Evolutionary Adaptation of the Essential tRNA Methyltransferase TrmD to the Signaling Molecule 3',5'-cAMP in Bacteria

Zhang, Yong; Agrebi, Rym; Bellows, Lauren E.; Collet, Jean-Francois; Kaever, Volkhard; Grundling, Angelika

Published in:
Journal of Biological Chemistry

DOI:
10.1074/jbc.M116.758896

Publication date:
2017

Document version
Publisher's PDF, also known as Version of record

Document license:
CC BY

Citation for published version (APA):
Evolutionary Adaptation of the Essential tRNA Methyltransferase TrmD to the Signaling Molecule 3′,5′-cAMP in Bacteria*§

Received for publication, September 15, 2016, and in revised form, November 21, 2016. Published, JBC Papers in Press, November 23, 2016, DOI 10.1074/jbc.M116.758896

Yong Zhang1,2†, Rym Agrebi3,4, Lauren E. Bellows1, Jean-François Collet5,6, Volkhard Kaever3,7, and Angelika Gründling1,2

From the 4 Section of Microbiology and MRC Centre for Molecular Bacteriology and Infection, Imperial College London, London SW7 2AZ, United Kingdom, 6WELBIO, Avenue Hippocrate 75, 1200 Brussels, Belgium, 5De Duve Institute, Université Catholique de Louvain, Avenue Hippocrate 75, 1200 Brussels, Belgium, and 7Research Core Unit Metabolomics, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany

Edited by Norma Allewell

The nucleotide signaling molecule 3′,5′-cyclic adenosine monophosphate (3′,5′-cAMP) plays important physiological roles, ranging from carbon catabolite repression in bacteria to mediating the action of hormones in higher eukaryotes, including human. However, it remains unclear whether 3′,5′-cAMP is universally present in the Firmicutes group of bacteria. We hypothesized that searching for proteins that bind 3′,5′-cAMP might provide new insight into this question. Accordingly, we performed a genome-wide screen and identified the essential Staphylococcus aureus tRNA m1G37 methyltransferase enzyme TrmD, which is conserved in all three domains of life as a tight 3′,5′-cAMP-binding protein. TrmD enzymes are known to use S-adenosyl-L-methionine (AdoMet) as substrate; we have shown that 3′,5′-cAMP binds competitively with AdoMet to the S. aureus TrmD protein, indicating an overlapping binding site. However, the physiological relevance of this discovery remained unclear, as we were unable to identify a functional adenylate cyclase in S. aureus and only detected 2′-3′-cAMP but not 3′,5′-cAMP in cellular extracts. Interestingly, TrmD proteins from Escherichia coli and Mycobacterium tuberculosis, organisms known to synthesize 3′,5′-cAMP, did not bind this signaling nucleotide. Comparative bioinformatics, mutagenesis, and biochemical analyses revealed that the highly conserved Tyr-86 residue in E. coli TrmD is essential to discriminate between 3′,5′-cAMP and the native substrate AdoMet. Combined with a phylogenetic analysis, these results suggest that amino acids in the substrate binding pocket of TrmD underwent an adaptive evolution to accommodate the emergence of adenylate cyclases and thus the signaling molecule 3′,5′-cAMP. Altogether this further indicates that S. aureus does not produce 3′,5′-cAMP, which would otherwise competitively inhibit an essential enzyme.

3′,5′-Cyclic adenosine monophosphate (3′,5′-cAMP) is a second messenger molecule found in all three domains of life (1). It is involved in the regulation of a variety of physiological processes ranging from carbon catabolite repression (CCR)3 in bacteria to mediating the action of hormones in eukaryotes (1). CCR exists in most bacteria and describes the phenomenon that certain carbon sources (usually glucose) are preferentially catabolized over other secondary carbon sources. This is achieved through complex positive and negative regulatory transcription networks (2). CCR is well studied in Escherichia coli, where 3′,5′-cAMP and the catabolite receptor protein (CRP) together form an active transcriptional factor that activates the expression of genes coding for proteins involved in catabalizing secondary carbon sources when glucose is exhausted (2). 3′,5′-cAMP is synthesized from ATP by adenylate cyclases (ACs), a large family of enzymes with divergent sequence, domain, and structural features (1). So far, six classes of ACs have been reported, with class III enzymes found in all domains of life and classes I, II, and IV only present in bacteria (1). The ACs identified in Prevotella ruminicola and Rhizobium etli are distinct from the existing families and were proposed to form Class V and VI enzymes, respectively (3, 4). Class I ACs are exemplified by the E. coli CyaA enzyme; class II ACs are bacterial toxins most often secreted into eukaryotic host cells where they perturb host cell functions (5). Lastly, class IV ACs are a unique group of proteins only found in bacteria but forming part of a larger protein family called CYTH domain proteins (6, 7). CYTH proteins are an ancient protein family that exists in all three domains of life and are named after the type IV AC CyaB from Aeromonas hydrophila and the human thiamine triphosphatase. It has been proposed that these enzymes were originally inorganic triplyphosphatases and subsequently evolved to contain other enzymatic activities such as adenylate cyclase, mRNA triphosphatase, and thiamine triphosphatase activity (6, 7). CYTH proteins contain a characteristic and highly conserved EXEXK amino acid motif at their N terminus and have a

* This work was supported by Wellcome Trust Grant 100289 and European Research Council Grant 260371 (to A. G.) and EMBIO long-term fellowship ALT 721-2013 (to Y. Z.). The authors declare that they have no conflict of interest with the contents of this article.

† Author’s Choice—Final version free via Creative Commons CC-BY license.

‡ Present address: Dept. of Biology, University of Copenhagen, DK-2200, Copenhagen, Denmark.

§ To whom correspondence should be addressed. E-mail: a.grundling@imperial.ac.uk.

The abbreviations used are: CCR, carbon catabolite repression; CRP, catabolite receptor protein; AC, adenylate cyclases; AdoMet, S-adenosyl-L-methionine; DRAcALA, differential radial capillary action of ligand assay; ITC, isothermal titration calorimetry; TSB, tryptic soy broth; MTase, methyltransferase; AdoHcy, adonesyl-L-homocysteine.
conserved fold with eight $\beta$-sheets forming a tunnel-like structure (6). Various other conserved charged amino acids with their side chains projecting into the tunnel have been identified, and these are involved in coordinating the different polyphosphate substrates or are involved in enzyme catalysis (6, 8–10).

