ABSTRACT All cells must adapt to changing conditions, and many use cyclic AMP (cAMP) as a second messenger to sense and respond to fluctuations in their environment. cAMP is made by adenylyl cyclases (ACs), and mycobacteria have an unusually large number of biochemically distinct ACs. cAMP is important for gene regulation in mycobacteria, and the ability to secrete cAMP into host macrophages during infection contributes to Mycobacterium tuberculosis pathogenesis. This article discusses the many roles of cAMP in mycobacteria and reviews what is known about the factors that contribute to production, destruction, and utilization of this important signal molecule. Special emphasis is placed on cAMP signaling in M. tuberculosis complex bacteria and its importance to M. tuberculosis during host infection.

CYCLIC AMP IS A UNIVERSAL SECOND MESSENGER USED BY PATHOGENS AND THEIR HOSTS

The ability to sense and respond to changing environments is essential for all organisms, and this process is mediated through signal transduction. The small molecules that relay signals from receptors to one or more effector proteins within the cell during signal transduction are called second messengers. Cyclic nucleotides, (p)ppGpp, Ca2+, inositol triphosphate, and diacylglycerol function as second messengers in different types of cells. Cyclic 3′,5′-AMP (cAMP) is one of the most widely used second messengers, and its presence in bacteria, archaea, fungi, eukaryotic parasites, and mammals provides numerous opportunities for cAMP-mediated modulation of host-pathogen interactions (1–5).

CAMP signaling in mammals controls biological processes ranging from metabolism to memory formation and innate immunity, although it was first discovered for its role in hormone signal transduction (1, 6, 7).

In bacteria, cAMP is best known for its role in mediating the “glucose response,” or catabolite repression in Escherichia coli (2, 8). However, cAMP is also a critical regulator of virulence for many bacterial and fungal pathogens (9). This chapter discusses the many roles of CAMP signaling in mycobacteria, including the regulation of gene expression and manipulation of host cell signaling during infection.

cAMP is generated from ATP by adenylyl cyclases (ACs) and hydrolytically degraded by phosphodiesterases (PDEs) (Fig. 1). ACs are distributed among six classes based on their primary amino acid sequences, with the well-studied bacterial AC from E. coli being a member of class I. The secreted AC toxins from Pseudomonas aeruginosa, Bacillus anthracis, and Bordetella pertussis (10–12) belong to class II, and classes IV to VI each contain a very small number of representatives from assorted bacteria (13–17). Class III comprises the largest and most diverse group of cyclases, including all known ACs from eukaryotes and many bacterial ACs (18).

Mycobacterium tuberculosis complex bacteria encode as many as 16 biochemically distinct class III ACs, which is highly unusual in the microbial world (Fig. 2).
This large number is especially striking when compared with the single AC of most bacteria and fungi, including *E. coli*, *Streptomyces griseus*, *Corynebacterium glutamicum*, *Candida albicans*, and *Cryptococcus neoformans* (20–23). In contrast to the abundance of ACs, only a single cAMP PDE, Rv0805, has been identified in mycobacteria, as discussed later in this article (24–27).

cAMP mediates its regulatory effects through its allosteric interactions with cAMP-binding proteins, which undergo conformational changes upon cAMP binding that alter their activation states. The best-studied outcome of cAMP signaling is regulation of gene expression. In the dominant bacterial paradigm, cAMP regulates transcription through cAMP-receptor protein (CRP) family transcription factors that are activated by direct binding of cAMP. Transcriptional regulation by cAMP in eukaryotes is less direct, because cAMP-mediated activation of eukaryotic transcription factors often occurs through a protein kinase A (PKA) complex intermediate. In this case, binding of cAMP to regulatory subunits in the PKA complex liberates catalytically active kinase subunits, which then activate downstream transcription factors by phosphorylation.

cAMP signaling in *M. tuberculosis* complex bacteria does not fit the classical catabolite repression paradigm that has been so well established in *E. coli*. This may not be surprising, because metabolic profiling has recently shown that *M. tuberculosis*'s ability to cocatabolize different carbon sources reduces its need for catabolite repression (28). In *E. coli*, the *lac* operon codes for proteins that allow lactose to be used as a secondary carbon source when glucose is not available. Glucose depletion leads to an increase in cAMP levels, allowing induction of the *lac* operon in the presence of lactose through binding of the cAMP-CRPEc complex to the *lac* operator. While cytoplasmic cAMP levels in *E. coli* drop 3- to 4-fold when ~0.2% glucose is substituted for glycerol as a carbon source (29, 30), cAMP levels in mycobacteria show little response to glucose. No significant change in the cytoplasmic cAMP levels of *Mycobacterium bovis* BCG occurred when cells were either provided 0.2% glucose (31) or starved for carbon (32). cAMP levels decrease in both fast- and slow-growing mycobacteria in response to very high levels of glucose (2%), but the biological significance of this result is not clear (33, 34). Rather, cAMP signaling in *M. tuberculosis* seems broadly important for metabolism, virulence,
and host interactions, a trend that is being increasingly recognized in other bacterial pathogens as well.

