Hypoxia-Activated Cytochrome *bd* Expression in *Mycobacterium smegmatis* Is Cyclic AMP Receptor Protein Dependent

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Mycobacteria are obligate aerobes and respire using two terminal respiratory oxidases, an *aa*₃-type cytochrome *c* oxidase and a cytochrome *bd*-type menaquinol oxidase. Cytochrome *bd* is encoded by *cydAB* from the *cydABDC* gene cluster that is conserved throughout the mycobacterial genus. Here we report that *cydAB* and *cydDC* in *Mycobacterium smegmatis* constitute two separate operons under hypoxic growth conditions. The transcriptional start sites of both operons were mapped, and a series of *cydA-lacZ* and *cydD-lacZ* transcriptional reporter fusions were made to identify regulatory promoter elements. A 51-bp region was identified in the *cydAB* promoter that was required for maximal *cydA-lacZ* expression in response to hypoxia. A cyclic AMP receptor protein (CRP)-binding site (*viz.* GTGAN₆CCACC) was identified in this region, and mutation of this site to CCCAN₆CTTTC abolished *cydA-lacZ* expression in response to hypoxia. Binding of purified CRP (MSMEG_0539) to the *cydAB* promoter DNA was analyzed using electrophoretic mobility shift assays. CRP binding was dependent on GTGAN₆CCACC and showed cyclic AMP (cAMP) dependency. No CRP site was present in the *cydDC* promoter, and a 10-bp inverted repeat (CGGTG GTACCGGTACCACCG) was required for maximal *cydD-lacZ* expression. Taken together, the data indicate that CRP is a direct regulator of *cydAB* expression in response to hypoxia and that the regulation of *cydDC* expression is CRP independent and under the control of an unknown regulator.

The electron transport chain in mycobacteria plays a vital role in growth by generating a proton motive force across the bacterial inner membrane to fuel energetic processes in the cell membrane. Electrons enter the electron transport chain primarily via NADH dehydrogenase and succinate dehydrogenase complexes with the menaquinone-menaquinol pool, and the chain terminates through the actions of two terminal oxidases: an *aa*₃-type cytochrome *c* oxidase, encoded by *ctaBCDEF* (1), and cytochrome *bd* oxidase, encoded by the *cydABDC* gene cluster (2). *In vitro* studies have shown that the *cydABDC* gene cluster in *Mycobacterium smegmatis* is upregulated >50-fold in response to 0.6% air saturation at a low growth rate (3) and that *cydA-lacZ* expression in *M. smegmatis* increases 2- to 3-fold between 5 and 0.5% air saturation (2), suggesting that cytochrome *bd* oxidase is adapted to function at low oxygen tensions in mycobacteria. However, no studies have reported the oxygen affinity of cytochrome *bd* in mycobacteria.

In vivo analyses have revealed that the copy numbers of the *cydA* and *cydC* transcripts from *Mycobacterium tuberculosis* increase during chronic infection in mouse lungs (4) and that mutations in *cydC* result in impaired growth in mice (4). Additionally, *M. tuberculosis cydC* mutants are impaired in persistence in mice treated with isoniazid (INH) (5), suggesting discrete functions for *cydAB* and *cydDC* in this obligate human pathogen. Despite the importance of *cydAB* and *cydDC* in mycobacterial physiology and disease, the transcriptional organization of this cluster and the function of *CydDC* remain to be determined.

In *Escherichia coli*, *cydAB* and *cydDC* are present at different locations in the genome (6–8), while the genetic arrangement of *cydABCD* in *Bacillus subtilis* is operonic (9). The expression of *cydAB* in *E. coli* is controlled by two global regulators in response to oxygen: the fumarate nitrate reductase (FNR) regulator and the two-component system ArcBA (10). In *B. subtilis*, the expression

of the *cydABCD* operon is controlled by multiple regulators, including CcpA, Rex, and ResD (11, 12). In *Streptomyces coelicolor* A3, a redox-sensing repressor (Rex) controls several respiratory genes, including *cydAB* (13). However, sequence homologues of these regulatory genes have not been identified in mycobacteria, and the regulation of this operon in mycobacteria remains unclear. Recent work by Roberts et al. revealed that RegX3 of SenX3-RegX3, a two-component regulatory system of *M. tuberculosis*, regulates *cydAB* in response to lower oxygen tension (14).

