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The role of transcriptional regulation in maintaining the availability of mycobacterial adenylate cyclases

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Abstract

Mycobacterium species have a complex cAMP regulatory network indicated by the high number of adenylate cyclases annotated in their genomes. However the need for a high level of redundancy in adenylate cyclase genes remains unknown. We have used semiquantitiative RT-PCR to examine the expression of the eight *Mycobacterium smegmatis* cyclases with orthologs in the human pathogen *Mycobacterium tuberculosis*, where cAMP has recently been shown to be important for virulence. All eight cyclases were transcribed in the cell in all environments tested, and only four demonstrated environmental-mediated changes in transcription. *M. smegmatis* genes MSMEG_0545 and MSMEG_4279 were upregulated during starvation conditions while MSMEG_0545 and MSMEG_4924 were downregulated in H_2O_2 and MSMEG_3780 was downregulated in low pH. Promoter fusion constructs containing *M. tuberculosis* H37Rv promoters showed consistent regulation compared to their *M. smegmatis* orthologs. Overall our

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findings indicate that while low levels of transcriptional regulation occur, regulation at the mRNA level does not play a major role in controlling cellular cyclase availability in a given environment.

Introduction

Cyclic adenosine monophosphate (cAMP) is an important second messenger that is produced by adenylate cyclase enzymes and controls a wide range of cellular responses in both prokaryotic and eukaryotic cells (Botsford & Harman 1992; Peterkofsky et al. 1993; Tang & Hurley 1998). cAMP signaling is critical for the regulation of virulence genes in several bacterial pathogens such as *Yersinia pestis* and *Pseudomonus aeruginosa*, and recent evidence suggests that cAMP also plays a role in the virulence of *Mycobacterium tuberculosis*, the causative agent of tuberculosis (Rickman et al. 2005; Petersen & Young 2002; Smith et al. 2004; Agarwal et al. 2009). Deletion of the cAMP-controlled transcription factor, cAMP Receptor Protein (CRP), from the Mtb genome causes attenuation of *M. tuberculosis* in a murine model and reduced bacterial growth rates in vitro and within macrophages (Rickman et al. 2005; Akhter et al. 2008). Additionally deletion of adenylate cyclase Rv0386 causes a loss of intramacrophage, bacterial-derived cAMP, which leads to decreased bacterial survival during mouse infection (Agarwal et al. 2009).

The classical model for cAMP regulation in prokaryotes is based on the well characterized cAMP response in *Escherichia coli* (Botsford & Harman 1992). *E. coli* contains a single class I adenylate cyclase which catalyzes the conversion of ATP to cAMP (Peterkofsky et al. 1993). By comparison, the *M. tuberculosis* H37Rv genome contains 15 class III adenylate cyclases (McCue et al. 2000), 10 of which have confirmed, biochemically distinct, activity

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(Castro et al. 2005; Linder et al. 2004; Linder et al. 2002; Reddy et al. 2001; Abdel Motaal et al. 2006; Cann et al. 2003; Shenoy & Visweswariah 2006; Guo et al. 2001; Sinha et al. 2005; Tews et al. 2005). Along with the high number of adenylate cyclases in the mycobacterial genome, there is also a wide diversity in their protein structure and domain compositions. Mycobacterial cyclases include receptor, membrane bound, and soluble type family members, and two (Rv1625c and Rv2435c) belong to the mammalian type adenylyl cyclase grouping (McCue et al. 2000; Shenoy & Visweswariah 2006; Reddy et al. 2001). The high number and diversity of mycobacterial cyclases suggests a large role for cAMP signaling in these species, and leads us to reason that the cAMP signaling paradigm in mycobacteria is more complex than the classical *E. coli* model. Besides *M. tuberculosis*, other mycobacterial species including the nonpathogenic *M. smegmatis*, actinobacteria, alphaproteobacteria, and cyanobacteria also contain high numbers and diversities of annotated adenylate cyclases signifying that the *E. coli* paradigm is not transferrable to all prokaryotes (McCue et al. 2000; Shenoy et al. 2004).

