Characterisation of the transcriptional regulator Rv3124 of *Mycobacterium tuberculosis* identifies it as a positive regulator of molybdopterin biosynthesis and defines the functional consequences of a nonsynonymous SNP in the *Mycobacterium bovis* BCG orthologue.

Pablo Mendoza Lopez[1, 2], Paul Golby[2], Esen Wooff[2], Javier Nunez Garcia[2], M. Carmen Garcia Pelayo[2], Kevin Conlon[5], Ana Gema Camacho[1], R. Glyn Hewinson[2], Julio Polaina[3], Antonio Suárez García[4], Stephen V. Gordon[2,5, 6, 7, 8].

[1]Vircell S.L., Pol. Ind. Dos de Octubre, Plaza Domínguez Ortiz, 1, 18320 - Santa Fé, Granada, Spain.

[2]TB Research Group, VLA Weybridge, New Haw, Surrey KT15 3NB, UK.

[3]Instituto de Agroquímica y Tecnología de Alimentos, CSIC, Polígono de la Coma s/n, Paterna, Valencia, 46980, Spain.

[4]Centro de Investigación Biomédica, Parque Tecnológico Ciencias de la Salud, Avda. Conocimiento sn, 18100, Armilla, Granada, Spain.

UCD Schools of [5]Agriculture, Food Science and Veterinary Medicine, [6]Medicine and Medical Science, and [7]Biomolecular and Biomedical Science, College of Life Sciences, and [8]UCD Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Dublin 4, Ireland.

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Corresponding author:

Prof. Stephen Gordon, Room 037, Veterinary Sciences Building, School Agriculture, Food Science and Veterinary Medicine, University College Dublin, Dublin 4, Ireland.

Tel: (+353) 1 7166181; E-mail: stephen.gordon@ucd.ie

ABSTRACT

A number of single nucleotide polymorphisms (SNPs) have been identified in the genome of *Mycobacterium bovis* BCG Pasteur compared to the sequenced strain *M. bovis* 2122/97. The functional consequences of many of these mutations remain to be described; however mutations in genes encoding regulators may be particularly relevant to global phenotypic changes such as loss of virulence, since alteration of a regulator's function will affect the expression of a wide range of genes. One such SNP falls in *bcg3145*, encoding a member of the AfsR/DnrI/SARP class of global transcriptional regulators, and replaces with glycine a highly conserved glutamic acid residue at position 159 (E159G) in a tetratricopeptide repeat (TPR) located in the bacterial transcriptional activation (BTA) domain of BCG3145. TPR domains are associated with protein-protein interactions, and a conserved core (helices T1–T7) of the BTA domain seems to be required for proper function of SARP-family proteins. Structural modelling predicted that the E159G mutation perturbs the third α-helix of the BTA domain and could therefore have functional consequences. The E159G SNP was found to be present in all BCG strains, but absent from virulent *M. bovis* and *M. tuberculosis* strains. By overexpressing BCG3145 and Rv3124 in BCG and H37Rv and monitoring transcriptome changes using microarrays, we determined that BCG3145/Rv3124 acts as a positive transcriptional regulator of the molybdopterin biosynthesis *moa1* locus, and suggest that *rv3124* be renamed *moaR1*. The SNP in *bcg3145* was found to have a subtle effect on the activity of MoaR1, suggesting that this mutation is not a key event in the attenuation of BCG.

INTRODUCTION

The Bacille Calmette-Guérin (BCG) vaccine is a live attenuated strain of *Mycobacterium bovis* derived by *in vitro* passage of an *M. bovis* strain from 1908 to 1921 (Calmette, 1927). This *in vitro* cultivation led to the accumulation of mutations in the BCG genome that attenuated the bacillus. BCG is the only vaccine available against tuberculosis, and with more than 3 billion individuals having been immunized with BCG it is the most widely used vaccine in the world (Brosch *et al*., 2007). Despite the widespread use of BCG, a precise catalogue of the genetic lesions that led to attenuation has not been defined. Knowledge of these mutations would shed light not only on the attenuation of BCG, but also on virulence factors of the tubercle bacilli.

Work towards defining the attenuating genetic lesions in BCG began with the identification of the RD1-RD3 loci using subtractive genomic hybridisation techniques (Mahairas *et al*., 1996). The RD1 locus was shown to be deleted from all BCG strains but present in all virulent strains of *M. bovis* and *M. tuberculosis* studied. Subsequent work has shown that this deletion played a major role in the attenuation of BCG (Lewis *et al*., 2003; Pym *et al*., 2002) although restoration of RD1 by knock-in did not restore virulence to wild-type levels in immunocompetent mice (Pym *et al*., 2002), indicating that other attenuating mutations exist. Comparative genome analyses using genomic libraries and DNA arrays further expanded the list of deleted regions and chromosomal rearrangements between BCG and virulent *M. bovis* (Behr *et al*., 1999; Gordon *et al*., 1999; Mostowy *et al*., 2003). However, it was with the completion of the genome sequence of *M. bovis* BCG Pasteur, and comparison to the genomes of *M. tuberculosis* (Cole *et al*., 1998) and *M. bovis* (Garcia Pelayo *et al*., 2009), that we obtained single-nucleotide resolution of mutations present in BCG Pasteur. From genome analysis, 736 SNPs were identified between BCG Pasteur and the virulent *M. bovis* strain AF2122/97 (Brosch *et al*., 2007). However, many of these SNPs may be specific to the BCG Pasteur or *M. bovis* AF2122/97 strains sequenced. To gain a "minimal" list of SNPs that differentiate attenuated BCG from virulent *M. bovis* strains, Garcia Pelayo *et al*. (Garcia Pelayo *et al*., 2009) screened all SNPs present in BCG

against a panel of 21 *M. bovis* strains and 13 BCG strains. This allowed the identification of 186 SNPs between virulent *M. bovis* strains and all BCG strains, with 115 of these SNPs being nonsynonymous (nsSNP) and hence having potential functional consequences.

Previous characterisation of nsSNPs in BCG has revealed key insights into gene function. The first study to link a SNP to a phenotypic change in BCG strains was shown with the *mma3* gene, where a nsSNP abolished methoxymycolate production in "late" BCG strains (derived post-1927, compared to 'early' strains that were derived pre-1927) (Behr *et al*., 2000; Belley *et al*., 2004). A nsSNP in the BCG gene encoding the CRP global gene regulator has been shown to affect global gene regulation, but play no role in the attenuation of BCG (Hunt *et al*., 2008). Furthermore a nsSNP in the *pykA* gene of BCG has been shown to permit growth of BCG on glycerol as a sole carbon source, a mutation that was selected during growth of the *M. bovis* progenitor on glycerinated potato-slices (Keating *et al*., 2005). Hence, determining the functional consequences of nsSNPs found in BCG can reveal much about biological function of the gene product as well as any role for the gene in virulence.

