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NON-TUBERCULOUS MYCOBACTERIA: GENERAL

Characterisation of a putative AraC transcriptional regulator from Mycobacterium smegmatis

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S U M M A R Y

MSMEG_0307 is annotated as a transcriptional regulator belonging to the AraC protein family and is located adjacent to the arylamine N-acetyltransferase (nat) gene in Mycobacterium smegmatis, in a gene cluster, conserved in most environmental mycobacterial species. In order to elucidate the function of the AraC protein from the nat operon in M. smegmatis, two conserved palindromic DNA motifs were identified using bioinformatics and tested for protein binding using electrophoretic mobility shift assays with a recombinant form of the AraC protein. We identified the formation of a DNA:AraC protein complex with one of the motifs as well as the presence of this motif in 20 loci across the whole genome of M. smegmatis, supporting the existence of an AraC controlled regulon. To characterise the effects of AraC in the regulation of the nat operon genes, as well as to gain further insight into its function, we generated a ΔaraC mutant strain where the araC gene was replaced by a hygromycin resistance marker. The level of expression of the nat and MSMEG_0308 genes was down-regulated in the ΔaraC strain when compared to the wild type strain indicating an activator effect of the AraC protein on the expression of the nat operon genes.

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1. Introduction

Transcriptional factors modulate gene expression through binding to a specific DNA sequence usually found upstream of the gene or the genomic area that they control. They are important proteins that can help cells acclimatise to challenging environments based on the changing external stimuli. The AraC/XylS protein family of transcriptional regulators, present in bacterial species is involved in a variety of cellular processes from carbon metabolism to stress responses and the regulation of virulence [1]. Common characteristics of the AraC proteins is the presence of a conserved region of 100 residues in the C-terminal region of the protein that form a helix-turn-helix structure responsible for DNA binding, a second region in the N-terminal region of the protein contains a ligand binding domain and a peptide-linker region connecting the two functional domains. The proteins that belong to the AraC/XylS family usually recognise palindromic DNA sequences and bind to them by forming dimers using the helix-turn-helix domain [2].

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB) can survive within macrophages as well as in the extreme environment found in granulomas during infection in the human body. For this reason, the genome of M. tuberculosis contains an exceptionally large number of transcriptional factors, including 13 sigma factors, 5 anti-sigma factors and 7 anti-anti-sigma factors [3] which assist its adaptation to different environments and stresses. Six of these 190 transcription factors belong to the AraC/XylS family (Rv1317, Rv1395, Rv1931c, Rv3082c, Rv3736 and Rv3833). Most of the M. tuberculosis AraC proteins characterised,

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such as the AraC proteins encoded by Rv1395, Rv1931c and Rv3082c genes are linked with virulence as their genetic alteration generates an attenuated phenotype either in vitro in macrophage infection model or in vivo in mice [4–8]. Until now there has been no functional information regarding the role of the Rv3736 and Rv3833 encoded AraC proteins in M. tuberculosis. Furthermore, Mycobacterium smegmatis, the saprophytic environmental species of the genus Mycobacterium and a common laboratory surrogate for molecular genetic studies of M. tuberculosis, contains 16 different AraC proteins encoded in its genome, indicating the need of this organism to adapt to multiple niches. One of these AraC proteins is encoded by the MSMEG_0307 gene which is located between the aryamine N-acetyltransferase (nat, MSMEG_0306) gene and a novel oxidoreductase (MSMEG_0308) that is believed to be involved in riboflavin biosynthesis [9].

The nat operon in M. tuberculosis has been validated as a likely therapeutic target due to its important endogenous roles in M. tuberculosis, related to cholesterol degradation, cell wall biogenesis, intracellular growth and altered drug susceptibility [10–12]. In M. tuberculosis, the hsaA (Rv3570c), hsaB (Rv3567c), hsaC (Rv3568c) and hsaD (Rv3569c) genes are co-transcribed with the nat (Rv3566c) gene and their corresponding proteins have been shown to be directly involved in the cholesterol metabolism pathway [13]. The NAT protein utilises acyl coenzymeA (CoA) catabolites, including acetyl CoA and n-propionyl CoA. These intermediates play a central role in metabolic support of cell wall biosynthesis [14,15]. Cholesterol is considered to be a vital energy source for M. tuberculosis cells growing within macrophages [16]. In addition, this nat gene cluster in M. tuberculosis is under the control of the kstR transcription factor (Rv3574) that controls a regulon of genes involved in lipid and cholesterol metabolism [17].

