Published in final edited form as:
Nat Rev Microbiol. ; 10(1): 27–38. doi:10.1038/nrmicro2688.

The myriad roles of cyclic AMP in microbial pathogens, from signal to sword

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Abstract

All organisms must sense and respond to their external environments, and this signal transduction is often done with second messengers such as cyclic nucleotides. Adenosine 3'5'-cyclic AMP is a universal second messenger that is used by diverse forms of life, including mammals, fungi, protozoa and bacteria. In this review, we discuss the many roles of cAMP in bacterial, fungal and protozoan pathogens and its contributions to microbial pathogenesis. These include coordination of intracellular processes such as virulence gene expression with extracellular signals from the host environment, and manipulation of host immunity by increasing cAMP levels in host cells during infection.

All organisms must respond to changing environments, and the signal transduction pathways that allow them to do so on a cellular level are essential for the control of processes ranging from chemotaxis to differentiation and apoptosis[1–4]. This signaling is mediated by a vast array of small soluble signaling molecules, both within and outside of cells[5,6]. Some of these molecules, such as the hormone-like acyl homoserine lactone (AHL) autoinducers that control quorum sensing in gram negative bacteria, diffuse across cell membranes from the extracellular millieu to directly control gene expression[5]. However, many environmental signaling pathways rely on 'second messenger' molecules to relay external signals from membrane receptors to one or more effectors within the cell. Second messengers include such diverse molecules as cyclic nucleotides, (p)ppGpp, Ca^{2+}, inositoltriphosphate and diacylglycerol[7,8–9–12].

The cyclic nucleotide adenosine 3'-5' cyclic monophosphate (cAMP) was the first second messenger to be described, and it is also the most broadly used by organisms. cAMP is generated from ATP by adenylyl cyclases (ACs), while phosphodiesterases (PDEs) catalyze its hydrolytic degradation (Figure 1). ACs are divided into six classes based on primary amino acid sequences. Class III is the largest and most diverse group of cyclases, and it includes all known ACs from eukaryotes, as well as many bacterial ACs[7,13,14,15]. However, the well-studied bacterial AC from *Escherichia coli* belongs to Class I. Downstream regulatory effects of cAMP are mediated through its allosteric interactions with cAMP-binding proteins, whose activation states are altered by conformational changes that are induced upon cAMP binding.

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Regulation of gene expression is a major outcome of cAMP signaling, but the mechanisms differ between bacteria and eukaryotic cells[16,17]. Transcription factors of the cAMP-receptor protein (CRP) family in bacteria are activated by direct binding of cAMP, while cAMP-mediated activation of the transcription factors in eukaryotic cells often requires protein kinase A (PKA) complex as an intermediate. In this case, cAMP binding to regulatory subunits in the PKA complex liberates catalytically active kinase subunits, which activate downstream transcription factors by phosphorylation.

cAMP was first discovered for its role in hormone signal transduction in eukaryotic cells[18]. Biological processes now known to be controlled by cAMP signaling in eukaryotes range from metabolism to memory formation and innate immunity[16,19]. Later, cAMP was also discovered in bacteria, and its role in mediating the 'glucose response', or catabolite repression, was extensively studied in *E. coli* over several decades[17,20]. During catabolite repression, the presence of glucose reduces cAMP production, which is needed for activation of the *lac* operon (which codes for proteins that allow lactose to be used as a secondary carbon source) through binding of the cAMP-Crp complex[21].

However, increasing recognition of the roles of cAMP in microbial virulence, ranging from potent toxin to master regulator of virulence gene expression, has generated new interest in this second messenger. The near universal use of cAMP signaling in life forms as diverse as bacteria, archaea, fungi, eukaryotic parasites and mammals provides unique opportunities for cAMP-mediated modulation of host-pathogen interactions. However, many of these interactions are only just being discovered. The essential roles of cAMP in eukaryotic signal transduction and bacterial carbon catabolite repression have been covered previously[14–17,21]. In this review, we focus on the multiple roles of cAMP in pathogen biology, with an emphasis on the importance of cAMP to virulence gene regulation, host-pathogen interactions and pathogen responses to their host environments.

Role of cAMP signaling in pathogenic bacteria

Host-dependent AC toxins, secreted by several pathogens into host cells during infection, provided the first examples of the role of cAMP in bacterial virulence[14,22]. More recently, it has become evident that cAMP is involved in processes other than catabolite repression and AC toxin action, as disruption of intracellular cAMP signaling attenuates virulence in numerous bacterial pathogens[23–27]. cAMP has central roles in regulating biofilm formation, type III secretion, carbon metabolism and virulence gene regulation in many pathogens. Several new strategies by which pathogens modulate cAMP levels within their host cells have also recently been discovered[28,29].

Glucose depletion induces AC activity in some, but not all, bacteria, and the use of CRP-family transcription factors as a primary means for relaying the message is common. Much of the cAMP signaling diversity within bacteria occurs at the level of the cAMP-associated regulatory targets, which vary between organisms. The effects of this cAMP signaling are amplified in some cases by cAMP-mediated co-regulation of other global regulators. For example, expression of the stress-associated global regulator RpoS is controlled by cAMP-Crp in *Vibrio vulnificus*, *E. coli* and *Salmonella enterica*[30–32]. In the case of *V. vulnificus*, transcriptional repression of *rpoS* occurs by direct binding of cAMP-Crp complex to each of two binding sites in the *rpoS* promoter[30]. Similarly, cAMP levels control biofilm production in *Vibrio cholerae*, partly through regulation of a diguanylate cyclase responsible for production of the second messenger cyclic di-GMP[33].

cAMP-CRP also appears to be adept at integrating horizontally acquired plasmid and island-based genes into existing regulatory networks, possibly due to its broad use across many unrelated species. For example, in *Yersinia pestis*, Crp directly activates expression of *pla*, a

plasmid-encoded virulence gene that facilitates infection following either flea bite or aerosol transmission[34]. Plasticity is evident in this adaptive process, as cAMP-Crp positively regulates protein secretion from each of three type III secretion systems (T3SSs) in *Yersinia. enterocolitica*[24], but directly down-regulates transcription of an orthologous T3SS, Ysc, in *Y. pestis*[35]. Similarly, the horizontally-acquired *tcpA-F* genes, which encode proteins needed for biogenesis of a virulence-associated toxin-coregulated pilus (TCP), are regulated by Crp in *V. cholerae*[36].

The following three cases provide examples of both the extent and significant diversity of cAMP-associated gene regulation in bacterial pathogens.

Pseudomonas aeruginosa

cAMP has an integral and multifaceted role in *P. aeruginosa* pathogenesis, as this bacterium produces three ACs: CyaA, CyaB and ExoY. ExoY is a host-activated AC toxin that is exported with several other proteins into host cells through a cAMP-regulated T3SS[37,38], and is discussed in a later section. However, CyaA and CyaB remain within the *P. aeruginosa* cytosol, where they increase intracellular cAMP levels to control virulence gene expression (Figure 2). Low Ca^{2+} concentration is a signal that leads to cAMP production and expression of all known T3SS components[39]. CyaB, which produces the bulk of intracellular cAMP in *P. aeruginosa*, may itself be a membrane sensor protein that detects low Ca^{2+} concentrations and/or contact with host cells[39]. Deletion of *cyaB* attenuates virulence[26,40] owing to a reduced expression of T3SS components, as virulence can be recovered by overexpression of a regulatory factor, ExsA, that specifically restores expression of T3SS components[26].

Transcriptional upregulation of T3SS genes is mediated by the cAMP-Vfr complex in *P. aeruginosa*. Vfr is a CRP-family transcription factor that is similar to, but not functionally interchangeable with, *E. coli* Crp[41]. While *vfr* complements an inactivating *crp* mutation in *E. coli*, *E. coli crp* cannot substitute for *vfr* in *P. aeruginosa*[42]. Vfr controls expression of many virulence-associated genes in *P. aeruginosa*, including those encoding exotoxin A, type IV pili, a T3SS, and the *las* quorum-sensing system[43]. All of these genes, except the *las* genes, are regulated by Vfr in a cAMP-dependent fashion. Surprisingly, expression of the *las* genes requires Vfr, but *las* regulation is not affected by intracellular cAMP levels[43]. Rather, Vfr appears to require a cAMP-independent cofactor for *las* induction, raising the possibility that Vfr functions as a dual regulator that is capable of controlling different gene sets in response to various environmental conditions. The cAMP-Vfr complex also upregulates expression of a 3',5'-cAMP PDE, CpdA[44], as well as a second cAMP-binding protein, CbpA, in *P. aeruginosa*[45]. CpdA degrades cAMP as a counterbalance to cAMP synthesis by the ACs, and is required for proper expression of virulence factors[44]. In contrast, the functions of CbpA are unknown, although its cAMP-dependent polar localization in the cell is intriguing and suggests that additional roles for cAMP in *P. aeruginosa* may be awaiting discovery.