In this communication, we report on the transcriptional organization of the *cydABDC* gene cluster in *M. smegmatis* and show that *cydAB* and *cydDC* are expressed as two transcriptional units. The expression of *cydAB* is controlled directly by cyclic AMP receptor protein (CRP) in response to hypoxia, but the regulator of *cydDC* remains to be identified.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* DH10B was grown in Luria-Bertani (LB) medium (15) at 37°C with agitation at 200 rpm or on LB agar plates. *M. smegmatis* mc²155 (16) and derived strains (see Table S1 in the supplemental material) were grown in LB medium supplemented with 0.05% (wt/vol) Tween 80 (Sigma-Aldrich) (LBT), in Hartmans-de Bont (HdB) minimal medium supplemented with 20 mM glycerol and 0.05% (wt/vol) Tween 80, unless otherwise stated, or on LBT

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agar plates. Cultures were grown in serum vials as described previously (17). To confirm oxygen depletion, 1.5 μ M (final concentration) methylene blue was added to cultures, and reduction was monitored visually (by decolorization). All *M. smegmatis* strains were inoculated to an initial optical density at 600 nm (OD_{600}) of 0.005 and grown at 37°C with agitation at 200 rpm. Samples (2 ml) to measure β -galactosidase expression were taken (see below), the optical density was measured, and then the cultures were stored at -20°C . All solid media contained 1.5% agar, and liquid media contained ampicillin (100 μ g ml^{-1}) and kanamycin (50 μ g ml^{-1} for *E. coli* and 20 μ g ml^{-1} for *M. smegmatis*). The OD_{600} was measured in a Jenway 6300 spectrometer.

RT-PCR. Reverse transcriptase PCR (RT-PCR) was carried out using Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The RT reactions were performed using 1 μ g of RNA extracted from an *M. smegmatis* mc²155 chemostat culture grown under hypoxic conditions (3). To determine if the *cydABDC* genes were cotranscribed, random hexamer primers (Invitrogen) and gene-specific primers were used (see Table S1 in the supplemental material). Control experiments to exclude DNA contamination were conducted as described above, using the same RNA samples as templates, but in the absence of reverse transcriptase. For positive controls, PCRs using the genomic DNA (gDNA) of *M. smegmatis* mc²155 as a template were performed using *Taq* DNA polymerase (Roche).

Mapping of *cydAB* and *cydDC* TSSs. The transcriptional start site (TSS) for the *cydAB* operon was mapped by 5' rapid amplification of cDNA ends (5'-RACE), using a 3'/5'-RACE kit (Roche) according to the manufacturer's instructions. First-strand cDNA was obtained by using Superscript III reverse transcriptase (Invitrogen), 1 μ g of total RNA (as described above), and the *cydA*-specific primer HLA11 (see Table S1 in the supplemental material). The resulting cDNA was purified, and a dA tail was added according to the kit instructions. Prior to the addition of terminal transferase, cDNA was heated (5 min, 94°C) and immediately placed on ice to denature RNA secondary structures. Purified dA-tailed cDNA was used as a template for a PCR using the oligo(dT) anchor forward primer and HLA12 (see Table S1). The resulting PCR product was used as a template for the second round of PCR, using the PCR anchor primer and HLA13 (see Table S1). The resulting PCR product was purified using a High Pure PCR product purification kit (Roche) according to the manufacturer's instructions and was sequenced using primer HLA13. Multiple rounds of 5'-RACE with biological replicates were performed. The last nucleotide before the poly(A) tail was aligned with the genome sequence to determine the transcriptional start site. The transcriptional start site of *cydDC* was also mapped as described above, using the primers HLA14, HLA15, and HLA16 (see Table S1).