In contrast, the gene organisation around nat differs in M. smegmatis and the hsaA-D genes (MSMEG_6035–MSMEG_6038) are clustered together in a region about 57 kb downstream from the nat locus. Nevertheless, kstR regulatory DNA sequences are present between the nat and the MSMEG_0305 genes (Figure 1) and upstream of the hsaA-D gene cluster in the genome of M. smegmatis indicating possible co-regulation. However, it has been shown previously that the nat gene cluster in M. smegmatis is not directly controlled by the kstR transcriptional regulator but rather the MSMEG_0305 is under the effect of the KstR regulator [17,18].

In the view of the presence of a transcription factor that belongs to the AraC protein family in the nat gene cluster in M. smegmatis it is important to assess whether it influences the expression of nat and the adjacent gene for the novel reductase (MSMEG_0308). The question of whether the AraC transcriptional factor has a role in the regulation of genes that are involved in lipid and cholesterol metabolism in M. smegmatis also needs to be addressed. We report here, the characterisation of the AraC-family transcriptional regulator MSMEG_0307 from M. smegmatis using biochemical assays, the characterisation of its regulon and DNA binding sites, as well as its influence on the regulation of gene expression of the nat gene cluster in M. smegmatis. In addition, we have characterised a protein–protein interaction network that is formed by the nat operon gene products.

Figure 1. The nat operon in M. smegmatis. A comparison between the nat operons in M. smegmatis and M. tuberculosis. In the nat operon of M. smegmatis the intergenic region between nat and MSMEG_0305 genes is shown in loop with the location of the kstR and araC motifs highlighted in the sequence. In the M. tuberculosis nat operon, the identified promoter and kstR sequences are shown with the arrows. RT-PCR amplification of the overlapping regions of the nat operon genes using specific primers, shown as numbered arrows on top of the genes. Lanes with (+) on the agarose gel correspond to cDNA whereas lanes with (−) corresponds to negative control, i.e. cDNA that produced without the addition of reverse transcriptase (RT) in order to distinguish possible genomic DNA contamination. Primer set 1: MSMEG_0305-nat, primer set 2: nat-MSMEG_0307, primer set 3: MSMEG_0305-MSMEG_0308 and primer set 4: MSMEG_0308-MSMEG_0309.
2. Materials and methods

2.1. Bacterial strains, growth conditions and plasmids

The bacterial strains and plasmids used in this study are listed in Table S1. The oligonucleotide primers are listed in the Supplementary Table S1. Escherichia coli strains JM109 and BL21(DE3) pLysS were grown in Luria–Bertani (LB) broth with rotation at 200 rpm or in LB agar at 37 °C, unless specified otherwise. M. smegmatis mc²155 [19] and the modified strains were grown in Middlebrook 7H9 broth supplemented with 0.2% (v/v) glycerol, 0.05% (w/v) Tween-80 and 10% ADC (Albumin-Dextrose-Catalase, BD) with rotation at 180 rpm or in Middlebrook 7H10 agar supplemented with 0.5% (v/v) of glycerol and 10% OADC (Oleic acid-Albumin-Dextrose-Catalase, BD) at 37 °C, unless specified otherwise. Comparative growth curves of M. smegmatis mc²155 and the Δnat, ΔMSMEG_0307 and ΔMSMEG_0308 strains were performed using mycobacterial minimal medium (0.5 g l⁻¹ of l-asparagine, 1 g l⁻¹ of KH₂PO₄, 2.5 g l⁻¹ of Na₂HPO₄, 50 mg l⁻¹ of ferric ammonium citrate, 0.5 g l⁻¹ of MgSO₄.7H₂O, 0.5 mg l⁻¹ of CaCl₂ and 0.1 mg l⁻¹ of ZnSO₄) supplemented with a carbon source (glycerol, glucose, melibiose or cholesterol) at 0.2% (v/v). Antibiotics were used at the following concentrations (μg ml⁻¹): kanamycin (30), chloramphenicol (34), hygromycin B (100 for E. coli and 50 for M. smegmatis) and trimethoprim (12). All chemicals were purchased from Sigma–Aldrich (Poole, UK) unless otherwise stated. High-fidelity Phusion DNA polymerase (NEB) was employed in all cloning related PCR reactions whereas Taq DNA polymerase (NEB) was used in all other PCR reactions in this study. Restriction digestion enzymes were purchased from New England Biolabs (Hitchin, UK). All constructs were confirmed by sequencing (Gene Service, UCL).