Although ACs enzymes are in general widely distributed among bacteria, there is conflicting evidence if $3',5'$-cAMP is produced and plays a physiological role in the Firmicutes group of bacteria. Although a bioinformatics analysis performed by Galperin et al. (11) on 555 complete bacterial and archaeal proteomes indicated that adenylate cyclase enzymes are absent in the majority of Firmicutes bacteria, including *Staphylococcus aureus*, a protein corresponding to SACOL1008 of *S. aureus* strain COL is nevertheless often annotated as adenylate cyclase. However, the predicted cyclase activity of this protein has never been tested. It has also been reported that $3',5'$-cAMP is present in *Bacillus subtilis* when grown under oxygen limitation conditions, and its level was shown to decrease in the presence of nitrate (12, 13). However, in these studies the molecule suggested to be $3',5'$-cAMP was identified only through chromatographic methods and its actual chemical structure was never confirmed by other methods, such as mass spectrometry, which is now routinely used. In *S. aureus*, ArcR (SACOL2653 in strain COL), a member of the CRP/FNR family of bacterial transcriptional regulators, plays a role in mediating catabolite repression (14). Furthermore, $3',5'$-cAMP was shown in *in vitro* assays to enhance the ability of ArcR to bind to the promoter region of the *lce* gene, coding for an L-lactate dehydrogenase (14). However, it was never tested if $3',5'$-cAMP is actually present in *S. aureus* and has a similar effect on ArcR in *vivo*. In this study we wanted to shed further light on whether or not $3',5'$-cAMP is produced and plays a physiological function in *S. aureus*. Using an *S. aureus* ORFeome protein expression library, we screened for $3',5'$-cAMP-binding proteins and identified TrmD as a tight $3',5'$-cAMP-binding protein. However, we were unable to detect $3',5'$-cAMP under various growth conditions nor a functional adenylate cyclase in *S. aureus*. TrmD is a highly conserved tRNA methyltransferase and present in all three domains of life (15). It converts Gly-37 into m$^1$G37 by transferring the methyl group from S-adenosylmethionine (AdoMet) to a subset of tRNA species (15, 16). We further found that TrmD proteins from *E. coli* and *Mycobacterium tuberculosis* do not bind $3',5'$-cAMP. Subsequent bioinformatics and extensive biochemical analyses suggested that $3',5'$-cAMP competes with AdoMet for binding, presumably inhibiting the essential function of TrmD in *S. aureus*. Together with a phylogeny analysis, our data suggest that $3',5'$-cAMP is absent in *Staphylococcus*. Finally, our work also highlights that the emergence of $3',5'$-cAMP as a signaling molecule in bacteria required essential evolutionary adaptations of AdoMet-binding proteins such as TrmD.

**Results**

Identification of the *S. aureus* TrmD Protein as a $3',5'$-cAMP-binding Protein—There is conflicting evidence whether or not $3',5'$-cAMP exists in the Firmicutes group of bacteria. We reasoned that if $3',5'$-cAMP is indeed present and functionally relevant in *S. aureus*, specific $3',5'$-cAMP-binding protein(s) must exist. To investigate this, we made use of a *S. aureus* ORFeome protein expression library and the differential radial capillary action of ligand assay (DRAcALA), a simple and fast method for the detection of small molecule-protein interactions (17–19). The DRAcALA method is based on the principle that free radiolabeled small ligand will diffuse outward once spotted on a nitrocellulose membrane but will stay as a tight spot when bound to a protein (17). In previous studies this assay and the ORFeome protein expression library was successfully used to identify c-di-AMP and ppGpp-binding proteins in *S. aureus* (18–20). The ORFeome protein expression library is a collection of 2337 *E. coli* strains allowing for the overproduction of 86% of the annotated *S. aureus* strain COL proteins as His-MBP-fusion proteins (18–20). To apply this assay to the identification of potential $3',5'$-cAMP-binding proteins, the expression of the *S. aureus* proteins was induced, and *E. coli* cell lysates were prepared. Next, radiolabeled $[^{32}P]cAMP$ was synthesized using a C-terminal truncated form of the *E. coli* adenylate cyclase enzyme CyA. As assessed by thin layer chromatography (TLC), 97% of the input [a-32P]ATP was converted to [a-32P]cAMP (data not shown). The genome wide DRAcALA screen was subsequently performed as previously described (18–20). Two technical replicates were performed, and the lysate from one strain expressing the *S. aureus* COL protein SACOL1256 (plate 13 well F05) gave a positive result for $3',5'$-cAMP binding (data not shown). SACOL1256 (or SAUSA300_1133 in the USA300 strain FPR3757) codes for the tRNA methyltransferase TrmD, termed from here on out as TrmD$_{SA}$. To further investigate if TrmD$_{SA}$ can bind $3',5'$-cAMP with a physiological relevant affinity, SAUSA300_1133 from the USA300 strain LAC$^+$ was cloned into vector pET28b, and the protein was expressed and purified as N-terminally His-tagged fusion protein. DRAcALAs were carried out with $[^{32}P]cAMP$ and serially diluted TrmD$_{SA}$ protein ranging from 200 $\mu$M to 1.5 nM and a $K_d$ of 1.97 $\pm$ 0.24 $\mu$M was determined (Fig. 1A). This binding affinity is in a similar range as the reported $K_d$ of 2 $\mu$M for the interaction between the *E. coli* transcription factor CRP and 3',5'-cAMP (21). To test if the binding is specific to $3',5'$-cAMP, an excess of the cold competitor nucleotides 3',5'-cAMP, 3',5'-cGMP, c-di-GMP, and c-di-AMP was added to the binding reaction. This analysis revealed that only cold 3',5'-cAMP but none of the other nucleotides tested could compete for binding with the radiolabeled $[^{32}P]cAMP$ (Fig. 1B). Two forms of CAMP have been detected in cells, 3',5'-cAMP, the classic signaling nucleotide, and 2',3'-cAMP, suggested to be a nucleotide intermediate formed during the RNA degradation process (22, 23). As revealed by competitive binding assays, only the classic signaling molecule 3',5'-cAMP but not 2',3'-cAMP could prevent the binding of radiolabeled 3',5'-cAMP to TrmD$_{SA}$ (Fig. 1B). Taken together, these data show that the *S. aureus* TrmD protein is able to bind the 3',5'-cAMP signaling nucleotide with high affinity. However, it is also of note that no interaction between 3',5'-cAMP and the *S. aureus* ArcR protein, a transcription factor with homology to the *E. coli* CRP protein, could be detected (Fig. 1C).
3',5'-cAMP Competitively Binds at the AdoMet Substrate Binding Site of the S. aureus TrmD Protein—TrmD is a highly conserved and essential enzyme and present in nearly all bacteria. TrmD is responsible for methylating the Gly-37 residue at the N1 position in a subset of tRNAs using AdoMet as methyl donor (15). Because 3',5'-cAMP is chemically similar to AdoMet and both contain an adenine moiety and a ribose ring (Fig. 1D), this raised the possibility that 3',5'-cAMP binds to the AdoMet substrate binding site of TrmD$_{S_{A}}$. Indeed, as revealed by a competitive binding assay, AdoMet could inhibit in a dose-dependent manner the binding of radiolabeled 3',5'-cAMP to TrmD$_{S_{A}}$ (Fig. 1B). Next, isothermal titration calorimetry (ITC) experiments were performed, and a $K_d$ of 121.4 $\mu$M was determined for AdoMet binding to TrmD$_{S_{A}}$. Of note, the TrmD$_{S_{A}}$ and AdoMet interaction was determined by ITC and not DRaCALA, as the latter method can only be used for high affinity binding interactions (low $\mu$M $K_d$ or below) and using a radiolabeled ligand. Taken together, these data indicate that 3',5'-cAMP binds at the same site as the natural substrate AdoMet, consistent with a competitive binding mechanism.

