Novel regulatory roles of cAMP receptor proteins in fast-growing environmental mycobacteria

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Mycobacterium smegmatis is a fast-growing, saprophytic, mycobacterial species that contains two cAMP-receptor protein (CRP) homologues designated herein as Crp1 and Crp2. Phylogenetic analysis suggests that Crp1 (Msmeg_0539) is uniquely present in fast-growing environmental mycobacteria, whereas Crp2 (Msmeg_6189) occurs in both fast- and slow-growing species. A crp1 mutant of M. smegmatis was readily obtained, but crp2 could not be deleted, suggesting it was essential for growth. A total of 239 genes were differentially regulated in response to crp1 deletion (loss of function), including genes coding for mycobacterial energy generation, solute transport and catabolism of carbon sources. To assess the role of Crp2 in M. smegmatis, the crp2 gene was overexpressed (gain of function) and transcriptional profiling studies revealed that 58 genes were differentially regulated. Identification of the CRP promoter consensus in M. smegmatis showed that both Crp1 and Crp2 recognized the same consensus sequence (TGTGNa8CACA). Comparison of the Crp1- and Crp2-regulated genes revealed distinct but overlapping regulons with 11 genes in common, including those of the succinate dehydrogenase operon (MSMEG_0417-0420, sdh1). Expression of the sdh1 operon was negatively regulated by Crp1 and positively regulated by Crp2. Electrophoretic mobility shift assays with purified Crp1 and Crp2 demonstrated that Crp1 binding to the sdh1 promoter was cAMP-independent whereas Crp2 binding was cAMP-dependent. These data suggest that Crp1 and Crp2 respond to distinct signalling pathways in M. smegmatis to coordinate gene expression in response to carbon and energy supply.

INTRODUCTION

cAMP receptor proteins (CRPs) are members of the CRP–FNR (fumarate nitrate reductase regulator) superfamily of transcription factors. The ~370 family members control a wide range of physiological functions in a diverse set of bacteria. Thus, different members of the family regulate carbon, sulfur and nitrogen metabolism, denitrification, nitrogen fixation, aerobic and anaerobic respiration and virulence gene expression in response to a range of environmental and metabolic signals (Green et al., 2001, 2014; Kolb et al., 1993; Shimada et al., 2011). Members of the CRP–FNR family are homodimeric proteins with a sensory domain and a helix–turn–helix DNA-binding domain located at the N- and C-terminal regions, respectively, of each protomer (Weber & Steitz, 1987). The archetypal CRP fold is a versatile structure and its N-terminal domain has

Abbreviations: CCR, carbon catabolite repression; CRP, cAMP-receptor protein; ED pathway; Entner–Doudoroff pathway; EMP pathway; Embden–Meyerhof–Parnas pathway; EMSA, electrophoretic mobility shift assay; FNR, fumarate nitrate reductase regulator; qRT-PCR, quantitative real-time PCR.

Three supplementary tables and six supplementary figures are available with the online Supplementary Material.

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evolved to accommodate different sensory modules to respond to a diverse range of signals (Green et al., 2001; Körner et al., 2003).

In *Escherichia coli*, CRP controls gene expression in response to changes in intracellular cAMP concentration. Carbon catabolite repression (CCR) is a vital part of a global control system in bacteria to achieve hierarchical carbon usage (Kovárová-Kovar & Egli, 1998). During this process, depletion of glucose leads to activation of adenylyl cyclase by the phosphorylated form of the EIIA protein of the phosphotransferase system (PTS) (Saier & Reizer, 1994). As a result of this activation, the levels of cAMP increase, which in turn binds to CRP, resulting in the formation of the cAMP–CRP binary complex (Saier & Reizer, 1994). The cAMP–CRP complex binds at promoters containing a specific DNA sequence (consensus 5′-TGTGAN₆TCACA-3′) to regulate expression of the downstream genes (Berg & von Hippel, 1988). In addition to mediating CCR, a recent study demonstrated that cAMP–CRP signalling is involved in coordinating the expression of catabolic proteins with biosynthetic (anabolic) and ribosomal proteins in response to cellular metabolic demands (You et al., 2013). There are currently estimated to be between 378 and ~500 *E. coli* genes under the control of CAMP–CRP, including those encoding the transporters and catabolism of glucose and the process of aerobic respiration, demonstrating that CRP is a global regulator of gene expression in *E. coli* (Perrenoud & Sauer, 2005; Shimada et al., 2011).

Most mycobacterial genomes harbour 10 or more genes encoding purported adenylyl cyclases in contrast to the single adenylyl cyclase found in *E. coli* (Shenoy & Visweswariah, 2006). For example, *Mycobacterium tuberculosis* harbours 16 genes encoding adenylyl cyclases (McCue et al., 2000; Shenoy et al., 2004). Consequently, intracellular cAMP concentrations in mycobacterial cells are considered to be ~100-fold higher than those of other bacteria (Dass et al., 2008; Padh & Venkitasubramanian, 1976, 1980), but the significance of this is not known. Secretion of CAMP directly into host macrophages after infection has been reported and is thought to be important in tuberculosis pathogenesis (Agarwal et al., 2009; Bai et al., 2009; Lowrie et al., 1975). *M. tuberculosis* H37Rv contains a single CRP–FNR homologue encoded by the gene *Rv3676* (Cole et al., 1998), which is 32% identical to the *E. coli* CRP over 189 amino acid residues. Most of the amino acid residues contributing to cAMP binding and DNA binding in *E. coli* CRP are conserved in *M. tuberculosis* CRP (Bai et al., 2005; Rickman et al., 2005). *M. tuberculosis* CRP has been reported to regulate expression of genes required to control metabolism and growth under hypoxic and starvation conditions (Bai et al., 2005). Furthermore, deletion of *Rv3676* (crp) resulted in impaired growth in macrophage cell lines and in mice, indicative of a role of CRP (Rv3676) in tuberculosis pathogenesis (Hunt et al., 2008; Rickman et al., 2005).