2.2. Identification promoter sites and DNA motifs

All mycobacterial genome sequences that were used in this study were obtained from the NCBI (http://www.ncbi.nlm.nih.gov), BLAST analysis was performed using the NCBI BLAST algorithm [20]. Multiple sequence alignments were done using ClustalW algorithm at the EBI server [21]. For viewing, annotating and comparing mycobacterial genomes, the java-based software packages Artemis and ACT, from the Sanger Institute, were used [22,23]. The sequence viewer, PromView (http://www.comlab.ox.ac.uk/activities/compbio/bioinformatics-software/index.html#PromView) was used for the search of consensus promoter sequences obtained from previously published mycobacterial promoters [24]. The MEME algorithm [25] was used to discover conserved palindromic DNA motifs among mycobacterial species and MAST [26] was used to identify the presence of these motifs in the genome of M. smegmatis mc²155.

2.3. Cloning, overexpression and purification of MS0307

The gene encoding the AraC protein (MSMEG_0307) from M. smegmatis mc²155 was PCR amplified from genomic DNA and cloned into pET28b (+) vector (Novagen). The N-terminus of the AraC-family transcriptional regulator MSMEG_0307 was co-transcribed with a thrombin cleavage site followed by a hexahistidine tag. The recombinant AraC-family transcriptional regulator MSMEG_0307 was produced in BL21(DE3)pLysS cells at 18 °C following induction with 0.5 mM IPTG overnight. Cells were lysed by sonication on wet ice (5 cycles of 45sec on, 45sec off) and the AraC-family transcriptional regulator MSMEG_0307 was purified using nickel affinity chromatography (Invitrogen). The His-tagged MSMEG_0307 protein was then further purified on a HiLoad 16/60 Superdex™ 75 pg (Pharmacia) preparative gel filtration column, equilibrated with 20 mM Tris–HCl pH 8, 100 mM NaCl. Fractions contained pure (>99%) His-tagged MSMEG_0307 protein was pooled and concentrated using an Amicon Ultra concentrator (Millipore) at 5 mg ml⁻¹ and stored in 50% (v/v) glycerol in –80 °C for further use.

2.4. Electrophoretic mobility shift assays (EMSAs)

The DNA fragments (~300bp) containing the binding sequence motifs were amplified using PCR and primers (Table S1) and further purified. The reactions had a final volume of 10 μl and contained 100 ng of DNA, 1 × EMSA buffer (20 mM Tris.HCl pH8, 75 mM NaCl, 10 mM MgCl₂) and increasing concentrations of recombinant His-tagged MSMEG_0307 protein (0.01 μg to 1 μg). The reactions were incubated at room temperature for 30 min and then were loaded onto a 5% (v/v) native polyacrylamide gel. Following electrophoresis the gels were stained with ethidium bromide and the bands were visualised using BioDoc-It™ imaging system (UVP, Cambridge, UK).

2.5. Generation of ΔaraC in M. smegmatis and complementation studies

The deletion of the MSMEG_0307 gene (MSMEG_0307) from M. smegmatis mc²155 was performed using the method of specialised transduction as described previously [27]. Briefly, the left (898 bp) and right (782 bp) arms of the MSMEG_0307 gene were PCR amplified using the primers given in Table S1 and cloned into the suicide delivery vector p0004S to create the allelic-exchange plasmid p0004S-MSMEG_0307. The p0004S-MSMEG_0307 was then PacI digested and packed into the temperature sensitive mycobacteriophage phAE159 to generate the allelic-exchange phage phΔaraC. Wild-type M. smegmatis mc²155 was transduced using high-titre phΔMSMEG_0307 phages as described [27]. Following the specialised transduction, hygromycin resistant colonies were screened by PCR using a gene internal and external primer set (Table S1) and ΔaraC mutants were further confirmed by DNA sequencing. Complementation of the ΔMSMEG_0307 as well as overexpression of the WT M. smegmatis mc²155 were performed using the pMV261 plasmid [28] with the native MSMEG_0307 expressed under hsp60 mycobacterial promoter located in the plasmid.