3',3'-cAMP, but Not 3',5'-cAMP, Can Be Detected in S. aureus Extracts—Previous work indicated that cAMP is produced in B. subtilis when grown without aeration under oxygen limitation conditions (12, 13). However, it should be noted that cAMP production was only assessed using chromatographic methods, and its chemical structure was never confirmed by NMR- or mass spectrometry-based methods (12, 13). Given the fact that we uncovered a 3',5'-cAMP-binding protein in S. aureus, we next set out to determine if and when 3',5'-cAMP is produced in S. aureus using a sensitive mass spectrometry-based method (24). Cytosolic extracts were prepared from the wild-type S. aureus strain JE2 and strain NE1299, containing a transposon insertion in SAUSA300_0905, coding for an uncharacterized protein often annotated as adenylyl cyclase. The strains were grown in tryptic soy broth (TSB) medium under aerobic or micro-aerobic conditions as well as in B-medium supplemented with either glucose or sucrose as the only carbon source to reflect carbon catabolite repression in S. aureus (25). Extracts were prepared from both exponential and stationary phase cultures, and nucleotides were detected by LC-MS/MS as described in Bähre and Kaever (24). Using this sensitive method, 3',5'-cAMP concentrations can be detected up to a lower limit of 0.412 pmol per sample and also discriminated from 2',3'-cAMP. Large amounts of 2',3'-cAMP were detected in all samples (Fig. 2). Normalization based on total protein concentrations revealed higher 2',3'-cAMP levels in extracts prepared from strains grown in TSB medium under micro-aerobic than under aerobic conditions (Fig. 2). The 2',3'-cAMP levels were even higher when bacteria were grown in B-medium (Fig. 2). Of note, more 2',3'-cAMP was also present in extracts prepared from stationary than exponential phase cells, when the bacteria were grown in B-medium supplemented with glucose. Although high levels of 2',3'-cAMP could be detected in all samples, 3',5'-cAMP was not detected in any of the extracts, suggesting that S. aureus does not produce 3',5'-cAMP at least under the conditions tested.
Protein Adaption with the Emergence of 3′,5′-cAMP

SAUSA300_0905 and Its Homologs Are Distinct From Type IV AC Enzymes—An initial bioinformatics analysis indicated that the predicted S. aureus adenylate cyclase SAUSA300_0905 (USA300 FPR3757 nomenclature) is most closely related to type IV AC enzymes. It is possible that the SAUSA300_0905 protein was not expressed or active under the growth conditions tested, and hence, we were unable to detect 3′,5′-cAMP in the S. aureus extracts. To test if SAUSA300_0905 is able to synthesize 3′,5′-cAMP in vitro, the protein was expressed and purified from E. coli as the N-terminal His-tag fusion protein. The E. coli CyaA2–446 protein was purified and used as positive control. Radiolabeled [α-32P]ATP was used as the substrate, and reactions were set up in three different buffers, as previously reported for in vitro enzyme assays with the E. coli CyaA (26) or the Yersinia pestis type IV AC CyaB (8). The enzyme reactions were incubated for 1 h or overnight at 37 °C, and the reaction products were subsequently analyzed by TLC. Within 1 h, the E. coli CyaA2–446 enzyme converted >91 and 61% of the ATP to cAMP in the Mg2+- and Mn2+-containing buffers, respectively; however, none of the [α-32P]ATP was converted by SAUSA300_0905 (Fig. 3A). Overnight reactions essentially yielded similar results (data not shown). Genes coding for active AC enzymes have previously been identified through their ability to complement an E. coli cyaA mutant strain using simple plate assays, as 3′,5′-cAMP-producing E. coli strains appear red or blue on MacConkey- or X-Gal-containing plates, respectively (3, 4, 27). To test if SAUSA300_0905 is able to produce 3′,5′-cAMP when expressed in E. coli, SAUSA300_0905 and as a positive control the E. coli cyaA2–446 gene were cloned with an N-terminal His tag in vector pBAD33 and expressed under the control of the arabinose-inducible promoter. The resulting plasmids, pBAD33-SUSAUSA300_0905-His6 and pBAD33-cyaA2–446-His6, and the empty vector pBAD33 as negative control were introduced into the E. coli strain DHM1 containing plasmid pBAD33, pBAD-cyaAEC-His6, or pBAD-SUSAUSA300_0905-His6 was propagated in LB medium without or with 0.02% arabinose. Whole cell lysates were prepared as described under “Experimental Procedures,” proteins were separated on a 12% SDS-PAGE gel, and His-tagged proteins were detected using a His-tag-specific antibody.

3′,5′-cAMP in extracts prepared from wild-type S. aureus strain JE2 and the isogenic SAUSA300_0905 mutant strain (Δ9005). Bacteria were grown in TSB medium with/without agitation (A) or in B-medium supplemented with 25 mM of glucose or sucrose (B). As indicated in the graph, extracts were either prepared from logarithmic (log) phase cultures or stationary (stat) phase cultures.

To investigate this further, we revisited the annotation of SAUSA300_0905 by performing a detailed bioinformatics analysis. SAUSA300_0905 was used in a BLASTP search, the result of which indicated that it belongs to the CYTH superfAMILY of genes.
A protein similar to bona fide type IV adenylate cyclases. Next, homologs of SAUSA300_0905 were retrieved from the NCBI non-redundant (nr) protein sequence database, and this yielded 998 sequences with a minimum of 30% sequence identity and 60% sequence coverage. To compare SAUSA300_0905 with a genuine type IV adenylate cyclase, CyaB from Y. pestis was used as a query sequence to retrieve its closest homologs from the NCBI nr database. This yielded 562 sequences with a minimum of 30% sequence identity and 60% sequence coverage. But none of the CyaB homologs were from the Firmicutes group of bacteria. Next, multiple sequence alignments were performed within each group of proteins, and sequence logos were generated (Fig. 4A). A multiple sequence alignment was also performed across the two groups of proteins. This analysis revealed that the EXEK signature motif of CYTH family protein was conserved in both groups of proteins. However, key residues required for the adenylate cyclase activity of type IV ACs, such as Lys-111 and Arg-113, which are essential for forming hydrogen bonds with the $\alpha$- and $\beta$-phosphate groups of ATP, or Phe-5, Cys-83, Arg-
113, and Glu-136 (green stars in Fig. 4A), which have been shown to be required for adenylate cyclase activity (8), are absent in SAUSA300_0905 and its homologs. On the other hand, a unique and highly conserved DXEXEXXE motif (yellow stars in Fig. 4A) was identified within the C-terminal region of SAUSA300_0905 and its homologs that is absent from type IV ACs. Phyre2 was then used to generate a structure model of SAUSA300_0905 (using bh2851, a putative adenylate cyclase from Bacillus halodurans, with PDB code 2GFG as template, 94% of residues modeled at >90% confidence). This analysis suggested that the S. aureus protein SAUSA300_0905 does assume a fold typical for CYTH proteins with a tunnel-like structure similar to CyaB (PDB code 3N0Y) (Fig. 4B). Despite the similar structural fold, the absence of key residues required for adenylate cyclase activity and the presence of other conserved residues at the C terminus of the SAUSA300_0905 indicates that this protein is distinct from type IV adenylate cyclases and, therefore, may have a different enzymatic activity. Lastly, using the E. coli CyaA, Bacillus anthracis CyaA P40136, Arthrobacter platensis CyaC O33293, P. ruminicola Cya O68902, and R. etli CyaC Q8KY20 proteins as representatives of type I, II, III, V, and VI ACs, respectively, in BLASTP searches, no proteins with significant similarity were found in Staphylococaceae. These data suggest that no AC enzyme for the production of the classic signaling nucleotide 3',5'-cAMP is encoded in S. aureus.

