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

Rebecca M. Corrigan, Ivan Campetto, Tharthika Jeganathan, Kevin G. Roelofs, Vincent T. Lee, and Angelika Gründling

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 a 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, a predicted cation/proton antiporter, CpaA, which as we show here also directly binds c-di-AMP. Because 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 PI-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.

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The authors declare no conflict of interest.

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systems are part of the RCK protein family and play an important role in transporter gating (23, 24). KtrASA is a typical RCK protein, with an RCK_N domain and an RCK_C domain (Fig. 1C). On the basis of a structural model, it is likely that KtrASA assumes a similar two-lobe 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 according to 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 KtrASA 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 whether c-di-AMP–protein interactions could be measured with this assay, we produced 32P-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-KtrASA protein and determined an interaction with a *K*_d of 64.4 ± 3.4 nM (Fig. 2B). Only an excess of unlabelled 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 KtrASA-D32A/D52A variant

**Fig. 1.** Identification of *S. aureus* KtrASA 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 (asterisk) was identified by mass spectrometry as *S. aureus* protein SAUSA300_0988 (KtrASA). (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 KtrASA 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.

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 and KtrD, form potassium transporters (Fig. 1B) (22). SAUSA300_0988 is the only KtrA/C-type protein in *S. aureus* and was renamed KtrASA. The cytoplasmic components of Ktr systems are part of the RCK protein family and play an important role in transporter gating (23, 24). KtrASA is a typical RCK protein, with an RCK_N domain and an RCK_C domain (Fig. 1C). On the basis of a structural model, it is likely that KtrASA assumes a similar two-lobe 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 according to 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).

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**Fig. 2.** Characterization of the c-di-AMP/KtrASA interaction by DRAcALa. (A) Schematic representation of the DRAcALa to study c-di-AMP protein interactions. (B) Binding curve and *K*_d determination for c-di-AMP and purified His-KtrASA. *K*_d values were determined from the curve as previously described (27). (C) DRAcALas with purified His-KtrASA protein and 32P-labeled c-di-AMP and an excess of cold competitor nucleotide as indicated above each spot. (D) DRAcALAs with purified His-KtrASA, His-KtrASA-D32A, or His-KtrASA-D32A/D52A and 32P-labeled c-di-AMP. (E) DRAcALAs with purified His-KtrASA-1-140 (RCK_N) or His-KtrASA-134-220 (RCK_C) and 32P-labeled c-di-AMP or 32P-labeled ATP as indicated below the spots. (F) Binding curves and *K*_d determination for c-di-AMP and purified His-KtrASA-134-220 protein containing only the RCK_C domain. The data were plotted, and the best-fit line was determined by nonlinear regression incorporating the hill equation using GraphPad Prism software.
with alanine substitutions of the two key nucleotide-binding residues within RCK N (Fig. 2D). To determine more specifically which portion of KtrASA interacts with c-di-AMP, the RCK N and RCK C domains were produced and purified separately. Although 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 F), 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 KtrASA variants in place of purified proteins (Fig. S5). Furthermore, *E. coli* extracts containing the full-length *B. subtilis* KtrA protein, but not a 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 whereby 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 KtrASA and c-di-AMP in the growth of *S. aureus* in low potassium conditions, the growth of ktrA and gdpP mutant strains was compared with that of the wild-type Lac* strain. The gdpP mutant strain has 15-fold higher levels of intracellular c-di-AMP due to loss of c-di-AMP activity (11), and therefore KtrA should be in the nucleotide-bound state under these conditions. Because 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 conditions, 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 B). 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 D). 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 KtrASA in potassium uptake in *S. aureus* strains and that c-di-AMP binding to KtrASA might inactivate channel activity, because 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. 32P-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 ORF (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 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 Isopropyl-β-D-thiogalactopyranoside (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 T7Q. Four percent of the library strains were subsequently analyzed by PCR and all found

