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Rv0494 is a starvation-inducible, auto-regulatory FadR-like regulator from *Mycobacterium tuberculosis*

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Fatty acid metabolism plays an important role in the survival and pathogenesis of *Mycobacterium tuberculosis*. Lipids are assumed to be the major source of energy during dormancy. Here, we report the characterization of a starvation-inducible, lipid-responsive transcriptional regulator, *Rv0494*, divergently transcribed from the *Rv0493c* probable operon. The striking difference in the transcriptional regulatory apparatus between mycobacteria and other well-studied organisms, such as *Escherichia coli*, is the organization of mycobacterial promoters. Mycobacterial promoters have diverse architectures and most of these promoters function inefficiently in *E. coli*. In this study, we characterized the promoter elements of *Rv0494* along with the sigma factors required for transcription initiation. *Rv0494* promoter activity increased under nutrient starvation conditions and was transcribed via two promoters: the promoter proximal to the translational start site was active under standard growth conditions, whilst both promoters contributed to the increased activity seen during starvation, with the major contribution from the distal promoter. Furthermore, Rv0494 translation initiated at a codon located 9 bp downstream of the annotated start codon. Rv0494 bound to its upstream sequence to auto-regulate its own expression; this binding was responsive to long-chain fatty acyl-CoA molecules. We further report Rv0494-mediated transcriptional regulation of the *Rv2326c* gene – a probable transmembrane ATP-binding transporter encoding gene.

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INTRODUCTION

The success of *Mycobacterium tuberculosis* as a pathogen lies in its ability to survive in an asymptomatic state within the host for long periods and reactivate when host immunity falters. During the course of infection, mycobacteria face harsh environmental conditions, such as hypoxia, low pH and nutrient starvation (Pandey & Sassetti, 2008; Rohde *et al.*, 2007; Via *et al.*, 2008). Direct investigation of the bacilli within the host is difficult; hence, different *in vitro* models have been generated which mimic the conditions present within the mammalian host. The nutrient starvation model as designed by Loebel has been used to study the physiology of mycobacteria that probably occurs within the mammalian host (Loebel *et al.*, 1933a, b; Betts *et al.*, 2002). The metabolic activity of starved bacteria is minimal; however, the bacilli are viable and these are thought to be the conditions that prevail during dormancy. Taken together, these observations indicate that mycobacteria utilize their resources by coordinating gene expression, efficiently in accordance with the prevalent conditions. Proper utilization of promoters in coordination with transcriptional regulators plays an important role in mycobacterial physiology. More than 150 transcriptional regulators are encoded by the genome of *M. tuberculosis* (Cole *et al.*, 1998), which can have diverse effects on the catalytic properties of RNA polymerase. However, the majority of these regulators are yet to be characterized (http://genolist.pasteur.fr/TubercuList/). FadR regulators are the most abundant amongst the GntR family of transcription regulators and consist of all α-helical C-terminal domains with a mean length of ~160 aa (Haydon & Guest, 1991; Rigali *et al.*, 2002; Vindal *et al.*, 2007a, b). Extensive studies have been carried on the FadRs of *Escherichia coli*, *Vibrio vulnificus* and *Corynebacterium glutamicum*, and they are reported to play important roles both in cellular physiology and virulence (Agari *et al.*, 2011; Brown & Gulig, 2008; Casali *et al.*, 2006; Georgi *et al.*, 2008; Santangelo *et al.*, 2008). However, current knowledge on the promoter architecture and regulation of these FadR-like proteins in mycobacteria is limited.

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Abbreviations: ABC, ATP-binding cassette; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; MU, Miller units; TSS, transcription start site.