TrmDs from E. coli and M. tuberculosis Do Not Bind 3',5'-cAMP—Our results so far indicate that 3',5'-cAMP can in vitro assays competitively bind to the AdoMet substrate binding site of the S. aureus TrmD protein. On the other hand, we were unable to detect 3',5'-cAMP in S. aureus extracts nor an enzyme that would be able to produce this signaling nucleotide. Hence, the interaction of 3',5'-cAMP and the S. aureus TrmD protein may not be of physiological relevance. However, TrmD is a highly conserved protein and present in a large number of bacteria that have been experimentally shown to produce 3',5'-cAMP. This raises the possibilities that in these organisms 3',5'-cAMP could either be a competitive inhibitor of TrmD enzyme or alternatively that TrmD proteins evolved to discriminate between the 3',5'-cAMP and AdoMet ligands. To address this question, TrmD proteins from the 3',5'-cAMP producing γ-proteobacterium E. coli (TrmD_{Ec}) and the actinobacterium M. tuberculosis (TrmD_{Mt}) were chosen for further analysis. The genes coding for the corresponding TrmD proteins were cloned in the pET28b vector, and the proteins were expressed and purified as N-terminal His-tagged proteins (Fig. 5A). DRaCALAs were performed with radiolabeled 3',5'-cAMP and the purified TrmD_{Ec} and TrmD_{Mt} proteins. This analysis revealed that neither protein could bind 3',5'-cAMP as tightly as the S. aureus TrmD_{SA} protein, and due to this weak interaction no actual $K_d$ value could be determined using the DRACAL method (Fig. 5B). These data suggest that TrmD proteins from organisms producing 3',5'-cAMP are able to discriminate between 3',5'-cAMP and the AdoMet ligand, likely preventing 3',5'-cAMP to act as competitive inhibitor and blocking the essential functions of TrmD.

**Tyr-86 Is Critical for Discriminating 3',5'-cAMP from AdoMet in E. coli TrmD**—The difference in the capacity of the S. aureus, E. coli, and M. tuberculosis TrmD proteins to bind 3',5'-cAMP indicates a critical difference in their primary sequences and tertiary structures. To gain further insight into this, each of the TrmD proteins was used to retrieve their close homologs from their respective groups (that is, Firmicutes, γ-Proteobacteria, and Actinobacteria). Multiple sequence alignments were performed individually within each group, and sequence logos were generated using Jalview (Fig. 6A) (28). This analysis revealed three highly conserved motifs termed here A, B, and C, which based on previous structural and functional analysis are known to form the active site and the AdoMet substrate binding pocket in TrmD proteins (Fig. 6B) (29, 30). Among these motifs, the motifs B and C with consensus sequences C(Y/F)G(D/E) and EExEGD(Y/F) respectively, are essentially the same in all three groups of bacteria (Fig. 6A). However, motif A differs significantly, with the consensus sequence YLSPQG in Proteobacteria, VPTPAG in Actinobacteria, and the degenerated consensus sequence (L/Y)(L/M)XP(Q/A)G in Firmicutes (Fig. 6A). In particular, the first three amino acids of motif A, referred to from here on out as motif A1, show very low conservation in the Firmicutes group of bacteria. To visualize the location of these motifs in a structural context, a multiple sequence alignment was created for the TrmD proteins from all three groups. The alignment was subsequently mapped on the crystal structure of the TrmD protein from Hemophilus influenzae (PDB code 1UAK), a member of the γ-Proteobacteria group and displayed in the Consurf view (31). As expected, the three motifs, including the A1 motif, form the binding pocket for AdoMet on
Protein Adaption with the Emergence of 3',5'-cAMP

Because of the weak binding, no WT TrmD and the WT TrmDEC and the TrmDECY86F variants had similar and high binding affinities for AdoMet with $K_d$ values of 21.5 $\mu M$ and 27.9 $\mu M$, respectively. Taken together, these data indicate that the highly conserved Tyr-86 residue in E. coli TrmD and likely also in other $\gamma$-Proteobacteria is important for discriminating between 3',5'-cAMP and AdoMet, preventing the binding of the former, which otherwise could competitively inhibit the enzyme.

**Coevolution of TrmD with the Emergence of the 3',5'-cAMP Signaling Pathway** —The fact that TrmD proteins from bacteria that have been shown to produce 3',5'-cAMP (E. coli and M. tuberculosis) do not bind 3',5'-cAMP with high affinity, whereas TrmD from S. aureus tightly binds 3',5'-cAMP raises the possibility that TrmD has evolved immunity to 3',5'-cAMP in bacteria producing this signaling nucleotide. To test this hypothesis, the phylogeny of TrmD was analyzed and compared with that of adenylate cyclases. To do this we searched for homologs of S. aureus TrmD in the 555 complete bacterial and archaeal proteomes used by Galperin et al. (11) (supplemental Table S1). BLASTP queries identified a total of 503 homologs with a large taxonomic distribution. Specifically, homologues are found in one copy in all bacterial phyla (except for Syntrophobacter fumaroxidans MPOB that have two TrmD proteins: YP_847151 and YP_847109; supplemental Table S1). Close homologs are absent from Archaea, consistent with previous report that TrmD from Archaea is more similar to eukaryotic counterparts. A phylogenetic tree of TrmD homologs was built (supplemental Fig. S1) as well as all sequences were aligned, and careful inspection of the alignment showed that motifs B and C of the AdoMet binding site are well conserved among all TrmD homologs (supplemental Fig. S2). However, the first three residues of motif A are variable (supplemental Fig. S2). Based on the data of Galperin et al. (11) concerning the presence/absence of adenylate cyclase in bacterial proteomes, there appears to be a positive correlation between the presence or absence of ACs

**FIGURE 6. Comparison of AdoMet binding site residues found in TrmD proteins from Firmicutes, $\gamma$-Proteobacteria, and Actinobacteria.** A, sequence logos motifs of AdoMet binding site residues found in TrmD proteins from Firmicutes, $\gamma$-Proteobacteria, and Actinobacteria. TrmD proteins from bacteria belonging to the Firmicutes, $\gamma$-Proteobacteria, and Actinobacteria were retrieved, and sequence alignments and logo motifs were prepared and displayed in the ClustalX default color scheme as described under "Experimental Procedures." Amino acid residues forming the AdoMet binding site were identified and labeled as motifs A (further split into A1 and A2), B, and C. Motifs A1, A2, B, and C are and are highly conserved between the three groups, whereas motif A1 is variable. Amino acid numbers indicated above the logo motif section is based on the S. aureus COL strain TrmD protein. B, ConSurf model of AdoMet binding site motifs of TrmD proteins. A multisequence alignment was generated for TrmD proteins found in Firmicutes, $\gamma$-Proteobacteria, and Actinobacteria and mapped using the ConSurf server onto the structure of the AdoMet-bound H. influenzae TrmD protein (PDB code 1UA6). Purple represents high, white represents medium, and turquoise represents low conservation. The AdoMet ligand is shown as a stick model, and areas with motif A1, A2, B, and C amino acids are circled.