Construction of transcriptional fusion constructs. Promoter activities for the *cydA* gene and *cydD* gene regions were studied using the *E. coli*-*Mycobacterium* shuttle vector pJEM15 (18). A series of transcriptional fusions to *lacZ* that progressively truncated the intergenic region were constructed (see Fig. 2). Primers used to amplify these fusions are listed in Table S1 in the supplemental material. The constructs were then cloned into *E. coli* and transformed into *M. smegmatis*. All amplified promoter regions were confirmed by DNA sequencing. Derived strains are listed in Table S1. β -Galactosidase assays were performed as described previously (19).

Expression and purification of CRP from *M. smegmatis* mc²155. The *crp* gene sequence of *M. smegmatis* mc²155 was amplified by a PCR using primers HLA64 and HLA65 (see Table S1 in the supplemental material) and cloned into pQE80L, which contains an N-terminal His tag coding sequence (Qiagen), using KpnI and HindIII restriction sites. The resulting plasmid, pHLA27, was then transformed into the expression strain *E. coli* BL21(Δ DE3 Δ *cydA*) (20). Cultures were grown in 2-liter flasks with 500 ml of LB medium supplemented with 100 μ g ml^{-1} ampicillin at 37°C with agitation at 200 rpm until an OD_{600} of approximately 0.5 was reached. Expression was then induced by the addition of isopropyl- β -D-thiogalactopyranoside (1 mM final concentration) prior to an additional 4 h of

growth. Cells were harvested by centrifugation (7,000 rpm, 4°C, 15 min), washed, and resuspended in lysis buffer (150 mM Tris-HCl, 2 mM MgCl_2 , 1% glycerol, 1 Complete Mini protease inhibitor cocktail tablet [Roche] per 7.5 ml, 5 mg DNase [Roche]) prior to cell disruption. Cells were disrupted by three passages through a French pressure cell (American Instrument Company) at 20,000 lb/in². Unbroken cells were then removed by centrifugation (10,000 rpm, 10 min, 4°C), and cell-free supernatants were collected from the membranes by ultracentrifugation (45,000 rpm, 45 min, 4°C). The supernatants containing cytoplasmic proteins were then loaded at a flow rate of 0.5 ml min^{-1} onto a 1-ml HisTrap column (GE Healthcare, Sweden) equilibrated with 10 column volumes of buffer A (20 mM Tris [pH 7.2], 500 mM NaCl, 20 mM imidazole, 10% glycerol [vol/vol], 1 Complete Mini protease inhibitor cocktail tablet [Roche] per 7.5 ml). Unbound samples were removed by washing with 5 column volumes of buffer A. The column was eluted with a gradient of buffer B (20 mM Tris [pH 7.2], 500 mM NaCl, 400 mM imidazole, 10% glycerol [vol/vol], 1 Complete Mini protease inhibitor cocktail tablet [Roche] per 7.5 ml) at a flow rate of 1 ml/min over 30 column volumes, to reach 100% buffer B. Eluted fractions were analyzed by SDS-PAGE (12.5%) and visualized with Simply blue stain (Invitrogen). Elution fractions containing 6 $\times$ His-CRP were pooled and concentrated using a centrifugal filter with a 10-kDa-molecular-mass-cutoff filter (Amicon). The protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce). The presence of the 6 $\times$ His tag was confirmed by immunoblotting using a horseradish peroxidase (HRP)-linked anti-His polyclonal antibody (AbCam, United Kingdom) and was visualized using chemiluminescence (SuperSignal West Pico chemiluminescence substrate; Thermo Scientific). The identification of CRP was further confirmed by matrix-assisted laser desorption ionization–tandem time of flight mass spectrometry (MALDI-TOF/TOF MS) on a model 4800 MALDI-TOF/TOF analyzer (AB Sciex).