**NUCLEOTIDE CYCLASES IN M. TUBERCULOSIS**

Initial sequencing of the *M. tuberculosis* H37Rv genome predicted five ACs (35), and Bayesian analysis of sequenced genomes by McCue et al. expanded this number to 15 (19). Ten of these H37Rv ACs have been shown to be biochemically active: Rv0386, Rv1264, Rv1318, Rv1319, Rv1320, Rv3645, Rv1625c, Rv1647, Rv1900c, and Rv2212 (reviewed in reference 36). Sequencing of additional *Mycobacterium* genomes showed variable AC conservation across species and identified a pseudogene in *M. tuberculosis* H37Rv (Rv1120c), as well as an AC in *M. tuberculosis* CDC1551 (MT1360) that is not present in *M. tuberculosis* H37Rv (21, 37). MT1360 is thought to have arisen from expansion of the Rv1318c ortholog in CDC1551. Rv1120 is inactivated by a single base mutation that causes a frame shift in H37Rv, but its ortholog in *M. avium* MAP_2672 lacks this mutation and appears to be a functional gene. Table 1 lists the cyclases in *M. tuberculosis* H37Rv, as well as potential orthologs in *M. bovis* BCG, *M. avium*, and *Mycobacterium smegmatis*.

There is a great deal of diversity among *M. tuberculosis* cyclases with respect to their associated functional domains, and the group includes both soluble and membrane-associated proteins. Surprisingly, two *M. tuberculosis* ACs (Rv1625c and Rv2435) have mammalian-like catalytic sites (19). Rv1625c is particularly unusual in that its six transmembrane helices topologically resemble those of a mammalian-like integral membrane AC. Rv1625c activity has been demonstrated in both mammalian epithelial cells and *E. coli* (19, 38, 39), which is consistent with the sequence-based predictions. While four *M. tuberculosis* putative ACs (Rv0891c, Rv1359, Rv1647, and Rv2212) contain only a recognizable catalytic domain, the majority also have
<table>
<thead>
<tr>
<th>Location</th>
<th>AC orthologs in Mycobacterium species&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Functionality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble Rv0891c</td>
<td>MT0915 Mb0915c BCG0943c NID&lt;sup&gt;d&lt;/sup&gt; NID NID NID</td>
<td>Activity&lt;sup&gt;b&lt;/sup&gt; Gene expression&lt;sup&gt;c&lt;/sup&gt; Signal&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Soluble Rv1120c</td>
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<td>Pseudogene&lt;sup&gt;24&lt;/sup&gt; ND</td>
</tr>
<tr>
<td>Soluble Rv1264</td>
<td>MT1302 Mb1295 BCG1323 MMAR4173 MAP2507c NID ML1111 NID NID MSMG5018 MSMG0545</td>
<td>Yes&lt;sup&gt;40&lt;/sup&gt; Hypoxia&lt;sup&gt;46&lt;/sup&gt; ND</td>
</tr>
<tr>
<td>Soluble Rv1359</td>
<td>MT1403 Mb1394 BCG1421 NID ML1111 NID NID</td>
<td>Low pH&lt;sup&gt;41&lt;/sup&gt;</td>
</tr>
<tr>
<td>Soluble Rv1647</td>
<td>MT1685 Mb1674 BCG1686 MMAR2454 MAP1357 ML1399 NID MSMG3780</td>
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</tr>
<tr>
<td>Soluble Rv1900c</td>
<td>MT1951 Mb1935c BCG1939c MMAR0286 NID ML2016 NID</td>
<td>High pH&lt;sup&gt;27&lt;/sup&gt;</td>
</tr>
<tr>
<td>Soluble Rv2212</td>
<td>MT2268 Mb2235 BCG2228 MMAR3257 NID MMAR4173 MMAR4279</td>
<td>Yes&lt;sup&gt;45&lt;/sup&gt; ND</td>
</tr>
<tr>
<td>Membrane-assoc. Rv1318c</td>
<td>MT1359 Mb1352c BCG1379c MMAR4078 MAP2440 ML1154 NID MSMG4924</td>
<td>Yes&lt;sup&gt;42&lt;/sup&gt; ND</td>
</tr>
<tr>
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</tr>
<tr>
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<td>MT1361 Mb1354c BCG1381c MMAR4079 NID NID NID</td>
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</tr>
<tr>
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<td>ND</td>
</tr>
<tr>
<td>Membrane-assoc. Rv2435c</td>
<td>MT2509 Mb2461c BCG2454c MMAR3757 MAP2250c NID ND ND</td>
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<td>MT3748 Mb3669 BCG3703 MMAR5137 MAP0426c NID ND ND</td>
<td>ND</td>
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<td>ND</td>
</tr>
<tr>
<td>Multidomain Rv1358</td>
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<td>Yes&lt;sup&gt;44&lt;/sup&gt; ND</td>
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<tr>
<td>Multidomain Rv2488c</td>
<td>MT2563 Mb2515c BCG2507c NID NID NID</td>
<td>ND</td>
</tr>
</tbody>
</table>