One of the 186 nsSNPs between BCG and virulent *M. bovis* was found in the *bcg3145* gene (an orthologue of *rv3124* in *M. tuberculosis*). BCG3145 is a member of the AfsR/DnrI/SARP (*Streptomyces* Antibiotic Regulatory Protein) class of transcriptional regulators. This class also contains EmbR, the regulator of three arabinosyltransferases that are the targets of the front-line tuberculosis drug ethambutol (Belanger *et al*., 1996). The structure of EmbR has been elucidated, revealing DNA binding, bacterial transcriptional activation (BTA), and forkhead-associated domains (Alderwick *et al*., 2006). The E159G mutation in BCG3145 mutates to glycine a conserved glutamic acid residue located in the BTA domain (region T3). However, whether the E159G mutation affects the ability of BCG3145 to regulate transcription, or the identity of the genes regulated by BCG3145, was unknown. Mutations in regulatory genes may be particularly relevant to global phenotypic changes such as loss of virulence, since alteration of a regulator's function will affect the expression of a wide range of genes. In order to define the function of

BCG3145/Rv3124, we describe here structural predictions, transcriptome analysis, site-directed mutagenesis, gel-shift assays, and promoter reporter assays that show BCG3145/Rv3124 to be a positive transcriptional regulator of the *moa1* locus.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Mycobacteria and *Escherichia coli* strains used in this study are listed in Table 1. Strains were grown in 7H9 Middlebrook broth (Becton Dickinson) containing albumin-dextrose-catalase (ADC, Becton Dickinson) (7H9-ADC), 0.2 % glycerol and 0.05% Tween 80. After electroporation of pSM96 and derivatives, mycobacterial transformants were selected on 7H11 medium containing 10% (v/v) oleic acid–ADC (OADC, Becton Dickinson), with antibiotics added as appropriate: kanamycin (Sigma) at 25μg/ml, and hygromycin (Invitrogen) at 150 μg/ml. *E.coli* strains were used as a general purpose cloning and expression host and grown at 37°C in Luria-Bertani (LB) medium with shaking, and on LB agar with ampicillin (Sigma) 100 μg/ml, hygromycin 150μg/ml, or kanamycin 25 μg/ml as appropriate.

Confirmation of SNP in *bcg3145*

A 500 bp fragment flanking the E159G nsSNP was amplified from a range of BCG, *M. bovis* and *M. tuberculosis* strains using Forward (ACCGTCCACACGTACATTTG) and Reverse (AGCGTGGACTTGAGTCTATG) primers (MWG). DNA from heat killed mycobacterial cells was used as a template for the amplifications, with DNA polymerase HotStar Taq (Qiagen). The PCR reaction cycle conditions were 95°C for 15 min, followed by 30 cycles of 95°C for 1 min, hybridization for 1 min at 58°C, extension for 1 min at 72°C, and a final incubation at 72°C for 5 min. The PCR product was purified and the point mutation was confirmed by cycle sequencing (Sequencing Service, University of Dundee, UK).

Modelling of the Rv3124 structure

The GenTHREADER program at the PSIPRED server (Bryson *et al*., 2005; Jones, 1999) was used to identify and align proteins structurally related to Rv3124. Only one protein with high resemblance (ca. 50% sequence identity) was retrieved from the search. This was EmbR, a transcriptional regulator from *M. tuberculosis* (PDB id: 2FF4) (Alderwick *et al*., 2006). Sequence alignment with EmbR was used to obtain a model of Rv3124 structure from the Swiss-Model server (Kiefer *et al*., 2009). Protein structures were analyzed with the Swiss-PDP Viewer (http://spdbv.vital-it.ch/).

Complementation of *M. bovis* BCG with *bcg3145* and *rv3124*

Using pSM96 as a vector (an *E. coli*-mycobacteria shuttle vector containing a *hsp60* promoter upstream of a multiple cloning site, constructed by Dr S. Michell, VLA Weybridge), constructs were generated with *rv3124* (pB1) or *bcg3145* (pB3, Table 1). For pB1, *rv3124* was amplified from *M. tuberculosis* H37Rv genomic DNA and for pB3, *M. bovis* BCG genomic DNA. PCR reactions used primers Rv3124BamF (ATGGGATCCGTGCA ATTCAACGTCTTAGGACCACTGGAAC, *Bam*HI site underlined) and Rv3124PstR (CATCTGCAGTTATGCCAAACCGATGGGAAGGAG, *Pst*I site underlined). The amplification conditions were 94°C for 15 s, then 30 cycles of 15 s at 94°C, hybridisation and extension at 68°C for 3 min, then a final extension for 3 min at 68ºC. pSM96 and PCR products were digested with *Bam*HI and *Pst*I, ligated following standard protocols, and transformed into *E. coli*. Sequencing was used to confirm the correct sequence and orientation of the cloned fragments. Plasmid constructs were purified from *E. coli* and electroporated into *M. bovis* BCG Pasteur using standard methods, with transformants selected on 7H11 plates containing kanamycin and hygromycin. Isolated colonies were selected from the plates, checked for the presence of the plasmid by PCR, and then grown in 7H9 containing the appropriate antibiotic for transcriptome analysis.

Transcriptome analysis

Strains were grown in 7H9-ADC medium with 0.05% Tween80 to an OD_{600} of ≈0.6, corresponding to mid-log growth. Each strain was cultured three times (3 biological replicates), with each culture split in two and RNA extracted (two technical replicates; hence six microarrays in total per strain). Total RNA from each strain was extracted, purified, reverse-transcribed, and labelled with Cy5-dCTP (Amersham Pharmacia) as previously described (Golby *et al*., 2007). Cy3-labeled DNA (*M. bovis* AF2122/97 and *M. tuberculosis* H37Rv) was used as a control. Probes were hybridised to whole-genome *M. bovis*/*M. tuberculosis* composite microarrays and scanned with an Affymetrix 428 scanner. The microarrays were developed by the Bacterial Microarray Group (St Georges, University of London) in collaboration with the Veterinary Laboratories Agency (Weybridge). The array design is available in BμG@Sbase (Accession No. A-BUGS-31; http://bugs.sgul.ac.uk/A-BUGS-31) and also ArrayExpress (Accession No. A-BUGS-31). Fully annotated microarray data have been deposited in BμG@Sbase (accession number E-BUGS-93; http://bugs.sgul.ac.uk/E-BUGS-93) and also ArrayExpress (accession number E-BUGS-93). Image processing and data normalisation steps were performed as previously described (Golby *et al*., 2008).

To select differentially expressed genes between strains, a T-test was applied to each gene with the Benjamini and Hochberg False Discovery Rate applied to adjust p-values and correct for multiple testing. Those differentially expressed genes (adjusted p-value smaller than 0.05) showing an expression difference greater than 3-fold between strains were selected for further investigation. Results for these genes were confirmed by quantitative RT-PCR analysis (qRT-PCR), as previously described (Golby *et al*., 2007). Briefly, RT-PCR was carried out using the QuantiTecT SYBR Green PCR kit (Qiagen). Each reaction was carried out in a 25 μl volume containing 12.5 μl of QuantiTect Master Mix, 0.4 μM of each primer and 5μl of diluted cDNA. All reactions were run in duplicate using a RotorGene 3000 instrument (Qiagen). The instrument was programmed to cycle at 50°C for 30 min; 95°C for 15 min; and then 50 cycles of 15 s at 95°C, 30 s at 55°C, and 30 s at 60°C. For each reaction the melting curve was analyzed and the PCR product was run on an

agarose gel in order to confirm the specificity of the RT-PCR reaction. Expression levels were normalised using the *sigA* gene as an internal reference. Primers used for qRT-PCR are listed in Table S1.