2.6. Drug susceptibility assay

The susceptibility of mycobacterial strains against various antibiotics was determined using the resazurin redox indicator assay as described previously [29]. Briefly, wild-type M. smegmatis mc²155 and the ΔMSMEG_0307 mutant were grown until mid-exponential phase (1 OD₆₀₀) and then 100 μl of diluted cells (10⁶ CFUs) were added into a 96 well plate that contained 100 μl of two-fold dilutions of antibiotics at various concentrations in μg/ml. [isoniazid (INH 50 to 0.09), pyrazinamide (PZA 50 to 0.09), rifampicin (RMP 50 to 0.09), ethambutol (EMB 150 to 0.29), streptomycin (STM 50 to 0.09), kanamycin (KAN 50 to 0.09), ampicillin (AMP 150 to 0.29) and chloramphenicol (CLP 50 to 0.09)]. The plates were incubated at 37 °C for 2 days. Following 24 h of incubation, 50 μl of 0.01% (w/v) sterile resazurin solution in presence of 1% (v/v) Tween–80 was added to all wells of the plate and left overnight at 37 °C. The minimum inhibitory concentrations (MICs) were defined as the lowest antibiotic concentration of the well where bacterial cells were not able to grow and thus did not reduce the resazurin dye.
2.7. RNA extraction and cDNA synthesis

Wild-type M. smegmatis mc²155, ΔMSMEG_0307, wild-type M. smegmatis mc²155 with either the empty vector (pMV261) or overexpressing MSMEG_0307 (pMVaraC) and the ΔMSMEG_0307 complemented mutant cells were grown in Middlebrook 7H9 broth until OD₆₀₀ was 0.8 and total RNA was extracted from using the GTC method as previously described [17] and cDNA was synthesised using SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer’s instructions. A control was set up to assess genomic DNA contamination by replacing the SuperScript III Reverse Transcriptase with water.

2.8. Operon analysis

The boundaries of the nat operon were identified by PCR amplification of the intergenic regions of the operon and adjacent genes using M. smegmatis mc²155 cDNA as a template and specific primers (Table S1) for each region. Positive (cDNA from M. smegmatis mc²155) and negative controls (cDNA made without Reverse Transcriptase) were also used. The amplicons were then analysed using agarose gel electrophoresis.

2.9. RT-qPCR

Real-time quantitative polymerase chain reactions (RT-qPCR) were performed using the DyNamo SYBR Green qPCR kit (NEB) on the MJ Research Bio-Rad Real Time PCR Opticon 2 System (GRI). M. smegmatis mc²155 gDNA was used for the generation of a standard curve and sigA (MSMEG_2758; a mycobacterial sigma factor) was used as a reference gene for the relative quantification method. Briefly, a 20 µl reaction was set up on ice containing 1× DNA Master SYBR Green 1 mix, 1 µl of cDNA and 0.3 µM of each primer (Table S1). The PCR reactions were initially heated to 95 °C for 10 min before 35 cycles of 95 °C for 30 s, 62 °C for 20 s, and 72 °C for 20 s were performed. Fluorescence was measured at the end of each cycle following a heating step to 80 °C to ensure the denaturation of any primer-dimers. At the end of the PCR, melting curve analysis was performed to verify the product specificity. The experiment was performed in duplicate and each gene was measured in triplicates (three biological replicates, two experimental replicates) giving a total of six data points per gene. Fold changes were calculated using the 2⁻ΔΔCt statistical method [30].