<sup>b</sup>Experimental evidence for AC activity.

<sup>c</sup>Conditions that affect expression of AC gene.

<sup>d</sup>Environmental conditions that affect AC functional activity.

<sup>e</sup>NID, not in database.

<sup>f</sup>ND, not determined.
other functional motifs. Most of these associated domains are expected to add new effector function capabilities and/or regulate cyclase activity in response to environmental signals. For example, Rv2435c has a chemotaxis receptor-like extracellular domain (19), while Rv1264 contains an N-terminal auto-regulatory domain that inhibits cyclase activity above pH 6.0 (40, 41).

Five other multidomain ACs (Rv1318c, Rv1319c, Rv1320c, Rv2435c, and Rv3645c) are membrane-associated ACs that contain HAMP (histidine kinases, adenyl cyclases, methyl binding proteins, and phosphatases) domains (42). HAMP domains are amphoteric alpha-helices that are often associated with two-component signal transduction pathways. These HAMP domains are thought to bridge the sensing of extracellular signals with the responding intracellular signaling domains (43). Three proteins (Rv0386, Rv1338, and Rv2488c) contain both ATPase and helix-turn-helix domains. While the role of its accessory domains is not clear, Rv0386 was identified as the M. tuberculosis AC responsible for elevating cAMP levels in infected macrophages, leading to an increase in tumor necrosis factor-α (TNF-α) production via the PKA and cAMP (44).

Rv1900c contains an αβ-hydrolase domain commonly found in hydrolytic enzymes. Crystallographic studies showed that Rv1900c forms asymmetric homodimers that form a closed conformation upon binding substrate ATP (45). Rv1900c also showed some guanyl cyclase activity (45).

**REGULATION OF cAMP PRODUCTION**

Expression of at least seven M. tuberculosis AC genes is likely modulated at the transcriptional level by environmental factors such as starvation and hypoxia (46, 47). However, cAMP production is often controlled largely at the posttranslational level through activation of the cyclases themselves (8, 18). Activity of M. tuberculosis ACs has been shown to respond to host-associated signals such as pH, fatty acids, ATP, and CO₂ levels (37, 41, 48–50) (Table 1), and it is likely that more signals will be identified. The activity of Rv1264 is directly responsive to pH, and the activity of Rv2212 is regulated in vitro by fatty acids, pH, and ATP concentration. Given the sheer number of ACs in M. tuberculosis H37Rv, it is not surprising that they respond to a wide variety of signals. Regulation by multiple signals provides an opportunity for ACs to coordinate M. tuberculosis’s response to complex microenvironments within the host. Identifying the signals that affect cAMP signaling in M. tuberculosis during infection promises to be a rich area of future investigation.

**DIVERSE ROLES FOR cAMP WITHIN MYCOBACTERIA**

Three of ten predicted cyclic nucleotide monophosphate (cNMP) binding proteins have been functionally characterized to date (Fig. 2), and each of these proteins is discussed in greater detail later in this article. Rv0998 (and its ortholog in M. smegmatis [MSMEG_5458]) contains a domain with similarity to the GNAT (GCN5-related N-acetyltransferases) family and functions as a cAMP-responsive protein lysine acetylase in mycobacteria (51). Rv3676 (referred to as CRPM₄, for cAMP responsive protein of M. tuberculosis) and Rv1675c (named Cmr, for cAMP and macrophage regulator) contain helix-turn-helix DNA binding domains and belong to the CRP-FNR family of transcription factors (19). Both CRPM₄ and Cmr function as cAMP-responsive transcription factors, although only CRPM₄ has been shown to directly bind cAMP (52–55).

Seven predicted cNMP binding proteins (Rv0073, Rv0104, Rv2434c, Rv2564, Rv2565, Rv3239c, and Rv3728) remain uncharacterized, although expression of Rv2565 is induced during human infection (56). Rv2565 contains a conserved esterase domain along with its putative cNMP-binding motif, but neither domain has been studied at the functional level. The remaining six predicted effector proteins also contain accessory domains with predicted transport and/or esterase activities (19, 36) that have not previously been associated with cAMP-mediated signal transduction in bacteria. Characterization of these proteins in mycobacteria is therefore likely to establish new roles for cAMP and/or cGMP signaling.