TrmD. The A1 motif area appears as white patch in the Consurf view, indicative of a lower conservation (Fig. 6B). To gain a better understanding of the location of the amino acids within the A1 motif in the context of the protein/AdoMet ligand interactions, a comparative structural alignment was performed with PyMOL using the available crystal structures of the S. aureus TrmD protein (PDB code 3KY7) and the H. influenzae TrmD protein (PDB code 1UA6), the latter of which has been crystallized in the presence of AdoMet. As shown in the H. influenzae TrmD-AdoMet complex structure, the Tyr residue at the beginning of the A1 motif (corresponding to Tyr-86 in E. coli TrmD$_{EC}$) and highly conserved in Proteobacteria, forms hydrogen bonds with the 3'-OH and 2'-OH of the ribose ring in AdoMet (Fig. 7A). The corresponding 3'-OH group in 3',5'-cAMP forms a phosphoester bond with the 5'-phosphate group (Fig. 7A) and hence would not be available for such a hydrogen bond interaction. This observation indicates that Tyr-86 in TrmD$_{EC}$ might play a critical role in discriminating AdoMet from 3',5'-cAMP by forming an additional hydrogen bond with AdoMet. Consistent with this idea, a Leu residue is present at the corresponding position in the A1 motif of the S. aureus TrmD$_{SA}$ protein, which could not form a hydrogen bond with the 3'-OH of AdoMet, and this may allow TrmD$_{SA}$ to bind AdoMet as well as 3',5'-cAMP. To test this hypothesis, the LMC amino acid residues of the TrmD$_{SA}$ A1 motif were replaced with YLS residues as found in TrmD$_{EC}$. This variant was expressed and purified as N-terminal His-tagged protein (Fig. 5A). As assessed by DRAcALAs, this variant showed a decreased binding affinity for 3',5'-cAMP (Fig. 7B). Conversely, the Tyr-86 residue in the TrmD$_{EC}$ protein was replaced with a Phe (lacking the phenol hydroxyl group of Tyr) or Leu residue as present in TrmD$_{SA}$, yielding the TrmD$_{EC}$Y86F and TrmD$_{EC}$Y86L variants, respectively (Fig. 5A). The TrmD$_{EC}$Y86F variant was still unable to bind 3',5'-cAMP (similar to WT TrmD$_{EC}$); however, the TrmD$_{EC}$Y86L variant had a much increased binding affinity for 3',5'-cAMP (Fig. 7C). These findings were corroborated further by ITC experiments. Because of the weak binding, no $K_d$ values could be determined for the interaction between 3',5'-cAMP and the WT TrmD$_{EC}$ protein and the TrmD$_{EC}$Y86F variant; however, the TrmD$_{EC}$Y86L variant was able to bind 3',5'-cAMP with a $K_d$ of 41.2 $\mu M$. On the other hand, this variant now had a decreased binding affinity for AdoMet with a $K_d$ of 69.9 $\mu M$, whereas the WT TrmD$_{EC}$ and the TrmD$_{EC}$Y86F variants had similar and high binding affinities for AdoMet with $K_d$ values of 21.5 $\mu M$ and 27.9 $\mu M$, respectively. Taken together, these data indicate that the highly conserved Tyr-86 residue in E. coli TrmD and likely also in other $\gamma$-Proteobacteria is important for discriminating between 3',5'-cAMP and AdoMet, preventing the binding of the former, which otherwise could competitively inhibit the enzyme.

Amino acid numbers indicated above the logo motif section is based on the...
Protein Adaption with the Emergence of 3',5'-cAMP

FIGURE 7. Tyr-86 in E. coli TrmD is critical to discriminate between 3',5'-cAMP and AdoMet. A, comparison of the AdoMet-binding pockets of the S. aureus and H. influenzae TrmD proteins. The apo structures of the S. aureus TrmD (PDB code 3KY7; shown in yellow) was overlaid in PyMOL with the AdoMet-bound structure of the H. influenzae TrmD (PDB 1UAK, show in gray). A zoomed-in view of the ligand binding pocket is shown in schematic representation, with the AdoMet, Tyr-86 of TrmDEC, and Leu84 of TrmDSA shown as a stick model. Tyr-86 of TrmDec formed two hydrogen bonds with 2'- and 3'-OH of the ribose ring of AdoMet, whereas Leu-84 of TrmDSA probably cannot. B and C, binding curve and Kd determination between 3',5'-cAMP and WT S. aureus TrmD and the indicated variant (B) and WT E. coli TrmD protein and the indicated variants (C). Radiolabeled cAMP and purified TrmD or the TrmD-LMC-YLS variant TrmDEC or the TrmDEC-Y86F and TrmDEC-Y86L variants ranging from 1.5 nm to 200 μM were used in DRaCALAs, the average fraction-bound values and S.D. of at least three values were plotted, the curve fitted, and Kd value determined as previously described (17). D, Logomotifs of the TrmD protein A motif amino acids of representative Firmicutes. The TrmD protein sequences from 78 representative Firmicutes, as described in Galperin et al. (11), were retrieved and grouped into sequences from bacteria likely containing an adenylate cyclase (with AC (n = 69)) or containing an adenylate cyclase (no AC (n = 69)) or containing an adenylate cyclase (with AC (n = 69)). The sequences were aligned separately, and logo motifs were generated and displayed in the ClustalX default color scheme. The TrmD A motif was split as described in Fig. 6A into motifs A1 and A2, and amino acid numbers indicated above each logo motif section is based on the S. aureus strain COL TrmD protein sequence.

A

B

C

D

TABLE 7

<table>
<thead>
<tr>
<th></th>
<th>A1</th>
<th>A2</th>
<th>A1</th>
<th>A2</th>
</tr>
</thead>
<tbody>
<tr>
<td>84</td>
<td>P</td>
<td>G</td>
<td>P</td>
<td>Q</td>
</tr>
<tr>
<td>no AC (n=69)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>with AC (n=9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

JANUARY 6, 2017

VOLUME 292 • NUMBER 1 • JOURNAL OF BIOLOGICAL CHEMISTRY

320

ASBMB

Downloaded from http://www.jbc.org/ at Copenhagen University Library on April 4, 2018
in several of the model Firmicutes bacteria (Bacillus, Listeria, and Staphylococcus) (2). Although CRP-like transcription factors are present in Firmicutes, they often lack key residues known to be required for the binding of 3',5'-cAMP (14). As shown in this study, ArcR, a CRP-family transcriptional factor in S. aureus, does not bind 3',5'-cAMP as determined by DRaCALA (Fig. 1C). Using a very sensitive LC-MS/MS-based method, 3',5'-cAMP could not be detected in S. aureus extracts prepared from cultures grown under several different conditions including micro-aerobic and catabolite repression conditions, where 3',5'-cAMP was believed to be produced (Fig. 2) (12, 13). In vitro and in vivo experiments on the predicted adenylate cyclase SAUSA300_0905 (SACOL1008) enzyme indicated that this protein is not a genuine adenylate cyclase as this protein does not hydrolyze ATP nor produce 3',5'-cAMP (Fig. 3, A and B). Close homologues to SAUSA300_0905 are found in many other bacteria belonging to the Firmicutes group. Despite the fact that this protein likely has a similar overall fold as type IV ACs, we identified in our bioinformatics analysis distinct sequence features in SAUSA300_0905 and its homologs compared with bona fide Class IV ACs (Fig. 4A). The physiological function of SAUSA300_0905, its enzymatic activity, and substrate specificity remain to be studied.

A genome wide DRaCALA screen for 3',5'-cAMP-binding proteins using an S. aureus ORFeome library identified the essential tRNA methyltransferase TrmD\(_{\text{SA}}\) as 3',5'-cAMP-binding protein (Fig. 1A). 3',5'-cAMP binds with high affinity to TrmD\(_{\text{SA}}\) and competes for the binding with the native substrate AdoMet (Fig. 1A). The difference in binding affinity is indicated by the limited capacity of AdoMet to compete with 3',5'-cAMP for binding to TrmD\(_{\text{SA}}\) (Fig. 1B). Given that TrmD binds its substrate tRNA species in an AdoMet-dependent manner (16), this difference in binding affinity suggests a potential inhibitory effect on the essential function of TrmD would 3',5'-cAMP be present in S. aureus. As shown in this work, a tyrosine residue found at position 86 in the E. coli TrmD and highly conserved among TrmD proteins from \(\gamma\)-Proteobacteria, aids in the discrimination and preferential binding of AdoMet over 3',5'-cAMP in E. coli (Figs. 6A and 7). This residue is absent in S. aureus and most other Firmicutes (Figs. 6A and 7A). However, some non-type Firmicutes strains, which are predicted to encode a genuine adenylate cyclase (11), such as Clostridium acetobutylicum ATCC 824, Clostridium perfringens str. 13, Caldicellulosiruptor saccharolyticus DSM 8903, Desulfofomicum acidothrix JF1, Natranaerobius thermophilus JW/NM-WN-LF, and their sub-strains, contain a Tyr or Phe residue at this position, suggesting a coupling of the presence of a functional adenylate cyclase and 3',5'-cAMP production with the presence of a Tyr/Phe amino acid residue at this position (Fig. 7D). Altogether the findings presented in this study strongly suggest that 3',5'-cAMP and a functional adenylate cyclase enzyme are absent in S. aureus and, although not all, likely also a large number of other bacteria belonging to the Firmicutes group.