With a high number of adenylate cyclases there is likely to be a high amount of redundancy in mycobacterial cAMP production. Enzymatic regulation of adenylate cyclases in response to changing environments has been identified as a regulatory mechanism in *M. tuberculosis* (Abdel Motaal et al. 2006; Linder et al. 2004; Cann et al. 2003; Linder et al. 2002). However we hypothesize that it is unlikely for all cyclases to be present in the cell at the same time. Instead we thought it more probable that regulation first occurs at the level of transcription with unique cyclases transcribed and translated under specific environmental or growth conditions. For instance, Dass et al. demonstrated that expression of MSMEG_3780 is downregulated under low pH conditions and that downregulation is tied to decreased production of cAMP in that environment (Dass et al. 2008). While Dass et al focused on characterizing the

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detailed regulation of one cyclase we have examined expression of all 8 Mtb cyclase orthologs found in nonpathogenic *M. smegmatis* to determine the role transcriptional regulation may have on the availability of cyclases in the cell.

Materials and Methods

Bacterial culture

M. smegmatis mc^{2}155 was grown in Tryptic Soy Broth (TSB) supplemented with 0.05% Tween-80. Cultures were grown in ambient air or 5% CO_2 at 37ºC in 25cm^2 tissue culture flasks rocking with gentle agitation. For gene regulation assays, late log phase cultures were exposed to low pH (TSB adjusted to pH 5.5 with 0.1 M HCl), starvation (incubation in Phosphate Buffered Saline), hydrogen peroxide (5 mM), or nitric oxide (10 mM DETA) for 4 hours and gene expression was compared to non-exposed cultures.

RNA preparation

Late log phase culture of *M. smegmatis* mc^{2}155 was pelleted and resuspended in RNase free water. Cells were mechanically disrupted using a bead beater (BioSpec Products) for 4 rounds of beating on high for 1 minute each, in a mixture of 0.1 mm zirconia-silica beads (BioSpec Products) 45% TRIzol (Invitrogen), 45% acid phenol, and 10% chloroform-isoamyl alcohol (24:1). RNA was precipitated with isopropanol/3 M sodium acetate (pH 5.2) and resuspended in RNase free water. RNeasy Mini Kit and RNase-free DNAse (Qiagen) were used to remove contaminating DNA following manufacturer specifications.

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97 Semiquantitiative RT-PCR

98 cDNA was prepared from 0.5 µg of RNA following iScript cDNA synthesis kit specifications
99 (BioRad). PCR was run using a series of cDNA dilutions (0 – 1:1000) as templates to ensure
100 reactions chosen for quantitation were in the linear range of the PCR (Table 1 for primers).
101 Reactions were performed at 94ºC for 1 min, 57ºC for 1 min and 72ºC for 1 min followed by a
102 10 min extension at 72ºC. Control reactions were performed against 16S rDNA using cDNA
103 diluted 10^{-5}. PCR products were separated on agarose gels and band densities quantified using
104 ImageJ software (Abramoff et al. 2004). The 16S PCR products from all growth conditions were
105 normalized to one another before quantitation of individual genes, to ensure equal levels of
106 starting RNA in each reaction. 16S RNA PCR was also performed using total RNA without
107 reverse transcription to ensure the absence of DNA contamination.
108
109 Gene reporter construction and assay
110 Promoter:GFP reporter strains were generated for gene expression analysis of *M. tuberculosis*
111 promoter regions in a *M. smegmatis* background. The intergeneic DNA sequences of the
112 adenylate cyclase genes were amplified by PCR (Table 1 for primer sequences) and amplified
113 DNA was cloned into pGFPoriM, which carries a promoterless *gfpmut2* gene as previously
114 described (Purkayastha et al. 2002; Florczyk et al. 2003). Constructed plasmids were
115 electroporated into *M. smegmatis* mc^2155 at 2500 mV (Eppendorf 2510 Electroporator). GFP
116 fluorescence from cultured cells was detected using GloMax Multi+ Detection System
117 (Promega) and normalized to 10^6 bacteria based on OD_{600}.
118
119 Results and Discussion

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121 Regulation of *M. smegmatis* adenylate cyclases