Coordinate regulation of some pathways by multiple cNMP binding proteins also seems likely. Recent studies from Stapleton et al. (57) showed that WhiB1 is a transcriptional repressor of groEL2 and that expression of WhiB1 is regulated by CRPM₄ in response to cAMP levels. Previous studies by Gazdik et al. (58) demonstrated Cmr binding to the upstream region of groEL2, as well as regulation within macrophages at 2 h postinfection. Stapleton et al. confirmed Cmr’s binding and regulation of the groEL2 promoter in M. tuberculosis (57). Thus, expression of groEL2 is regulated directly by Cmr, while also being indirectly modulated by CRPM₄ through WhiB1. Similarly, Pelly et al. suggested dual cAMP-mediated regulation by Cmr and CRPM₄ of the small noncoding RNA ncr11264c and/or Rv1265 (59), which was previously shown to be directly regulated by Cmr (58). A third
A possible example of multiple cNMP effector proteins affecting a regulatory pathway is illustrated in Fig. 3. The upstream region of the acetyl CoA-synthase gene Rv3667 (acs) contains putative CRP_Mt binding motifs (G. S. Knapp et al., unpublished), raising the possibility that expression of Rv3667 is also regulated by CRP_Mt. Such coordinate regulation by cAMP-dependent factors provides another exciting new area of investigation.

**FIGURE 3** cAMP-dependent regulation of acetylation in *E. coli* (A) and *Mycobacterium* (B). In *E. coli*, cAMP’s role in regulation is at the transcriptional level, whereas in *M. tuberculosis*, cAMP binds to the acetyltransferase, PatA, directly. cAMP-CRP complexes regulate acs and patZ at the transcriptional level in *E. coli*, while the role of CRP_Mt in regulation of acs and patA is unknown at this time. CobB is a NAD^+^-dependent sirtuin, as is Rv1151c.


**EMERGING ROLES FOR cAMP SIGNALING IN MYCOBACTERIAL GENE REGULATION AND METABOLISM**

The role of cAMP in modulating gene expression in mycobacteria was first demonstrated by assessing changes in the *M. bovis* BCG proteome following the addition of dibutyryl cAMP [60]. Dibutyryl cAMP crosses the bacterial cell membrane more readily than...
cAMP and increases cellular cAMP levels when it is converted to cAMP and butyrate in the bacterial cytoplasm. cAMP’s role in the resulting protein expression changes was confirmed by treating control samples with dibutyrate alone. Subsequent studies showed that similar results could be obtained by increasing cAMP levels endogenously through expression of the catalytic domain of the Rv1264 AC, because Rv1264 is constitutively active in the absence of its repressor domain (58). Proteomic analyses identified five of these cAMP-regulated proteins: GroEL2, Rv2971, PE_PGRS6a, Mdh, and Rv1265 (60). Cmr was later shown to regulate expression of the genes encoding all five proteins, although in some cases the regulation is indirect (58).

There is a growing body of literature demonstrating the role of cAMP in regulating the metabolism of mycobacteria, at the transcriptional through posttranslational levels. Both CRPMt and Cmr contribute to M. tuberculosis–host interactions through their effects on M. tuberculosis gene regulation, while Rv0998 posttranslationally regulates M. tuberculosis metabolism through its cAMP-dependent acetylase activity. Deletion of the CRPMt encoding gene crp attenuates M. tuberculosis virulence in a murine model and reduces bacterial growth rates in vitro and within macrophages (61). While Cmr regulates M. tuberculosis gene expression within macrophages, cmr-deleted M. tuberculosis is not defective for growth in vitro or within resting macrophages (58). Cmr may therefore have a more specialized role than CRPMt in M. tuberculosis biology. However, Cmr’s regulation of mdh, as noted above, suggests that Cmr also plays a role in carbon metabolism. Likewise, Rv0889’s regulation of acetyl coenzyme A (CoA) synthase activity is consistent with a role for this cAMP binding protein in central metabolism. While each of these effector proteins is discussed in more detail below, cAMP’s emerging role in the regulation of small noncoding RNAs deserves mention.