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**Fig. 3.** Effect of potassium on growth of wild-type, ktrA, and gdpP *S. aureus* strains. (A and B) The indicated *S. aureus* strains were grown overnight in 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 of wild-type, 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. OD readings were determined after 24 h growth and plotted as % growth compared with the growth in the absence of nigericin. Experiments were repeated a minimum of five times. When grown in 2.5 mM KCl the ktrA mutant consistently showed a twofold reduced MIC for each experiment. The MIC for all of the strains varied between experiments from 0.1 to 0.8 μM for the wild-type and complemented strain and 0.05-0.4 μM for the mutant strains.
signal adenylate transduction protein kinase), respectively. To determine whether these proteins are indeed bona fide c-di-AMP receptor proteins. Subsequently extracts were prepared and used in DRaCALAs with 32P-labeled c-di-AMP and E. coli extracts prepared from the vector control strain (pET28b) or strains overproducing His-CpaASA-402-614 (RCK_N and RCK_C) or His-CpaAUA-513-614 (RCK_C). Cold c-di-AMP was added as a competitor where indicated.

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 ~70% of the lysates. Finally, these extracts, arrayed in 25 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 cutoff value for positive interactions was set at 1.4 times this average fraction 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 whether 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, whereas 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 cutoff (Fig. S4 and S7). When no interaction was observed with Adk after cloning, 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 cloning (Fig. S7), and both proteins interacted specifically with c-di-AMP because 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. 5 B and C). CpaA was not identified in this screen because 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 bona fide 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 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 KdpABC and the two-component system KdpDE, which is 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 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 on the basis of 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 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 copper 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). Because 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. Although 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 tryptic soy broth (TSB) or CDom at 37 °C with aeration. CDM was prepared as previously reported (38), with the following modifications: KH2PO4 was substituted with Na phosphate buffer, and KCl was added at concentrations stated in the text. In addition, Gly 50 mg/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-Val 30 mg/L; L-Tyr 50 mg/L; and thymine 20 mg/L were added. Information on strain construction is provided in SI Methods. Strains and primers used are listed in Tables S1 and S2, and the methicillin-resistant S. aureus (MRSAs), Strain COL Gateway Clone Set, Recombinant in E. coli, Plates 1–25, NR-19277 were obtained through BEI Resources, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH).

Affinity Pull-Down Assay. Twenty milliliters of an S. aureus LAC* culture with an OD600 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 s at setting 6 (MP Biomedicals). Samples were centrifuged for 5 min at 17,000 × g and subsequently for 1 h at 100,000 × g to obtain cytoplasmic protein extracts. Forty microliters streptavidin dynabeads (Invitrogen) coupled with 2.4 μM biotinylated c-di-AMP (BioLog) were incubated with 1.5 mg cytoplasmic proteins in 1.5 mL 50 mM HCl (pH 7.5), 230 mM NaCl, 0.5 mM DTT, and 4 mM EDTA containing 50 μg/mL BSA for 30 min at room temperature. Samples were washed four 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% (wt/vol) 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 to 4 L E. coli cultures. Cultures were grown to an OD600 of 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 A280 readings.

Fig. 5. Identification of PstA and KdpD as specific c-di-AMP target proteins. (A) For the whole-genome DRaCALA screen, 32P-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 by 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 ± 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± 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 ± background). (8 and C) DRaCALAs were performed with E. coli extracts prepared from strains overproducing His-PstA (B) or KdpD-His (C) and 32P-labeled c-di-AMP and an excess of cold competitor nucleotide as indicated above each spot. Fraction of bound nucleotide was determined as described by Roelofs et al. (27), and values from three independent experiments were plotted with SDs.
Minimum Inhibitory Concentrations. Overnight cultures of S. aureus strains in CDM containing 2.5 mM KCl were adjusted to an OD600 of 0.05, serially diluted, and 5 μL S. aureus (BEI Resources, NIAID, NIH) were grown in 1.5 mL LB-M9 in 2-mL 96-well plates at 37 °C for 24 h. Minimum inhibitory concentrations (MICs) were determined as the antiminocarial concentration at which growth was inhibited by >75% compared with 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 OD600 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. A total of 2,343 E. coli strains containing pDONR212 vectors with S. aureus strain COL ORFs (BEI Resources, NIAID, NIH) were grown in 1.5 mL LB-M9 in 2-mL 96-well plates at 37 °C for 24 h. Minimum inhibitory concentrations (MICs) were determined as the protein-metabolite interactions.

Preparation of E. coli Whole-Cell Lyssates. BL21(DE3) pET28b-containing strains or T7Q 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 1/10 of their original volume in 40 mM Tris (pH 7.5), 100 mM NaCl, 10 mM MgCl2 binding buffer containing 2 mM PMSF, 20 μg/mL DNase, and 0.5 mg/mL lysozyme. Cells were lysed by three freeze/thaw cycles. Lyssates were directly used in binding assays or stored at −20 °C.