Unlike *M. tuberculosis*, the genome of the fast-growing environmental saprophyte *Mycobacterium smegmatis* mc²155 encodes two purported CRPs, MSMEG_0539 (Crp1) and MSMEG_6189 (Crp2) (http://cmr.jcvi.org/). The presence of multiple copies of CRP in bacterial genomes is unusual and warrants investigation. In this communication, we show that Crp1 and Crp2 of *M. smegmatis* have distinct roles in controlling transport and catabolism of carbon sources and components of the electron transport chain for energy generation.

### METHODS

**Bacterial strains and cultivation conditions.** *E. coli* DH10B was grown in Luria–Bertani (LB) (Sambrook & Russell, 2001) medium at 37 °C with agitation at 200 r.p.m. or on LB agar plates (Table S1, available in the online Supplementary Material). *M. smegmatis* mc²155 (Snapper et al., 1990) and derived strains (Table S1) were grown in LB supplemented with 0.05% (w/v) Tween 80 (Sigma Aldrich) (LBT), Hartmans–de Bont (HdB) minimal medium supplemented with 10 mM glycerol and 0.05% (w/v) Tween 80 unless otherwise stated or on LBT agar plates. All *M. smegmatis* strains were inoculated to an initial optical density of 0.005 and grown at 37 °C with agitation at 200 r.p.m. Samples (2 ml) to measure β-galactosidase expression were taken (see below), the optical density was measured and samples were then stored at −20 °C. All solid media contained 1.5% agar and liquid media contained ampicillin (100 µg ml⁻¹), kanamycin (50 µg ml⁻¹ for *E. coli*; 20 µg ml⁻¹ for *M. smegmatis*), hygromycin B (200 µg ml⁻¹ for *E. coli*; 50 µg ml⁻¹ for *M. smegmatis*) or tetracycline (20 ng ml⁻¹ for *M. smegmatis*). The optical density was measured at 600 nm (OD₆₀₀) in a Jenway 6300 spectrometer. All strains, plasmids and primers used in this study are listed in Table S1.

**Phylogenetic analysis.** Protein sequences of *crp* homologues were imported into MEGA 5 software (Tamura et al., 2011) from MicrobesOnline (Dehal et al., 2010) and the Comprehensive Microbial Resource (Peterson et al., 2001). Evolutionary history was reconstructed using the neighbour-joining method with a bootstrap test (1000 replicates) to determine the percentage of replicate trees in which the associated taxa clustered together. Branch lengths were represented in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Phylogenetic analyses were performed using MEGA 5 (Tamura et al., 2011).

**Construction of *M. smegmatis* mutants and complementation vector.** All molecular biology techniques were performed according to standard procedures (Sambrook & Russell, 2001). Restriction enzymes and other molecular biology reagents were obtained from Roche Diagnostics or New England Biolabs. Genomic DNA of *M. smegmatis* was isolated as described previously (Gebhard et al., 1990) and derived strains (Table S1) were used as a template for overlap extension PCR (Ho et al., 1989). Similarly, a 1012 bp fragment upstream of *crp1* including a 115 bp coding sequence was amplified with primers HLA39 and HLA40, and a 926 bp fragment downstream of *crp1* including a 37 bp coding sequence was amplified with primers HLA38 and HLA39. The 102 bp coding sequence was amplified with primers HLA36 and HLA37. These two PCR products were used as a template for overlap extension PCR (Ho et al., 1989) and the product was then cloned into the SpeI site of the pX33 vector (Gebhard et al., 2006), the pPR23-derived vector (Pelicic et al., 1997), resulting in pHLA13, and transformed into *M. smegmatis* mc²155. The *crp1* gene was then deleted using the two-step method for integration and excision of the plasmid as described previously (Tran & Cook, 2005). Similarly, a 1012 bp fragment upstream of *crp2* including a 156 bp coding sequence was amplified with primers HLA39 and HLA40, and a 926 bp fragment downstream of *crp2* including a 102 bp coding sequence was amplified with primers.
HLA40 and HLA41. The construct for making a crp2 deletion strain was made as described above, resulting in pHLA14, and deletion was performed as described above. In addition, a fragment containing a 500 bp coding sequence of crp2 was amplified and cloned into the SpeI site of the pX33 vector, resulting in pHLA15. The deletion of genes was confirmed by Southern analysis using the Amersham Gene Images AlkPhos Direct Labelling and Detection System with CDP-Star detection reagent (GE Healthcare) according to the manufacturer’s protocol.

To complement the crp1 deletion mutant with the crp1 gene, crp1 was amplified from genomic DNA using the primers HLA54 and HLA55 (Table S1) and the Phusion High-Fidelity PCR kit (New England Biolabs). The product was then cloned into the multiple cloning site of E. coli-mycobacteria shuttle vector pMV261 (Stover et al., 1991) using restriction sites EcoRI and HindIII to create pHLA21. The plasmid was then used to transform E. coli DH10B for amplification, and the sequence of the plasmid was checked using the PCR primers described above. The correct construct was used to transform the M. smegmatis crp1 mutant strain. In addition, the vector pMV261 was used to transform the M. smegmatis wild-type and mutant strains.

Construction of tetracycline inducible construct. To create the Crp2 tetracycline inducible expression construct, a 678 bp PCR product of the crp2 gene was amplified from M. smegmatis mc²155 using primers HLA52, containing a consensus ribosome-binding site sequence GGAGG upstream of crp2, and HLA53. The product was digested with NdeI and SpeI and ligated into the tetracycline inducible vector pMind (Blokker et al., 2005), digested with the same restriction enzymes. The resulting construct was confirmed via sequencing and was designated pHLA20. M. smegmatis mc²155 strains HLA126 and HLA107, harbouring plasmids pMind and pHLA20, respectively, were grown in HdB minimal medium supplemented with 10 mM glycerol and 0.05 % (w/v) Tween 80 to an OD₆₀₀ of 0.3, and expression was then induced with tetracycline (20 ng ml⁻¹). All cultures were supplemented with hygromycin B.