122 The genome of *M. smegmatis* contains ten annotated adenylate cyclases, eight of which
123 have orthologs in pathogenic *M. tuberculosis* (Kapopoulou et al. 2011). In order to determine the
124 role of gene expression in adenylate cyclase availability we systematically examined
125 transcription of all eight orthologs using semi-quantitative RT-PCR. Gene expression was
126 examined with a focus on *M. tuberculosis* orthologs, and in a variety of environments chosen
127 based on those known to be relevant for *M. tuberculosis* infection, due to the recent connection
128 between cAMP production and *M. tuberculosis* virulence. Conditions examined include
129 starvation, low pH, oxidative and nitrosative stress and 5% CO_2 (Smith 2003). Expression under
130 control conditions (growth in TSB + Tween-80) indicated that all 8 adenylate cyclase genes are
131 transcribed at the same time in the cell, albeit with varied levels of transcription (Figure 1). Out
132 of all conditions tested the greatest level of regulation was observed during starvation and
133 oxidative stress (H_2O_2 exposure), while no difference in expression of any genes was seen during
134 nitrosative stress or the presence of CO_2. Two genes (MSMEG_0545 and MSMEG_4279) were
135 upregulated 2-3 fold after 4 hrs of starvation while one gene (MSMEG_3780) was observed to
136 be downregulated approximately 2 fold in each of starvation and low pH. Additionally two genes
137 (MSMEG_0545 and MSMEG_4924) were downregulated approximately 2 fold under oxidative
138 stress (Figures 1 and 2).

139 Interestingly only four of the eight adenylate cyclase genes showed changes in expression
140 in any environments examined. While statistical, these observed changes were not drastic shifts
141 in expression, leading only to 2-3 times higher or lower levels of mRNA in any given
142 environment. Combined with the result that all eight genes were transcribed at various levels in

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all environments tested we conclude that our results counter our hypothesis and indicate that transcriptional regulation plays only a minor role in controlling the availability of various adenylate cyclases in the cell. Three of the four genes that did demonstrate transcriptional regulation encode for soluble adenylate cyclase proteins suggesting that transcriptional regulation may play a larger role in the availability of soluble cyclases as opposed to the membrane associated and multi-domain structures. It is likely that biochemical regulation of various protein domains has the dominant role in regulation of cAMP production by the redundant cyclases.

Regulation of *M. tuberculosis* adenylate cyclases

Eight of the ten *M. smegmatis* cyclases are orthologs of adenylate cyclases in *M. tuberculosis*, representing just over half of the annotated cyclases in H37Rv (Kapopoulou et al. 2011). Promoter regions of each ortholog pair, represented by the 500 nucleotides upstream of the ATG start site of each gene, were compared for sequence similarities using the sequence alignment program T-Coffee (http://www.ebi.ac.uk/Tools/msa/tcoffee/) (Notredame et al. 2000). Percent identities ranged from 51.94% to 82.08% (Table 2), indicating enough similarity to hypothesize that regulation would be similar between orthologs.

In order to determine if regulation in *M. smegmatis* was similar to that of the pathogenic orthologs we generated GFP:promoter fusions using the intergenic regions amplified from H37Rv chromosomal DNA. Overall, regulation between species orthologs was very similar. Rv1359 showed similar regulatory patterns of upregulation in starvation and downregulation in oxidative stress as did MSMEG_0545 (Figure 3). Interestingly Rv1359 is one of five H37Rv annotated adenylate cyclases that has not been shown to have biochemical activity and is

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166 predicted to be enzymatically inactive (Shenoy et al. 2004). Transcriptional regulation of this
167 gene indicates that even if it is not a functional adenylate cyclase, it is transcribed and likely has
168 an unidentified function in the cell. Additionally Rv1647 showed similar regulatory patterns of
169 downregulation in both starvation and low pH similarly to MSMEG_3780, supporting the
170 observation by Dass et al (Dass et al. 2008) who reported similar regulation (Figure 3).
171 The last regulatory similarity observed was the oxidative stress regulation of both
172 MSMEG_4924 and the Rv1320c promoter region. MSMEG_4924 has three predicted orthologs
173 in Mtb, Rv1318c, Rv1319c and Rv1320c. Rv1319c and Rv1320c are predicted to be an operon
174 with only 13 nucleotides between the two genes, thus both genes are represented by the Rv1320c
175 promoter region. The regulatory similarity of MSMEG_4924 to Rv1320c and not Rv1318c
176 correlates with the higher percent similarity to the Rv1320 promoter (82.08%) than the Rv1318c
177 promoter (54.6%) (Table 2).
178
179 Conclusions
180 Mycobacterial species contain a high number of functional adenylate cyclases when
181 compared to *E. coli*, the typical prokaryotic model system. The high level of cyclase redundancy
182 led us to hypothesize that only specific cyclases would be expressed in the cell under any given
183 condition. However, the results in this study counter that hypothesis, indicating that all eight *M.*
184 *smegmatis* cyclases are transcribed in the cell at one time. Observed changes in transcription
185 levels in response to varying environments was minor, but conserved between mycobacterial
186 orthologs, validating the use of *M. smegmatis* as a model system for studying the complex
187 mycobacterial adenylate cyclase/cAMP network. While cAMP has been shown to be important

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for *M. tuberculosis* pathogenesis, adenylate cyclase transcriptional regulation does not appear to have a major role in regulating the availability of cyclases in the cell.