Differential Radial Capillary Action of Ligand Assay. The principle of the DRaCALA is described by 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 −1 nM 32P-labeled c-di-AMP, synthesized as described in SI Methods, or 0.5 nM [32P]-labeled ATP and incubated at room temperature for 5 min. For the whole-genome screen the 32P-labeled c-di-AMP was dispensed into lystate-containing 96-well plates using a MultiDrop Microplate Dispenser (BioTek) and the mixture spotted onto nitrocellulose membranes 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 Kd values were calculated as previously described (27).

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Supporting Information

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SI Methods

Plasmid and Strain Construction. Strains used in this study are listed in Table S1, primers are listed in Table S2, and the methicillin-resistant *Staphylococcus aureus* (MRSA), Strain COL Gateway Clone Set, Recombinant in *Escherichia coli*, plates 1–25, NR-19277 were obtained through BEI Resources, National Institute of Allergy and Infectious Diseases, National Institutes of Health. Plasmid pET28b-His-ktrA<sub>AS</sub> was constructed by amplifying the *ktrA<sub>AS</sub>* gene from LAC* genomic DNA using primer pair ANG1473/ANG1474 and inserted into NdeI/HindIII sites of pET28b. Plasmids pET28b-His-ktrA<sub>BS</sub>-D32A, pET28b-His-ktrA<sub>SA</sub>-D52A, and pET28b-His-ktrA<sub>BS</sub>-D32A/D52A were produced by splicing overlap extension PCR using plasmid pET28b-His-ktrA<sub>AS</sub> as the template. Initial PCR products were amplified using primers ANG111/ANG1502 and ANG1503/ANG112 for the D32A mutation, ANG111/ANG1504 and ANG1505/ANG112 for the D52A mutation, and ANG111/ANG1524 and ANG1505/ANG112 for the D32A/D52A mutation. The resulting PCR products were fused using primers ANG1473/ANG1474, digested with NdeI/HindIII and cloned into pET28b. The plasmids pET28b-His-ktrA<sub>BS</sub>-1-40 and pET28b-His-ktrA<sub>SA</sub>-134-220 were created by amplifying the corresponding DNA fragments of the *ktrA<sub>AS</sub>* gene from LAC* chromosomal DNA using primers ANG1473/ANG1493 and ANG1595/ANG1474, respectively. The resulting PCR products were digested with NdeI and HindIII and cloned into pET28b that had been digested with the same enzymes. pET28b-ktrA<sub>AS</sub> was produced by amplifying the *ktrA<sub>AS</sub>* gene from *Bacillus subtilis* 168 chromosomal DNA using primers ANG1495/ANG1497 and the PCR product was cut with the enzymes NheI and EcoRI and ligated into pET28b. Plasmid pET28b-ktrA<sub>AS</sub>-C22V for the expression of a cysteine-less KtrA variant previously used in structural studies (1) was produced as follows: *B. subtilis* 168 chromosomal DNA and primer pairs ANG1494/ANG1499 and ANG1498/ANG1497 were used for the first round of PCRs to introduce the C22V mutation. These DNA fragments were then fused using primers ANG1495/1497, the resultant PCR product digested with NheI/EcoRI and cloned into pET28b. Plasmid pET28b-ktrA<sub>AS</sub> C22V-1-144 was constructed using primers ANG1494/ANG1499 and ANG1498/ANG1496 and *B. subtilis* 168 chromosomal DNA as the template for the first round of PCRs and primers ANG1495/ANG1496 for the fusion PCR. The resulting PCR product was digested with NheI and EcoRI and cloned into pET28b cut with the same enzymes. The plasmids pET28b-His-cpaA-402-614, pET28b-His-cpaA-531-614, pET28b-His-<i>adk</i>, and pET28b-His-<i>pstA</i> were constructed using LAC* chromosomal DNA and primers pairs ANG1617/ANG1615, ANG1616/ANGB1615, ANG1585/1586, and ANG1591/ANG1592, respectively. All PCR products were digested with NdeI and EcoRI and cloned into pET28b. Plasmid pET28b-k<sup></sup><i>d</i>-His<sub>4</sub> was constructed by amplifying the <i>k<sup></i>d</i>-<i>His</i><sub>4</sub> gene from LAC* chromosomal DNA using primers ANG1579/ANG1580 and the resulting PCR product was cut with NdeI and EcoRI and inserted into pET28b. The plasmids pCL55-k<sub>AS</sub>A and pCL55ITET862-gdpP were created by amplifying the <i>ktrA</i> and gdp<sub>P</sub> genes from LAC* chromosomal DNA with primer pairs ANG1500/ANG1506 and ANG1139/ANG1140, respectively. The resulting fragments were cloned into pCL55 and pCL55ITET862 that had been cut with BamHI/SmaI and AvrII/BglII, respectively. All plasmids were initially transformed into <i>E. coli</i> strain BL21 (DE3).