Construction of promoter transcriptional fusions. To measure promoter activity, crp1–lacZ, MSSMEG_0420–lacZ and sdhB–lacZ fusion constructs were created using the primers listed in Table S1. The 500 bp upstream of the crp1 translational start site, 300 bp upstream of the crp2 translational start site, 227 bp upstream of the MSSMEG_0420 translational start site or 441 bp upstream of the sdhB translational start site were PCR amplified with the primers listed in Table S1 and ligated into the BamHI and KpnI restriction sites of the vector pEM15 to create plasmids pHLA25, pHLA5, pEM007 and pEM113, respectively. All the promoter regions amplified by PCR were confirmed by DNA sequencing. The plasmids were used to transform M. smegmatis mc²155 by electroporation. β-Galactosidase assays were performed as described previously (Gebhard et al., 2006).

Carbon source concentration assay. Cultures were grown in HdB with glycerol and glucose, glycerol and pyruvate or glucose and pyruvate. Supernatant samples were taken after initial inoculation to determine the starting carbon concentration of each individual flask and at specified time points during growth. All culture samples were centrifuged (13 000 r.p.m. 16 000 g, 5 min) to obtain cell-free supernatant for use in carbon source concentration assay and stored at −20 °C. Glycerol concentration was measured by detecting NADH oxidation (A₅₃₀) using the methods described by Pinter et al. (1967). The assay solution contained: 0.5 U glycerokinase ml⁻¹ from E. coli (Sigma Aldrich), 0.5 U pyruvate kinase ml⁻¹ from rabbit muscle (Sigma Aldrich), 1 U lactate dehydrogenase from ml⁻¹ from rabbit muscle (Roche), 50 mM Tris/HCl (pH 8), 2 mM MgCl₂, 0.25 mM NADH, 3 mM phosphoenolpyruvate and 3 mM ATP. Approximately 100 µl of each sample was added to 1 ml of assay solution, mixed and incubated at 37 °C for 15 min to allow the enzyme assay to reach an end point. Similarly, glucose levels were determined using a hexokinase-based assay (Bergmeyer et al., 1983). The assay solution contained: 25 ml of a 50 mM Tris/HCl (pH 7.5) solution, 20 mg MgCl₂, 10 mg ATP, 10 mg NADP and 75 µl of a hexokinase/glucose 6-phosphate dehydrogenase (G6P-DH) enzyme mix (Roche) that is equivalent to approximately 25 U hexokinase activity and 13 U glucose 6-phosphate dehydrogenase activity. Approximately 150 µl of each sample was added to 1 ml of assay solution, mixed and incubated at 25 °C for 30 min to allow the enzyme assay to reach an end point. Pyruvate levels were determined by detecting NADH oxidation (A₅₃₀) using a modified version of the method of Pinter et al. (1967). The assay solution contained: 1 U lactic dehydrogenase ml⁻¹ from rabbit muscle (Roche), 50 mM Tris/HCl (pH 8), 2 mM MgCl₂, 0.25 mM NADH and 3 mM ATP. Approximately 100 µl of each sample was added to 1 ml of assay solution, mixed and incubated at 37 °C for 15 min to allow the enzyme assay to reach an end point. The A₅₃₀ of each sample was measured and a standard curve of absorbance versus carbon source concentration was analysed with each set of samples, which was prepared from either 10 mM glycerol, 10 mM glucose or 10 mM pyruvate stock.

RNA extraction, microarray analysis and quantitative real-time PCR (qRT-PCR). To extract total RNA, cells were resuspended in TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Cells were lysed with two rounds of bead beating in a Mini-Beadbeater (Biospec) at 5000 r.p.m. for 30 s. DNA was removed from the RNA preparation by treatment with 2 U RNase-free DNase using the TURBO DNA-free kit (Ambion) according to the manufacturer’s instructions. The quality of RNA was checked on a 1% agarose gel and the concentration was determined using a NanoDrop ND-1000 spectrophotometer. Microarray analysis was performed as described previously (Berney & Cook, 2010; Berney et al., 2012) using arrays provided by the Pathogen Functional Genomics Research Centre funded by the National Institute of Allergy and Infectious Diseases using protocols SOP# M007 and M008 from The Institute of Genomic Research. Gene expression was calculated from the normalized signal intensities from replicate spots within a single slide and averaged for each set of biological replicates before expression ratios were calculated. The results from four biological replicates, which included two dye swaps, were then subjected to a t-test without false discovery correction in The Institute of Genomic Research MeV software (version 4.3.02). The mean value to be tested against was set to 1 with a critical P-value of 0.05. The analysis was used as a ranking method. For a general overview, genes with expression ratios ≥1.4 and ≤0.7 and a P-value of ≤0.05 were used for data interpretation. All data have been deposited at Gene Expression Omnibus (NCBI) with accession number GSE56057. qRT-PCR was performed as described previously (Berney & Cook, 2010). The sigA gene was used as an internal standard and the ΔACT method was used for calculation of gene expression ratios. Error bars represent standard deviations from three biological replicates.