Central role for cAMP signaling in *Vibrio cholerae*

Cholera toxin (CT) disrupts cAMP signaling in host intestinal epithelial cells during infection, causing the watery diarrhea that characterizes cholera disease (discussed below). However, the extensive role of cAMP signaling within the cells of *V. cholerae*, throughout the entire infection cycle, is much less appreciated. Recent studies show that cAMP signaling within *V. cholerae* integrates carbon source availability with population density, biofilm formation, bacteriophage sensitivity and virulence gene expression (Figure 3).

Vibrio spp. have a single AC, CyaA, that mediates signaling through the CRP family cAMP-binding transcription factor, Crp[46]. Glucose limitation stimulates production of cAMP by CyaA[47], and this cAMP binds with Crp to form the active transcription factor, cAMP-Crp. cAMP-Crp regulates quorum sensing, biofilm formation and expression of the cholera toxin (CT) and toxin co-regulated pilus (TCP) in *V. cholerae* by increasing levels of the master quorum sensing regulator, HapR[47]. HapR up-regulates quorum sensing, while indirectly down-regulating biofilm formation and expression of the *ctxAB* and *tcpA-F* genes, which encode CT and TCP proteins respectively[48,49]. HapR represses expression of the biofilm gene activator, VpsR[50]. Likewise, HapR reduces CT and TCP production by downregulating expression of AphA, a positive regulator of the coordinately expressed CT and TCP genes[51].

HapR expression requires the presence of two autoinducers, CAI-1 (cholera autoinducer 1) and AI-2 (autoinducer 2), which are extracellular signaling molecules secreted by *V. cholerae*[52]. In this bacterium, cAMP-Crp promotes CAI-1 production by positively regulating expression of the gene encoding the CAI-1 synthase, CqsA. The key role of cAMP in linking carbon source to population density in *V. cholerae* was further confirmed by showing that the cell density needed to activate HapR was increased when the intracellular levels of cAMP were decreased by the addition of glucose to the culture[53]. Surprisingly, the regulation of CqsA expression by cAMP-Crp seems to act at the level of mRNA stability, although the mechanism for this CRP-mediated post-transcriptional regulation has not been determined[53].

In addition, cAMP modulates *V. cholerae* pathogenesis through HapR-independent mechanisms, which may be important in El Tor strains that lack functional HapR due to a frameshift mutation[54]. cAMP-Crp downregulates expression of CdgA, a cyclic diguanylate cyclase that promotes *V. cholerae* biofilm formation through its production of c-di-GMP[33]. Moreover, cAMP-Crp participates with transcription factor ToxT in forming a bistable regulatory switch that controls CT and TCP expression during infection[36]. This bistable switch may allow a subpopulation of *V. cholerae* cells in rice water stool to remain in a hyper-infectious state by retaining their expression of CT and TCP for up to five hours outside the host. This strategy would allow increased transmission during epidemics without compromising the fitness of *V. cholerae* outside the host. The role of cAMP-Crp signaling in promoting resistance of *V. cholerae* O1 strains to environmental bacteriophages provides another means by which cAMP may contribute to *V. cholerae* fitness outside the host[55]. Resistance occurs at the level of phage adsorption, and may be due to loss or masking of a lipid or protein-based phage receptor on the bacterial cell surface. This possibility is consistent with the known role of cAMP-Crp in regulating expression of the outer membrane protein OmpT in *V. cholerae*. In this case, Crp competes with the ToxR repressor for binding to the *ompT* promoter, and leads to enhanced transcription of *ompT*[56].

Multiple cyclases and effector proteins in *M. tuberculosis*

The ACs of mycobacteria and their effector proteins have been recently reviewed[28,57], so they will be discussed only briefly here. *Mycobacterium tuberculosis* has 16 AC-like proteins, 10 of which have confirmed AC activity[58,59] (Figure 4). All are Class III cyclases, but the group includes soluble, membrane bound and integral membrane enzymes. Many *M. tuberculosis* ACs contain functional domains other than the AC catalytic core region, including receptor, inhibitory, DNA-binding and HAMP (histidine kinases, ACs, methyl binding proteins and phosphatases) domains[58,60–62]. These accessory domains probably regulate the AC activity, and may in some cases add additional functions to the enzyme. Glucose, except when added at very high concentrations, has little effect on *M. tuberculosis* cAMP levels[63,64]. Instead, *M. tuberculosis* ACs are regulated at multiple levels by a range of conditions encountered within the host. For example, the activity of *M. tuberculosis* ACs is directly affected by pH, fatty acids, and carbon dioxide (CO_2), whereas hypoxia and

starvation affect the expression of AC-encoding genes[28]. In particular, macrophage passage stimulates AC activity within *M. tuberculosis*, raising cAMP levels as much as 50 fold in two hours, as compared to incubation in tissue culture media alone[63]. Viable mycobacteria also secrete cAMP into their host macrophages during infection, as discussed below. One AC, Rv0386, has been specifically linked to production and secretion of cAMP within macrophages, and deletion of the corresponding gene decreases *M. tuberculosis* virulence and pathology in a murine infection model[65].

In addition to its complex array of ACs, *M. tuberculosis* encodes 10 putative cAMP binding proteins[28,57,58]. These include two CRP family transcription factors, and a protein lysine acetylase[25,66–68]. The remaining seven putative cAMP binding proteins have yet to be characterized. One of the two transcription factors, Crp, controls a regulon of over 100 genes and its DNA binding activity is similar to that of *E. coli* Crp[66]. Deletion of *crp* attenuates *M. tuberculosis* virulence in a murine model[25], and current efforts are aimed at determining which of its regulon members specifically contribute to virulence. The second transcription factor, Cmr, controls expression of a different set of genes in response to cAMP levels and macrophage passage[67]. The biological role of the cAMP-responsive acetylase is not known.

Bacterial manipulation of host cAMP levels

Disruption of signal transduction within host cells is an effective virulence strategy used by many pathogens, and the myriad ways in which cAMP levels affect immune function make cAMP signaling a frequent target within host cells[19] (Figure 5). Elevated cAMP levels can suppress innate immune functions by modulating inflammatory mediator expression, dampening the phagocytic response and reducing intracellular killing of ingested pathogens[19]. Another powerful effect of elevated cAMP levels in host intestinal epithelial cells is the excessive fluid secretion that causes the watery diarrhea associated with cholera and some toxigenic *E. coli* infections[69,70].

The most studied microbial strategy for elevation of host cAMP levels is the production of certain toxins by bacteria[40,69,70]. These toxins can be ACs themselves, as is the case with the AC toxin of *Bordetella pertussis*, the edema factor (EF) toxin of *Bacillus anthracis* and ExoY of *P. aeruginosa*. Alternatively, toxins such as cholera toxin (CT) of *V. cholerae*, pertussis toxin (PT) of *B. pertussis* or labile toxin (LT) of *E. coli* can modulate the activity of the endogenous host ACs by altering the function of heterotrimeric G proteins that in turn regulate AC activity. Elevation of cAMP concentrations by bacterial pathogens also occurs by toxin-independent mechanisms, as discussed below. Some bacterial pathogens even use multiple simultaneous strategies to elevate host cAMP levels. For example, *B. pertussis* secretes two toxins (CyaA and PT), each of which increases cAMP synthesis in the host cell by a different mechanism. The end result of such a two-pronged approach with *B. pertussis* is reduced phagocytosis and decreased production of reactive oxygen intermediates by intoxicated macrophages, along with suppression of neutrophil recruitment to the lungs during infection[71,72].

AC toxins

B. anthracis and *B. pertussis* both produce and export calmodulin-dependent AC toxins, which are active only in their host cells. Activation of these ACs requires binding with the calcium-binding protein calmodulin, a host protein[69]. *P. aeruginosa* also exports a host-dependent AC toxin, ExoY, that is activated by an unidentified host factor, different from calmodulin[37]. A secreted AC has also been identified in *Y. pestis*, although it has not been characterized[73].

The AC toxins from *B. pertussis* (CyaA) and *B. anthracis* (EF) have numerous and varied effects on phagocytosis, apoptosis, and migration of leukocytes[69,71,74–76]. Despite mechanistic similarities, CyaA and EF show differences in protein structure, enzyme kinetics, localization in the host cell and interactions with calmodulin[77,78]. CyaA is a single polypeptide that inserts into the plasma membrane of target cells[79]. In contrast, EF is the toxic component of an A-B toxin (ET) that is taken up by receptor-mediated endocytosis and ultimately released into the cytosol, where it typically resides in a perinuclear localization[80]. CyaA and EF both specifically promote a Th1 to Th2 immunological switch at low levels of toxin, while all T cell differentiation is suppressed at high levels of toxin[77]. The Th1-to-Th2 shift occurs when the cAMP produced by the toxins activates a PKA pathway that increases production of Th2 cytokines such as IL10 and IL4. However, this same pathway also turns down expression of IL12, a key cytokine involved in development of the kind of Th1-type cell-mediated immune response that is required for clearance of many pathogens. Structural comparisons of EF with host ACs has lead to the identification of two inhibitors that specifically target EF and may be candidates for post-exposure treatment[81].