There is increasing recognition of the gene regulatory roles played by small noncoding RNAs (sRNAs) in bacteria subjected to stressful conditions (reviewed by Gottesman and Storz [62]). Recent studies by several groups have identified numerous sRNAs in mycobacteria, including M. smegmatis, M. bovis BCG, and M. tuberculosis, but very few have been biologically characterized (63–65). Expression of one of these sRNAs, ncRv11264c, is regulated by cAMP (59). ncRv11264c was initially identified as Mcr11, MTS0997, and ncrMT1302 by different groups but is referred to here using a consensus nomenclature proposed by these investigators (66). ncRv11264c is located in the intergenic region between the pH-sensitive AC Rv1264 and Rv1265, discussed above as a cAMP- and Cmr-regulated gene of unknown function (40, 41, 58, 63). Regulation of ncRv11264c was shown to be both cAMP and growth phase dependent (59). Dysregulation of ncRv11264c expression in an M. tuberculosis ΔRv1264 mutant establishes a regulatory link between ncRv11264c and Rv1264 gene expression, but the underlying mechanism has not been established. Similarly, the role of ncRv11264c in M. tuberculosis awaits discovery.

**Cyclic AMP Signaling in Mycobacteria**

**Cyclic AMP-Associated Transcription Factors: Cmr and CRPMt**

Relatively little is known about Cmr compared to CRPMt, particularly with respect to its biochemical properties. In contrast to CRPMt, Cmr has not been shown to directly bind cAMP in vitro but directly regulates three of the five Cmr-regulated genes described above (mdh, groEL2, and Rv1265) in response to cAMP levels (58). Expression of all three genes is also regulated by Cmr in M. tuberculosis and M. bovis BCG upon macrophage uptake (58) and in response to low pH in M. bovis BCG, but not in M. tuberculosis. It is possible that Cmr’s sensitivity to cAMP levels only occurs in vivo or that a second factor mediates Cmr’s CAMP-associated regulatory behavior. Alternatively, coregulation by a second transcription factor, as in the case of groEL2’s dual regulation by Cmr and WhiB1 mentioned above, could contribute to Cmr’s apparent CAMP responsiveness.

CRPMt is by far the better studied of the two cAMP-responsive transcription factors in M. tuberculosis, although much remains to be learned. CRPMt is required for M. tuberculosis pathogenesis, and crp mutants grow more slowly than wild type in vitro (61). The CRPMt ortholog in M. bovis BCG (CRPBCG) differs from CRPMt at two amino acid positions (L47P and E178K) (67) but is fully functional in DNA binding and virulence assays (52, 68). CRPMt’s putative regulon includes over 100 genes (discussed below), and it is not known which of these genes contributes to pathogenesis. However, recent studies indicate that serine deficiency contributes to the in vitro growth defect in the crp mutant.

CRPMt directly upregulates expression of serC, which encodes a phosphoserine aminotransferase required for serine production (69, 70). In E. coli, serC is required for bacterial growth in the absence of serine supplementation. Exogenous addition of serine, glycine, or cysteine (which can be converted to serine) restores wild type growth of M. bovis BCG and M. tuberculosis crp deletion mutants in vitro. Constitutive expression of serC in
M. bovis BCG and M. tuberculosis crp mutants also restored growth in vitro, but not within macrophages. Therefore, serC deficiency is responsible for the slow growth phenotype of Δcrp mutants, but CRP-regulated genes other than serC are also required for M. tuberculosis growth within macrophages and, likely, mammalian hosts.

There are many other genes in the CRPMt putative regulon that may contribute to M. tuberculosis virulence and/or persistence. For example, expression of rpfA, which encodes a potent resuscitation-promoting factor (Rpf), is directly activated by CRPMt (61). Rpf is a growth factor that stimulates the growth of aged M. tuberculosis cultures and may contribute to reactivation of dormant M. tuberculosis (71). Regulation of rpfA by CRPMt is consistent with CRPMt having a role in persistence and/or reactivation of tuberculosis CRPMt, and it will be exciting to learn if this is the case. However, CRPMt has also been shown or predicted to regulate numerous other biologically important genes, including those encoding proteins involved in respiration, nitrogen assimilation, fatty acid and carbohydrate metabolism, and members of the highly repetitive glycine rich PE_PGRS family of surface proteins (53, 61, 72, 73). Biological studies focused on the regulation and function of these genes should reveal a great deal about the roles of CRPMt in mycobacterial biology.

CRPMt can also function as both a repressor and an activator, and some promoters contain multiple CRPMt binding sites of differing affinities. For example, expression of M. tuberculosis whiB1, a member of the Wbl (WhiB-like) family, is both positively and negatively regulated by CRPMt (55, 61, 74), depending on cAMP levels and which of the multiple binding sites are bound. As mentioned above, WhiB1 has recently been shown to coregulate groEL2 with Cmr, resulting in its indirect regulation by CRPMt, so it is clear that we can expect a great deal of regulatory complexity from CRPMt.