EMSAs. Purified *M. smegmatis* CRP was used in electrophoretic mobility shift assays (EMSAs), performed using a DIG Gel-Shift kit, 2nd generation (Roche), to 3' end label target DNA with digoxigenin-11-ddUTP (DIG). A 233-bp probe encompassing the *cydA* promoter, designated *cydA*^{WT}, was obtained using primers HLA21 and HLA27 (see Table S1 in the supplemental material) and labeled with DIG. For mutagenesis of the CRP-binding site, mutations (CCC and TTT) of the conserved GTG and CAC nucleotides in the CRP-binding site were generated using primers HLA23 and HLA26, creating the 233-bp probe *cydA*^{mutant} (see Table S1). Binding reactions were performed by incubating 0.4 ng DIG-labeled DNA with increasing amounts of CRP (0 to 7.7 μ M) in the presence of 0.2 mM cyclic AMP (cAMP) in 10- μ l reaction volumes, unless otherwise stated. The gel shift reaction mixtures were then loaded into a 6% native acrylamide gel (37.5:1 acrylamide:bis) and were electrophoresed in 0.5 $\times$ Tris-borate-EDTA (TBE) at 300 V for 20 min. Protein-bound DIG-labeled DNA was then blotted using an Xcell II blot module (Invitrogen), fixed to a nylon N⁺ membrane (GE Healthcare), detected by a chemiluminescence immunoassay, and visualized by use of an Odyssey Fc dual-mode imaging system (Licor).

RESULTS AND DISCUSSION

Transcriptional organization of the *cydAB* and *cydDC* genes in *M. smegmatis*. Inspection of the genomes of various mycobacterial species reveals that the organization of the *cydABDC* gene cluster is conserved in species ranging from pathogenic to soil-dwelling mycobacteria (see Fig. S1 in the supplemental material). In addition, this operon-like organization is found in closely related species, such as *Rhodococcus* sp., *Nocardia farcinica*, *Corynebacterium glutamicum*, and *Streptomyces coelicolor* (see Fig. S1). To validate the *cydABDC* operon structure in mycobacteria, we performed RT-PCR to amplify the intergenic region for each of the genes of the *cydABDC* cluster in *M. smegmatis*, using RNAs extracted from *M. smegmatis* mc²155 chemostat cultures grown un-

FIG 1 RT-PCR analysis of *cydABCD* transcription. (A) Schematic of RT-PCRs performed. Open triangles indicate the annealing positions of the primers used, and sizes of expected products are indicated. Numbers below the lines correspond to the PCR products for the intergenic regions, as indicated in panel B. (B) RT-PCR analysis of the *cydABDC* operon. RT-PCR in the absence of reverse transcriptase or PCR with genomic DNA of *M. smegmatis* as a template was performed as a control reaction. gDNA, genomic DNA; +RT, RT-PCRs in the presence of reverse transcriptase; -RT, RT-PCRs in the absence of reverse transcriptase. The New England BioLabs ϕ X174 DNA HaeIII digest molecular marker and its sizes (bp) are shown on the left. Transcriptional start sites of *cydAB* (C) and *cydDC* (D) were determined by 5'-RACE (traces are given for the reverse sequences), and +1 nucleotides are indicated in bold. The start codons of *cydA* and *cydD* are indicated with arrows, putative -10 and -35 elements of the promoters are shown in bold, and putative ribosome-binding sites (*rbs*) are underlined.

der hypoxic conditions (3) (Fig. 1A). Positive controls were amplified by PCR using genomic DNA (gDNA) as the template. RT-PCR products obtained were of the same size as those from the positive controls (Fig. 1B). In order to rule out DNA contamination in RNA preparations, RT-PCR in the absence of reverse transcriptase was performed, and no products were formed (Fig. 1B),

indicating that RNA was free of genomic DNA contamination. The RT-PCR results indicated that *cydA* and *cydB*, as well as *cydD* and *cydC*, were cotranscribed (Fig. 1B). A PCR product was not detected using primers (see Table S1) spanning the intergenic region between *cydB* and *cydD* (Fig. 1B), demonstrating that *cydAB* and *cydDC* were not cotranscribed as an operon of four genes

under the conditions tested here. Previously, Megehee and Lundrigan performed RNase protection assays using a probe designed to span a 12-bp intergenic region between the *cydA* and *cydB* genes (21). Their work revealed that the probe was protected, suggesting that *cydA* and *cydB* form an operon, consistent with our data. The *cydAB* and *cydDC* genes of Gram-negative bacteria are generally found at different loci in the genome (6, 8, 22, 23). In contrast, Gram-positive bacteria usually contain *cydABDC* operons. For example, the *cydABCD* operon is polycistronic in *C. glutamicum* (24) and *B. subtilis* (9).