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291 Table 1.Primers used throughout this study

RT-PCR		Promoter:Reporter Fusions	
Gene	Sequence[a]	Gene	Sequence
MSMEG_0545	F-GATCGAGCCGAAGAACTGTG	Rv1264	F-NNNNGGATCCGACGATGTCGACGTAGTTGT
	R-ATTGAGGGCGATCAAGTGAG		R-NNNNGGTACCGCGCACGTGGTCTGTCAC
MSMEG_3578	F-CGATCGTCAACAAACTGGTG	Rv1318c	F-NNNNGGATCCAGATGCCCGAGGTCCAAG
	R-CAGGTATCCGTTGTGCAGTG		R-NNNNGGTACCGTGCTCTTGGCCGACAT
MSMEG_3780	F-CATACTCTTGCGCCTGTGAA	Rv 1359	F-NNNNGGATCCGGAGGTTCGCCACAAGATT
	R-CCCTGAGGTCTTTCGTGCT		R-NNNNGGTACCCGATACCTTCCGGCTAAGCA
MSMEG_4279	F-CGACCTGTCGGATTTCACC	Rv1647	F-NNNNGGATCCAGCGGGAACCGCTAGGG
	R-CATCTGATGCCGCAGAACT		R-NNNNGGTACCGTAGGTGGTGCGGGCTGAG
MSMEG_4477	F-AGCCTGGCGTATCAGCTCT	Rv1900c	F-NNNNGGATCCACCGGATCGATCACTTGC
	R- ACGGTCCAGAACAATTCGAC		R-NNNNGGTACCATGGTCGAGGCGGATCAC
MSMEG_4924	F-GTGACGCTGGAGAACCTGAC	Rv2212	F-NNNNGGATCCGCAGATTGGTGATGCTCAGA
	R-AAGATGAAGCCGAACACCAG		R-NNNNGGTACCGACCATAGCAGGACGTCACC
MSMEG_5018	F-ATCCAGCCACTCCTGGAAG	Rv2435c	F-NNNNGGATCCGTCTGAGTGCGTCGTCGTT
	R-TGAGCAGCCAGTTGATCAGT		R-GGTACCTACCGAGTCCAGTGCCTCAC
MSMEG_6154	F-CCTGCTCAACGAGTTCTTCC	Rv3645	F-NNNNGGATCCAATCACCACGATCTGCCAGT
	R-GCGTCACCCTGGAACTTGT		R-NNNNGGTACCGCATGCTCAGCGAGAACAG
16S rDNA	F-GCGATACGGGCAGACTAGAG	tuf	F-NNNNGGATCCGTGCGGAAGTAGAACTGCGG
	R-CCTCCTCCTGATATCTGCGCATT		R-NNNNGGTACCAGGAAGTTGAGATCGTCGGC
sigA	F-TCGAGGACGAGGAAGAAGAA		
	R-CCTCCAGCAGATGGTTTTTG		

292 [a] F – forward primer, R – reverse primer

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Table 2. Percent identity between Mtb and M. smegmatis ortholog promoters[a]

MSMEG gene	H37Rv gene	Percent Identity[b]
0545	Rv1359	57.02
3578	Rv2435	46.3
3780	Rv1647	51.94
4279	Rv2212	78.97
4477	Rv1900	54.42
4924	Rv1318c	54.6
4924	Rv1319c/ Rv1320c	82.08
5018	Rv1264	54.01
6154	Rv2645	69.28

[a] Promoter region defined as the 500 nucleotides upstream of translation start site