The *ktrA* mutant *S. aureus* strain NE788 containing a transposon insertion with an erythromycin resistance marker within the *ktrA* gene was obtained from the Nebraska Transposon Mutant Library held at the Network on Antimicrobial Resistance in <i>S. aureus</i> (NARSA) strain collection (2). The transposon insertion site within the *ktrA* gene, and more specifically 16 bases downstream of the *ktrA* start site, was confirmed by sequencing. The *ktrA* transposon insertion was transduced with Phi5 into the erythromycin-sensitive CA-MRSA strain LAC<sup>*</sup> and <i>S. aureus</i> Newman derived strain NM<sub>A</sub><sup>ΔΦ</sup> was produced from ANG2753 and ANG2614. Insertion of the transposon within the *ktrA* gene in all strains was again confirmed by sequencing. For complementation analysis, the plasmids pCL55, pCL55-k<sub>AS</sub>A, pCL55ITET862, and pCL55ITET862-gdpP were initially electroporated into *S. aureus* strain SE11. The integrated plasmids were then transduced with Phi5 into the appropriate LAC<sup>*</sup> and the Newman-derived strain NM<sub>A</sub><sup>ΔΦ</sup> yielding strains ANG2569, ANG2570, ANG2817, ANG2806, ANG2821, and ANG2820 (Table S1). Strain NM<sub>A</sub><sup>ΔΦ</sup> was constructed by deleting phage NM4 contained within the gdh gene of strain Newman, which allows subsequent chromosomal integration of pCL55-derived plasmids. Strain NM<sub>A</sub><sup>ΔΦ</sup> was constructed by allelic exchange using plasmid pKOR1-Δ<sup>D</sup>NM4 and a previously described method (3, 4).

Synthesis of 32<sup>P</sup>Cyclic Diadenosine Monophosphate. 32<sup>P</sup>-labeled cyclic diadenosine monophosphate (c-di-AMP) was synthesized from α-[32<sup>P</sup>]<i>P</i>ATP (Perkin-Elmer) by incubating 55.5 nM α-[32<sup>P</sup>]<i>P</i>ATP with 20 μM of the diadenylate cyclase protein DisA in 40 mM Tris (pH 7.5), 100 mM NaCl, 10 mM MgCl<sub>2</sub> binding buffer at 30 °C overnight. The sample was incubated for 5 min at 95 °C and the DisA protein removed by centrifugation. To remove unconverted ATP, samples were incubated with 20 μM H-Ras protein for 1 h at room temperature. The ATP-bound H-Ras protein was separated from the radiolabeled c-di-AMP by filtration on 3-kDa cutoff spin column yielding 32<sup>P</sup>c-di-AMP of 88.8% purity. Reaction products were visualized by spotting 2 μL on POLYGRAM CEL300 PEI TLC plates (Macherey-Nagel) and separation in 1:1.5 (vol/vol) saturated NH₄SO₄ and 1.5 M KH₂PO₄ (pH 3.6). The radioactive spots were visualized using a FLA5000 Fujifilm PhosphorImager, and data were quantified using AIDA software. On the basis of densitometry analysis, an average final concentration of 9.99 nM 32<sup>P</sup>c-di-AMP was obtained.