Construction of an *M. bovis* BCGΔ*bcg3145* knockout

Generation of the *M. bovis* BCGΔ*bcg3145* followed a previously described phage protocol (Bardarov *et al*., 2002) with *bcg3145* disrupted by a hygromycin cassette using a shuttle phasmid. Briefly, an approximately 1 kb region flanking *bcg3145* was amplified by PCR. For the downstream region, the primers RIGHT PBCGPPEF (ATGAAGCTTCTACGCCCCTCAATGCAAGCG; *Hind*III site underlined) and PBCGPPER (ATACTAGTCAGCTGGCGGCCATTCAGGCTC; *Spe*I site underlined) were used; after *Hind*III and *Spe*I digestion the fragment was cloned into the cosmid pYUB854, to generate p3145right. The upstream region of *bcg3145* was amplified using the primers PBCG3144F (ATTCTAGAGGCGGTCAACGAGGCGATCTGC; *Xba*I site underlined) and PBCG31444R (ATTCTAGAAGCACCTGCCGTCGTTGCGTTC; *Xba*I site underlined); after *Xba*I digestion this fragment was cloned into p3145right to generate the cosmid p3124KO. The correct orientation of the fragments was determined by restriction enzyme digestion and sequencing. The *in vitro* packaging reaction of the cosmid was done by *Pac*I digestion and using the *in vitro* packaging kit Giga XL (Stratagene). After transduction of *E. coli* HB101 cells, transductants were selected on plates containing hygromycin and positive colonies were checked by *Pac*I digestion.

M. smegmatis mc^2155 was transformed with purified phasmid DNA by electroporation followed by selection on agar plates for 4 days at 30ºC. The phages generated were titred, and a ratio of 10 phage/bacteria was used for the transduction of *M. bovis* BCG at the non-permissive temperature of 37^{o}C. Hygromycin resistant transformants were screened for loss of *bcg3145* by PCR and Southern blot analysis. Southern blotting (Figure S1) was performed using a nonradioactive 435 bp probe and the DIG High Prime DNA Labelling and Detection Starter Kit II (Roche Applied Science).

Site-directed mutagenesis

Site-directed mutagenesis (SDM) on *rv3124* was carried out using the Stratagene Quikchange II XL site-directed mutagenesis kit. The primer pairs used are listed in Table S2. As a template for the reaction, *rv3124* was cloned into the *Bam*HI and *Pst*I sites of pUC19 after amplification using primers Rv3124BamF (ATGGGATCCGTGCAATTCAACGTC TTAGGACCACTGGAAC) and Rv3124PstR (CATCTGCAGTTATGCCAAACCGATGG GAAGGAG), generating pUC3124. The SDM reactions were carried out in a total volume of 50 µl containing 5 µl reaction buffer, 10 ng of pUC3124, 125 ng of each oligonucleotide, 1 µl dNTP mix and 2.5 units of *Pfu* Ultra HF DNA polymerase. The thermocycling programme used was 95ºC for 30 seconds, followed by 16 cycles of 95ºC for 30 seconds, 55ºC for 1 min, and 68ºC for 5 min. After cooling the tubes on ice for 2 min, template was degraded using 10 units of *Dpn*I restriction enzyme at 37ºC for 1 hour. One microlitre of each reaction was used to transform competent *E. coli* XL1-Blue cells and transformants were selected on LB-amplicilin plates. Mutant colonies were isolated and sequence verified (The DNA Sequencing & Services, University of Dundee, UK).

Purification of recombinant Rv3124

To purify Rv3124, MBPpET, an in-house double fusion tag vector (Vircell), was used using an MBP-Rv3124-His_6 orientation with the Maltose binding protein (MBP) solubility enhancer and a 6-His tag flanking Rv3124. Fusion of *rv3124* to MBPpET was accomplished using PCR to generate DNA fragments from *M. tuberculosis* gDNA containing *Bam*HI and *Xho*I sites. The resulting DNA fragments were ligated to the *Bam*HI and *Xho*I sites of the MBPpET vector. Correct nucleotide sequence was confirmed by DNA sequencing performed at the "Lopez Neyra" Institute of Parasitology and Biomedicine in Granada (Spain). For expression of the MBP-Rv3124-His fusion protein, the construct was transformed into *E. coli* strain BL21 (Invitrogen), grown at 37ºC in LB containing 50µg/ml kanamycin to an OD_{600} of 0.6, and protein expression was induced with the addition of IPTG and arabinose to a final concentration of 1 mM and 0.2% respectively,

with cultures then grown for an additional three hours. Cells were harvested by centrifugation at 1,789 x *g*, and pellets that were not used immediately were frozen at –80°C. For the scale-up purification procedure, frozen pellets from 1 litre of BL21 cells overproducing Rv3124 were thawed and resuspended in lysis buffer (200 mM Tris-HCl [pH 7.5], 200 mM sodium chloride, 1 mM EDTA and 10 mM β-mercaptoethanol). Cells were lysed using a French press cell disrupter (Thermo). The crude extract was centrifuged at 50,000 x *g* for 30 min at 4°C, and the Rv3124 protein present in the supernatant was purified using Amylose Resin matrix according to the manufacturer's instructions (New England Biolabs). Final protein concentration was determined using a Bradford Protein Assay Kit (BioRad) with BSA (Pierce) as a standard.

Electrophoretic mobility shift assay (EMSA)

The gel mobility shift assays were performed using a 222 bp DNA fragment (Rv3109pro) containing 190 bp upstream of the *moaA1* gene and the first 32 bp of the *moaA1* gene. The Rv3109pro fragment was generated by PCR using the primers F1 (AACGAAAGCTCGCACATGAGTGGTC) and R3 (ATGGAGCTACCATATCAGGCAG), and end-labelled with [γ-32P]-dATP (PerkinElmer) using polynucleotide kinase. Binding reactions (10 µl) contained 1 µl BSA, 1 µl poly (dI–dC), 1 µl 10x binding buffer (40% glycerol, 50 mM DTT, 500 mM NaCl, 100 mM Tris-HCl, pH 7,5, 10 mM $MgCl_2$, 5mM EDTA), purified Rv3124 protein of varying concentrations and a constant concentration of labelled substrate DNA. When indicated cold Rv3109 probe was added as competitor DNA with a 10 and 100 fold-molar excess. After incubation at 37ºC for 30 min, protein-bound and free DNA were separated by electrophoresis at ambient temperature on a 4.5% native polyacrylamide gel running at 7 mA for three hours. The gels were dried for 2 hours and exposed to X-ray film overnight or longer at -80ºC.

Analysis of promoter activity

rv3109 (*moaA1*) promoter PCR fragments were cloned upstream of *lacZ* in the L5-based integrating vector pSM128 (Dussurget *et al*., 1999), and then sequenced to verify the sequence and correct orientation of the promoter-*lacZ* fusions. To generate constructs, three forward primers

were used, namely Fg1 (TAGTACTAACGAAAGCTCGCACATGAGTGGTC; *Sca*I site underlined), Fg2 (TAGTACTCCACGATGCGCCGATGCATTTCGG) and Fg3 (TAGTACTTAACCGACTGCGTCCAAAGT), and one reverse primer Rev (TAGTACTATGGAGCTACCATATCAG). PCR products and pSM128 were *Sca*I digested and ligated to generate pB31, pB32 and pB33, respectively. Plasmids were electroporated into *M. smegmatis* mc^2155 previously transformed with pB1 and kanamycin- and streptomycin-resistant transformants were isolated. Three independent transformants of each construct were selected for measurements of promoter activity. Cell extracts were prepared using a previously described method (Sala *et al*., 2008). β-galactosidase assays were performed on *M. smegmatis* mc^2155 cells grown at 37ºC to an OD_{600} = 1. Cells were collected by centrifugation, resuspended in 500µl of TEDP (0.1 M Tris-HCl, 1 mM EDTA, 1 mM DTT and 1 mM PMSF), and disrupted by sonication using 2 pulses of 20 seconds at 40% amplitude (Bandelin Sonoplus GM 70, Bandelin). β–galactosidase activity was assayed as described by Miller (Miller, 1972). *M. smegmatis* transformed with promoter probe vector alone (pSM128) was used as a negative control. The enzyme activities were expressed as nanomoles of *o*-nitrophenol-β-galactopyranoside converted to *o*-nitrophenol $minute^{-1}$ milligram of $protein^{-1}$.