[b] Determined with T-Coffee multi-sequence alignment

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Figure 1. Regulation of *M. smegmatis* adenylate cyclase genes under starvation conditions. Semi-quantitative RT-PCR was used to compare adenylate cyclase mRNA levels between late-log phase cultures incubated for 4 hours in mycomedia (control) or PBS (starvation). (A) A representative depiction of PCR amplified cDNA separated using agarose gel electrophoresis. (B) Data obtained from ImageJ quantifying the band densities observed on the depicted agarose gel comparing the cDNA levels under control (black bars) and starvation (white bars) conditions. (C) Average of three different experiments represented as fold differences of starvation compared to control expression. * indicates statistically significant difference between expression in control and starvation for an individual gene ($p<0.05$)

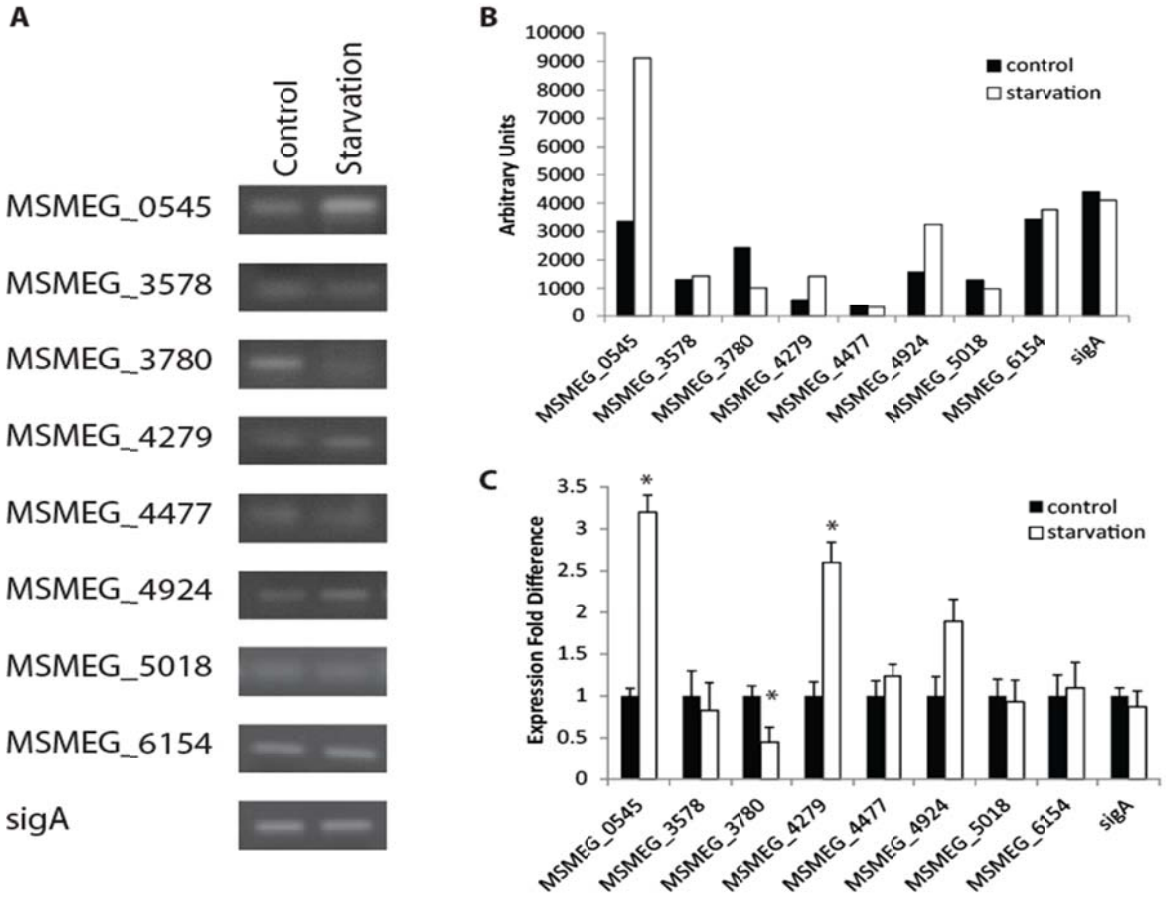

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Figure 2. Regulation of *M. smegmatis* adenylate cyclase genes under low pH and oxidative stress. Semi-quantitative RT-PCR was used to compare adenylate cyclase mRNA levels between late-log phase cultures incubated for 4 hours in mycomedia (control, black bars) , mycomedia adjusted to pH 5.5 (low pH, hatched bars), and mycomedia containing H_2O_2 (H2O2, grey bars). Results are the average of three independent experiments and are expressed in fold difference comparing experimental condition to control. * indicates conditions with statistically significant differences in expression compared to control for an individual gene ($p<0.05$)