Toxins that upregulate the activity of host ACs

A second approach for increasing host cell cAMP levels is to modify the alpha subunits of heterotrimeric G proteins that regulate the activity of host ACs[19,40,70]. *V. cholerae* CT, *E. coli* LT and *B. pertussis* PT are ADP ribosylating enzymes that target different regulatory G protein subunits. CT and LT target the stimulatory G_s alpha subunits, effectively locking them into an active mode that causes continuous production of cAMP by host ACs. In contrast, the ADP-ribosylation activity of PT inactivates the inhibitory G_i alpha subunits, preventing them from down-regulating AC activity. In all cases, the result is constitutive AC activity that leads to accumulation of cAMP within targeted cells.

Secretion of cAMP into host cells

M. tuberculosis secretes cAMP directly into host macrophages upon infection. cAMP levels within macrophages increase 2–3 fold upon infection with viable, but not heat-killed *M. tuberculosis*-complex bacteria[28,63,65]. Increased levels of cAMP within infected macrophages correlates with decreased phagolysosome fusion[82], reduced pathology and attenuated virulence[65]. The increased cAMP was shown to arise from the bacteria rather than the host macrophages by using [^{14}C]-radiolabeled *M. tuberculosis* and is largely due to synthesis by a specific AC, Rv0386[65]. The mechanism for cAMP secretion is not known but it is regulated separately from cAMP production[63]. Albumin inhibits both the production and secretion of cAMP in *M. tuberculosis*-complex bacteria grown *in vitro*. Oleic acid restores cAMP production, but not secretion, in these bacteria, resulting in very high levels of cytoplasmic cAMP[63]. Identification of the secretory mechanism and its regulation is key to unraveling the specific roles of cAMP in tuberculosis infection.

Exploiting host signaling via complement-TLR crosstalk

Porphyromonas gingivalis provides an additional example of a means by which a pathogen can directly increase cAMP levels in host cells. *P. gingivalis* expresses a protease with C5 convertase-like activity, which cleaves the host complement protein C5 into its active C5a and C5b components. The resulting C5b is degraded to prevent stimulation of an inflammatory response. However, C5a binds to its receptor C5aR while intact *P. gingivalis* bacteria bind to a Toll-like receptor, TLR2, and/or the chemokine receptor CXCR4 on macrophages. Coordinate stimulation of these two receptors triggers cAMP production in the host cell, with subsequent PKA activation that leads to down-regulation of the iNos inducible nitric oxide killing pathway and a concomitant reduction in the concentration of antimicrobial nitric oxide[29].

cAMP signaling in pathogenic fungi

Eukaryotes do not possess CRP-like transcription factors, and protein kinase A (PKA) is the primary cAMP-activated intermediary between ACs and downstream gene regulatory events. Mammalian cells carry two distinct types of ACs: transmembrane ACs (tmAC) are activated by heterotrimeric G proteins in response to signals received through G protein coupled receptors (GPCRs), while soluble ACs (sAC) are directly activated by CO_2/HCO_3^-[15]. Fungal ACs combine features of both tmAC and sACs, as they are membrane-bound ACs that can be activated by both G proteins and CO_2/HCO_3^-. In contrast to mammalian cells that contain many different ACs, each of which may be controlled by a specific signal, fungi carry only one extremely versatile AC that is capable of processing and integrating environmental signals from a variety of sources. This single AC is required for virulence of many pathogenic fungi, including the human pathogens *Cryptococcus neoformans*[83], *Candida albicans*[84] and *Aspergillus fumigatus*[85], as well as the rice pathogen *Magnaporthe oryzae*[86]. The role of cAMP/PKA signaling in virulence has been best studied in *C. neoformans* and *C. albicans*[83,87].

Cryptococcus neoformans

C. neoformans is a dimorphic basidiomycete that causes fungal meningoencephalitis, particularly in immunocompromised individuals[88]. Essential virulence determinants of *C. neoformans* include the antioxidant melanin and an antiphagocytic capsule[83]. The single AC present in *C. neoformans* is a membrane-bound protein, Cac1, that is dispensable for *in vitro* growth but essential for virulence[89]. Genetic analyses have shown that cAMP production by Cac1 is activated by the heterotrimeric G-protein G subunit Gpa1, following stimulation of the GPCR Gpr4 by amino acids[90] (Figure 6). Transcriptome analyses have shown that the Ras and cAMP/PKA signaling pathways are largely independent from one another in *C. neoformans*, although both contribute to antifungal drug susceptibility[91]. Nonetheless, regulation of mating and hyphal differentiation requires the AC-associated protein Aca1, which is a positive regulator of Cac1, and this may involve input from the Ras signaling pathway.

In the absence of cAMP, PKA exists as an inactive tetrameric complex that includes two regulatory (Pkr1) and two catalytic (Pka1) subunits. cAMP binding to PKA causes Pkr1 subunits to release active Pka1 that then phosphorylates effector proteins such as the transcription factor Nrg1, which regulates downstream functions such as virulence gene expression[92,93]. Maintaining homeostasis of the cAMP/PKA system also requires ways to decrease cAMP levels. Such negative inputs include inhibition of Gpa1 by the GTPase activator protein (GAP) Crg2 to reduce cAMP production, and hydrolysis of cAMP by a PDE, Pde1[83].

Despite the absence of much information on the downstream targets of cAMP-PKA, the essentiality of this signaling pathway for virulence gene regulation in *C. neoformans* serotype A is clear. Deletion of any of the genes encoding components such as Gpa1, Cac1 and Pka1 of the regulatory cascade results in loss of melanin, capsule production and virulence[83,94]. Similarly, *pkr1*-deleted mutants of *C. neoformans* are hypervirulent[89,95].

An alternative way to activate the cAMP/PKA pathway in *C. neoformans* is with CO_2/HCO_3^-, which is consistent with the role of CO_2 in stimulating capsule production[96]. Cac1 is directly activated by CO_2/HCO_3^-, and this effect is independent of G-protein mediated regulation of Cac1 by Gpa1[97,98]. CO_2 exists in equilibrium with HCO_3^-, and the conversion of low CO_2 concentrations into HCO_3^- is catalyzed by carbonic anhydrases (CAs). *C. neoformans* produces two CAs, Can1 and Can2[99]. Only Can2 is required for growth *in vitro*, where CO_2 levels are low (~0.033%), and this dependence on Can2 is relieved by high

levels of CO_2. Palmitate supplementation also rescues *in vitro* growth of *can2*-deleted mutants, suggesting a role for Can2 in fatty acid metabolism[97,99]. However, neither CA is required for virulence, presumably because of the high levels of CO_2 (~5%) present within mammalian hosts[99]. Future studies are needed to address the specific roles of CO_2 versus HCO_3^- in Cac1 activation, and whether the means by which the cAMP/PKA pathway is stimulated confers any specificity on the downstream effector functions that are affected.

Candida albicans

The cAMP/PKA signaling pathway is highly conserved in all fungi, but each system has its own signaling specificities and controls different functions (Figure 6). *C. albicans* is a commensal ascomycete that causes serious disease in immunocompromised individuals[100]. The yeast-to-filament transition that is crucial for *C. albicans* virulence is regulated by the cAMP/PKA pathway, although using G-protein pathways that are different from those used by *C. neoformans*.

In contrast to *C. neoformans*, the single AC (Cyr1, previously called Cdc35) of *C. albicans* is directly activated by the G-protein Ras1 in response to glucose, serum and *N*-acetylglucosamine, leading to hyphal growth[84]. This direct activation takes place by Ras1-GTP binding to a Ras-association (RA) domain of Cyr1, and therefore differs from what has been observed in *C. neoformans*, in which the cAMP/PKA pathway appears to be largely independent of Ras signaling[91,101]. Two catalytic subunits of PKA, Tpk1 and Tpk2, regulate downstream events in response to cAMP production in *C. albicans*. Tpk2 is the primary kinase responsible for hyphal growth and virulence, while Tpk1 controls stress responses[102,103]. Following activation by Tpk2, the Efg1 and Flo8 transcription factors are responsible for virulence gene regulation[104]. The AC-cAMP signaling pathway in *C. albicans* can also be activated *in vitro* by amino acids through the heterotrimeric GPCR Gpr1 and its associated G-protein, Gpa2[105]. However, the mechanism by which this activation pathway causes hyphal growth in *C. albicans* has not been described.

In contrast to their differential use of G-protein activation pathways, AC activation by CO_2/HCO_3^- and the importance of CAs at low CO_2 concentrations is similar in *C. albicans* and *C. neoformans*[98,99], as demonstrated by the successful complementation of the hyphal growth deficiency of a *C. albicans cyr1*-deleted mutant by expressing Cac1 from *C. neoformans*[97]. The activation of Cyr1 by CO_2/HCO_3^- is independent of G-protein signaling and occurs by direct binding to the catalytic domain of Cyr1.