DEFINING THE CRPMt REGULON AND BINDING MOTIF

A putative regulon for CRPMt includes over 100 genes and was initially identified by microarray analysis and by using a combination of affinity capture and Bayesian-based computational approaches (53, 61). Hypoxia-regulated (14%) and starvation-regulated (30%) genes comprise the two largest groups of genes in this predicted CRPMt regulon, which is consistent with CRPMt’s proposed role in M. tuberculosis persistence (46, 47, 53). However, the percentages of hypoxia- and starvation-regulated genes in the putative CRPMt regulon are similar to what has been reported from microarray studies overall, making it difficult to assess the particular significance of these regulatory signals. Nonetheless, CRP’s regulatory network in E. coli also includes many genes that respond to starvation and anaerobiosis (2), making this an important avenue of future investigation.

A combination of E. coli CRP binding sites and M. tuberculosis DNA sequences recovered by affinity capture using CRPMt were initially used to predict CRPMt’s palindromic binding motif (CTGTGANNNNNNTCACG/A), which was based on 58 predicted binding sites from the M. tuberculosis genome (53). Six of seven intergenic putative CRPMt binding sites tested bound CRPMt and CRPBCG both in vitro and in vivo (52). In addition, mutation of nucleotides G2 or C15, which are conserved in all predicted binding sites, abolished binding with CRPMt. Recent in silico studies have predicted many new CRPMt targets, and as many as 145 promoters have been proposed to be directly regulated by CRPMt binding (72, 73, 75). Experimental validation of these sequence predictions is needed to accurately establish the CRPMt regulon. However, the addition of indirect regulatory effects and downstream genes in regulated operons could greatly expand the number of genes whose expression is affected by CRPMt.

STRUCTURE AND FUNCTION OF CRPMt

CRPMt and E. coli CRP have similarities, including a strong resemblance in their DNA binding sites, that suggest functional parallels despite their low levels of identity at the sequence level (52, 53, 61, 67, 68, 74). Both bend their target DNA upon binding and increase their affinity for DNA when bound with cAMP (2, 53). Several structural studies have investigated the mechanism by which cAMP binding affects CRPMt’s ability to bind DNA. While it is likely that a cAMP-induced repositioning of CRPMt’s helix-turn-helix domain affects its DNA binding properties, questions remain about the extent of this helix reorientation, the amount of asymmetry between subunits, and the effects of this asymmetry (54, 76, 77).

Functional differences between CRPMt and E. coli CRP may also complicate cross-species structural analyses. For example, CRPMt cannot substitute for CRP in E. coli, possibly because it fails to interact properly with E. coli’s RNA polymerase (53, 67). Moreover, CRPMt binds DNA strongly and specifically even in the absence of cAMP, shows only a modest increase in DNA binding
affinity (2- to 10-fold) when bound with cAMP, and has relatively low affinity for cAMP compared with *E. coli* CRP (53, 55, 61). CRP<sub>Mt</sub> also appears to bind cAMP in a closed structure, while cAMP binding converts *E. coli* CRP to an open structure (53, 54, 61). Some of these features of CRP<sub>Mt</sub> are similar to those of the mutant cAMP-independent *E. coli* CRP<sup>*</sup> protein, although the structural bases of these differences are not clear (53, 78). Structural studies that directly compare apo and holo forms of CRP<sub>Mt</sub> with those of *E. coli* CRP would therefore be quite valuable in understanding the complexity and range of cAMP signaling mediated through this highly conserved transcription factor.

THE PROTEIN LYSINE ACETYLASE Rv0998

The recent finding that Rv0998 and its ortholog in *M. smegmatis*, MSMEG_5458, are cAMP-dependent lysine acetylases is an exciting new development in the cAMP signaling field (51). Protein lysine acetylation, a posttranslational modification, is evolutionarily conserved among eukaryotes and prokaryotes. In *E. coli* and *Salmonella*, acetylation of proteins is altered in response to carbon source availability, leading to differences in carbon and energy source usage and changing the metabolism of fatty acids and nucleotides (79–82). Additionally, acetylation plays a role in complex signal transduction systems that regulate cell division and flagellum synthesis (83).

Rv0998 and MSMEG_5458 share 56% identity at the amino acid level, and each contain a cyclic nucleotide binding domain fused to a GNAT family motif. Nambi et al. (51) identified a member of the universal stress protein (USP) family MSMEG_4207 as a target for acetylation in *M. smegmatis*. Purified MSMEG_5458 or Rv0998 acetylated the epsilon amino group of a lysine in this USP, and acetylation levels were higher in the presence of cAMP than in cGMP. They also demonstrated cAMP binding by both acetylases and showed that a point mutation, R95K, in the cNMP binding domain of MSMEG_5458 abolished cAMP binding (51).