Mapping of the *cydAB* and *cydDC* promoters. The intergenic spacing between the stop codon of *cydB* and the start codon of *cydD* was 95 bp in *M. smegmatis*, suggesting a potential promoter region. In order to determine the transcriptional start sites (TSSs) of the *cydAB* and *cydDC* operons of *M. smegmatis*, 5'-RACE analysis was performed, and multiple replicates yielded a single 5'-RACE product. The same TSS (+1) was identified in all replicates, located in the promoter region of *cydAB*, at an adenine residue 33 bp upstream of the translational start site (Fig. 1C). Putative -10 and -35 promoter elements were determined, allowing for the design of *cydA-lacZ* promoter fusions (Fig. 1C). The TSS of *cydDC* was mapped for both replicates to a cytosine residue 9 bp upstream of the *cydD* translational start site (Fig. 1D), further validating the finding that *cydAB* and *cydDC* are not operonic in *M. smegmatis* under hypoxic conditions.

To identify the transcriptional elements controlling *cydAB* expression, a series of *cydA-lacZ* fusion constructs containing progressively truncated promoter sequences upstream of the identified transcriptional start site were constructed and introduced into *M. smegmatis* mc²155 (Fig. 2C). Cells harboring *cydA-lacZ* were grown in HdB medium with 20 mM glycerol (carbon excess) in serum vials (17) until hypoxic conditions were reached (~45 to 50 h; detected by methylene blue reduction), and *cydA-lacZ* expression was measured (Fig. 2A). As cells entered hypoxia, the level of *cydA-lacZ* increased approximately 2-fold and remained high (Fig. 2A). As the *cydA-lacZ* promoter constructs were progressively truncated from -270 to +133 bp relative to the *cydA* TSS, a significant reduction in *cydA-lacZ* expression, from 80 Miller units (MU) to 15 MU, was observed (Fig. 2C). A 51-bp region located at bp -102 to -51 upstream of the *cydA* TSS was identified as being essential for maximal *cydA-lacZ* expression in response to hypoxia (Fig. 2C).

The expression of *cydD-lacZ* increased approximately 4-fold as cells entered hypoxia (Fig. 2B). The *cydD-lacZ* constructs covering the promoter region between -290 and +130 bp relative to the *cydD* TSS were used to map the regulatory region of the *cydDC* operon (Fig. 2D). A significant reduction in *cydD-lacZ* expression, from 35 MU to 3 MU, was observed from bp -99 to -43 upstream of the *cydD* TSS (Fig. 2D), indicating that a 56-bp region was required for expression of the *cydDC* operon. Sequence analysis of the 56-bp region revealed a 10-bp inverted repeat (CGGTGGTACCGGTACCACCG) centered at -61 bp relative to the transcriptional start site.

***cydAB* is regulated by CRP in response to hypoxia.** To elucidate further genetic elements that regulate the expression of the *cydAB* operon, sequence analysis of the *cydAB* promoter was performed. Sequence analysis of the region from bp -102 to -51 revealed a possible binding site (GTGAGCTAACCCACC) for

FIG 2 Expression of *cydA-lacZ* and *cydD-lacZ* in *M. smegmatis*. (A and B) Growth and *cydA-lacZ* expression (A) or *cydD-lacZ* expression (B) of *M. smegmatis* mc²155 grown in HdB minimal medium supplemented with 20 mM glycerol in rubber-stoppered serum vials. The dotted parallel lines in panels A and B indicate the point at which methylene blue was decolorized and hence hypoxic conditions were achieved. Vertical arrows indicate the time points at which samples were taken for measurement of either *cydA-lacZ* expression or *cydD-lacZ* expression. (C and D) Physical maps of *cydA-lacZ* (C) and *cydD-lacZ* (D) fusion constructs used in expression experiments. Horizontal lines with numbers indicate promoter positions with respect to the transcriptional start site. The expression of *cydA-lacZ* and *cydD-lacZ* fusions (open bars) is reported in Miller units (MU). Results are shown as means $\pm$ standard deviations (SD) for three biological replicates. Plasmid constructs (pHLA) are listed in Table S1 in the supplemental material.