CO_2 induces the development of pseudohyphae and the invasion of underlying agar in *C. albicans* but not in non-pathogenic *Candida* spp., making it a specific biomarker for pathogenicity[98]. CO_2 generated by *C. albicans* cells can accumulate to levels sufficient to induce invasive hyphal growth, providing numerous ways in which CO_2 can contribute to pathogenesis[87]. While hyphal growth is essential for virulence, the ability of *C. albicans* to switch between yeast and hyphal forms is also critical for pathogenesis, as mutations that lock cells in either yeast or filamentous forms leads to attenuation[106]. Similarly, *set3*-deleted mutants, in which loss of the Set3C histone deacetylase complex causes hypersensitivity to filamentation signals, are avirulent[107]. The multiple ways in which disruption of the cAMP/PKA signaling pathway attenuates virulence makes it a promising target for antifungal drug development.

Serum is one of the most potent stimulators of hyphal growth in *C. albicans*, and it was recently shown that muramyl dipeptides (MDP) derived from the breakdown of cell wall peptidoglycan from co-infecting or commensal bacteria are critical for this activation[108]. MDPs stimulate cAMP production by directly binding leucine-repeat regions (LRR) within Cyr1. Ras1 contributes to Cyr1 activation in response to serum, but full activation of hyphal

growth via this pathway also involves the sensing of G-actin pools, as extensive actin cytoskeleton remodeling is required for hyphae development. Cyr1 does not directly bind G-actin, but is able to integrate the serum, MDP and actin signals as part of a tripartite complex with G-actin and the cyclase-associated protein Cap1[87,109]. Cap1 binds both Cyr1 and G-actin, serving as a bridge between them. Discovery of MDPs from commensal and/or co-infecting bacteria as direct enhancers of *C. albicans* pathogenesis is a reminder of the complexity of the microbial interactions within the human host. It also has important implications for treatment of both bacterial and fungal infections, as antibiotic therapies that increase bacterial cell wall fragmentation may specifically trigger or exacerbate a fungal infection.

cAMP signaling in parasitic protozoa

Parasites also need accurate signaling mechanisms to sense and respond to new conditions as they move between mammalian hosts, insect vectors and the environment. Frequently, a membrane receptor senses the environment and activates signaling pathways to trigger an immediate response, such as motility or exocytosis, but also a delayed response, such as cell division or differentiation into the next developmental stage. cAMP is emerging as a central signaling mediator that regulates essential processes in parasites. Evidence for an evolutionarily conserved cAMP signaling pathway is abundant in protozoa of the genera *Plasmodium*, *Leishmania*, *Trypanosoma*, *Toxoplasma* and *Entamoeba*, as well as in the parasitic worm *Schistosoma*, which together cause the majority of human mortality and morbidity worldwide.

Modulation of cAMP levels in the host cell

Intracellular parasites frequently induce signaling cascades in the host cell that facilitate invasion. An example of this behavior is *Trypanosoma cruzi*, which stimulates Ca^{2+} and cAMP signaling in the host cell to induce the recruitment and fusion of host cell lysosomes that the parasite requires for invasion[110]. Another parasite strategy for survival is the evasion of the host immune system. In this case, cAMP has been implicated in *Plasmodium* evasion of Kupffer cells, where parasites induce an increase in cAMP that mediates the inhibition of the microbicidal respiratory burst and as a consequence, results in the survival of the parasite[111].

Host cell invasion by the malaria parasite

Plasmodium spp., the causative agents of malaria, start infection in the hepatocytes of the host, but continue by invading and replicating in erythrocytes. In both stages, liver and blood, *Plasmodium* uses cAMP pathways to regulate processes required to establish a successful infection. During the liver stage, activation of an AC, AC □, in *Plasmodium* sporozoites increases intracellular cAMP and induces exocytosis that results in the exposure of proteins required for hepatocyte invasion[112] (Figure 7). The AC □protein of *Plasmodium* contains a domain with homology to K^+ channels, which is thought to be functionally linked to the AC activity. Additionally, motility induced by albumin in sporozoites is regulated by cAMP[113].

In the erythrocytic stage, cAMP signaling pathways have been implicated in intra-erythrocyte survival, regulation of the parasite cell cycle and invasion of host cells. At this stage, *Plasmodium* replicates in a synchronous way, which is responsible for the cyclical fevers that characterize malaria. The mechanism underlying this phenomenon is a melatonin-activated cAMP-signaling cascade in the parasite, which regulates cell cycle, resulting in the synchronized replication[114,115]. The permeability of the host erythrocyte membrane, which allows intake of nutrients required for parasite survival and replication, is

also regulated by cAMP. A cAMP-dependent PKA regulates anion channel conductance in the infected erythrocyte membrane, a process that controls parasite growth[116]. Invasion of host erythrocytes is also regulated by PKA, which phosphorylates apical membrane antigen 1 (AMA1), a protein present on the parasite surface that mediates erythrocyte invasion[117]. Besides the role in host cell invasion, cAMP is also required in *Plasmodium* for the transition from the asexual form to gametocytes, which appears to be regulated by cAMP signaling pathways[118–120]. PKA has also been implicated in stage transition from an actively replicating form into the quiescent chronic stage in *Toxoplasma gondii*[121], a parasite related to *Plasmodium*.

cAMP in stage transition of trypanosomatids and other parasites

cAMP signaling pathways have been described in *T. cruzi*, which causes Chagas disease, and *Trypanosoma brucei*, the cause of human African trypanosomiasis. In *T. cruzi*, cAMP regulates PKA with striking effects on cell survival[122] and cell proliferation through the inhibition of DNA, RNA and protein synthesis[123]. cAMP has also been implicated in the transition of the human-infective form to the insect-infective form of *T. cruzi*[124,125]. Additionally, a PDE regulates osmosis in the insect-infective form of this parasite[126].

In *T. brucei*, cAMP regulates parasite virulence[127] and induces the cell cycle arrest that occurs in the differentiation from the human-infective stage into the insect-infective stage[128]. In the related parasite *Leishmania*, cAMP also appears to regulate stage transition[129] and motility[130]. *Entamoeba*, the causative agent of amebiasis, is another example of a parasite where cAMP-dependent pathways seem to regulate stage transition, in this case from the human intestinal form into the free-living cyst[131]. In *Schistosoma*, a parasitic worm, cAMP-mediated pathways are also present and essential for parasite survival[132].

There is growing evidence for the use of cAMP signaling pathways in processes required for parasite survival. Although there is a great diversity in the functional processes involved, transition into a different stage of the life cycle and invasion of the host cell appear as common processes regulated by cAMP. This is not surprising, since both events require a specific response to the environment that will facilitate survival of the parasite. From this point of view, cAMP-regulated pathways, which involve proteins related, but not identical, to the human host, appear as promising pharmacological targets for drug development.

Conclusions and future directions

cAMP signaling controls a surprisingly diverse range of processes in pathogenic bacteria, fungi and protozoa, and the critical roles for cAMP in microbial pathogenesis extend from within the pathogens themselves to their mammalian host cells. cAMP is a key regulator of virulence gene expression in bacteria and fungi, through its influence on the activity of transcription factors. Regulation of virulence gene expression by cAMP-dependent transcription factors, coupled with the environmental control of cAMP production, allows pathogens to coordinate their gene expression programs with the presence of appropriate environmental signals from their hosts. Modulation of cAMP levels within host cells by bacteria and protozoa contributes to their pathogenesis by suppressing immune responses while facilitating pathogen invasion, colonization and transmission. The success of this strategy is evident from the number of bacterial species that have independently evolved such mechanisms, which range from direct secretion of cAMP and bacterial ACs to manipulation of host cAMP production pathways. The frequent integration of horizontally-acquired virulence genes into existing cAMP-controlled regulatory circuits also suggests that cAMP-mediated signaling may facilitate virulence evolution in some pathogenic microbes.

Despite some general themes, e.g., the presence of CRP family transcription factors in bacteria, and cAMP-PKA regulated transcription factors in fungi and protozoa, cAMP signaling pathways are complex, and there exists a great deal of diversity among cAMP-associated regulatory circuits in different pathogens. Much of this diversity likely arises from the participation of regulatory cofactors, and recent evidence suggests that many cofactors involved in the activation of ACs as well as cAMP-dependent gene regulation remain to be discovered. The recent findings of cAMP-regulated transcriptional coactivators (CRTC) and PKA-independent cAMP-responsive EPAC (exchange proteins activated by cAMP) family transcription factors in eukaryotes[15,16,75], as well as the presence of cAMP binding proteins other than transcription factors in bacteria such as *P. aeruginosa* and *M. tuberculosis*, promise additional levels of complexity that should be investigated. Characterization of these factors and deciphering their regulatory functions is likely to establish new cAMP signaling paradigms in both bacteria and fungi.

cAMP signaling also plays an important role in sensing and responding to host signals. In some, but not all cases, CRP-mediated gene expression is coordinated with carbon source availability in the host. CO_2 and Ca^{2+} are additional signals used by multiple pathogens to sense the host environment through their AC/cAMP signaling systems. Fungi and mycobacteria express CO_2-responsive ACs, while ACs from *P. aeruginosa, B. pertussis* and *B. anthracis* are Ca^{2+} sensitive. A further level of cAMP signaling being used by pathogens to sense the host environment is the peptidoglycan sensitivity of *C. albicans*. This ability of an opportunistic pathogen to sense and exploit a polymicrobial infection is especially intriguing and has important implications for treatment. The possibility that other pathogens may have similar abilities should be investigated.