RESULTS

The *bcg3145* SNP was an early event in the derivation of *M. bovis* BCG.

The E159G SNP in *bcg3145* is present in the genome-sequenced strain *M. bovis* BCG Pasteur 1173P2 relative to the virulent sequenced strains *M. tuberculosis* H37Rv and *M. bovis* AF2122/97 (the nucleotide sequences of the *mb3147* and *rv3124* orthologues are 100% identical). In order to confirm the presence of this mutation in other BCG strains by conventional sequencing, the gene was amplified and sequenced from "early" and "late" BCG strains (Behr *et al*., 1999). The *bcg3145* sequence from all *M. bovis* BCG strains tested (Pasteur, Denmark, Frappier, Russia, Tokyo, Sweden and Tice) differed from the *M. bovis* or *M. tuberculosis* orthologues in an adenine to guanine nsSNP that produces a glutamic acid to glycine change in amino acid residue 159 of the mature protein (E159G) relative to the orthologue in virulent strains (Figure S2). Using BLAST

we also identified the non-mutated residue in other genome-sequenced *M. tuberculosis* strains (CDC1551, KZN 1435, C, Haarlem, F11 and H37Ra). Therefore the E159G mutation was an early event during the derivation of BCG.

Modelling the structure of Rv3124 and predicted effects of the E159G mutation

To assess the function of Rv3124/BCG3145 and the possible effect of SNPs, the structure of Rv3124 was modelled using *M. tuberculosis* EmbR as the template. The sequences of these two proteins share 54% identity and 73% similarity. The resulting model (Figure 1a) shows that Rv3124 is a two-domain protein composed of an amino-terminal DNA-binding domain (DBD) and a carboxy-terminal Bacterial Transcriptional Activation (BTA) domain, the same modular arrangement present in other members of the SARP (*Streptomyces* antibiotic regulatory proteins) family of transcription factors, such as *Streptomyces peuceticus* DnrI (Sheldon *et al*., 2002). Rv3124 lacks domains present in some other SARP proteins, such as forkhead-associated domains (Alderwick *et al*., 2006). Residue E159 is located in the third α-helix of the BTA domain of Rv3124 (Figure 1b). The replacement of glutamic acid at position 159 with glycine perturbs the helix, and therefore would be predicted to have functional effects on the activity of BCG3145.

Transcriptome analysis

In order to define the *in vivo* function of BCG3145/Rv3124, the *M. tuberculosis* regulator was overexpressed in *M. bovis* BCG Pasteur under the control of the *hsp60* promoter, and the effect on the transcriptome analysed using microarrays and qRT-PCR. Overexpression of *bcg3145* or *rv3124* in BCG did not result in any change in the *in vitro* growth profile of the recombinant BCG strains in 7H9 liquid media compared to wild type BCG (data not shown). The results obtained from microarray analyses showed that 10 genes were significantly overexpressed and 11 genes were down-regulated (minimum of 3-fold differential expression) when *rv3124* was overexpressed in *M. bovis* BCG (Table 2) compared to a vector-only control. A cluster of 6 differentially expressed genes were localised in the *moa1* locus of the *M. bovis* BCG genome,

including the *moa1* locus: *moaA1* (*rv3109*), *moaB1* (*rv3110*), *moaC1* (*rv3111*) and *moaD1* (*rv3112*). To validate the microarray data for those genes with up-regulated expression, quantitative real time PCR (qRT-PCR) analysis was performed. Figure 2 compares the fold changes in expression in *M. bovis* BCG measured by microarray and qRT-PCR for the *moa1* locus. Both methods displayed broad agreement in expression levels for each gene, confirming the experimental procedures and statistical approaches used in this study. Overexpression of *rv3124* in *M. tuberculosis* H37Rv also lead to upregulation of the *moa1* locus as determined by microarray analysis (data not shown).

M. bovis BCG Δ*bcg3145* mutant construction

To assess the effects of inactivation of BCG3145 on *M. bovis* BCG, a knockout mutant was generated by inserting a hygromycin cassette into the *bcg3145* gene. Allelic exchange was confirmed in *M. bovis* BCG by PCR (data not shown) and Southern blotting (Figure S2). To initially characterize the *M. bovis* BCG Δ*bcg3145* mutant strain the growth profiles of the knockout and the wild type were measured under *in vitro* standard culture conditions and they showed similar doubling times (data not shown). As our transcriptome analysis had shown that the *moa1* locus was regulated by Rv3124, we measured the expression of these genes in the wild type and mutant by qRT-PCR, and no significant difference was found. This was probably to be expected, as BCG3145/Rv3124 both show low-level expression in mid-log phase cultures of *M. tuberculosis* or *M. bovis* BCG (Brosch *et al*., 2007; Golby *et al*., 2007).

Effect of the BCG3145 mutation on transcription of the *moa1* locus

To elucidate the effects of the E159G SNP on the function of the transcriptional regulator BCG3145, *M. bovis* BCG was complemented with a plasmid over-expressing *bcg3145* or wild-type *rv3124* under the *hsp60* promoter. Comparison of global expression across these two strains by microarray analysis revealed a set of differentially expressed genes (Table 3). As the expression level of Rv3124 was not equal across the BCG/pB1 and BCG/pB3 recombinants, it was necessary

to normalise fold-changes to Rv3124 expression levels. The final normalized fold change was therefore calculated by dividing the BCG/pB1 vs. BCG/pB3 fold changes by 2.84, the increased level of expression of Rv3124 seen in the BCG/pB1 compared to BCG/pB3. Overexpression of Rv3124 produced an 8.5- to 2.77-fold higher level of induction of the genes in the *moa1* locus compared to BCG3145 (Table 3). Microarray results were confirmed by qRT-PCR (Table S3). Hence it appears the mutation in BCG3145 reduces the ability of the regulator to induce expression of the *moa1* locus.

EMSA and *moaA1* promoter characterization

Specific *in vitro* binding of Rv3124 to the *rv3108-moaA1* region was assessed by electrophoretic mobility shift assays (EMSAs). We evaluated binding of Rv3124 to an intergenic region of 202 bp, upstream of *moaA1*/*rv3109*, which was selected as a putative binding sequence. Purified Rv3124 bound to radiolabelled Rv3109pro promoter probe DNA and retarded its mobility (Figure 3b). The extent of binding increased with Rv3124 protein concentration, with a mobility shift detected using 0.15 µg of protein. Binding to the labelled promoter could be specifically competed with a 10 and 100-fold excess of the corresponding unlabelled DNA. Bioinformatic screens for regulatory binding motifs upstream of *rv3124* and *moaA1* did not reveal any significant matches.