TrmD is a highly conserved tRNA methyltransferase found in all three domains of life that converts Gly-37 into m'\(^{3}\)G37 by transferring the methyl group from AdoMet to a subset of tRNAs (15, 32). This modification on tRNAs is essential for maintaining the correct reading frame during protein translation (15, 33). Abolishing the function of TrmD increases +1 frameshift events during protein translation, and growth defects have been observed in its absence in bacteria and yeast (15, 33–35). To bind the AdoMet substrate, TrmD proteins assumes a particular protein fold composed of a deep trefoil knot that is a characteristic of SPOUT family RNA methyltransferases (MTases). SPOUT family MTases methylate ribosomal RNAs or tRNAs on a base or ribose ring using AdoMet as the methyl donor (36). The conserved AdoMet binding site and unique protein fold of SPOUT MTases raises the question as to why other MTases from S. aureus were not identified as 3',5'-cAMP-binding proteins. Several MTases have been crystallized in complex with AdoMet, its analogue sinefungin, or S-adenosyl-\(l\)-homocysteine (AdoHcy) (29, 37–39). Careful inspection of the substrate binding sites of these other MTases, namely the tRNA MTases TrmL (PDB code 1MXI), TrmH (PDB code 1V2X), MjNep1 (PDB code 3BBH), and the RNA MTases ScNep1 (PDB code 2V3K) and RsmE (PDB code 2Z0Y) (Fig. 8), revealed that in these cases a main chain peptide amine of a highly conserved glycine residue forms hydrogen bonds with the 2′- and 3′-OH of AdoMet/AdoHcy/sinefungin. This difference might explain why only TrmD\(_{\text{SA}}\) was found to interact with 3',5'-cAMP, as other MTases in S. aureus are likely able to discriminate between 3',5'-cAMP and AdoMet.

In \(\gamma\)-Proteobacteria and Firmicutes, we found bioinformatic ally and experimentally a good correlation between the ability of TrmD proteins to discriminate between 3',5'-cAMP and AdoMet and the presence of a tyrosine or phenylalanine residue at a position equivalent to position 86 in the E. coli TrmD protein (Figs. 6 and 7). These data suggest that TrmD proteins in bacteria producing 3',5'-cAMP adapted to confer “immunity” to 3',5'-cAMP, binding of which would otherwise inhibit the essential function of TrmD. However, this simple correlation was not obvious in all groups of bacteria. In particular, we found that TrmD proteins from the Actinobacteria group had a unique set of highly conserved amino acid residues VPT in the motif A1 (Fig. 6A). Considering the numerous adenylate cyclases reported in this group of bacteria (up to 16 ACs in M. tuberculosis) (1), it is possible that the TrmD proteins underwent a global whole-protein adaption and optimization to properly function in the unique physiological conditions imposed by these ACs. It remains to be investigated how these “unusual” TrmD proteins cope with the presence of physiological concentrations of 3',5'-cAMP. Such studies could also have translational impact, as TrmD proteins from bacteria are distinct from those found in Eukaryotes and Archaea and thus remain attractive drug targets.

TrmD has been extensively studied, especially in pathogenic bacteria, where a number of biochemical and structural characterization were performed (16, 29) in order to pinpoint unique features that are drug-able. Indeed, the TrmD\(_{\text{MT}}\) protein was highlighted as an excellent drug target candidate in M. tuberculosis (40), and screens for small molecule TrmD enzyme inhibitor were performed (41, 42). Lahoud et al. (42) found that adenosine and methionine fragments of AdoMet preferentially inhibit bacterial TrmD proteins over those of eukaryotic-Archaea origins. Hill et al. (41) screened a large col-
Protein Adaption with the Emergence of 3',5'-cAMP

FIGURE 8. Schematic representation of the hydrogen-bonding networks between S-adenosylmethionine or its analogs and bacterial tRNA/rRNA methyltransferases. The substrate binding pockets are shown. A, tRNA MTases TrmD (tRNA (guanine-N1)-methyltransferase, in complex with AdoMet, PDB code 1UAK). B, TrmH (tRNA (guanosine18)-2'-O-methyltransferase, in complex with AdoMet, PDB code 1V2X). C, MjNep1 (ribosomal RNA small subunit methyltransferase, in complex with AdoHcy, PDB code 3BBH). D, TrmL (tRNA (cytidine34)-2'-O-methyltransferase in complex with AdoHcy, PDB code 1MXI). E, rRNA MTases ScNep1 (ribosomal RNA small subunit methyltransferase Nep1, in complex with AdoMet, PDB code 2V3K). F, RsmE (ribosomal RNA small subunit methyltransferase E, in complex with AdoMet, PDB code 220Y). AdoMet, AdoHcy, and sinefungin and key protein amino acid residues involved in hydrogen bond (yellow dotted lines) formation with the 2’ and 3’-OH in the substrate are shown in stick models (carbon, white; oxygen, red; nitrogen, blue; sulfur, orange). Small gray dots indicate water molecules. Images were generated in PyMOL.
H37Rv chromosomal DNA were used to amplify the trmD<sub>E</sub> gene. The PCR product was digested with NdeI and HindIII and ligated with plasmid pET28b. For construction of plasmid pET28b-His<sub>6</sub>-cyaA<sub>EC</sub> (2–446) primer pair, ANG1788/1789 and NheI and EcoRI and ligated with plasmid pET28b. For construction and use of this ORFeome library has been described previously (19, 20). Protein expression and the preparation of whole cell lysates and the subsequent DRaCALA were performed as previously described, with the modification that 32P-labeled 3'-5'-cAMP was used as the nucleotide ligand (17–19). For the determination of Kₐ values by DRaCALA, 2-fold serial dilutions of purified His-TrmD<sub>Sa</sub>, His-TrmD<sub>EC</sub>, His-TrmD<sub>Tb</sub>, or the different variants were prepared in binding buffer (40 mM Tris, pH 7.5, 100 mM NaCl, 10 mM MgCl₂) starting at a concentration of 200 μM and subsequently mixed with ~2 nm 32P-labeled 3'-5'-cAMP. The mixtures were incubated for 5 min at room temperature before spotting 2.5 μl of the reactions on nitrocellulose membranes (Amersham Biosciences Hybond-ECL; GE Healthcare). The fraction of ligand bound and Kₐ values were calculated as previously described (17). For nucleotide competition assays, the specified purified protein at a final concentration of 100 μM was incubated with ~2 nm 32P-labeled 3',5'-cAMP. The mixtures were incubated for 5 min at room temperature, 2.5 μl was spotted onto nitrocellulose membranes, and fraction-bound values were determined as described above.