Recombinant expression and purification of Crp1 and Crp2 from M. smegmatis mc²155. The crp1 gene sequence of M. smegmatis mc²155 was amplified by PCR using primers HLA64 and HLA65 and cloned into pQE80L, which contains an N-terminal His-tag (Qiagen), using KpnI and HindIII restriction sites (Table S1). The resulting plasmid pHLA27 was used to transform the expression strain JRG5876 E. coli BL21(DE3Acyn) which is unable to synthesize cAMP (Stapleton et al., 2010). The authors validated that the recombinant protein was not bound to cAMP (Stapleton et al., 2010). Cultures were grown in 1 liters flasks with 500 ml LB medium supplemented with 100 µg ampicillin ml⁻¹ at 37 °C with agitation at 200 r.p.m. until an OD₆₀₀ of approximately 0.5 was reached. Expression was then induced by the addition of IPTG (1 mM final concentration) prior to an additional 4 h of growth. Cells were harvested by centrifugation (7500 r.p.m. 7519 g, 4 °C, 15 min), washed and resuspended in lysis buffer (150 mM Tris/HCl, 2 mM
MGCl₂ 1 % glycerol, one Complete Mini protease inhibitors cocktail tablet (Roche) per 7.5 ml and 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 p.s.i. (138 MPa). Unbroken cells were then removed by centrifugation (10 000 r.p.m. 11 872 g, 10 min, 4 °C) and the cell-free supernatant was collected from the membranes by ultracentrifugation (45 000 r.p.m. 207 871 g, 45 min, 4 °C). The supernatant containing cytoplasmic proteins was then loaded at a flow rate of 0.5 ml min⁻¹ onto a 1 ml HisTrap (GE Healthcare) equilibrated with 10 column volumes of buffer A [20 mM Tris (pH 7.2), 500 mM NaCl, 20 mM imidazole, 10 % glycerol (v/v)], one Complete Mini protease inhibitors cocktail tablet (Roche) per 7.5 ml. Unbound samples were removed by washing with five 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 (v/v), one Complete Mini protease inhibitors cocktail tablet (Roche) per 7.5 ml] at a flow rate of 1 ml min⁻¹ over 30 column volumes, reaching 100 % B. Eluted fractions were analysed by SDS-PAGE (12.5 %) and visualized with Simply blue stain (Invitrogen). Elution fractions containing hexa-histidine-tagged Cp1 were pooled and concentrated using a centrifugal filter with a 10 kDA molecular mass cut-off filter (Amicon). Protein concentration was determined using a BCA protein assay kit (Pierce). The presence of the 6 × His tag was confirmed by immunoblotting using a horseradish peroxidase-linked anti-His polyclonal antibody (AbCam) and visualized using chemiluminescence (SuperSignal West Pico chemiluminescent substrate; Thermo Scientific). The identification of Cp1 was further confirmed by MALDI TOF/TOF MS on a 4800 MALDI TOF/TOF analyser (AB Scienx).

For Cp2 expression, the expression plasmid pNS24, a PET28a (Invitrogen) derivative encoding a His₆–MSMEG_6189 fusion protein, was constructed using primers PRNS_17F and PRNS_17R (Table S1). The plasmid was used to transform electro-competent E. coli strain JRG5876 (Stapleton et al., 2010) and the Cp2 protein was typically overproduced by culturing the resulting strain in a 2 l flask containing 500 ml Lennox Broth (5 g NaCl, 5 g yeast extract, 10 g tryptone) with 200 µg ampicillin ml⁻¹, inoculated 1:100 from an overnight culture. The cultures were grown aerobically at 37 °C with shaking (250 r.p.m.) until an OD₆₀₀ of ~0.6 was reached. IPTG was then added (up to 120 µM ml⁻¹ final concentration). Cultures were then incubated at 37 °C with shaking (250 r.p.m.) for 2–3 h before collecting the bacteria by centrifugation at 11 900 g for 30 min at 4 °C. Cell pellets were resuspended in 10 ml of binding buffer (20 mM sodium phosphate, 0.5 M NaCl, pH 7.4) and lysed by two passages through a French pressure cell at 20 000 p.s.i. (138 MPa). The soluble and insoluble fractions were separated by centrifugation at 39 190 g for 15 min at 4 °C. The soluble fraction was used immediately for purification of His-tagged Cp2 by nickel affinity chromatography. Purification was performed using the His-tag purification program for His tags on a 1 ml HiTrap chelating column of the AKTA prime machine (GE Healthcare) according to the manufacturer’s instructions. The bound His-tagged Cp2 protein was eluted by applying an imidazole gradient (0–0.5 M in 20 mM sodium phosphate, 0.5 M NaCl, pH 7.4). The fractions collected were stored at 4 °C and samples were analysed by SDS-PAGE to locate the target protein.

Electrophoretic mobility shift assays. Purified M. smegmatis Cp1 was used in electrophoretic mobility shift assays (EMSA) using a 2nd Generation DIG Gel-Shift kit (Roche) to 3′-end-label target DNA with DIG. The 227 and 431 bp probes designated sdh1 and shd2 were obtained using the primers described in Table S1. Binding reactions were performed by incubating 0.4 ng DIG-labelled DNA with Cp1 in the presence or absence of 0.2 mM cAMP in 10 μl reaction volumes. The gel-shift reactions were then loaded into a 6 % native acrylamide gel (37.5:1 acrylamide/bis) and were electrophoresed in 0.5 × TBE (44.5 mM Tris, 44.4 mM borate and 10 mM EDTA) at 300 V for 20 min. Protein-bound DIG-labelled DNA was then blotted using an Xcell II Blot Module (Invitrogen) and fixed to a nylon N⁺ membrane (GE Healthcare) and was detected by a chemiluminescent immunoassay and visualized using an ODYSSEY Fc Dual Mode Imaging System (LiCOR).

To assess Cp2 binding, promoter regions were radiolabelled by addition of [α-32P]-dCTP [1 μCi (37 GBq) at ~3000 Ci mmol⁻¹] to PCRs containing M. smegmatis genomic DNA and the desired primers (Table S1). Amplified radiolabelled DNA was purified using a Qiagen PCR purification kit. EMSAs were carried out with radiolabelled promoter DNA (200 bp sdh1 and 200 bp shd2) and varying concentrations of Cp2. The Cp2 protein was pre-incubated at 20 °C for 30 min, with or without cAMP as indicated, in 100 mM NaCl, 50 mM Tris (pH 7.5), 10 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT and 0.25 mg BSA ml⁻¹, before the addition of radiolabelled DNA (~3 ng) for a further 10 min (total reaction volume of 15 μl). Calf thymus DNA (~1 μg) was used as a non-specific competitor in all assays. After incubation, loading buffer (2 μl of 50 % glycerol, 0.25 % bromophenol blue) was added to the samples immediately before applying to 6 or 10 % polyacrylamide gels buffered with 0.5 × TBE. Electrophoresis was carried out at 30 mA for ~30 min with 0.5 × TBE running buffer. The gels were then dried and visualized by autoradiography.