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Here, we present a detailed view of the transcriptional regulation of *M. tuberculosis* Rv0494 – one of the proteins of the FadR subfamily, classified and annotated previously in our laboratory. Rv0494 has orthologues present in most of the mycobacterial genera. However, it seems that *Mycobacterium smegmatis* does not have a functional orthologue of Rv0494; the closest one has 32 % identity at a query coverage of 61 %. The nutrient starvation model as designed by Loebel was used to study the effect of nutrient starvation on the transcriptional activity of *Rv0494*. Growth arrest due to nutrient starvation is the major factor which drives the activity of this promoter, although an alternate promoter also exists to drive the expression during growth in rich media. The Rv0494 protein binds to its upstream sequence in the region of the proximal promoter to repress its expression; the DNA-binding ability is inhibited in the presence of long-chain fatty acyl-CoA molecules. An Rv0494-like binding motif exists upstream of the *Rv2326*c gene, which is in a probable operon with *Rv2325*c. Rv0494 binds to this motif and represses the activity of this promoter.

METHODS

Bacterial strains and growth conditions. *E. coli* strain DH5α was used for plasmid propagation and for all cloning purposes. *M. smegmatis* mc^{2}155 and *Mycobacterium bovis* BCG were grown in Middlebrook 7H9 media supplemented with OADC (oleic albumin dextrose catalase) and 0.05 % Tween 80 (stock 20 %). Antibiotics were used at the following concentrations: kanamycin (30 μg ml^{-1}), hygromycin (50 μg ml^{-1}) and ampicillin (100 μg ml^{-1}). All oligonucleotides and plasmids used in this study are listed in Tables 1 and 2. Restriction enzymes were procured from Fermentas. Site-directed mutagenesis was carried out using the overlapping extension PCR method. All clones generated were confirmed by sequencing and restriction digestion.

β-Galactosidase assay. For β-galactosidase assay, all the constructs as well as vector were used to transform *M. smegmatis* mc^{2}155 and transformants were either grown in rich media or subjected to starvation in PBS. The activity was measured in Miller units (MU). The promoter activity for all constructs was measured in triplicate, in three different experiments.

Mapping of the transcription start site (TSS). *M. bovis* BCG was grown in 7H9 complete media and total cellular RNA was isolated with a Qiagen RNeasy kit as per the manufacturer's instructions. Total RNA (10–15 μg) was reverse transcribed using SuperScript III reverse transcriptase (Life technologies) as per the manufacturer's instructions and [γ-^{32}P]ATP (BRIT)-labelled primer. A dideoxy sequencing ladder was generated for pUC19 plasmid as template and M13 universal forward primer (USB; 70140 KT). The products of primer extension and the chain termination reaction were resolved on 6 % polyacrylamide/8 M urea gel in Tris/borate-EDTA (TBE) buffer.

Identification of sigma factors required for Rv0494 expression by promoter-specific pull-down assay. Identification of sigma factors was carried out essentially as described by Bharati *et al.* (2013). In brief, a 350 bp fragment encompassing the promoter region of *Rv0494* was PCR amplified using ARpEJ494for and biotin-labelled reverse primer AR494pulldownbiotin. The amplified PCR product was incubated overnight with streptavidin-agarose beads (Life technologies). RNA polymerase with associated sigma factors was partially purified from *M. bovis* BCG. Bacterial cells were harvested by centrifugation; suspended in lysis buffer (50 mM Tris/HCl, pH 7.9,