In Vitro Adenylyl Cyclase Assay—The purified His<sub>6</sub>-SACOL1008 protein was tested for potential adenylyl cyclase activity using previously described in vitro assay systems (8, 26). The purified His-CyaA<sub>EC</sub> (2–446) protein was used as the positive control, and enzyme assays were set up in three different buffer systems. Buffer 1 consisted of 40 mM Tris, pH 7.5, 100 mM NaCl, and 10 mM MgCl₂ and was previously used to measure the activity of the E. coli CyaA enzyme (26). Buffer 2 con-
Protein Adaption with the Emergence of 3',5'-cAMP

TABLE 2

<table>
<thead>
<tr>
<th>Number</th>
<th>Name</th>
<th>Restriction sites</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANG1918</td>
<td>5-NheI-MG1655 TrmD</td>
<td>CTAGGCTAGCTGGATTGGCATAATTAGCCTGTTTCCTGAAATG</td>
<td></td>
</tr>
<tr>
<td>ANG1919</td>
<td>3-EcoRI-MG1655 TrmD</td>
<td>CCGAATTCCTGACATCGCCATGTTTATGTTGTTGCTG</td>
<td></td>
</tr>
<tr>
<td>ANG1920</td>
<td>5-NheI-SACOL1256 TrmD</td>
<td>CTTAGCTGCAAAATTTAGATTTATTATTTTATTTTCTTGGT</td>
<td></td>
</tr>
<tr>
<td>ANG1921</td>
<td>3-EcoRI-SACOL1256 TrmD</td>
<td>CCGAATTCCTGACATCGCCATGTTTATGTTGTTGCTG</td>
<td></td>
</tr>
<tr>
<td>ANG2152</td>
<td>EcTrmDY86F-R</td>
<td>TGAAGGTATTCCGGCGACACTGCTGTTTACGCTGTTTATGTTGCTG</td>
<td></td>
</tr>
<tr>
<td>ANG2153</td>
<td>EcTrmDY86F-L</td>
<td>TGAAGGTATTCCGGCGACACTGCTGTTTACGCTGTTTATGTTGCTG</td>
<td></td>
</tr>
<tr>
<td>ANG2154</td>
<td>EcTrmDY86L-R</td>
<td>TGAAGGTATTCCGGCGACACTGCTGTTTACGCTGTTTATGTTGCTG</td>
<td></td>
</tr>
<tr>
<td>ANG2155</td>
<td>EcTrmDY86L-F</td>
<td>TGAAGGTATTCCGGCGACACTGCTGTTTACGCTGTTTATGTTGCTG</td>
<td></td>
</tr>
<tr>
<td>ANG2156</td>
<td>SaTmDMLMC-YLS-R</td>
<td>CGGAGATTTATACCGCCTTTTTCGAAAAATATTGCTGTAATAGC</td>
<td></td>
</tr>
<tr>
<td>ANG2157</td>
<td>SaTmDMLMC-YLS-F</td>
<td>CGCCTTTAGGACCAATACCAACCCACACACATCGATATCGTGAC</td>
<td></td>
</tr>
<tr>
<td>ANG2158</td>
<td>RV2906c Forward</td>
<td>GCCCATATGGCACACCACCACCACCACATGCGCATCGATATCGTGAC</td>
<td></td>
</tr>
<tr>
<td>ANG2159</td>
<td>RV2906c Reverse</td>
<td>GCCCATATGGCACACCACCACCACCACATGCGCATCGATATCGTGAC</td>
<td></td>
</tr>
<tr>
<td>ANG2003</td>
<td>KpnI-CyaAEc.H6-F</td>
<td>CGCCTTTAGGACCAATACCAACCCACACACATCGATATCGTGAC</td>
<td></td>
</tr>
<tr>
<td>ANG2004</td>
<td>XbaI-CyaAEc.H6-R</td>
<td>GCCCATATGGCACACCACCACCACCACATGCGCATCGATATCGTGAC</td>
<td></td>
</tr>
<tr>
<td>ANG2005</td>
<td>KpnI-CyaSA.Sa.H6-F</td>
<td>GCCCATATGGCACACCACCACCACCACATGCGCATCGATATCGTGAC</td>
<td></td>
</tr>
<tr>
<td>ANG2006</td>
<td>XbaI-CyaSA.Sa.H6-R</td>
<td>GCCCATATGGCACACCACCACCACCACATGCGCATCGATATCGTGAC</td>
<td></td>
</tr>
<tr>
<td>ANG1788</td>
<td>5-NheI-CyaA (2–446)</td>
<td>CTAGGCTAGCTGACATCGCCATGTTTATGTTGTTGCTG</td>
<td></td>
</tr>
<tr>
<td>ANG1789</td>
<td>3-EcoRI-CyaA (2–446)</td>
<td>CCGAATTCCTGACATCGCCATGTTTATGTTGTTGCTG</td>
<td></td>
</tr>
</tbody>
</table>

sisted of 50 mM Tris, pH 8.8, 20 mM MgCl₂, and 1 mM DTT, and buffer 3 was similar to buffer 2 but 20 mM MnCl₂ was replaced with 20 mM MgCl₂. Buffers 2 and 3 have been previously used to assess the activity of type IV ACs (8). 4 μM purified protein was used in a 10-μl reaction volume with 333.3 nM [α-32P]ATP added. The reactions were incubated at 37 °C for 1 h or overnight, heat-inactivated, and subsequently analyzed by TLC as described above.

In Vivo Adenylate Cyclase Activity Assay—The ability of SAUSA300_0905 (SACOL1008 homolog) to synthesize 3',5'-cAMP in E. coli was tested by introducing the plasmid pBAD33-SAUSA300_0905-His₆ into the E. coli cyaA mutant strain DHM1. Plasmids pBAD33-cyaA(2–446)-His₆ and pBAD33 were also introduced into strain DHM1, and the resulting strains were used as positive and negative controls, respectively. After transformation into DHM1, ~30 colonies from each transformation plate were inoculated into LB medium and incubated at 37 °C overnight. The next morning they were subcultured in fresh LB medium at 37 °C and grown to an A₆₀₀ of ~0.5. One ml of each culture was harvested and adjusted to A₆₀₀ of 5, and 5 μl were spotted on LB plates supplemented with 0.02% arabinose and 40 μg/ml X-Gal and the appropriate antibiotics. Plates were incubated overnight at 37 °C, and photos were taken with a Nikon camera.

Preparation of Cell Extract and Detection of Nucleotides by LC-MS/MS—S. aureus strains JE2 and JE2 Tn::SAUSA300_0905 (NE1299, ANG3894) were grown overnight in TSB medium as well as in B-medium supplemented with 25 mM concentrations of either glucose or sucrose (for stationary phase samples). The next day cultures were also back-diluted 1:50 into fresh medium and incubated for 3 h at 37 °C (for exponential phase samples). Bacterial cells corresponding to a 1-ml culture of A₆₀₀ of 10 were harvested by centrifugation, and cell extracts for LC-MS/MS analysis were prepared as described below. For cells grown under micro-aerobic conditions, overnight cultures were diluted 1:50 into 50-ml Falcon tubes filled up to the top with fresh TSB medium and incubated at 37 °C without agitation for 24 or 48 h (for exponential and post-exponential phase samples). To ensure that the bacteria did not aerobically respire under these growth conditions, resazurin was added to the cultures at a final concentration of 0.001% w/v as previously described (43). The reduction potential of resazurin at pH 7.0 and 25 °C is +380 mV, sitting between oxygen gas (+820 mV) and cytochromes (+290 to +80). This makes this compound suitable for detecting aerobic respiration activity or lack thereof, as the former would lead to the reduction of resazurin to resorufin and a blue to pink color change (45). Next, bacteria were collected by centrifugation, and the pellets were suspended immediately in 1 ml of nucleotide extraction buffer containing acetonitrile-methanol-water (2:2:1, v/v) and heated for 15 min at 95 °C to minimize the effect of oxygen on the cell physiology and metabolites. Six samples were prepared for each culture condition for strains JE2 and JE2 Tn::SAUSA300_0905. For three of these samples the extraction buffer was spiked with 92.8 ng/ml isotope-labeled 3',5'-cAMP. To generate the isotope-labeled 3',5'-cAMP, 5 mM 13C,15N-ATP was converted into 13C,15N-cAMP with 5 μM E. coli CyaA(2–446) in 40 mM Tris, pH 7.5, 100 mM NaCl, and 10 mM MgCl₂ buffer, and the sample was incubated at 37 °C overnight. The conversion rate was determined as 93.4% by LC-MS/MS analysis. S. aureus extracts were prepared, and 3',5'-cAMP and 2',3'-cAMP was detected by LC-MS/MS as described previously (24).