RESULTS AND DISCUSSION

Fast-growing environmental mycobacteria possess two functional CRPs

The CRP protein from E. coli is the founding member of the CRP–FNR superfamily of transcriptional regulators. Cp1 and Cp2 of M. smegmatis are 34 and 32 % identical to E. coli CRP over 189 aa, respectively, and possess several conserved residues involved in both cAMP binding and DNA recognition (Schultz et al., 1991) (Fig. 1). Cp1 and Cp2 of M. smegmatis are, respectively, 76 and 97 % identical to M. tuberculosis CRP (Fig. 1). Amino acid residues (from Arg218 to Asn224) in the C-terminal region of Cp1 and Cp2 form an additional helix (G-helix) that is absent in E. coli CRP (Fig. 1). Multiple sequence alignment of CRP from different bacterial species across different phyla demonstrated that the additional C-terminal helix is present only in actinomycetes, including Mycobacterium, Rhodococcus, Gordonia, Corynebacterium and Streptomyces, but is absent in non-actinomycetes, including E. coli, Vibrio cholerae and Pseudomonas aeruginosa (Fig. S1). The role of this helix remains to be elucidated in actinomycetes. In M. tuberculosis, deletion of this C-terminal helix resulted in an insoluble form of the protein, demonstrating that it was required for correct folding of CRP, but making it difficult for functional studies to be performed (Kumar et al., 2010).

To establish a phylogenetic relationship for Cp1 and Cp2 in M. smegmatis, the amino acid sequences of the CRPs from other mycobacterial species, as well as from those belonging to the genera Streptomyces, Corynebacterium and Escherichia were compared using MEGA 5 (Tamura et al., 2011) (Fig. S2). The resulting tree was calculated based on the similarity of the CRP sequences and created by the neighbour-joining method (Saitou & Nei, 1987). It is noteworthy that only fast-growing environmental mycobacteria such as M.
smegmatis and Mycobacterium gilvum possessed two CRPs, whilst slow-growing pathogenic mycobacteria had only one CRP (Fig. S2). M. tuberculosis harbours an additional cAMP-binding protein, designated Cmr (Rv1675c) (cAMP and macrophage regulator) (Gazdik & McDonough, 2005; Gazdik et al., 2009). Orthologues of Cmr are present in other slow-growing mycobacteria and the Cmr regulon is distinct from the M. tuberculosis CRP regulon (Gazdik et al., 2009). Phylogenetic analysis revealed that Cmr from slow-growing mycobacterial species forms a distinct cluster (Fig. S2) and both Crp1 and Crp2 of M. smegmatis, as well as CRP of M. tuberculosis Cmr. These observations suggest that Crp1 found only in fast-growing environmental mycobacteria is distinct from Cmr.

**Mutational analyses of Crp1 and Crp2 in M. smegmatis indicate an essential role for Crp2**

To establish the expression profile of the crp1 and crp2 genes in M. smegmatis, we first constructed transcriptional crp1–lacZ and crp2–lacZ fusions and studied gene expression throughout growth using HdB minimal medium supplemented with 10 mM glycerol. Expression of crp1–lacZ was constitutive throughout the growth cycle, with a mean promoter activity of 67 MU. Similar to crp1 expression, that of crp2–lacZ was constitutive throughout the growth cycle with a mean promoter activity of 153 MU. To determine the physiological roles of Crp1 and Crp2 in M. smegmatis, we set out to construct unmarked non-polar deletions of each gene. A Dcrp1 mutant was readily obtained (Fig. S3), but a Dcrp2 deletion mutant could not be obtained using the same method. Southern hybridization of putative Dcrp2 mutants was consistent with the presence of wild-type crp2, even after several attempts and screening a large number of colonies (131) (Fig. S3). To further validate the essentiality of Crp2, we employed a single cross-over strategy that would integrate into the chromosome within the coding sequence of crp2, resulting in disruption of the crp2 gene (Fig. S3f). We hypothesized that if the crp2 gene was non-essential for
growth, a crp2 integrant would be obtained after the recombination event. No integrant was obtained despite prolonged incubation at 37 °C for 10 days, suggesting that Crp2 was essential for growth of M. smegmatis under the conditions tested herein. In M. tuberculosis, Crp2 is non-essential for growth, but Δcrp2 mutants show impaired growth in macrophages and in mice (Bai et al., 2011; Rickman et al., 2005).

Next, the phenotypic effect of crp1 and crp2 was assessed using HdB minimal medium supplemented with either 5 mM glucose or 10 mM glycerol under aerobic and hypoxic growth conditions (Fig. S4). As crp2 could not be deleted using conventional allelic exchange mutagenesis, we overexpressed Crp2 using a tetracycline inducible plasmid pMind (Blokpoo et al., 2005) and performed growth analysis. Crp2 was induced with tetracycline (20 ng ml⁻¹) in HdB minimal medium supplemented with either 5 mM glucose or 10 mM glycerol at an OD₆₀₀ of 0.3 and monitoring of growth. No significant phenotypic effect of crp2 overexpression was observed under aerobic growth conditions (Fig. S4a and b), whereas there was a strong growth phenotype with the Δcrp1 mutant on glucose (Fig. 2c), but not on glycerol (Fig. S5a). Growth analysis of both the crp1 deletion mutant and the crp2 overexpression mutant in HdB minimal medium supplemented with 10 mM glycerol under hypoxic conditions was also performed as described previously (Aung et al., 2014; Berney et al., 2012). However, no profound phenotypic effect was observed under these conditions (Fig. S4c and d).