3. Results

3.1. Defining the nat operon in M. smegmatis

In order to define the presence and extent of a nat operon in M. smegmatis, RT-PCR analysis was performed on the basis that any amplicons obtained using intergenic primers would indicate that the two genes are co-transcribed together and thus belong to the same operon (Figure 1). Using this rationale, we obtained amplicons for the intergenic regions between nat-MSMEG_0307 (MSMEG_0306-MSMEG_0307) and MSMEG_0307-MSMEG_0308 (MSMEG_0307-MSMEG_0308) but not between the MSMEG_0305-nat and MSMEG_0308-MSMEG_0309, indicating that in M. smegmatis the nat operon consists of three genes (Figure 1). Using bioinformatic analyses, we search for a putative promoter sequence using as input known mycobacterial promoters; however we were not able to identify a conserved promoter sequence upstream of nat gene at the start of the operon. A comparative analysis on different mycobacterial genomes indicated that a similar gene organisation was seen in the nat operon among fast-growing mycobacteria as opposed to the nat and hsaA-D clusters found in slow-growing mycobacteria (Figure S1).

Thorough sequence analysis using the MEME algorithm [25] was carried out on the M. smegmatis nat operon as well as a 1.5 kb DNA fragment upstream of the nat gene in order to identify the presence of regulatory DNA sequences that could be affected by the MSMEG_0307 gene product. Comparative genome analyses were performed on the closely related fast-growing mycobacterial species that have similar nat gene clusters and an MSMEG_0307 orthologue (M. smegmatis, Mycobacterium gilvum, Mycobacterium vanbaalenii and Mycobacterium sp. MCS/KMS/JLS). This approach identified two different regulatory motifs. The motif designated from now on as Motif 1 is located between −25 and −5 upstream of the nat gene start codon (Figure 2A) and another motif designated from now on as Motif 2 is located between −57 and −34 upstream of the MSMEG_0307 gene (Figure 2A). Motif 1 consists of a 20 bp palindromic DNA sequence (ACCTGCACGAGTCGAGTT) (Figure 2A) and the Motif 2 consists of a 23 bp DNA sequence (GTCAGACGACTTTCTTCTT) (Figure 2A). In order to identify the presence of these two motifs elsewhere in the genome of M. smegmatis as well as possible sites of action of the MSMEG_0307 protein, the MAST algorithm was employed to search a database of intergenic regions of M. smegmatis genome [26]. Motif 1 was found to be present in twenty additional instances in the M. smegmatis genome (Table S2) and the Motif 2 in five additional instances (Table S3). This indicates the existence of a regulon controlled by the AraC transcription regulator MSMEG_0307.

3.2. Binding of AraCMsmeg to the identified DNA motifs

In order to determine whether the MSMEG_0307 protein binds to any of the two identified motifs, the MSMEG_0307 protein from M. smegmatis mc²155 was cloned, over-expressed and purified as a recombinant protein (Figure S2). DNA fragments containing Motif 1 or 2 were amplified using PCR and used for Electrophoretic Mobility Shift Assays (EMSA). As shown in Figure 2B the presence of recombinant MSMEG_0307 was able to produce a band shift indicating the binding of the protein to Motif 1. Interestingly, in a parallel experiment no DNA band shift was observed when Motif 2 was used as a substrate for the MSMEG_0307 (Figure 2B) indicating that the MSMEG_0307 protein binds specifically to the Motif 1.

3.3. Characterisation of a ΔaraC strain

In order to investigate the role of the MSMEG_0307 gene product of the nat operon in M. smegmatis the gene (MSMEG_0307) was deleted from the genome using specialised transduction [27]. Resistant colonies were screened for the presence of the deletion by PCR using one set of primers which amplified either an internal region of the gene or a region flanking the deleted MSMEG_0307 gene. As expected, the ΔMSMEG_0307 strain did not show any PCR amplification using the internal set and had an amplicon with a size difference using the external primer set indicating the presence of the hygromycin resistance cassette in the genome in the place of the MSMEG_0307 gene (Figure S3). A similar methodology was applied for the generation of the MSMEG_0308 gene deletion mutant (ΔMSMEG_0308) as well.

The ability to generate a ΔMSMEG_0307 strain indicates that the MSMEG_0307 gene is not essential for in vitro growth of M. smegmatis. Comparative growth curves in enriched media between wild type and the ΔMSMEG_0307 strain indicated that the loss of the MSMEG_0307 gene did not significantly affect the growth of the mutant strain (Figure 3A). Subsequently the ability of the ΔMSMEG_0307 strain to grow in minimal media with glycerol,
glucose, melibiose [31] was tested as several bacterial AraC transcription factors are known to control genes that are responsible for the degradation of complex carbon sources, such as arabinose metabolism in E. coli [32]. It was found that the deletion of the MSMEG_0307 transcription regulator had little effect on the growth on these carbon sources (Figure 3B).