To further define the sequence elements recognized by Rv3124, we conducted EMSAs with 5′ deleted fragments of Rv3109pro probe, and generated promoter-reporter vector constructs. For the latter, fragments of 190 bp, 116 bp and 45 bp upstream *moaA1* were cloned into the promoterless integrative vector pSM128 (see Methods section), creating transcriptional fusions with the β-galactosidase gene (Dussurget *et al*., 1999); these constructs were then transformed into an *M. smegmatis* strain overexpressing Rv3124, and reporter activity measured (Figure 3c). β-galactosidase activity of all constructs was significantly higher than the control pSM128; however β-galactosidase background was seen in recombinants with the 190 bp fragment-*lacZ* fusion in the

absence of Rv3124 overexpression, possibly due to read-through or due to the presence of a functionally similar transcriptional regulator in *M. smegmatis*. Nevertheless, the presence of Rv3124 increased the promoter activity three-fold. The levels of expression of the 116 bp construct were approximately 50% lower than those showed for the 190 bp fragment. The smallest fragment (45 bp) only generated background activity levels.

EMSA was performed with Rv3124 and the same *moaA1* promoter fragments used in the β-galactosidase assays (Figure 3d). Fragments were generated by PCR amplification using the same primers pairs used for the promoter-probe vector construction and gel-purified. Bound protein was detected using the larger fragment Rv3109pro-190bp. The Rv3109pro-116bp probe was weakly bound by protein, and no binding was detectable with the Rv3109pro-45bp probe. Thus Rv3124 binds directly to the *moaA1* promoter.

Rv3124 domain characterization

The E159G mutation in BCG3145 occurs in the bacterial transcriptional activator (BTA) domain of BCG3145. The transcriptome results from strains overexpressing BCG3145 or Rv3124 suggested that the E159G SNP had a subtle effect on gene regulation, so we sought to determine how other mutations at the E159 locus would affect the regulator's activity. To identify other functionally important residues, BCG3145/Rv3124 was compared to other SARP family member proteins (Figure S1). Previously it had been shown that alanine substitution of the conserved residues G90 and Y91 in the DNA binding domain (DBD) of the related SARP-family regulator DnrI abrogated protein-DNA binding (Sheldon *et al*., 2002), so we sought to determine whether similar mutations in Rv3124 would block its activity.

Residues G90, Y91 and E159 in Rv3124 were subjected to site-directed mutagenesis. The range of mutations generated was G90A, Y91A, E159I, E159W, E159D, E159Q. The mutant proteins were overexpressed in the *M. bovis* BCGΔ*bcg3145* knockout strain to ensure no interference from a chromosomal copy of the gene, and alterations in the transcription levels of the

moa1 locus were determined by qRT-PCR, normalizing the *moa1* levels in the different strains to the mutated Rv3124 expression levels. Figure 4a shows the downregulation of the *moa1* locus in BCG Pasteur strains overexpressing mutated versions of Rv3124 compared with BCG overexpressing the non-mutated Rv3124.

The mutations introduced in the DNA binding domain (DBD) had divergent effects on protein function. While the G90A substitution had no effect on the transcription of the *moa1* locus, the Y91A mutation significantly reduced locus transcription. This would appear to conflict with the previously reported results obtained with the DnrI regulator (Sheldon *et al*., 2002). To determine the structural basis for these results, the G90A and Y91A mutation were mapped to the Rv3124 structural model (Fig 4b). The DBD domain is highly conserved among gene regulators of the OmpR/PhoB family (Alderwick *et al*., 2006), and is formed by a three helix bundle flanked by two beta-sheets. Residues G90 and Y91 are located in one of the two sheets. The role of G90 seems not to be critical since the bend in the short coil intersection between the two strands that form the sheet is maintained by two contiguous conserved prolines at positions 88 and 89. Therefore the mutation G90A does not have a significant effect in the activity of the protein. On the other hand, residue Y91 seems to have a more important function in the overall stability of the DBD by its interaction with another conserved residue (H63) located in a neighbouring helix. Hence the change of tyrosine to alanine in Y91A causes a perturbation of the local structure that compromises binding to DNA, and hence explains the decrease in the induction of the *moa1* locus by the Y90A variant of Rv3124.

To assess the importance of residue 159 in the BTA domain, several mutations were introduced. First, we were interested in evaluating the effect of a complete change in residue properties; hence, glutamic acid was substituted for isoleucine or tryptophan. These mutations completely disrupted protein function, as shown by the dramatic decrease of RNA levels for the *moa1* locus (Figure 4a). To assess the functional impact of more subtle changes, Glu159 was substituted with aspartic acid or glutamine. qRT-PCR analysis revealed that the aspartic acid

mutation did not significantly alter protein function, with the E159D Rv3124 protein able to complement the knockout strain to wild type levels. However, complementation with the E159Q mutated version of Rv3124 was not able to induce the *moa1* locus to the same degree as the wild type protein. Again using the Rv3124 structural model to map the mutations, it was evident that E159G, E159I, E159W caused a perturbation of the helix that affects the function of the BTA domain. Mutations E159D and E159Q have a lesser effect on the structure and hence maintain the functionality of the domain.

DISCUSSION

Comparison of the genome of *M. bovis* BCG Pasteur with *M. bovis* and *M. tuberculosis* has revealed a range of deletions and SNPs between the vaccine strain and virulent tubercle bacilli that may play a role in the virulence attenuation of BCG. However, while clear functional links have been made for some mutations in BCG, such as the RD1 deletion or SNPs in the *mmaA3* and *pykA* genes, linking genetic differences to phenotypic changes is far from trivial.

A comprehensive survey of SNP differences across BCG vaccine strains and *M. bovis* strains from the UK and France revealed 186 SNPs that differentiated virulent *M. bovis* from BCG, with 115 of these SNPs nonsynonymous. An amplification of this mutational difference is achieved when nsSNPs occur in regulatory genes, as perforce each one affects the expression of a wide range of genes. Amongst these nsSNPs was a mutation in *bcg3145*, encoding a putative transcriptional regulator of the AfsR/DnrI/SARP family. The predicted secondary structure of BCG3145 revealed that the protein consists of two conserved domains. The N-terminal region is a DNA binding domain (residues 1-96) formed by three α-helices packed against two antiparallel β-sheets forming a winged helix-turn-helix (Martinez-Hackert & Stock, 1997a), and a C-terminal region (residues 97-270) which is a Bacterial Transcriptional Activation domain formed with seven α-helixes (Alderwick *et al*., 2006). It has been suggested that the region of α-loop connecting the recognition helix α3 and the positioning helix α2 which are conserved in the DNA binding domain of the SARP family interacts with the C-terminal domain of the α subunit of RNA polymerase (Martinez-Hackert & Stock, 1997b; Tanaka *et al*., 2007). The E159G mutation in BCG3145 mutates to glycine a conserved glutamic acid residue located in a tetratricopeptide repeat (TRP) in the BTA domain (region T3). TRP domains are associated with protein-protein interactions (D'Andrea & Regan, 2003), while a conserved core (helices T1 to T7) of the BTA domain seems to be required for proper function of SARP family proteins (D'Andrea & Regan, 2003; Sheldon *et al*., 2002).