The specificity of cAMP signaling is a critical issue for pathogens with multiple ACs and for the host cells. *M. tuberculosis* may be the pathogen that presents the most challenging situation in this regard, as it has so many ACs. Linking individual ACs with specific regulatory outcomes is important for understanding cAMP signal transduction from a mechanistic perspective, as well as tuberculosis pathogenesis.

Perhaps the most important new frontier will be in understanding the effects of pathogen-mediated cAMP increases in host cells. The large number of pathogens that use this strategy, and the diversity of mechanisms that are used, point to the effectiveness of this approach. Understanding the consequences of pathogen-mediated manipulation of host cAMP may provide new antimicrobial targets as well as important new insights into the natural roles of cAMP in regulating innate immunity, as these pathogens can be used to probe the system in health and disease.

The myriad roles of cAMP signaling in microbial pathogenesis have only recently begun to be recognized, but it is clear that the universality of this second messenger provides tremendous opportunities for pathogens to modulate their host interactions during infection. Likewise, investigation of the diverse roles of cAMP in pathogen and host biology holds enormous potential for better understanding the interface between host and pathogen during infection, with the possibility of identifying new antimicrobial interventions.

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Glossary

Second messenger	a small molecule inside cells that relays signals from a receptor to one or more targets. The signal often originates outside the cell, although intracellular signals are also transmitted by second messengers in bacteria
Effector proteins	the downstream functional targets that are activated by second messengers in response to specific signal transduction events.

	Effector proteins may also be virulence factors that are injected into host cells by bacterial secretion systems
CRP (cAMP receptor protein) family transcription factors	global regulatory factors that contain cAMP and DNA binding domains and are associated with positive and negative gene regulation in some bacteria. Binding of cAMP typically induces a conformational change that increases the protein's DNA binding
Cyclic di-GMP (cyclic diguanylate)	a second messenger used exclusively in bacteria that has been associated with regulation of biofilm formation, motility and expression of virulence factors
Bistable switch	a regulatory mechanism that allows reversible toggling between two stable states. Such switching can occur in response to a transient signal, allowing stable maintenance of either state without continued presence of the inducer, and the generation of multiple subpopulations in a homogeneous environment
Regulon	a set of genes or operons whose expression is controlled by a common regulatory factor
***M. tuberculosis*-complex**	includes five mycobacterial species that are capable of causing tuberculosis (TB) disease. This group includes *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. canetti* and *M. microti*
Ras family G proteins or guanosine-nucleotide-binding proteins	a subfamily of small GTPases that control signaling cascades in eukaryotic cells, resulting in transmission of regulatory signals from outside the cell to the nucleus
G-actin	or globular actin, subunits polymerize into the long F-actin filaments that contribute to many eukaryotic processes, including cell structure, motility, cell division and cell signaling
Pseudohyphae	a filamentous form of yeast growth that occurs when budding yeast cells remain connected, forming a string of connected cells. They differ from true hyphae by their method of growth
Toll-like receptors	a class of transmembrane proteins expressed on the surface of many immune cells. These pattern recognition receptors stimulate innate immune defenses upon recognizing and binding any of a broad range of specific pathogen-derived molecules
Th1 and Th2 responses	lie at opposite ends of an immunological response spectrum and are driven by the activation of different subsets of helper T cells (Th). A Th1 response promotes killing of pathogens within host cells, while a Th2 response drives production of antibodies to neutralize the impact of toxins or extracellular pathogens. Each pathway amplifies itself while inhibiting the other. Thus, pathogens can evade host immunity by tipping the Th1/Th2 balance towards the type of immune response that is least effective against them
A-B toxins	are comprised of active (A) and binding (B) protein subunits. The A subunits are responsible for all of the toxic activity, but they require the B subunits for delivery into their target cells

Sporozoite	Stage of the *Plasmodium* parasite that is generated in the mosquito and is transmitted to the mammalian host, where it infects hepatocytes;
Gametocyte	Sexual stage of the *Plasmodium* parasite that is generated within infected erythrocytes in the mammalian host and it is transmitted to the mosquito.

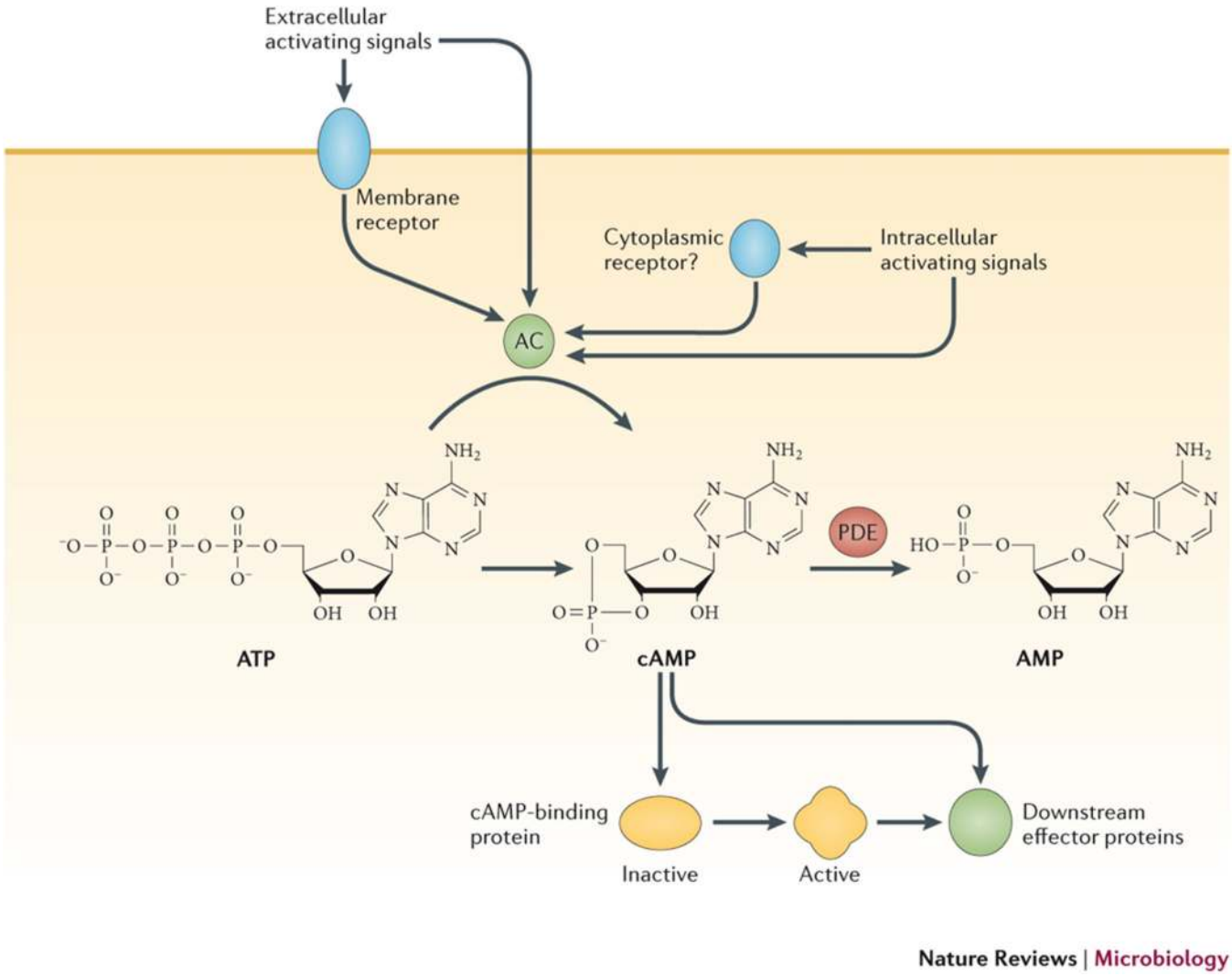

Fig. 1. cAMP relays environmental signals to regulatory outcomes

Adenylyl cyclases (ACs) can be directly or indirectly activated at the post-translational level by a number of environmental signals. Indirect activation pathways often involve membrane receptors that transmit extracellular signals to the AC by a phosphorylation event, although other cytoplasmic factors ("Factor X" in the figure) may also be required. cAMP then relays the signal to downstream effector proteins (DEP) either directly or indirectly by allosterically activating one or more cAMP-binding proteins. These cAMP-binding proteins may themselves be effector proteins, such as transcription factors or cyclic nucleotide gated channel (CNG) proteins, or they may be intermediaries, such as the regulatory subunits of PKA, which control activation of effector proteins further downstream. The inset shows the synthesis of cAMP by ACs, which catalyze conversion of ATP into cAMP and inorganic pyrophosphate while linking the remaining 5' phosphate with the 3' ribose carbon. cAMP is degraded by phosphodiesterases (PDEs) that catalyze the hydrolysis of the 3' linkage (marked with arrowhead), leaving adenosine 5' phosphate (not shown).