As the genes of the nat operon in M. tuberculosis are involved in cholesterol metabolism [13,33], the ability of the ΔMSMEG_0307 transcription regulator had little effect on the growth on these carbon sources (Figure 3B–E). As the genes of the nat operon in M. tuberculosis are involved in cholesterol metabolism [13,33], the ability of the ΔMSMEG_0307 transcription regulator had little effect on the growth on these carbon sources (Figure 3B–E).

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3.4. MSMEG_0307 protein regulates the expression of the nat operon in M. smegmatis

As the recombinant MSMEG_0307, a putative transcription regulator, was able to bind specifically to the Motif 1 upstream of the nat operon in M. smegmatis, the effect of the MSMEG_0307 gene deletion on the expression of the other genes of the operon was studied using RT-qPCR. The comparison of the relative expression levels of nat (MSMEG_0306) and MSMEG_0308 genes between the WT and the ΔMSMEG_0307 mutant revealed that the deletion of the MSMEG_0307 gene has a significant effect on the expression of the other operon genes (Figure 4) suggesting that the AraC protein controls the gene expression of the operon. Both nat and MSMEG_0308 genes were down-regulated in the ΔMSMEG_0307 mutant by 18 and 13 fold respectively indicating that the AraC transcription factor acts as an activator for the expression of nat and MSMEG_0308 genes. On the contrary, overexpression of the MSMEG_0307 gene in the wild-type M. smegmatis from the pMVaraC plasmid increased the expression of both nat and MSMEG_0308 by 23 and 16 fold respectively (Figure 4) confirming the activator effect of the MSMEG_0307 protein on the operon. The overexpression of the MSMEG_0307 gene using the pMVaraC...
construct in M. smegmatis was also confirmed by qPCR (data not shown). Complementation of the ΔMSMEG_0307 strain with the same plasmid (pMVaraC) was able to compensate for the down regulation of the nat gene resulting in a 7 fold up regulation; however, this was not the case for the MSMEG_0308 gene that remained down regulated 3 fold. The presence of the internal cassette in place of MSMEG_0307 gene might partially explain the reason why MSMEG_0308 gene expression remained down regulated in the complemented ΔMSMEG_0307 strain.

4. Discussion

The regulation of gene expression is essential for all living organisms to adapt to various environmental and physiological...
and they are believed to act in a similar manner to the two component system enhancing the adaptation of bacteria under different physiological stages and pathogenesis [40]. It is clear that the binding of the MSMEG_0307 protein to the conserved Motif 1 located upstream of the nat gene has a direct effect on the expression of nat and MSMEG_0308 transcripts.

Our preliminary studies using protein-fragment complementation [41] revealed the presence of a small protein complex made from the gene products of the nat operon (Figure S5). We also hypothesise that the MSMEG_0307, transcription factor, might also interact with sigma factors as parts of the RNA polymerase in order to support the initiation of the transcription on this genomic area.

Although there is no clear evidence of an orthologue of the MSMEG_0307 AraC protein in M. tuberculosis it is clear from comparative genomic analyses that all fast-growing environmental mycobacteria sequenced to date possess a similar gene architecture in their nat gene clusters and that the AraC-family transcriptional regulator MSMEG_0307 protein and its preferred DNA binding motif are highly conserved, suggesting that this genomic area plays an important role in the adaptation of these mycobacterial species to their specific environment. It will be interesting to identify the external stimuli as well as the ligands that bind to the MSMEG_0307-protein. Furthermore, uncertaining the biological significance of the MSMEG_0307 protein being situated next to NAT in environmental mycobacteria and providing an explanation of the different evolutionary pathways adopted by fast-growing environmental and slow-growing pathogenic mycobacteria, will give us an insight into the unique characteristics of the adaptation of this genus to multiple environments. This is the first report of the role of the AraC-family transcriptional regulator MSMEG_0307 protein from the M. smegmatis nat operon.

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Ethical approval: Not required.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tube.2014.08.007.

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