, serially diluted and 5 µl spotted onto CDM agar plates containing an extra 0.75 M NaCl. Plates were incubated at 37°C for 24-36 h.

Construction of the *S. aureus* ORFeome expression library. 2,343 *E. coli* strains containing pDONR221 vectors with *S. aureus* strain COL ORFs (BEI Resources, NIAID, NIH) were grown in 1.5 ml LB-M9 in 2 ml 96-well deep dishes (Greiner) selecting for kanamycin resistance. The cultures were centrifuged and the plasmids extracted using 96-well MultiScreen$_{HTS}$ PLASMID plates (Millipore). The *S. aureus* gateway ORFeome library was shuttled from the pDONR221 entry plasmids into the protein overexpression destination vector pDEST17 using LR clonase enzyme II as per manufacturer's guidelines (Invitrogen). Subsequently, the destination plasmid library was introduced into *E. coli* strain T7IQ (NEB) selecting for carbenicillin resistance.

Preparation of *E. coli* whole cell lysates. BL21(DE3) pET28b-containing strains or T7IQ pDEST17 containing library expression strains were grown in LB-M9 medium overnight at 30°C and subsequently induced for 6 h with 1 mM IPTG for protein induction. Bacteria were collected by centrifugation and suspended in 1/10th of their original volume in 40 mM Tris pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$ binding buffer containing 2 mM PMSF, 20 µg/ml DNase and 0.5 mg/ml lysozyme. Cells were lysed by 3 freeze/thaw cycles. Lysates were directly used in binding assays or stored at -20°C.

Differential radial capillary action of ligand assay (DRaCALA). The principle of the DRaCALA is described in Roelofs *et al* (27). Briefly, *E. coli* whole-cell lysates, 20 µM purified protein (for standard assays) or 12.5 µM protein (for competition assays) in binding buffer were mixed with approximately 1 nM ^{32}P-labeled c-di-AMP, synthesized as described in the supplementary information section, or 5.5 nM ^{32}P-labeled ATP and incubated at room temperature for 5 min. For the whole genome screen the ^{32}P-labeled c-di-AMP was dispensed into lysate-containing 96 well plates using a Multiflo Microplate Dispenser (BioTek) and the mixture spotted onto nitrocellulose membrane using a 96 well pin tool (V&P Scientific). For competition assays, 100 or 400 µM cold nucleotides (ATP, GTP, cAMP, cGMP, NAD, NADH, NADP, NADPH; Sigma. c-di-AMP, c-di-GMP; BioLog) were added to the initial mixture and 2.5 µl of reactions were spotted onto nitrocellulose membranes (Amersham Hybond-ECL; GE Healthcare), air-dried and radioactivity signals detected as described above. The fraction of ligand bound and K_d values were calculated as previously described (27).

Acknowledgments. This research was supported by the European Research Council grant 260371 and the Wellcome Trust grant 100289 to A.G. and the EMBO short-fellowship 401-2011 to R.M.C. .

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