**Identification of genes under Crp1 and Crp2 control in M. smegmatis**

Microarray analysis was performed to establish the Crp1 regulon using RNA extracted from exponential phase (OD₆₀₀ of ~0.5) cultures of M. smegmatis wild-type and the Δcrp1 mutant grown on HdB minimal medium supplemented with 10 mM glycerol (Fig. S5a). Microarray analysis revealed 239 genes that were differentially regulated, including 170 that were upregulated (≥1.4-fold) and 69 that were downregulated (≤1.4-fold) in response to the loss of crp1 function (P<0.05) (Table S2). The M. smegmatis Crp1 consensus sequence was identified as described by Hümpel et al., 2010) and designated TGTGN₈CACA. A histogram of genes differentially expressed in response to crp1 deletion within functional categories is shown in Fig. S5(c).

**Crp1 regulation of solute transport and carbon metabolism in M. smegmatis**

Microarray analysis revealed that 15% of the genes that were differentially regulated in response to crp1 deletion are involved in the transport and catabolism of carbohydrates (Table S2; Fig. S5c). Many of these proposed genes were upregulated in response to deletion of crp1, suggesting Crp1 repressed the expression of these genes (Table S2). These included operons for five carbon sugar transporters (namely MSMEG_1370–1374, MSMEG_1704–1706, MSMEG_1713–1715), a glucose, trehalose, N-acetylglucosamine transporter belonging to the sugar PTS family (MSMEG_2116–2119) and a glycerol transporter of the PTS family encoded by MSMEG_2121–2124 (Table S2) (functions reviewed by Tigtgemyer et al., 2007). To determine the physiological effect of the de-repression of genes involved in transport and catabolism of carbohydrates, growth analysis was performed using HdB minimal medium supplemented with either arabinose, xylose or glucose (Fig. 2) because the transporters...
for these sugars were upregulated in the Δcrp1 strain (Table S2). The Δcrp1 mutant had a faster growth rate on xylose, arabinose and glucose compared with the isogenic wild-type strain (Fig. 2). The wild-type phenotype could be restored in the Δcrp1 mutant by in trans complementation with crp1, suggesting that the observed growth phenotype was due to the deletion of crp1 (Fig. 2). Glycolysis is one of the main pathways of central carbon metabolism in mycobacteria. A limited number of genes encoding the enzymes involved in glycolysis were downregulated in response to crp1 deletion. These included glucose 6-phosphate isomerase, fructose biphosphate aldolase, triosephosphate isomerase (tpiA) and phosphoglycerate kinase (pgk) (Table S2). In addition, genes involved in the transport of amino acids, polyamines and spermidine were upregulated in response to crp1 deletion (Table S2).

The Entner–Doudoroff (ED) pathway is present in M. smegmatis (Bai et al., 1976), and transcripts encoding enzymes of this pathway [MSMEG_0314 (zwf), MSMEG_0313 (edd), MSMEG_0312 (eda)] were upregulated in the Δcrp1 mutant (Table S2). Prokaryotes often contain several glycolytic pathways, of which the ED pathway is the most common after the canonical Embden–Meyerhof–Parnas (EMP) glycolytic pathway. The microarray data suggest that Crp1 controls the differential expression of the ED and EMP pathways. In wild-type cells, Crp1 appears to repress the ED pathway to ensure carbon is routed through the EMP pathway. Flamholz et al. (2013) showed that the EMP pathway incurs a 3.5-fold higher protein cost compared with the ED pathway, but this is offset by the higher ATP yield of the EMP pathway versus the ED pathway. CRP mediates the hierarchical utilization of carbon sources in many bacteria, resulting in a diauxic pattern

**Fig. 3.** Growth and pattern of carbon source utilization by M. smegmatis wild-type (a, c, e) and Δcrp1 mutant (b, d, f) in HdB medium supplemented with 2.5 mM glucose and 5 mM glycerol (a, b), 2.5 mM glucose and 5 mM pyruvate (c, d) or 5 mM glycerol and 5 mM pyruvate (e, f). M. smegmatis wild-type and Δcrp1 mutant were grown in flasks at 37 °C for 39 h under aerobic conditions. Quantification of substrates from culture supernatants was performed using an enzyme assay (see Methods). Results shown are means ± SD of three biological replicates.
of growth (Saier, 1989). The ability of *M. smegmatis* wild-type and the Δcrp1 mutant to metabolize gluconate, a substrate of the ED pathway, was assessed (Fig. S6a). Both the wild-type and the Δcrp1 mutant were able to grow on gluconate and no significant difference in growth rate was observed (Fig. S6a), whereas growth analysis with arabinose and xylose revealed differences in growth rate (Fig. S6b and c). Despite Crp1 controlling expression of genes involved in the glycolytic flux through the EMP and ED pathways, Crp1 had no effect on the pattern of sugar utilization in *M. smegmatis* (Fig. 3). For example, when *M. smegmatis* (wild-type versus Δcrp1) was grown on equimolar mixtures of glycerol and glucose (Fig. 3a and b), glucose and pyruvate (Fig. 3c and d) or glycerol and pyruvate (Fig. 3e and f) the same pattern of carbon source utilization was noted between the wild-type strain and the Δcrp1 mutant. In most cases the carbon sources were used simultaneously and no diauxic was noted (Fig. 3). Simultaneous use of carbon substrates and an apparent lack of diauxic growth has been reported previously in *M. tuberculosis* (de Carvalho et al., 2010).