FIG 3 (A) *M. smegmatis* strain HLA107 (wild-type CRP-binding site; pHLA5) or strain HLA109 (mutated CRP-binding site; pHLA7) was grown in HdB minimal medium supplemented with 20 mM glycerol in serum vials (the growth curve is shown in panel B). Samples were taken at 42 h, and *cydA-lacZ* expression was measured (open bars). Expression of the control (pJEM15 [empty vector]) is shown. (B) *cydA-lacZ* expression in strain HLA107 (white bars; wild type) and strain HLA109 (black bars; mutant CRP motif) throughout growth. The dotted parallel line indicates the point at which methylene blue was decolorized and hence hypoxic conditions were achieved. *cydA-lacZ* expression was measured in Miller units, and results are shown as means $\pm$ SD for three biological replicates. Cell density (open squares) was monitored by measuring the OD₆₀₀. The growth curves of *M. smegmatis* strains HLA107 and HLA109 were similar, and only one of them is shown.

CRP centered at -65 bp relative to the transcriptional start site (Fig. 3A). This CRP-binding site matches the proposed *M. tuberculosis* CRP consensus sequence (GTGAN₆CCACA) at seven of eight defined bases (25). To define the significance of this putative CRP-binding site, a *cydA-lacZ* expression construct with a mutated CRP-binding site was constructed. Previously, it was shown that GTG and CAC in the CRP-binding motif (GTGAN₆CCACA) are highly conserved and are important for *M. tuberculosis* CRP binding to the promoter region (20, 25, 26). Therefore, a *cydA-lacZ* expression construct with substitutions (CCC and TTT) for these conserved GTG and CAC nucleotides was generated (i.e., CCCAN₆CTTTC). The resulting plasmid, pHLA7, was transformed into *M. smegmatis* mc²155, creating strain HLA109, and *cydA-lacZ* expression was measured in serum vials (Fig. 3). An increase in *cydA-lacZ* expression in wild-type strain HLA107 (25 MU to 80 MU) was observed upon entry into hypoxia (Fig. 3). In contrast, the *cydA-lacZ* promoter strain (HLA109) carrying the mutated CRP-binding site showed no induction of *cydA-lacZ* in response to hypoxia (Fig. 3), demonstrating that the CRP-binding site is required for the expression of the *cydAB* operon in response to hypoxia.

To determine if CRP interacts directly with the *cydAB* CRP-binding site, EMSAs were carried out with purified CRP and *cydA* promoter DNA (a 233-bp probe designated *cydA*^{WT}). EMSAs demonstrated CRP binding to *cydA*^{WT} with increasing CRP concentrations, and addition of cAMP enhanced the DNA binding of CRP (Fig. 4A and B). In order to further confirm that CRP binds to the putative CRP-binding site, the *cydA* promoter with the in-

troduced substitutions (CCC and TTT) for the conserved GTG and CAC nucleotides at the CRP-binding site was used as a probe (a 233-bp probe designated *cydA*^{mutant}). Mutations in the CRP-binding site abolished CRP binding to the *cydA*^{mutant} probe, indicating a direct and specific interaction between CRP and the CRP motif (Fig. 4B). The latter experiments also ruled out nonspecific binding of CRP to the *cydA* promoter, as binding was observed only with the 233-bp *cydA*^{WT} probe.