Table 1. Primers used in this study

Primer	Sequence 5′→3′
ARpEJ494upfor	CTAGTCTAGATCGAACCAGGCGGGGCCGGCCG
ARpEJ494uprev	CCCAAGCTTATCCACCCGCTGCCGATCCGGCGGC
AR494 pextn2	AGGATGGCGTCGGCGATCGT
AR494pET23afor	GGAATTCCATATGGTTGAGCCAATGAACCAGTCAAG
AR494pET23arev	CCGCTCGAGAGCGCGGTCGCGGTAGGACTT
ARPROMSDMFOR1	ATTCGCCCACCATATTGGTTGAGCCA
ARPROMSDMREV1	TGGCTCAACCAATATGGTGGGCGAAT
ARPROMSDMFOR2	ATTCGCCCACTCTATTGGTTGAGCCA
ARPROMSDMREV2	TGGCTCAACCAATAGAGTGGGCGAAT
ARPROMSDMFOR3	ATTCGCCCACCCTATCGGTTGAGCCA
ARPROMSDMREV3	TGGCTCAACCGATAGGGTGGGCGAAT
ARpEJ494ptnfor	CTAGTCTAGACCCGGCGGGGCATCCGGTGGAAACCA
ARpEJ494ptnRev	CCCAAGCTTCTAAGCGCGGTCGCGGTAGGACTTGACCA
ARpEJ494ttgoutfor	CTATATTGGTTGAGCAATGAACCAG
ARpEJ494ttgoutrev	CTGGTTCATTGCTCAACCAATATAG
ARpEJ494ATGoutfor	GCCAATGAACAGTCAAGTGTCTTTC
ARpEJ494ATGoutREv	GAAAGACACTTGACTGTTCATTGGC
AR494pulldownbiotin	Biotin-ATCCACCCGCTGCCGATCCGGCGGC
ARSEQFOR	GGAATTCCATATGGTCGCATACCAGACTTCCAGGCGCCC
ARSEQREV	CCGCTCGAGCTCTGCCAGGTCTCGCTCGG
AR494PVV16REV	CCCAAGCTTCTAGCGGTCGCGGTAGGACTT
ARpEJ2326For	CTAGTCTAGACCAGCGTCCTCGAGTCGTCGTAC
ARpEJ2326Rev	CCCAAGCTTCAGGCCCGCACACGGCGCAGCAC

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Table 2. Plasmids used in this study

Plasmid/strain	Description	Reference
pEJ414	Kanamycin-resistant *lacZ* reporter vector	Papavinasasundaram *et al.* (2001)
pEJ494up	297 bp of Rv0494 upstream region and 60 bp downstream of TTG cloned in pEJ414	This study
pEJ494upM1	Same as pEJ494up but with TATATT→CATATT mutation	This study
pEJ494M2	Same as pEJ494up but with TATATT→TCTATT mutation	This study
pEJ494M3	Same as pEJ494up but with TATATT→CCTATC mutation	This study
pEJ494up-orf	240 bp upstream region and total ORF for protein production under native promoter	This study
pEJ494ttgout	Same as pEJ494up-orf but with deletion at base 7 with respect to annotated start codon	This study
pEJ494ATGout	Same as pEJ494up-orf but with deletion at base 15 with respect to annotated start codon	This study
pET23a	Expression vector with ampicillin resistance	Novagen
pET23a-494	Overexpression of Rv0494 protein	This study
pVV16	Hygromycin, kanamycin-resistant mycobacterial overexpression plasmid with hsp60 promoter	Kordulákova *et al.* (2002)
pVV494	Rv0494 ORF cloned in pVV16 for overexpression	This study
pEJ2326	Upstream 300 bp and 24 bp downstream from the translation start codon of the *Rv2326c* gene	This study

2 mM EDTA, pH 8.0, 0.1 mM DTT, 300 mM NaCl, 5 % glycerol, 1 mg lysozyme ml^{-1}and 23 μg PMSF ml^{-1}). The cells were lysed by bead beating and nucleic acid binding proteins were precipitated by polyethyleneimine at 4 °C. Following centrifugation, the pellet was dissolved in 10 ml Buffer C (20 mM Tris/HCl, pH 7.9, 100 mM NaCl, 0.2 mM EDTA and 0.1 mM DTT) containing 1 M NaCl. After another round of centrifugation, 50 % ammonium sulphate precipitation was carried out. The pellet was resuspended in Buffer B (20 mM Tris/HCl, pH 7.4, 5 mM $MgCl_2$, 100 mM potassium glutamate, 10 % glycerol, 0.5 mM EDTA and 0.1 % Triton X-100), mixed with 100 μl promoter-streptavidin-agarose beads and further incubated overnight at 4 °C with constant rotation. Following overnight incubation, the beads were allowed to settle and the supernatant was carefully aspirated. The beads were washed twice with Buffer B and the washes were collected for SDS-PAGE analysis. The bound proteins were eluted with Buffer C (containing 0.4 M NaCl). The washes along with the eluted proteins were subjected to SDS-PAGE, followed by Western blotting. The sigma factors were identified with anti-sigma antibodies (AstraZeneca).