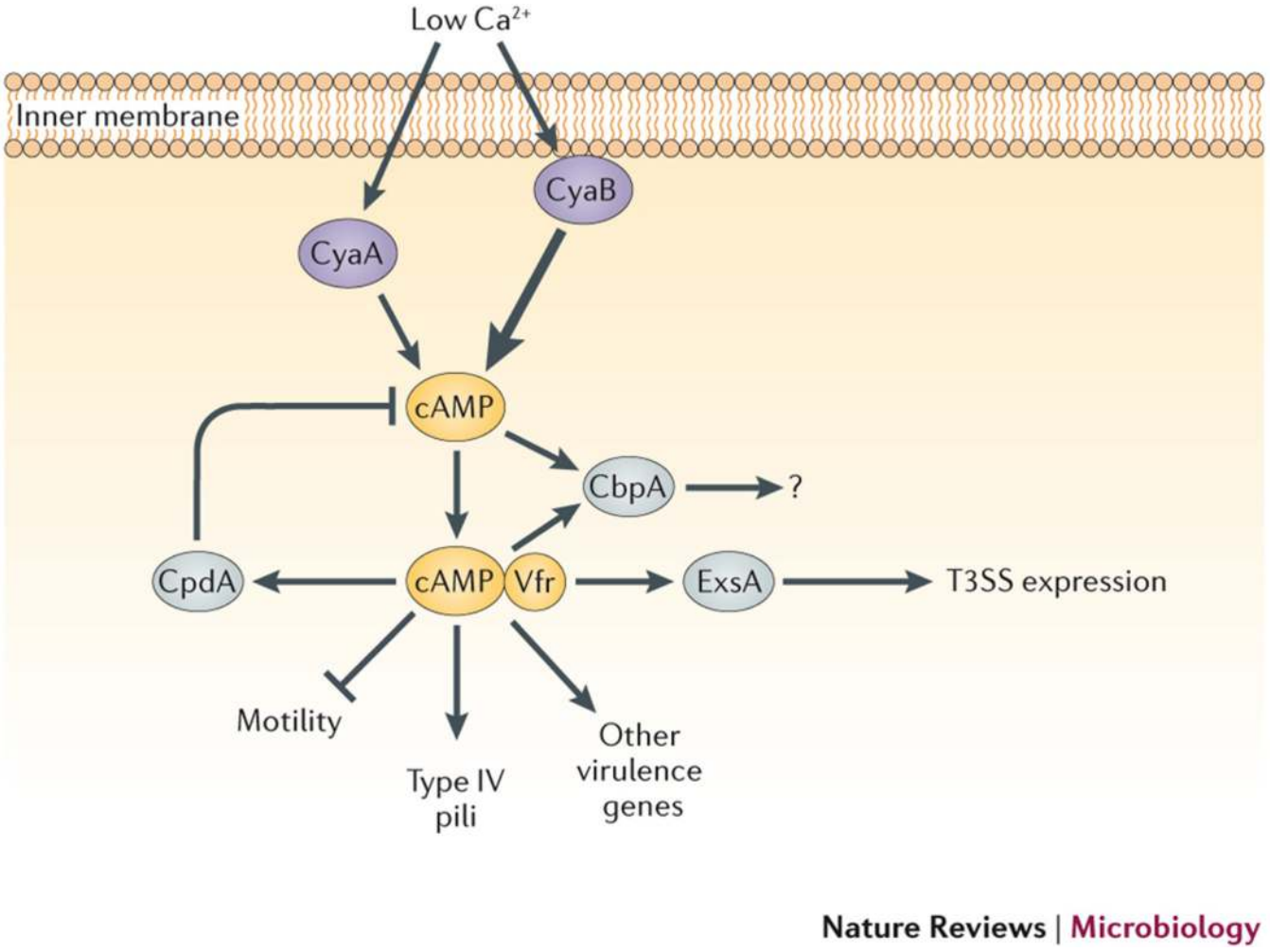

Fig. 2. Effects of cAMP signaling on gene regulation in *Pseudomonas aeruginosa*

Two ACs in *P. aeruginosa*, CyaA and CyaB, are induced by low Ca^{2+} concentrations, although CyaB is the dominant producer of cAMP, as indicated by the thicker arrow. cAMP binding to the CRP family transcription factor, Vfr (virulence factor regulator), regulates expression of a broad range of phenotypes. Intermediate regulatory factors are noted, where known, including LasI (LuxI-type acyl homoserine lactone (AHL) synthase) and LasR (regulator of the Las quorum sensing system), as well as the transcription regulator ExsA (exoenzyme secretion protein A). Arrows indicate positive regulation, while blunted lines indicate repression.

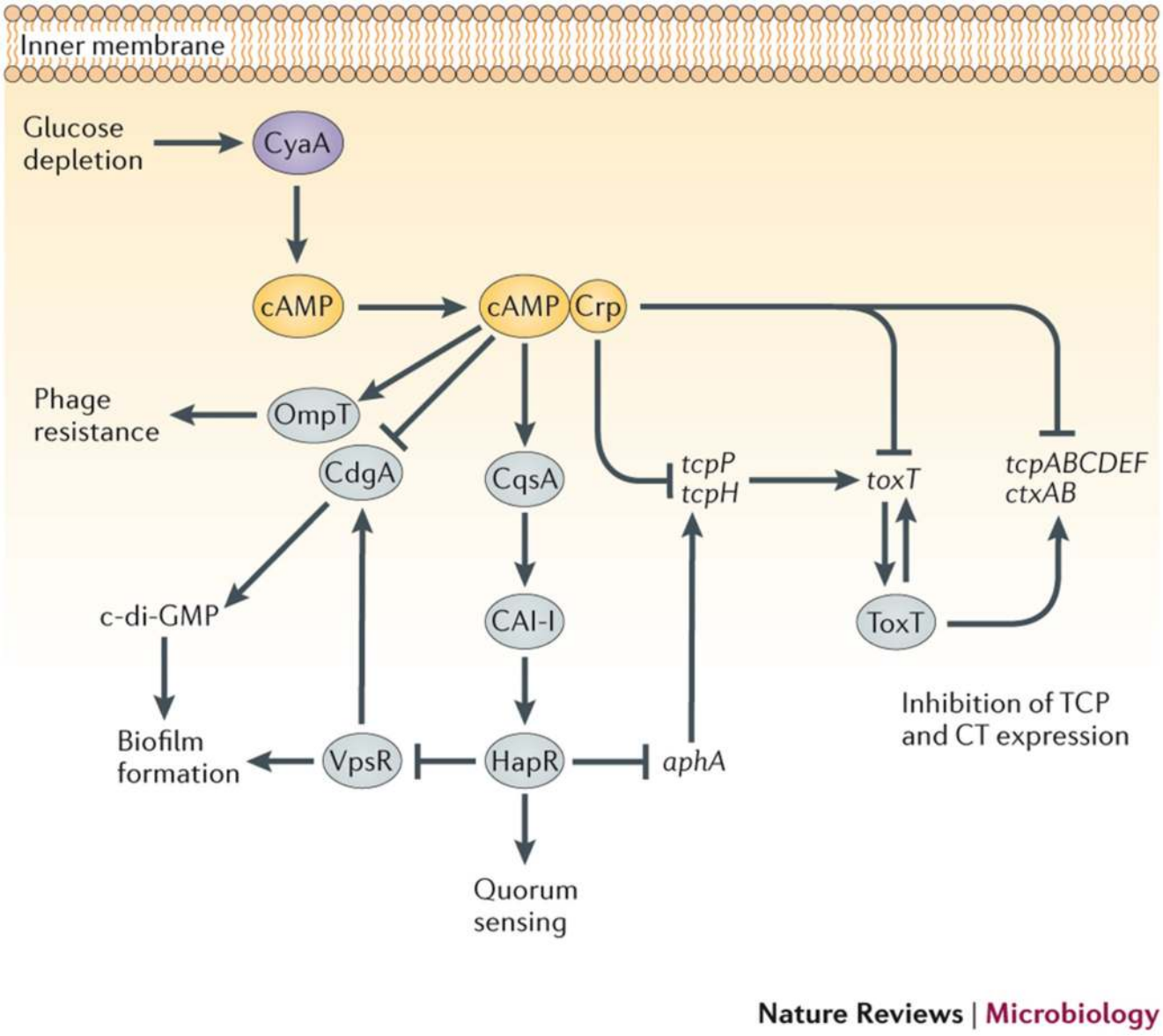

Fig. 3. Effects of cAMP signaling on gene regulation in *Vibrio cholerae*

cAMP signaling integrates carbon source information with a wide range of pathogenesis-associated processes in *V. cholerae*, through its effects on gene regulation. Biofilm formation, quorum sensing, virulence gene expression and phage sensitivity are all regulated either directly or indirectly by cAMP-CRP. cAMP-CRP regulates several individual processes by multiple pathways, emphasizing the likely importance of cAMP signaling throughout the infection cycle. Gene names: *ompT*, outer membrane protein T; *cqsA*, CAI-1 synthase ; *vpsR, Vibrio* polysaccharide regulator; *ahpAB*, transcriptional regulators; *toxT*, toxin regulator T; *ctxAB*, cholera toxin A and B (operon); *tcpA-F*, toxin-co-regulated pilus genes A to F (operon). Protein names: CyaA, adenylyl cyclase A; CrpVc, cAMP receptor protein; ToxR, toxin regulator R; CdgA, cyclic diguanylate cyclase A; HapR, hemagglutinin protease regulator; ToxT, toxin regulator T. Small molecules: di-cGMP, di-cyclic GMP; CAI-I, cholera autoinducer 1; HCO_3^-, bicarbonate ion.