Equilibrium Dialysis. Proteins (10 μM) in 40 mM Tris (pH 7.5), 100 mM NaCl, and 10 mM MgCl<sub>2</sub> binding buffer were injected into one cell of a Dispo-Dialyzer cassette with a 5-kDa cutoff membrane (The Nest Group), and c-di-AMP solutions in binding buffer varying in concentration from 0 to 80 μM were injected into the opposite cell. The reactions were allowed to equilibrate for 24 h at room temperature with moderate shaking. Next, samples were removed from each side of the cassette, boiled for 5 min to denature the protein, and centrifuged. The concentrations of c-di-AMP in the supernatants were determined by separating 15 μL of the samples by HPLC (Agilent LC1200) using a Luna 15062, 3-μm particle size RP C-18 column, and a 0.1-M triethylamine acetic acid (pH 6.1) (buffer A) and 80% (vol/vol) acetonitrile containing 20% (vol/vol) buffer A (buffer B) solvent system. The column temperature was set to 35°C and the flow rate to 0.25 mL/min, and a constant buffer B concentration of 6% (vol/vol) was used for the runs. Nucleotides were detected at A<sub>260</sub>, and authentic c-di-AMP (BioLog) was used as a standard to determine nucleotide-specific retention times. The concentrations of c-di-AMP present were...
calculated according to integrated nucleotide peak areas. Two independent experiments were performed (using proteins from two separate purifications) and the concentration of free nucleotide was plotted against the concentration of bound [c-di-AMP]/[protein]. The best-fit nonlinear regression line and $K_d$ values were determined using GraphPad Prism software.

**Modeling, Alignments, and WebLogo Motif.** Modeling experiments were performed using MODELER v. 9.10 (1) and sequence alignments with ClustalW (5). A structural model for the RCK_N domain of KtrA SA was generated using the *B. subtilis* RCK_N domain structure [Protein Data Bank (PDB) code 2HMW]. A model for full-length KtrA SA was generated using the *Methanobacterium thermoautotrophicus* potassium channel protein MthK structure (PDB code 2AEM). Geometry of the models was accessed by inspecting the Ramachandran plot in COOT v. 0.7 (2). Images were produced with PYMOL v. 1.5. The coordinates for all models are available from the authors on request.


**Fig. S1.** Comparative homology models of KtrA SA and RCK domain structures. (A) Structural model of the full-length KtrA SA protein with the RCK_N domain shown in blue and RCK_C domain in orange. The model is based on the structure of the RCK domain found in the *M. thermoautotrophicus* potassium channel protein MthK, which is shown in gray (PDB code 2AEM). (B) Structural model of the N-terminal KtrA SA RCK_N (blue) and the ATP-bound *B. subtilis* RCK_N domain structure (gray, PDB code 2HMW). (C) Enlarged image of the nucleotide-binding site with the location of the conserved GxGxxG motif highlighted in cyan and KtrA SA residues D32 and D52, identified as crucial nucleotide binding residues, indicated. Models were produced with MODELER v. 9.10 (1) and image with PYMOL v. 1.5. Geometry of the models was accessed by inspection of the Ramachandran plot in COOT v. 0.7 (2).


**Fig. S2.** Production of radiolabeled c-di-AMP. $^{32}$P-labeled ATP was converted with recombinant DisA protein to $^{32}$P-labeled c-di-AMP as described in Methods. Aliquots of the input ATP (lane 1) and the reaction products (lane 2) were analyzed by TLC. Residual ATP was removed by the addition of purified H-Ras protein (lanes 3–6), and in the presence of 20 μM H-Ras, $^{32}$P-c-di-AMP of 88.75% purity was obtained (lane 5). This material was further purified and used in differential radial capillary action of ligand assays (DRaCALAs).

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Fig. S3. Validation of DRaCALA to study c-di-AMP/protein interactions. (A) DRaCALAs with the *S. aureus* c-di-AMP phosphodiesterase GdpP. Purified GdpP protein (12.5 μM) was incubated with \(^{32}\)P-labeled c-di-AMP alone (spot 1) or in the presence of 100 μM of the indicated cold competitor nucleotide. These reactions (2.5 μL) were spotted on nitrocellulose membranes and subsequently analyzed with a phosphorimager. Fraction of bound nucleotide was determined as described by Roelofs et al. (1), and values from three independent experiments are plotted with SDs. A c-di-AMP/GdpP interaction could be observed, and as expected only an excess of cold c-di-AMP competed for binding with the \(^{32}\)P-labeled c-di-AMP. (B) DRaCALAs with the *B. subtilis* c-di-AMP cyclase DisA. Assays were set up as described in A but using purified DisA protein. A c-di-AMP/DisA interaction was observed, and as expected only an excess of cold c-di-AMP and ATP (the substrate for DisA) competed for binding with \(^{32}\)P-labeled c-di-AMP.