Concluding remarks. Our analysis of the *cydABDC* gene cluster from *M. smegmatis* provides insight into the differential regulation of this gene cluster. The *cydABDC* gene cluster was upregulated in response to hypoxia, and *cydAB* and *cydDC* were expressed as individual operons under these conditions. The expression of *cydAB* was directly regulated by the CRP protein in response to hypoxia, but the regulator of *cydDC* remains to be identified. The control of *cydAB* expression by CRP in response to oxygen-limited environments has been reported for *Shewanella oneidensis* (27), but the molecular mechanism of sensing is not known. In *M. tuberculosis*, the *cydABDC* operon is differentially expressed in response to deletion of CRP during routine *in vitro* (normoxic) growth (26). Inspection of the *cydA* promoter region revealed a putative CRP-binding site (CGTGGTGATCGGCA CA), suggesting that CRP might also be a direct regulator of *cydABDC* expression in *M. tuberculosis*. The expression of *cydA* in *M. tuberculosis* was 2.6-fold higher in the Δ *crp* (Rv3676) mutant than in the wild type during aerobic growth, suggesting that CRP is a negative regulator of *cydA* expression under these conditions. Whether CRP regulates *cydABDC* expression in response to hyp-

FIG 4 EMSAs of CRP binding to the *cydA* promoter. (A) The DIG-labeled *cydA*^{WT} DNA probe (233 bp) was incubated with increasing concentrations of CRP in the absence (lanes 2 to 5) or presence (lanes 6 to 9) of cAMP (0.2 mM). Lane 1, no CRP; lanes 2 and 6, 1 μ M CRP; lanes 3 and 7, 1.9 μ M CRP; lanes 4 and 8, 3.8 μ M CRP; lanes 5 and 9, 7.7 μ M CRP. (B) The DIG-labeled *cydA*^{WT} (left) and *cydA*^{mutant} (right) DNA probes were incubated with increasing concentrations of CRP (lanes 2 to 8) in the presence of cAMP (0.2 mM). Lanes 1 and 5, no CRP; lanes 2 and 6, 1.9 μ M CRP; lanes 3 and 7, 3.8 μ M CRP; lanes 4 and 8, 7.7 μ M CRP. The gel shift reaction mixtures were electrophoresed in a 6% native acrylamide gel (37.5:1 acrylamide:bis) at 300 V for 20 min. Protein-bound DIG-labeled DNA was visualized using an Odyssey Fc dual-mode imaging system (Licor).

oxia remains to be determined experimentally for *M. tuberculosis*. Mutation of the CRP-binding site in the *M. smegmatis* *cydAB* promoter abolished *cydAB* induction in response to hypoxia but did not prevent expression under normoxia, suggesting that additional regulatory proteins may bind at this promoter. This is consistent with previous reports that RegX3 of the SenX3-RegX3 two-component regulatory system of *M. tuberculosis* is required for maximal *cydA-lacZ* expression (14).

The signal(s) that CRP senses in response to hypoxia in mycobacterial species remains unclear. Mycobacterial CRP lacks typical redox-sensing domains, and *crp* expression is not under the control of DosR, a regulator of hypoxic gene expression in mycobacteria. Taken together, these observations suggest that CRP might not sense hypoxia *per se*. In *E. coli*, the traditional view of cAMP-CRP signaling has centered on carbon catabolite repression (CCR) to achieve hierarchical carbon usage. However, a recent study demonstrated that cAMP-CRP signaling mediates more than CCR and is involved in coordinating the expression of catabolic proteins with biosynthetic (anabolic) and ribosomal proteins in response to cellular metabolic demands (28). This study suggests that CRP is able to sense the anabolic demands of the cell and to allocate resources appropriately in response to growth rate. In *M. smegmatis*, CRP-dependent *cydAB* expression is increased 2-fold in response to hypoxia when carbon (glycerol) is in excess. Under extreme carbon (glycerol) limitation in continuous culture, however, *cydAB* is upregulated 50-fold in response to hypoxia (3). These data demonstrate that the expression of *cydAB* in *M. smegmatis* is linked not only to hypoxia but also to the anabolic and catabolic rates of the cell, suggesting a more complex molecular signaling network that remains to be elucidated.

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