**Crp1 regulates respiratory energy metabolism in *M. smegmatis***

Microarray analysis revealed that 15% of the differentially regulated genes in the *M. smegmatis* Δcrp1 were involved in energy generation, including genes that encode components of the oxidative phosphorylation (Table S2). These included gene clusters for the F1F0-ATP synthase, type I proton-translocating NADH–quinone oxidoreductase (*nuo*), succinate dehydrogenase 1 (MSMEG_0417–0420, Sdh1), succinate dehydrogenase 2 (MSMEG_1669–1672, Sdh2) and the cytochrome bd terminal oxidase (Table S2). To further validate this observation, qRT-PCR was performed on selected genes encoding components of the mycobacterial electron transport chain for the wild-type and Δcrp1 mutant (Fig. 4a). These experiments confirmed the regulatory patterns revealed by the microarray analysis of the *M. smegmatis* Δcrp1 mutant.

To further investigate Crp1 regulation of the Sdh1 and Sdh2 operons, *sdh* promoter activity was measured using promoter–lacZ fusions in HdB minimal medium supplemented with 10 mM glycerol (Fig. 4b). Consistent with the microarray and qRT-PCR data (Fig. 4b), there was a 5.0-fold increase in promoter activity of the *sdh1* construct in the Δcrp1 strain, indicating that Crp1 was a repressor of *sdh1* expression (Fig. 4b). In contrast, the promoter activity of *sdh2* construct was approximately 1.4-fold lowered in the Δcrp1 strain, suggesting Crp1 was an activator of *sdh2* expression (Fig. 4b).

**Crp2 regulon of *M. smegmatis* reveals diverse gene functions**

As described above, *crp2* could not be deleted using conventional allelic exchange mutagenesis, and we therefore overexpressed Crp2 using a tetracycline inducible plasmid pMind (Blokpoe et al., 2005) and performed transcriptional profiling analysis from RNA extracted from exponential phase (OD600 of ~0.5) cultures grown in HdB minimal medium supplemented with 10 mM glycerol to determine the molecular response to the overexpression of *crp2* in *M. smegmatis* mc^155^ (Fig. S5b). The analysis confirmed that *crp2* was upregulated 6.0-fold in response to tetracycline induction (Table S3). The microarray results

**Fig. 4.** Crp1 and Crp2 regulation of electron transport chain components in *M. smegmatis*. (a) Validation of Crp1 microarray analysis by qRT-PCR. Results shown represent gene expression ratio in response to deletion of *crp1* from *M. smegmatis* and as means ± sd of four (microarray) or three (qRT-PCR) independent biological replicates. *P*<0.05, **P*<0.01, ***P*<0.001. (b) Promoter activity of succinate dehydrogenase 1 (*sdh1*) and 2 (*sdh2*) in wild-type and the Δcrp1 mutant of *M. smegmatis*. Promoter activity was measured as described in Methods. pJEM15, empty vector. Results are shown as means ± sd of three biological replicates. **P*<0.01, ***P*<0.001. (c) Validation of Crp2 microarray analysis by qRT-PCR. Results shown represent gene expression ratio in response to overexpression of *crp2* from *M. smegmatis* and as means ± sd of four (microarray) or three (qRT-PCR) independent biological replicates. *P*<0.05, **P*<0.01, ***P*<0.001.
revealed 58 genes that were differentially regulated, including 49 that were upregulated (≥1.4-fold) and nine that were downregulated (≤1.4-fold) in response to the overexpression of crp2 (P≤0.05). A Crp2 consensus sequence was also identified as described above and designated TGTGNaCACa.

A histogram of genes differentially expressed in response to overexpression of crp2 within functional categories is provided in Fig. S5(d). Microarray analysis showed that Crp2 regulates genes with diverse biological functions, including genes encoding Sdh1, the WhiB4 and WhiB6 of WhiB family proteins, which have been implicated in many cellular processes (Larsson et al., 2012), and RpfE, one of five resuscitation-promoting factor proteins (RpfFs) that are required for the resuscitation of dormant cells (Kana et al., 2008) (Table S3). The M. tuberculosis genome encodes seven WhiB proteins (WhiB1–7) and their homologues are present in M. smegmatis. Recently, it was shown that cAMP induced both whiB4 and whiB6 in M. tuberculosis (Larsson et al., 2012). The authors also identified a putative CRP-binding site present in the 5′ untranslated region of both whiB4 and whiB6 and suggested that CRP may influence the expression of these whiB genes (Larsson et al., 2012). One of the whiB genes, whiB1, is directly regulated by CRP in M. tuberculosis (Stapleton et al., 2010). A CRP-binding site is also present in the promoter regions of both whiB4 and whiB6 genes in M. smegmatis, suggesting that Crp2 could directly regulate these whiB genes. M. tuberculosis possesses five rpf genes, rpfA–E (Downing et al., 2004, 2005; Kana et al., 2008). In M. tuberculosis, a CRP-binding site was identified in the promoter region of rpfA, and rpfA is directly regulated by CRP (Rickman et al., 2005). A putative CRP-binding site was also identified in the promoter region of rpfE in M. smegmatis, and Crp2 could regulate rpfE. The reason why Crp2 appears to be essential for growth of M. smegmatis remains unknown. Analysis of the genes regulated by Crp2 (Table S3) reveals that a homologue of MSMEG_6041 in M. tuberculosis encoding fadE34 is essential for growth on cholesterol (Griffin et al., 2011). Whether MSMEG_6041 is essential for growth of M. smegmatis is not known.

Eleven genes were found in both the Crp1 and Crp2 regulons (Table 1). These genes included the sdh1 operon but not the sdh2 operon. To determine whether Crp2 also regulates sdh1 expression, qRT-PCR was performed using three genes coding for three different electron transport chain components (MSMEG_0420) for Sdh1, sdhB for Sdh2 and cydB for cytochrome bd oxidase, and atpB for the F1F0 ATPase (Fig. 4c). Expression levels observed by qRT-PCR correlated well with those from the microarray analysis for Crp2 regulation.