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Fig. S4. Determination of the affinity between c-di-AMP and KtrA-SA by equilibrium dialysis. The affinity between c-di-AMP and (A) His-KtrA-SA or (B) His-KtrA-SA-134-220 was measured by equilibrium dialysis. For this, 10 μM protein in binding buffer was injected into Dispo-Biodialyzer cassettes and dialyzed for 24 h with c-di-AMP ranging from 80 μM to 0 μM. The c-di-AMP concentration on either side of the cassette was subsequently determined by HPLC analysis. Binding assays were performed twice and the concentration of free nucleotide plotted against bound c-di-AMP/protein. The best-fit nonlinear regression line incorporating the hill equation was determined using GraphPad Prism software, and corresponding \(K_d\) values are given.

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(a) H₂O, c-di-AMP, ATP, GMP, c-di-GMP, GTP, CMP

(b) H₂O, c-di-AMP, ATP, GMP, c-di-GMP, GTP, CMP
Fig. 55.  c-di-AMP binding assays with _E. coli_ extracts. (A) DRaCALAs were performed with $^{32}$P-labeled c-di-AMP and _E. coli_ extracts prepared from strains containing the empty pET28b vector or expressing _S. aureus_ KtrA proteins His-KtrA<sub>SA</sub>, His-KtrA<sub>SA</sub>-D32A, His-KtrA<sub>SA</sub>-D52A, and His-KtrA<sub>SA</sub>-D32A/D52A. (B) DRaCALAs were performed with $^{32}$P-labeled c-di-AMP or $^{32}$P-labeled ATP as indicated below the spots and _E. coli_ extracts expressing either the His-KtrA<sub>SA</sub>-1-140 (RCK<sub>N</sub> domain) or His-KtrA<sub>SA</sub>-134-220 (RCK<sub>C</sub> domain). (C) DRaCALAs were performed with $^{32}$P-labeled c-di-AMP and _E. coli_ extracts prepared from strains containing the empty pET28b vector or expressing the full-length _B. subtilis_ KtrA proteins His-KtrA<sub>BS</sub> and His-KtrA<sub>BS</sub>-C22V or the RCK<sub>N</sub> fragment His-KtrA<sub>BS</sub>-C22V-1-144. Of note, some of the _B. subtilis_ proteins contained the Cys to Val mutation at amino acid position 22 (C22V), a variant that was also used in previous structural studies (1).

Fig. S6. Effect of potassium on growth of *S. aureus* strains Newman, Newman *ktrA*, and *ktrA*-complemented 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 an extra 0.75 M NaCl and containing either 2.5 mM or 250 mM potassium. (C and D) Nigericin sensitivity curves of WT, *ktrA*, and complemented *S. aureus* Newman 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 with the growth in the absence of nigericin. Experiments were repeated a minimum of five times. When grown in 2.5 mM KCl the *ktrA* mutant consistently showed a twofold reduced minimum inhibitory concentration (MIC) for each experiment; however, the exact MIC for all of the strains, mutant and wild-type alike, varied between experiments from 0.2 to 0.8 μM for the wild-type and complemented strain and from 0.1 to 0.4 μM for the mutant strains.

Fig. S7. Targeted DRaCALAs with whole-genome screen results. DRaCALAs were performed with ^32^P-labeled c-di-AMP and *E. coli* extracts prepared from the vector control strain (pET28b) or strains overproducing His-KtrAα, His-PstA, KdpD-His, or His-Adk as indicated.
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Antibiotics were used at the following concentrations: for E. coli cultures: kanamycin (KanR) 30 μg/mL, ampicillin (AmpR) 100 μg/mL, carbenicillin 50 μg/mL; for S. aureus cultures: erythromycin (ErmR) 10 μg/mL, kanamycin (KanR) 90 μg/mL, chloramphenicol (CamR) 7.5 μg/mL, isopropyl β-D-1-thiogalactopyranoside (IPTG) at 1 mM.
Table S2. Primers used in this study

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Restriction sites in primer sequences are underlined.