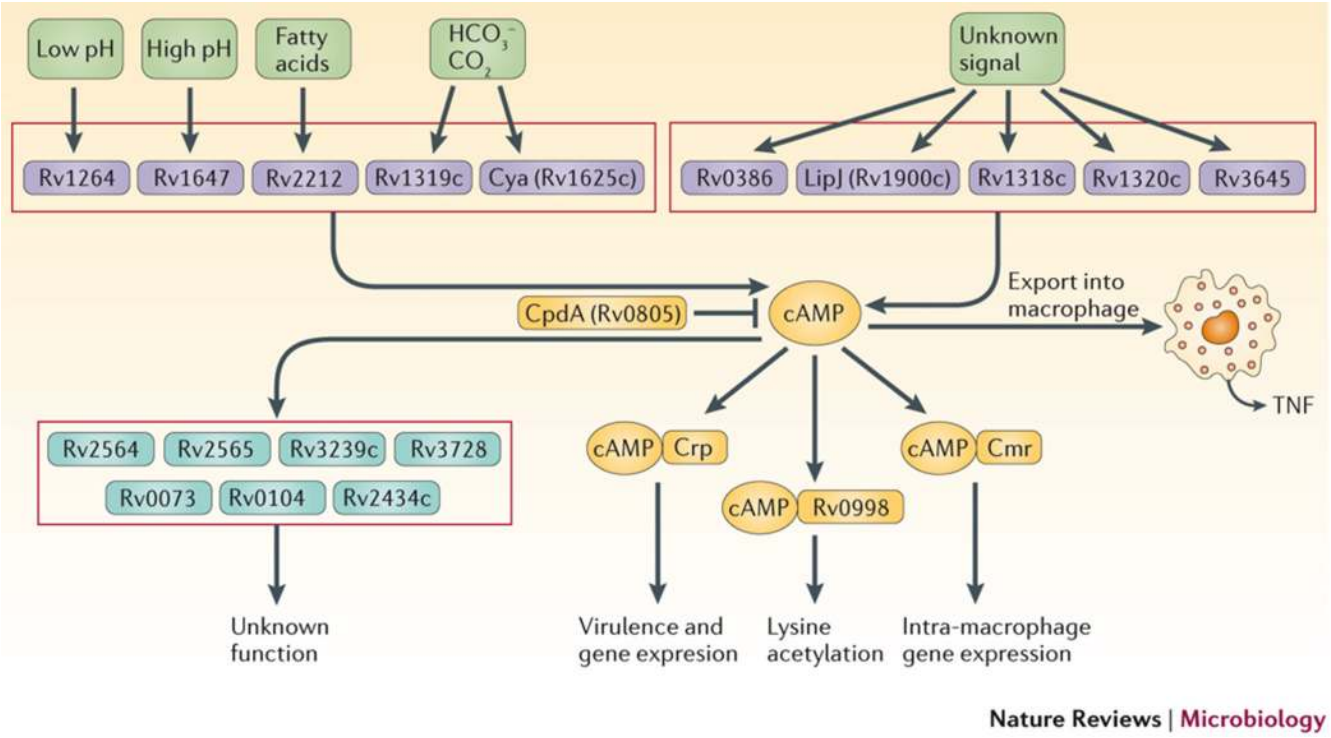

Fig. 4. Effects of cAMP signaling on gene regulation in *Mycobacterium tuberculosis*

cAMP signaling in *M. tuberculosis* is complex owing to its large number of adenylyl cyclases (ACs). The 'Rv' numbers listed in top section without boxes refer to the names of ten individual *M. tuberculosis* ACs that have demonstrated activity. Six additional putative ACs are not shown. Green boxes (top) include signals that activate the specific ACs indicated by arrows. Crp, Cmr and Rv0998 are cAMP-dependent effector proteins, which are activated by association with cAMP. However, a direct binding of cAMP to Cmr has not been demonstrated. Crp_{Mt} and Cmr are transcription factors, while Rv0998 is a protein acetylase. The other 'Rv' numbers in yellow boxes (middle section) correspond to seven other putative cAMP binding proteins. Blue boxes indicate the respective functions of specific cAMP binding proteins. Secretion of cAMP into macrophages is indicated by an arrow that connects cAMP (middle) to a blue box. Rv0805 is a phosphodiesterase that has low activity on cAMP. Arrows indicate positive activation, while the blunt ended line indicates degradation.

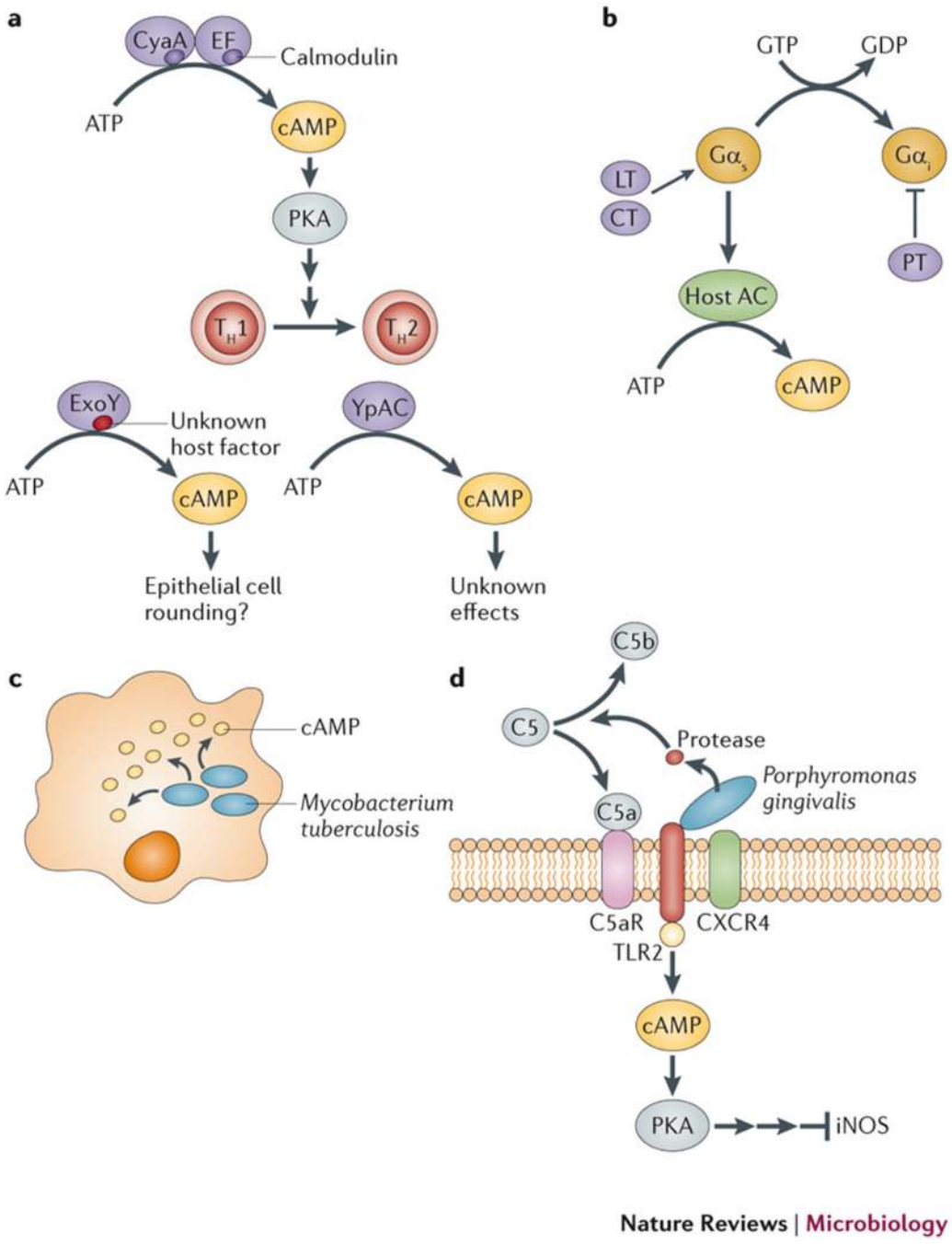

Fig. 5. Bacterial manipulation of host cAMP levels

Bacterial pathogens use numerous strategies to elevate cAMP levels within host cells during infection. Shown are the ADP-ribosylating exotoxins that increase the activity of endogenous host ACs (top, AC Modulating Toxins); the exported host-activated bacterial ACs produced by *Bordetella pertussis* (CyaA), *Bacillus anthracis* (EF), *Pseudomonas aeruginosa* (ExoY) and *Yersinia pestis* (Yp AC), (middle, AC Toxins); the activation of host ACs through coordinate signaling of complement C5a receptor C5aR (pink) and Toll-like receptor TLR2 (orange) stimulated by *Porphyromonas gingivalis*; and the direct secretion of cAMP by *Mycobacterium tuberculosis* (bottom). Red triangles represent calmodulin, red squares represent an unknown host activation factor, a purple diamond is C5a and the yellow

diamonds are cAMP. G α_s and G α_i are stimulatory and inhitory subunits of heterotrimeric G proteins, respectively.