Table 1. Genes that are common in both Crp1 and Crp2 regulons

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene symbol</th>
<th>Description</th>
<th>Crp1*</th>
<th>Crp2†</th>
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<tr>
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<td>Succinate dehydrogenase subunit B</td>
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<td>1.0</td>
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<td>1.6</td>
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<tr>
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<td>1.8</td>
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<tr>
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<td>Glycerol uptake facilitator, MIP major intrinsic</td>
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<tr>
<td></td>
<td></td>
<td>protein channel</td>
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<td></td>
<td>Transcriptional regulator, TetR family protein</td>
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<td>1.87</td>
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</tbody>
</table>

*Mean gene expression ratio (four biological replicates) in the crp1 mutant compared to the wild-type. Bold type indicates a ≥1.4-fold change in gene expression, P≤0.05.
†Mean gene expression ratio (four biological replicates) in crp2 overexpression compared to the wild-type. Bold type indicates a ≥1.4-fold change in gene expression, P≤0.05.
of \textit{sdh1} by Crp2 is direct or indirect, EMSAs were conducted using the promoter regions of \textit{sdh1} or \textit{sdh2} with purified Crp2 (Fig. 6a). The results revealed that Crp2 binds at the promoter of \textit{sdh1} in a cAMP-dependent manner, but did not bind at the promoter of \textit{sdh2} (Fig. 6a), consistent with the gene expression profiles obtained by microarray and qRT-PCR analyses (Fig. 4c). To further validate this, EMSAs of Crp2 with the \textit{sdh1} promoter in the presence of increasing concentrations of cAMP were performed (Fig. 6b). Crp2 caused a mobility shift of the \textit{sdh1} promoter at cAMP concentrations of 6.7 \text{ mM} and above, with a complete shift at 133 \text{ mM} (Fig. 6b), suggesting that binding is cAMP-dependent (Fig. 6b). EMSAs of Crp2 at the \textit{sdh1} promoter with increasing concentrations of Crp2 in the presence of cAMP were further performed to validate that the specific binding of Crp2 at the \textit{sdh1} promoter is cAMP-dependent (Fig. 6c). The direct binding of CRP to \textit{sdh1} (Rv0249c–Rv0247c) in \textit{M. tuberculosis} has not yet been studied, but CRP is computationally predicted to bind this promoter region (Bai et al., 2005; Krawczyk et al., 2009).
CONCLUSION

The CRPs of *M. smegmatis* play central roles in regulating metabolic and respiratory activity and function as bona fide DNA-binding proteins and regulators of gene expression (Fig. 7). We show that Crp1 regulates the electron chain components of *M. smegmatis*, and for the *sdh* operons, the binding of Crp1 was cAMP-independent. In contrast, Crp2 bound to the *sdh1* promoter in a CAMP-dependent manner and no binding of Crp2 to the *sdh2* promoter was detected. These data highlight the complex regulation of the *sdh* operons by Crp1 and Crp2 in *M. smegmatis*. The *sdh* operons in *M. smegmatis* are differentially regulated in response to energy limitation and hypoxia (Berney & Cook, 2010; Pecsi et al., 2014), and the involvement of CRP in this regulation points to Crp1 and Crp2 being able to integrate diverse signals to control gene expression. The involvement of CRP in regulation of aerobic respiration has also been reported in *E. coli* (Shimada et al., 2011), highlighting an important regulatory role for CRP in prokaryotic energy metabolism. We have recently reported that Crp1 regulates the expression of *cydAB* coding for the terminal oxidase cytochrome *bd* in *M. smegmatis* in response to hypoxia (Aung et al., 2014). The lack of the classical Fnr and ArcBA regulation of respiratory metabolism in mycobacteria is intriguing and suggests that CRP may have been adapted for this function. Moreover, none of the electron transport chain components in *M. smegmatis* is under control of the hypoxic regulator DosR (Berney et al., 2014), suggesting unique regulation of respiratory metabolism in mycobacteria.

Crp1 and Crp2 recognized the same consensus sequence (TGTGN₈CAC), and this CRP binding is similar to that reported for Crp2 of *M. tuberculosis* (GTGN₈CAC) (Kahramanoglou et al., 2014). A CAMP-independent Crp1 DNA-binding activity suggests that Crp1 may be evolutionarily adapted to interact with DNA in the absence of CAMP, given the high levels of CAMP reported in mycobacteria (Dass et al., 2008; Padh & Venkitasubramanian, 1976, 1980). 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. In *E. coli*, the traditional view of CAMP–CRP signalling has centred on CCR to achieve hierarchical carbon usage (KovarÏvaŠ-Kovar & Egli, 1998; Saier, 1989; Saier & Reizer, 1994). Crp1 in *M. smegmatis* had no effect on the pattern of carbon source utilization, pointing to other roles in mycobacterial physiology. You et al. (2013) report that CAMP–CRP signalling mediates more than CCR and coordinates the expression of catabolic proteins with biosynthetic and ribosomal proteins in response to cellular metabolic demands, implying that CRP senses the anabolic demands of the cell and allocates resources appropriately in response to growth rate. The *M. smegmatis* Crp1 regulon and the growth rate (i.e. fast versus slow) regulon in *M. smegmatis* (Berney & Cook, 2010) both contain genes encoding components of the respiratory chain, suggesting that as mycobacteria make the transition

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**Fig. 7.** Regulatory roles of Crp1 and Crp2 in *M. smegmatis*. Crp1 regulates three pathways of sugar transport (MFS, PTS and ABC), the Entner–Doudoroff (ED) pathway for glycolysis, and major components of the electron transport chain and F₁Fₒ-ATP synthase. Regulation by Crp1 is indicated by grey shading. Both Crp1 and Crp2 regulate succinate dehydrogenase I and this is indicated by absence of shading. ABC, ATP-binding cassette family; MFS, major facilitator superfamily; NDH-1, NADH-quinone oxidoreductase; PTS, the phosphotransferase system; SDH, succinate dehydrogenase.
from fast to slow growth rate, the cell adjusts its metabolic machinery to match physiological demand for energy via Crp1. This warrants further investigation to elucidate the complex and unique molecular signalling network involving Crp1 and Crp2 in *M. smegmatis*.

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