The *M. smegmatis* USP MSMEG_4207 is not conserved in *M. tuberculosis*, but a later study identified acetyl-CoA synthetase (ACS) as another biologically relevant target of both acetylases that is also present in *M. tuberculosis* (84). ACS converts acetate into the high-energy compound acetyl-CoA, which is central to many aspects of carbon metabolism. ACS is inactivated by acetylation at amino acid K167, and a reversible protein acetylation system was established when Rv1151c was shown to deacetylate ACS. Crystallographic studies have also demonstrated acetyl-CoA binding to Rv0998 (85).

ACS is an interesting target for acetylation in the broad scope of metabolism. In *E. coli*, ACS fixes acetate into acetyl-CoA when environmental concentrations of acetate are low (Fig. 3) (86). In this case, PatZ is the acetyl transferase, while CobB is the deacetylase that converts ACS back into its active form (87). The overall activity of ACS in *E. coli* is also modulated by cAMP, because both PatZ and ACS are encoded by CRP-regulated genes, *patZ* and *acs*, respectively (Fig. 3) (88). This contrasts with cAMP’s direct role in regulating the enzymatic activity of Rv0998 and MSMEG_5458 at the posttranslational level in mycobacteria. The role of CRP<sub>Mt</sub> in the regulation of Rv0998 or *acs* has not been investigated, but we have identified potential CRP<sub>Mt</sub> binding sites upstream of *acs* in *M. tuberculosis* (G. S. Knapp, unpublished).

Allosteric regulation of Rv0998 by cAMP involves conformational rearrangements that affect communication between the cNMP and GNAT domains, and Rv0998 activity is auto-inhibited in two ways in the absence of cAMP (83). In one case the C-terminal end of the peptide can insert as a helix within the cAMP-binding site, blocking cAMP binding. In the other, a large loop structure is formed that serves as a lid to block the catalytic active site. The major structural changes that occur in the presence of cAMP release the lid to expose the catalytic site while stabilizing the active form of the protein (85).

MORE MAKING THAN BREAKING OF cAMP IN MYCOBACTERIA?

Control of cytoplasmic cAMP levels can occur at the levels of production, degradation, or secretion; however, degradation is the primary way of balancing production in most organisms. Mycobacteria are unusual in this regard, because their cAMP secretion levels are high and reported degradation levels are weak. Despite the extraordinary number of ACs capable of generating cAMP in mycobacteria, Rv0805, a class III PDE that is structurally related to the metallophosphoesterase family (26, 36, 89), is the only cAMP PDE identified to date and is present only in slow-growing pathogenic mycobacteria. Rv0805 has significantly more activity (~150 times) against 2′,3′-cAMP than cAMP (26), and its over-expression increases the sensitivity of *M. smegmatis* to hydrophobic cytotoxic compounds, independent of its catalytic function (27). Thus, Rv0805’s poorly under-
stood role within mycobacteria represents an emerging story from two perspectives.

cAMP levels are reduced by ∼30% in M. smegmatis (24), and ∼50% in M. tuberculosis (44), compared with corresponding vector controls when Rv0805 is over-expressed. Seven amino acids contribute to Rv0805 demerization and are required for coordination of active site metals (25). Asp21, His23, Asp63, and His209 mediate Fe3+ binding, while Asn97, His169, Asp63, and His207 coordinate Mn2+ binding (25), and a version of Rv0805 with an N97A mutation lacks cAMP PDE activity (24, 27). Rv0805’s 2′,3′-cAMP PDE activity is greatly reduced by amino acid substitutions H98A or H98D (26), suggesting overlap of the functional sites for Rv0805’s 2′,3′-cAMP and 3′,5′-cAMP PDE activities. While Rv0805 has not been successfully crystallized with cAMP, a structural model of Rv0805’s active site accommodates cAMP docking (25, 27). However, Rv0805’s ability to hydrolyze cAMP (V_{max} = 42 nmol cAMP hydrolyzed/min/mg protein; K_m = 153 μM) (24) is only a fraction of E. coli CpdA’s activity for cAMP (V_{max} = 2.0 μmole/min/mg; K_m = 0.5 mM) (90), making it difficult to understand how mycobacteria maintain cAMP homeostasis.