In the present study we found that BCG3145/Rv3124 is a positive transcriptional regulator of the *moa1* locus, promoting the expression of the *moaA1B1C1D1* genes required for molybdopterin biosynthesis. Microarray results were confirmed independently by qRT-PCR, while direct binding of Rv3124 to the upstream region of *moaA1*, the first gene of the *moa1* locus, was confirmed by EMSAs. The *moaA1B1C1D1* genes encode key enzymes in the synthesis of molybdopterin, a pterin-based molybdenum-binding cofactor of molybdoenzymes such as nitrate reductase. MoaA1 and MoaC1 are predicted to catalyse the conversion of GTP into cyclic pyranopterin monophosphate (cPMP), the first step in the synthesis of molybdopterin; cPMP is then converted to the metal-binding pterin dithiolate by molybdopterin synthase, a heterotetrameric complex of MoaD1 and MoaE1 (Rv3119). MoaB1 is predicted to be involved in insertion of molybdenum into molybdopterin to form the molybdenum cofactor (Schwarz *et al* 2009).

In *E. coli*, the *moa* operon shows increased expression under anaerobiosis (Baker & Boxer, 1991), a situation which requires the production of several essential molybdoenzymes for anaerobic respiration (Self *et al*., 1999). Oxygen limitation is considered to be a key factor affecting the metabolism of *M. tuberculosis* in the hypoxic environment of the granuloma (Parish & Brown, 2008). In genome-wide expression analysis of *M. tuberculosis* isolated from lung biopsy samples, a 13-fold upregulation of *rv3124* was observed in granuloma tissues of patients with active tuberculosis compared to *in vitro* cultures of *M. tuberculosis* (Rachman *et al*., 2006). This condition is reproduced *in vitro* by the Wayne model (Wayne & Hayes, 1996) where oxygen is depleted gradually and *M. tuberculosis* growth shows two phases, a microaerobic state, called nonreplicating persistent stage 1 (NRP-1), and an anaerobic stage, NRP-2. *rv3124* has been shown to be upregulated in both NRP-1 and NRP-2 states in *M. tuberculosis* compared to aerobic roller cultures (Muttucumaru *et al*., 2004). Hence, regulation of *rv3124* appears to be modulated by oxygen availability, and this is an area that we are investigating further.

Sequence alignments and protein modelling suggested that the E159G mutation in BCG3145 would have structural consequences, with microarray analysis showing that the

mutation had a subtle effect on the ability of BCG3145 to activate *moa1* locus transcription. Overexpression of mutated variants of Rv3124 in BCG, with subsequent qRT-PCR on the constituent genes of the *moa1* locus, showed that E159I and E159W substitutions had profound effects on protein function, as would be expected, with E159Q showing more subtle effects, while mutation of the Tyr91 reside in the DNA-binding domain also abrogated the regulator's activity.

Conclusion

We have identified Rv3124 as a positive regulator of molybdopterin biosynthesis, and defined the functional consequences of a mutation in the BCG3145 orthologue. As Rv3124 regulates expression of the *moa1* locus, we suggest *rv3124* be renamed *moaR1*. The E159G mutation in BCG3145 was shown to decrease, but not ablate, the ability of the regulator to induce expression of the *moa1* locus. Given our results, and the fact that Rv3124 was not identified as a virulence factor in *M. tuberculosis* using saturation mutagenesis screens (Sassetti & Rubin, 2003), it would appear that the mutation in BCG3145 is not a key attenuating genetic lesion in BCG.

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Table 1. Bacterial Strains and plasmid used in this study

Bacterial strain/plasmid	Relevant genotype or characteristics	Reference or source
M. bovis BCG	Pasteur strain	VLA Weybridge-UK
M. bovis AF2122/97	Virulent isolate from a cow in Cornwall, UK.	VLA Weybridge-UK
M. tuberculosis H37Rv	Virulent strain isolated in 1905	VLA Weybridge-UK
M. smegmatis mc^2 155	Mutant with high plasmid transformation efficiency	Prof. W.R. Jacobs, AECOM, NY, USA
M. bovis BCG Δbcg3145	Knockout strain of *M. bovis* BCG Pasteur with *bcg3145* gene replaced by hygromycin cassette.	This study
E. coli DH5α	General cloning strain	Invitrogen
E. coli BL21(DE3)	Strain used for protein expression	Invitrogen
pSM96	Mycobacterial expression vector with *hsp60* promoter	VLA Weybridge-UK (Wooff *et al*., 2002)
pB1	*rv3124* in pSM96	This study
pB3	*bcg3145* in pSM96	This study
MBPpET	MBP-His fusion vector used in protein purification	Vircell, SL.
pYUB854	Cosmid vector used for mutant construction. It contains the hygromycin cassette and a multiple cloning site.	(Bardarov *et al*., 2002)
p3145KO	Cosmid used in the *bcg3145* knockout containing the gene flanking regions into pYUB854	This study
pSM128	Mycobacteriophage L5-based vector carrying a *lacZ* reporter	(Dussurget *et al*., 1999)
pB31	190bp upstream of *rv3109* in pSM128	This study
pB32	116bp upstream of *rv3109* in pSM128	This study
pB33	45bp upstream of *rv3109* in pSM128	This study
pB10	G90A mutation in the DNA binding domain	This study

pB11	Y91A mutation in the DNA binding domain	This study
pB15	E159I mutation in the BTA domain	This study
pB17	E159W mutation in the BTA domain	This study
pB23	E159D mutation in the BTA domain	This study
pB25	E159Q in the BTA domain	This study

Table 2. Microarray identification of differentially expressed genes in BCG overexpressing Rv3124 compared to BCG/pSM96 vector control

Rv number	Gene	Product	Fold Change*
rv0991		Hypothetical protein	3.0
rv2428	*ahpC*	Alkyl hydroperoxide reductase C	5.4
rv2636		Hypothetical protein	3.4
rv2931	*ppsA*	Phenolpthiocerol synthesis	3.0
rv3109	*moaA1*	Molybdopterin biosynthesis	36.8
rv3110	*moaB1*	Molybdopterin biosynthesis	21.5
rv3111	*moaC1*	Molybdopterin biosynthesis	16.4
rv3112	*moaD1*	Molybdopterin biosynthesis	9.3
rv3113		Conserved phosphatase	15.2
rv3114		Hypothetical protein	5.1
rv0823c		Transcriptional regulator	0.26
rv0824c	*desA1*	Fatty acid desaturase	0.33
rv1094	*desA2*	Fatty acid desaturase	0.31
rv1798		Conserved hypothetical protein	0.32
rv2836c	*dinF*	DNA-damage-inducible protein	0.33
rv2987	*leuD*	3-isopropylmalate dehydratase	0.33
rv2988	*leuC*	3-isopropylmalate dehydratase	0.16
rv2989		Transcriptional regulator	0.18
rv3212		Alanine-valine rich protein	0.32
rv3229c	*desA3*	Fatty acid desaturase	0.32
rv3919	*gid*	Glucose-inhibited division protein	0.28

***** Values <1 are down regulated in BCG overexpresssing Rv3124 compared to BCG/pSM96

Table 3. Genes differently expressed in BCG overexpressing Rv3124 (BCG/pB1) or BCG3145 (BCG/pB3)

		BCG/pB1*			BCG/pB3			Fold change without normalization to Rv3124 expression level	Normalized fold change[¶]
Rv	**Gene**	**Expression level (log_2)**	**Minimum level (log_2)**	**Maximum level (log_2)**	**Expression level (log_2)**	**Minumum level (log_2)**	**Maximum level (log_2)**		
rv2933	*ppsC*	-0.45	-0.51	-0.35	-2.17	-2.41	-2.06	3.39	1.15
rv2932	*ppsB*	-0.69	-0.81	-0.62	-0.35	-0.44	-0.32	3.08	2.35
rv3058		1.69	1.57	1.89	0.07	0.05	0.08	3.28	1.08
rv3109	*moaA1*	1.87	1.56	2.02	-2.70	-2.86	-2.64	23.91	8.41
rv3110	*moaB1*	2.29	2.16	2.43	-2.31	-2.48	-2.08	24.36	8.57
rv3111	*moaC1*	0.97	0.95	1.12	-2.88	-2.94	-2.45	14.51	5.11
rv3112	*moaD1*	1.62	1.43	1.97	-1.35	-1.71	-1.06	7.89	2.77
rv3113		0.57	0.35	0.74	-2.89	-3.26	-2.83	11.02	3.88
rv3114		0.24	0.13	0.32	-1.67	-1.81	-1.18	3.78	1.33

*Expression levels for each gene are the median of three independent microarray experiments. Minimum and maximum expression levels for the three replicates are shown.