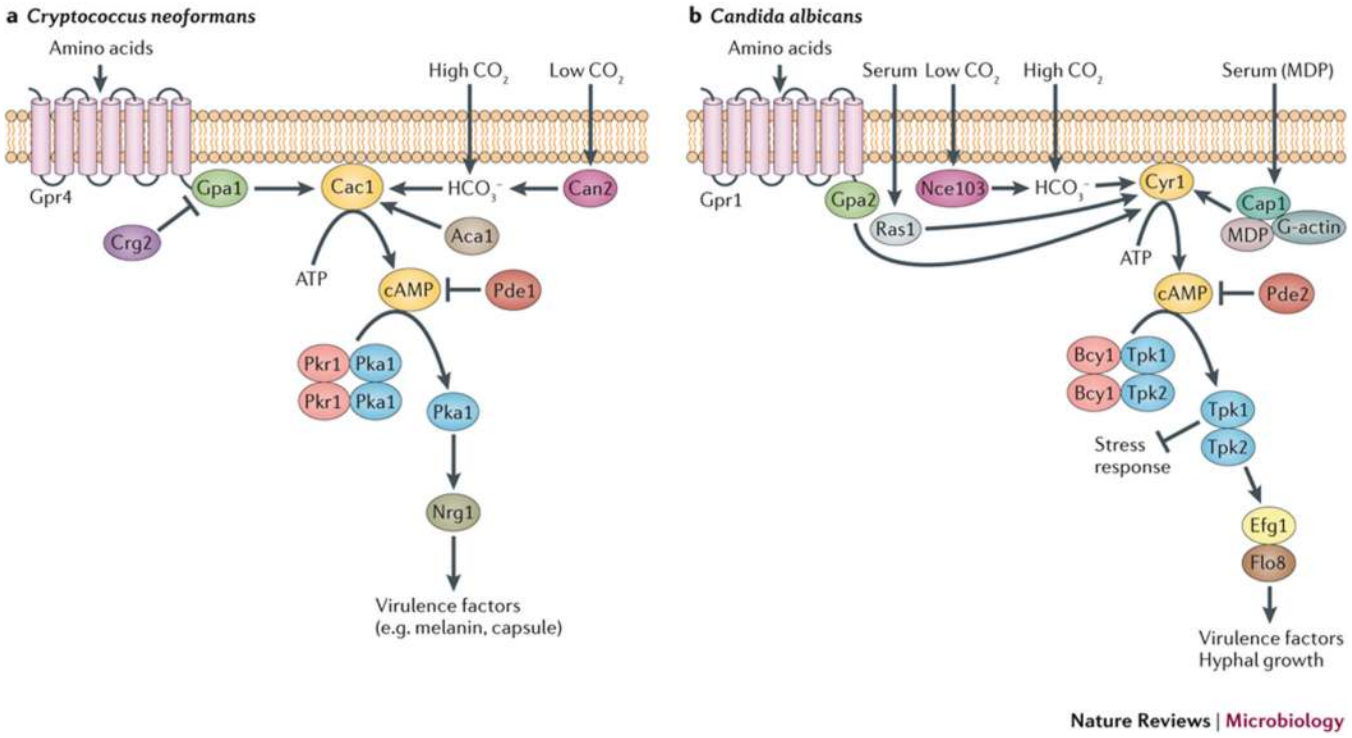

Fig. 6. cAMP signaling pathways in fungal pathogens

Both *Cryptococcus neoformans* (left) and *Candida albicans* (right) use their highly conserved cAMP/PKA pathways to regulate expression of key virulence determinants. However, the functions of these pathways differ. Matching shapes and colors are used to indicate orthologs in both fungi. Arrows denote positive regulatory effects while flat ended lines indicate inhibitory effects. Protein abbreviations for *C. neoformans*: Gpr4, G protein receptor; Gpa1, G protein G alpha subunit; Crg2, regulator of G protein signaling; Pkr1, PKA regulatory subunit; Cac1, adenylyl cyclase; Pka1, PKA catalytic subunit; Nrg1, transcription factor ; Can2, carbonic anhydrase; Ras1, guanosine nucleotide binding protein; Aca1, AC-associated protein; Pde1, phosphodiesterase. Protein abbreviations for *C. albicans*: Gpr1, G protein-coupled receptor; Gpa2, G protein alpha subunit; GAP, GTPase activator protein; Cyr1, adenylyl cyclase; Tpk1 and Tpk2, protein kinases; Nce103, carbonic anhydrase; Ras1, guanosine nucleotide binding protein; Cap1, cyclase associated protein; Pde2, phosphodiesterase; Bcy1, PKA regulatory subunit; Efg1 and Flo8, transcription factors.

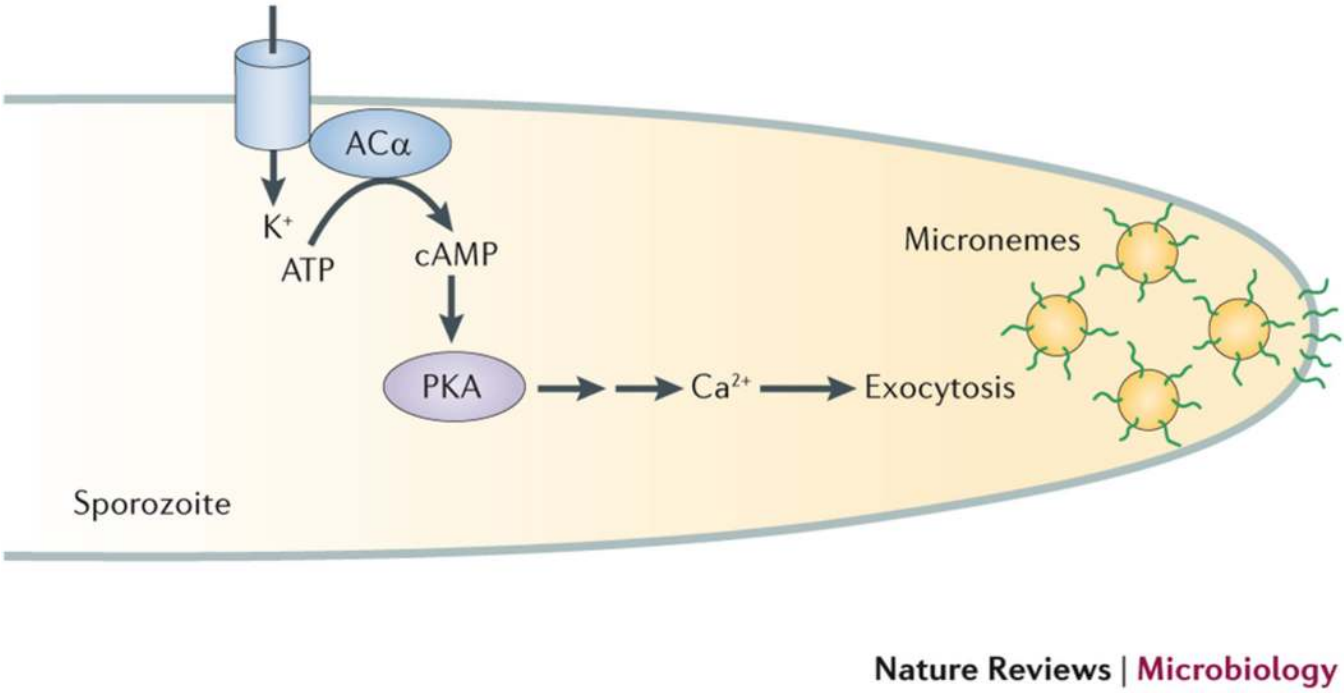

Fig. 7. Model for cAMP signaling in *Plasmodium* sporozoites leading to exocytosis

AC contains a domain with a putative K^+ channel in *Plasmodium*. Extracellular K^+ influx (1) activates AC activity, resulting in the increase of cAMP (2). Elevation of cAMP levels activates PKA (3) and leads to exocytosis of apical granules (micronemes) (4) that carry transmembrane proteins, which are necessary for invasion of host hepatocytes by *Plasmodium* sporozoites.