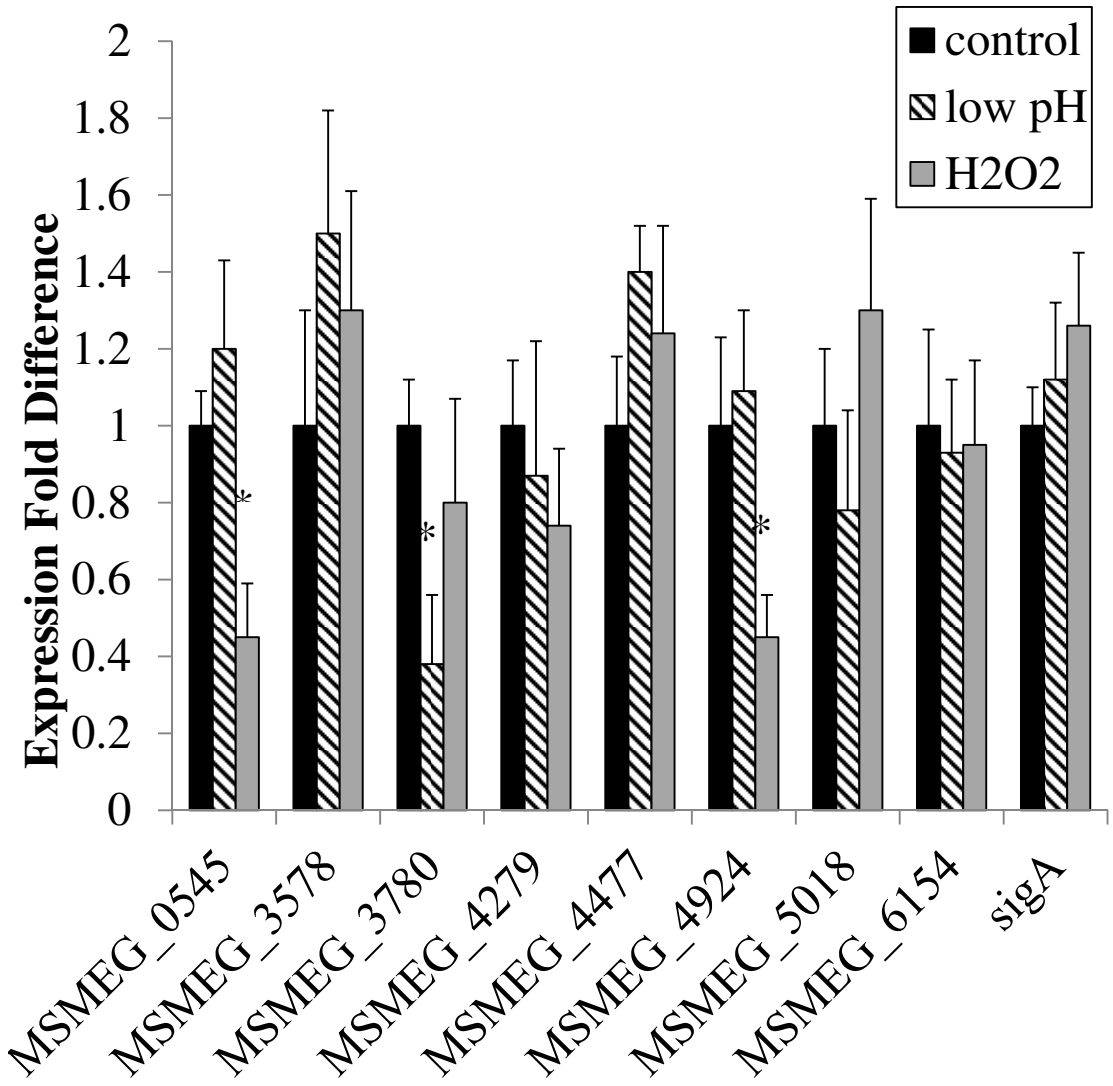

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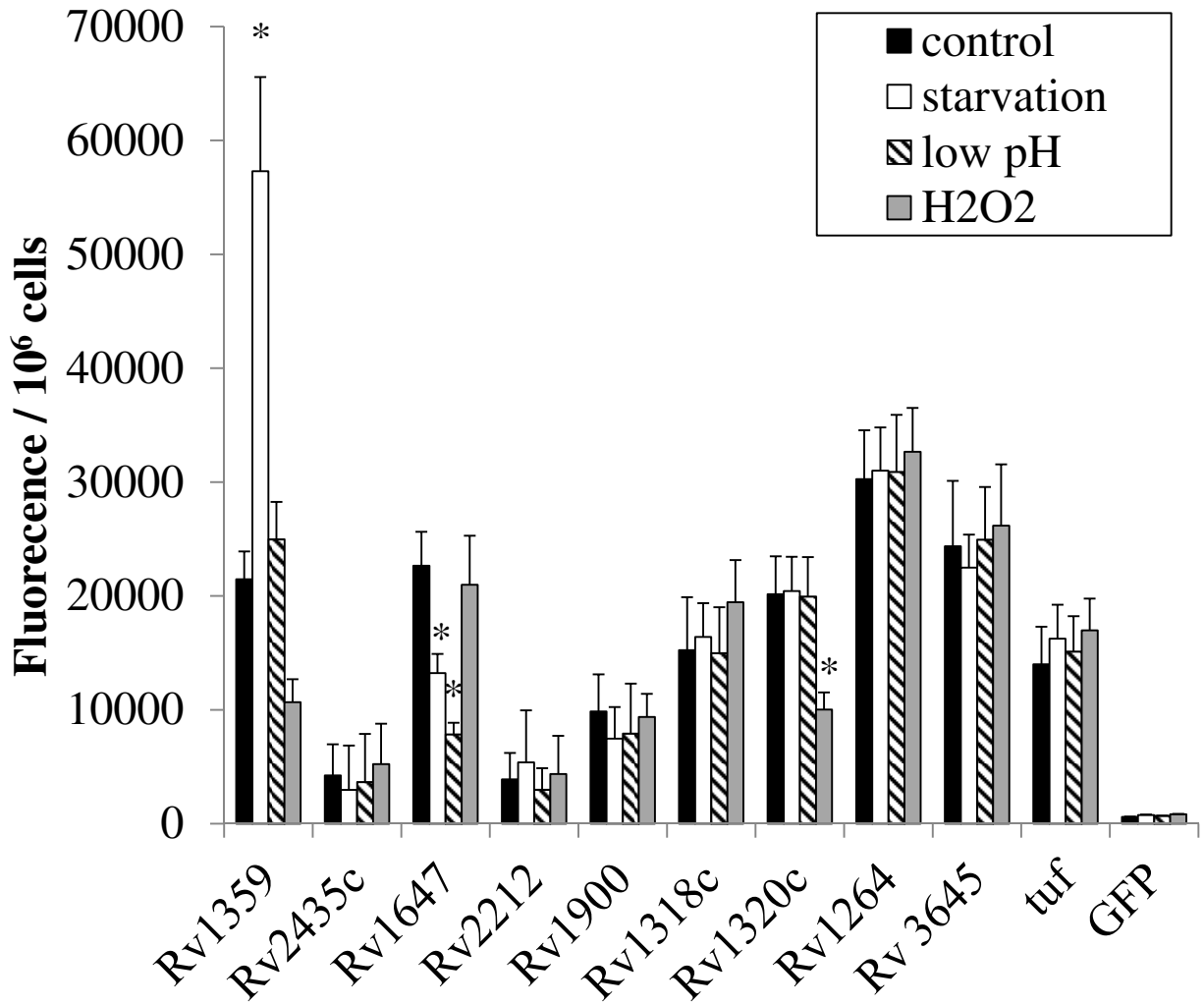
Figure 3. Regulation of *M. tuberculosis* adenylate cyclase genes. GFP: promoter fusions containing *M. tuberculosis* promoters were electroporated into *M. smegmatis* and used to examine adenylate cyclase expression in late log phase cultures after 4 hour incubation in mycomedia (control, black bar), PBS (starvation, white bar), mycomedia adjusted to pH 5.5 (low pH, hatched bars), and mycomedia containing H_2O_2 (H2O2, grey bars). Fluorescence was normalized to 10^6 cells and represented as the average of three independent experiments. * indicates conditions with statistically significant differences in expression compared to control for an individual gene ($p<0.05$)



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