[¶] Rv3124 was expressed at 2.84-fold higher in the BCG/pB1 compared to BCG/pB3

Figure Legends

Figure 1.

(a) Rv3124 structural model based on EmbR molecular structure; in green the DNA binding domain, in purple the BTA domain. Residue E159, located in the third α-helix of the BTA domain is shown in red. **(b)** Close up of the third α-helix of the BTA domain taken from a different angle. In blue, the side chains of residues that form the helix, except for E159, which is shown in red. Intra-chain H bonds are represented by green dashed lines. Replacement of Glu159 by Gly perturbs the helix.

Figure 2. Confirmation of microarray results by qRT-PCR. The *moa1* locus showed a higher level of expression in *M. bovis* BCG overexpressing Rv3124 than *M. bovis* BCG wild type as measured by microarray (white bars) and by qRT-PCR (black bars). Fold changes are the mean ratios ± standard deviation of gene expression from two independent experiments.

Figure 3. Binding of Rv3124 to *rv3108-moa1A* intergenic region.

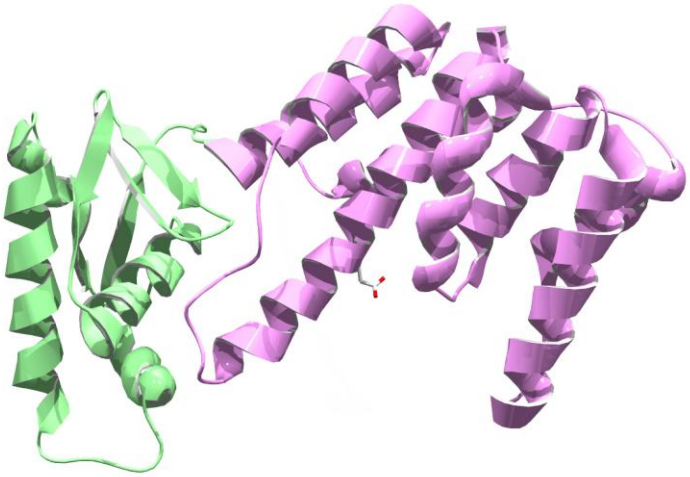
(a) The *moaA1* locus showing locus organisation and positions of oligonucleotides used in EMSA and *lacZ* fusions. Primers F1 and R3 were used to generate the Rv3109pro product used in EMSA assays; primers Fg1, Fg2, Fg3 and Rev were used to generate products for *lacZ*-transcriptional fusions. **(b)** EMSA was performed in the absence of Rv3124 (lane 1) or in the presence of 0.15 µg, 0.3 µg, 0.6 µg or 1.2 µg of purified Rv3124 (lanes 2-5). Competition was performed with Rv3124 (2.4 µg) with no competitor (lane 6) or with unlabeled specific competitor *rv3109* probe (10 and 100 fold-molar excess, lanes 7 and lane 8, respectively). **(c)** β-galactosidase activity of promoter probe constructs in wild type *M. smegmatis* mc^2155 (denoted by "-") and *M. smegmatis* overexpressing Rv3124 (denoted by "+"). Activity was monitored by plating strains on media containing X-gal and by quantitative β-galactosidase assay. Data are shown as Miller Units, and are the mean ± standard deviation of three replicates. **(d)** Binding assay with full length and truncated versions of the Rv3109pro probe (190bp, 116 bp, and 45bp) in the absence of Rv3124 (lane 1) or in the presence of

0.6 μg and 1.2 μg of Rv3124 (lanes 2-3 respectively). Decreased Rv3124 binding to the shorter probes correlates with reduced β-galactosidase activity in the corresponding *lacZ* transcriptional fusions.

Figure 4. (a) Fold change of the *moa1* locus in BCG strains overexpressing mutated versions of Rv3124. The expression of the *moa1* locus in BCG strains complemented with the mutated Rv3124 is compared to *M*. *bovis* BCG overexpressing the non-mutated Rv3124. Data from two independent experiments for each strain was first normalised to Rv3124 and then normalized to *sigA* RNA levels. **(b)** Residues G90 and Y91 (depicted in blue) are located in one of the two beta-sheets that form the DBD of Rv3124. Residue G90 seems not to be critical, explaining why its mutation had no effect on the regulator's activity. However, residue Y91 interacts with a conserved residue (H63) located in a neighbouring helix, showing why mutation of this residue in MoaR1 caused a decrease the induction of the *moa1* locus.

Figure 1

(a)



(b)