The biological significance of Rv0805’s activity against 2′,3′-cNMPs is also unclear. It is possible that these nucleotides are used for signaling or nucleotide acquisition. However, RNA cleavage by endonucleases associated with some CRISPR and TA toxin mRNA interferase systems generates 2′,3′-cyclic ends (91–93), and it is also possible that such RNA molecules serve as natural substrates for Rv0805 in M. tuberculosis.

cAMP PRODUCTION, SECRETION, AND HOST CELL INTERACTIONS

The large ratio of active ACs to PDEs may present an opportunity for accumulation of high levels of CAMP relative to other organisms, and this remains an area of current investigation (36, 55, 61, 94, 95). However, cAMP levels can vary greatly with conditions, and the use of different normalization methods (e.g., wet versus dry weight [34, 95]) further complicates inter-study comparisons. cAMP levels reported for mycobacteria (4.5 pmol/mg wet weight and 18.7 to 1,000 pmol/mg dry weight) are surprisingly comparable to those of E. coli and Salmonella typhimurium (5.0 pmol/mg wet weight and 130 to 450 pmol/mg dry weight). However, mycobacterial cytoplasmic cAMP levels from recent studies normalized to the number of bacteria rather than weight (0.5 to 400 pmol/10^8 bacteria) show that a tremendous range of cAMP accumulation is possible depending on the environmental conditions (31, 32, 44, 75).

Conditions that affect cAMP levels in mycobacteria include those associated with the host environment. For example, macrophage passage increases cAMP levels in TB-complex mycobacteria ∼50-fold relative to bacteria in tissue culture medium alone (31). Low pH also increases cAMP levels in both M. tuberculosis and M. bovis BCG (58), while albumin is inhibitory for M. bovis BCG’s cAMP production (31). Albumin’s inhibitory effects can be overcome by the presence of oleic acid, but the cAMP produced in the presence of oleic acid is secreted rather than retained within the cells (31).

Mycobacteria appear to secrete far more cAMP than other bacteria (8, 25), and this secretion contributes to M. tuberculosis pathogenesis (44). However, the factors that regulate cAMP export and the outcomes of this secretion have not been fully defined (31, 75). Modulation of host cell cAMP levels is a common strategy of many bacterial pathogens, but M. tuberculosis is the first shown to secrete premade cAMP (2, 44). B. anthracis, B. pertussis, and P. aeruginosa export host-dependent ACs that serve as toxins (12, 96). An exported AC has also been reported for Yersinia pestis, although it is yet to be characterized (14). An alternative strategy is used by Vibrio cholerae, E. coli, and B. pertussis, which inactivate their host cells by secreting enzymes that ADP-ribosylate the alpha subunits of heterotrimeric G proteins that regulate the activity of host ACs, leading to increased cAMP production in their host cells (7, 97, 98).

Live, but not heat-killed, Mycobacterium microti, M. bovis BCG, and M. tuberculosis directly export cAMP to increase the cAMP levels of their host macrophages (31, 44, 99, 100). The M. tuberculosis AC Rv0386 is responsible for this cAMP intoxication, which results in TNF-α production via the PKA and cAMP response-element-binding (CREB) protein pathway (44). Mutation of Rv0386 decreases survival and immunopathology in a mouse infection model, demonstrating the importance of cAMP intoxication for M. tuberculosis pathogenesis. Other reports indicate that CREB-mediated TNF-α production is also increased in macrophages infected with M. smegmatis, but not with M. avium (101, 102). These studies suggest that there are multiple ways in which mycobacteria can stimulate the CREB pathway in macrophages, because there is no Rv0386 ortholog in M. smegmatis. M. tuberculosis may also use CAMP to modulate phagosome trafficking, because there is some evidence that elevated
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cAMP levels in infected macrophages can inhibit phagosome-lysosome fusion (100, 103). CAMP secretion and signaling is clearly a promising new frontier at the interface of mycobacteria-host interactions.

CONCLUSIONS AND FUTURE DIRECTIONS

CAMP signaling is a complex and exciting area of investigation in mycobacteria, and the pace of research has increased greatly in recent years. Nonetheless, many critical questions remain. It will be especially important to identify the factors that regulate levels of CAMP within mycobacteria and the environmental signals to which they respond, as well as the biological roles of Cmr and CRP_Mt genes. Similarly, the impact of CAMP-regulated lysine acetylation on mycobacterial metabolism has yet to be explored, and the functions of most putative cNMP-binding effector proteins await discovery. The unexpected biological roles of Rv0805 and its lack of robust CAMP phosphodiesterase activity are particularly intriguing findings that warrant investigation. Elucidating the mechanism by which CAMP is secreted from mycobacterial cells, and the way in which this CAMP affects the host response to M. tuberculosis infection will be critical to our understanding of M. tuberculosis pathogenesis, as is the question of CAMP signaling specificity, given the large number of ACs in mycobacteria. There is clearly much to be done to unravel the multifactorial roles of CAMP signaling in mycobacteria at the molecular, genetic, and biological levels, and rewards of pursuing this journey hold great promise.

REFERENCES

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