Kₐ Determination by ITC—A MicroCal iTC200 instrument (GE Healthcare) was used to determine the dissociation constants of 3',5'-cAMP or AdoMet and the E. coli or S. aureus wild-type TrmD proteins or TrmD variants. To minimize the dilution effect, the purified TrmD proteins were dialyzed for 24 h at 4 °C against 4 liters of binding buffer (40 mM Tris, pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 5% v/v glycerol). Subsequently, the samples were spun down at 17,000 x g at 4 °C for 10 min to remove any insoluble material, the supernatant was transferred to new tubes, and the protein concentrations were measured using a BCA assay kit (Pierce). An aliquot of the dialysis buffer was used to make 1 mM 3',5'-cAMP and AdoMet solutions and also used to set the TrmD proteins to a concentration of 100 μM. After initial trials, the MicroCal iTC200 was set to a reference power of 6 μcal/s, a stirring speed of 500 rpm, and a temperature of 25 °C, and 20 injections were made at 180-s intervals. At least two technical replicates were performed with each
TrmD protein. As the negative control, the ligands were titrated against the dialysis buffer, and the obtained values were subtracted from the experimental data. Curve-fitting, data analysis, and 

**Western Blot—**The expression of SAUSA300_0905-His6 and CyaAEC(2–446)-His6 from the pBAD33 vectors in DHM1 was confirmed by Western blot. Briefly, cultures of strain DHM1 containing the different pBAD33-derivived vectors were grown overnight in LB medium at 30 °C. The next day the overnight cultures were induced with 0.2% arabinose for 3 h. Bacteria from 1-ml culture aliquots before and after the induction were collected by centrifugation, and cells were suspended in 1× SDS sample buffer to get a final A600 nm of 40. Samples were heated for 10 min at 95 °C, and proteins separated on a 12% SDS-PAGE gel. Proteins were then transferred to a PVDF membrane, and His-tagged proteins were detected using a monoclonal anti-poly-His-peroxidase antibody (Sigma A-7058).

**Sequence and Structure Analysis—**Homologs of the S. aureus protein SAUSA300_0905, the Y. pestis CyaB protein, and the TrmD proteins from E. coli, S. aureus, and M. tuberculosis proteins were identified as follows. For SAUSA300_0905 and CyaB from Y. pestis, the respective protein sequences were used as query sequences in BLASTP searches in the NCBI non-redundant (nr) protein sequence database using default settings. For the TrmD proteins, the respective protein sequences were used as query sequence in a BLASTP search confined to their respective groups of bacteria, namely E. coli TrmD for γ-Proteobacteria, M. tuberculosis TrmD for Actinobacteria, and S. aureus TrmD for Firmicutes. Subsequently, the identified protein homologs with a maximum expect (e) values below 3e-04 and a minimum sequence coverage and sequence identity of 60 and 30%, respectively, were retrieved and used for further analysis in Jalview (28). A multisequence alignment was generated for each group of proteins by running 20 iterations, and a conserved logo-sequence was generated with Cluster Omega (46). To compare the sequences of the SAUSA300_0905 and CyaB homologs or the E. coli, S. aureus, and M. tuberculosis TrmD homologs, the respective groups of sequences were combined, and a multi-sequence alignment and/or a logo-sequence was generated as described above. A multiple sequence alignment of all TrmD proteins was also generated and subsequently used as the input sequence on the ConSurf server to visualize the AdoMet binding site in a structural context (31). To this end, chain A of the AdoMet-bound H. influenzae TrmD protein was used as the structural template (PDB code 1UAK). PyMOL (v1.7.4.4 Edu Enhanced for Mac OS X, Schrödinger, LLC.) was used to display the ConSurf data and also for the structural comparison of the AdoMet binding site of the S. aureus TrmD (PDB code 3KY7) and H. influenzae TrmD (PDB code 1UAK) proteins. A structural model of SAUSA300_0905 was generated in Phyre2 (47) and viewed in PyMOL.

**Phylogeny Analysis of TrmD and Adenylate Cyclases—**A local protein database containing the 555 complete bacterial and archaeal proteomes used by Galperin et al. (11) in his study on the distribution of bacterial signal transduction systems was built. This database was queried with the BLASTP program (default parameters; Ref. 48) using the full-length sequence of TrmD protein of S. aureus strain N315 as a seed (Ref_seq: NP_374356, Locus_tag: SA1083). The distinction between homologous and non-homologous sequences was assessed by visual inspection of the BLASTP output (no arbitrary cut-offs on E-value or score). To ensure the exhaustive sampling of homologs, iterative BLASTP queries were performed using homologs identified at each step as new seeds. The absence of a homolog in any complete proteome in the local database was systematically verified by TBLASTN queries against the nucleotide sequence of the corresponding genome. For each candidate protein, the retrieved homologs were added to the dataset. The retrieved sequences were aligned using MAFFT v7.045b (default parameters; Ref. 49). Regions where the homology between amino acid positions was doubtful were removed using the BMGE software (BLOSUM30 option; Ref. 50). Bayesian analyses were performed using MrBayes version 3.2.2 (51) with a mixed model of amino acid substitution including a gamma distribution (4 discrete categories) and an estimated proportion of invariant sites. MrBayes was run with 4 chains for 1 million generations, and trees were sampled every 100 generations. To construct the consensus tree, the first 200 trees were discarded as “burn in” (51). The Sequence-logos of TrmD the alignments were generated using Phylo-mLogo visualization tool to highlight the three motifs involved in the AdoMet binding (52).

**Author Contributions—**Y. Z. and A. G. designed the study, acquired funding, and wrote the manuscript. Y. Z. and L. E. B. acquired the experimental data, Y. Z. and R. A. performed the bioinformatics analyses, Y. Z., R. A., L. E. B., J. C., V. K., and A. G. analyzed the data.

**Acknowledgments—**We thank Geralad Larrouy-Maumus for providing M. tuberculosis H37Rv chromosomal DNA, Annette Garbe for the nucleotide analysis by mass spectrometry, and Mark Bennet for the preliminary analysis of 2’,3’-cAMP by mass spectrometry. We also thank Steven Harre for assistance with the ITC experiments.

**References**


Downloaded from http://www.jbc.org/ at Copenhagen University Library on April 4, 2018
Protein Adaption with the Emergence of 3',5'-cAMP


Evolutionary Adaptation of the Essential tRNA Methyltransferase TrmD to the Signaling Molecule 3′,5′-cAMP in Bacteria
Yong Zhang, Rym Agrebi, Lauren E. Bellows, Jean-François Collet, Volkhard Kaever and Angelika Gründling

doi: 10.1074/jbc.M116.758896 originally published online November 23, 2016

Access the most updated version of this article at doi: 10.1074/jbc.M116.758896

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2016/11/23/M116.758896.DC1

This article cites 55 references, 21 of which can be accessed free at http://www.jbc.org/content/292/1/313.full.html#ref-list-1