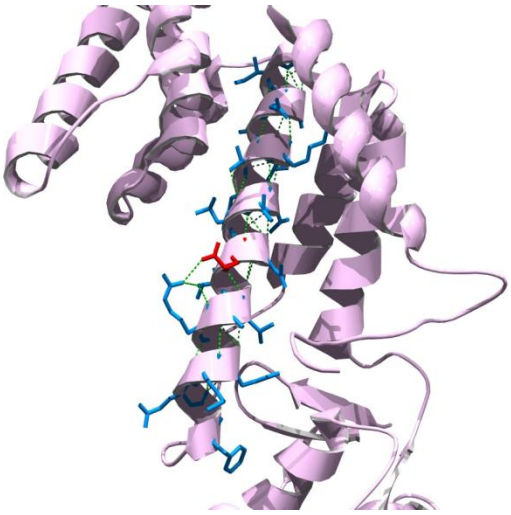


Figure 2

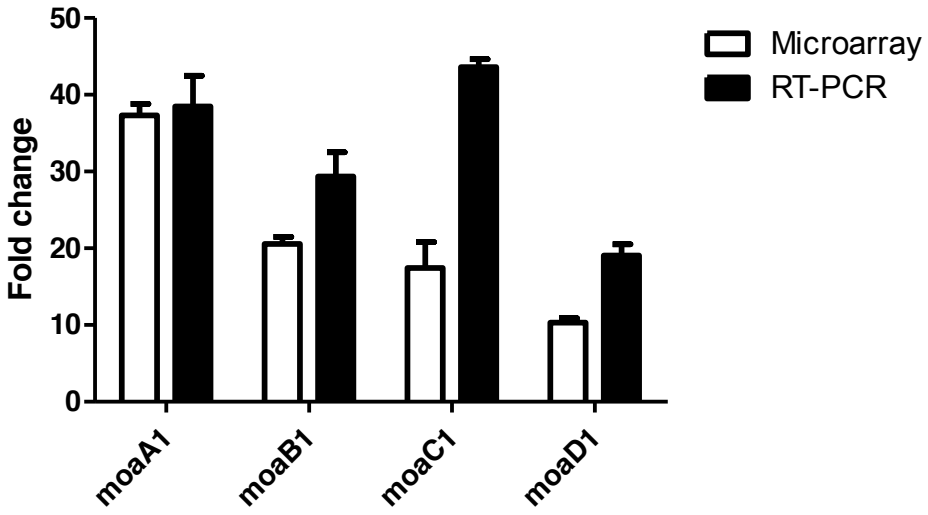
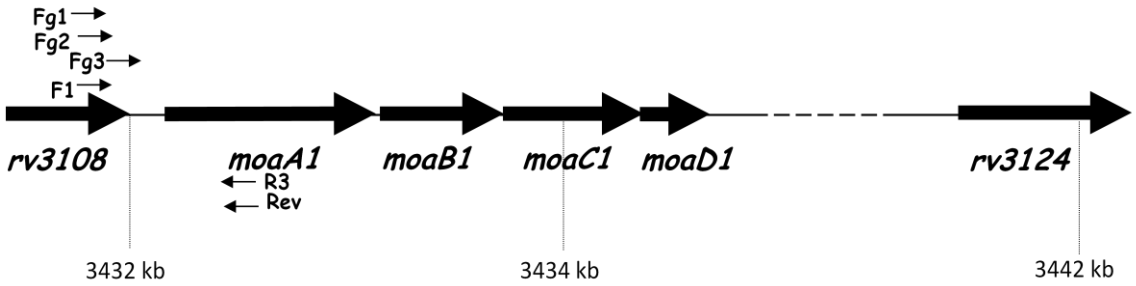
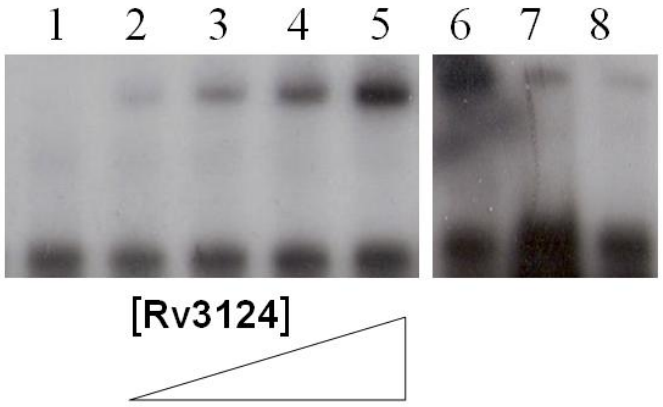

Fold change
50
40
30
20
10
0
moaA1
moaB1
moaC1
moaD1
Microarray
RT-PCR

Figure 3

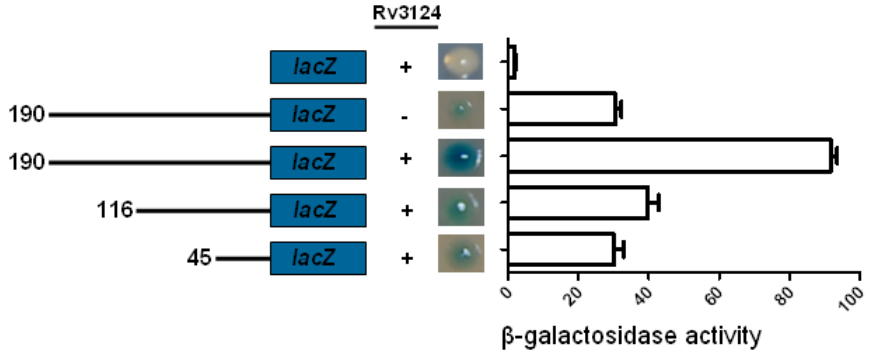
(a)


Fg1
Fg2
Fg3
F1
rv3108
moaA1
R3
Rev
moaB1
moaC1
moaD1
rv3124
3432 kb
3434 kb
3442 kb

(b)


1 2 3 4 5 6 7 8
[Rv3124]

(c)


Rv3124
lacZ
+
190
lacZ
-
190
lacZ
+
116
lacZ
+
45
lacZ
+
0
20
40
60
80
100
β-galactosidase activity

(d)

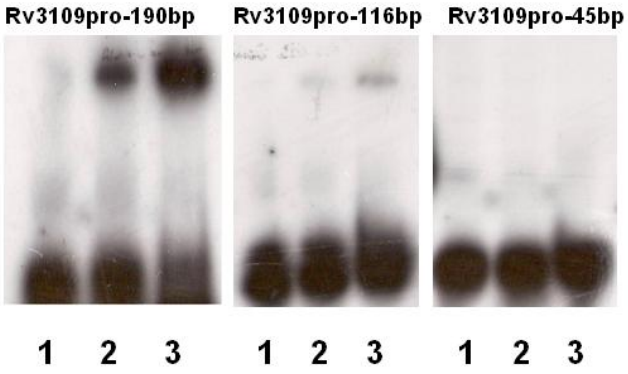
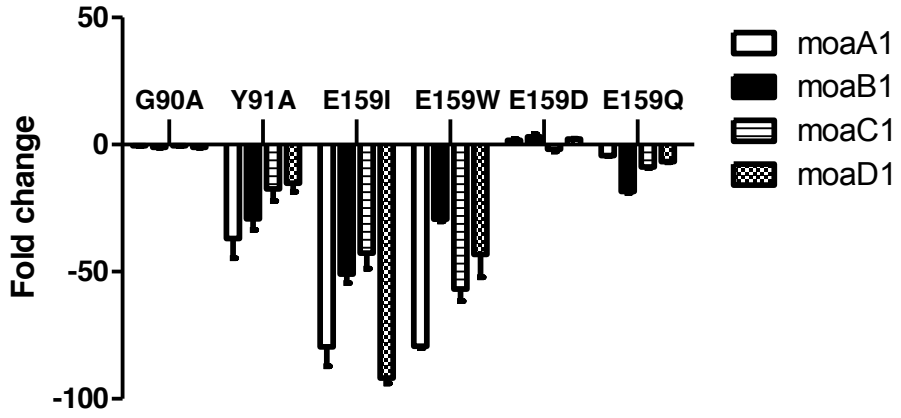
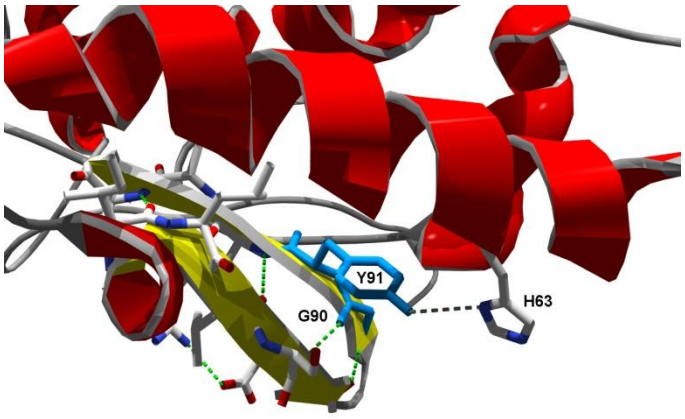

Rv3109pro-190bp
Rv3109pro-116bp
Rv3109pro-45bp
1 2 3 1 2 3 1 2 3

Figure 4

(a)


50
0
-50
-100
Fold change
G90A
Y91A
E159I
E159W
E159D
E159Q
moaA1
moaB1
moaC1
moaD1

(b)


Y91
G90
H63