Orthologue prediction, multiple sequence alignment and prediction of the FadR-binding site in the upstream region of orthologues. The RBBH (Reciprocal Best BLAST Hit) method was used to identify the putative orthologous proteins between each of the two proteomes, using the BLASTP program with an *E* value cut-off of 10^{-6} for both directions, followed by a manual improvement according to the predicted secondary structure (Altschul *et al.*, 1990; Fulton *et al.*, 2006). Upstream and certain portions of the downstream sequences from all the orthologues were aligned, and the conserved sequences showing the nucleotide preference as exhibited by FadR-like binding sites were identified.

Cloning, expression and purification of Rv0494. The DNA fragment encoding Rv0494 was PCR amplified from *M. tuberculosis* H37Rv genomic DNA using gene-specific primers, AR494pET23arev and AR494pET23afor. The PCR fragment was ligated to pET-23a expression vector (Novagen), between the *Nde*I and *Xho*I sites. The verified recombinant clone was used for protein overexpression in *E. coli* strain BL21(DE3). The recombinant His_6-tagged protein was produced with 1 mM IPTG at 37 °C and purified using Ni-NTA affinity chromatography.

Electrophoretic mobility shift assay (EMSA). EMSA was carried out to verify protein–DNA interaction. DNA fragments were [γ-^{32}P]ATP (3000 Ci mmol^{-1}) labelled using T4 polynucleotide kinase as per the manufacturer's instructions (NEB). Labelled DNA fragments were purified through a Sephadex G50 (Sigma) column. The labelled DNA fragment in a total volume of 20 μl reaction was incubated with increasing concentrations of recombinant Rv0494 protein in binding buffer (10 mM Tris/HCl, pH 8, 10 mM NaCl, 1 mM EDTA, 5 % glycerol, 10 μg poly(dI–dC) ml^{-1} and 5 μg BSA ml^{-1}). The protein–DNA complex was resolved on 6 % non-denaturing polyacrylamide gel in 0.5 × TBE buffer. Gel electrophoresis was run for sufficient time to allow resolution between the protein–DNA complex and the free DNA probe.

DNase I footprinting. A uniquely radiolabelled Rv0494 fragment (~25 000 c.p.m.) encompassing the binding site was incubated for 45 min at room temperature with recombinant Rv0494 in 50 μl EMSA binding buffer. DNA digestion was carried out with 3 μg DNase I (Sigma) for 90 s at room temperature, after which the reaction was stopped with DNase I stop solution (Sigma). After ethanol precipitation and a 70 % alcohol wash, the pellet was dissolved in formamide dye mix, heat denatured for 5 min at 95 °C and resolved on 6 % acrylamide/8 M urea gel. A dideoxy sequencing ladder corresponding to the coding strand was also run along with the samples.

Chromatin immunoprecipitation (ChIP) assay. The ChIP assay was done using *M. bovis* BCG. In brief, the cells were grown and fixed with 1 % formaldehyde for 30 min at room temperature. The cross-linking was terminated by adding 125 mM glycine. The cross-linked cells were washed three times with PBS and finally suspended in resuspension buffer (0.5 M sucrose, 20 mM HEPES/KOH, pH 7.4, 2 mM EDTA and 7 mM β-mercaptoethanol). Protease inhibitor cocktail (Roche Diagnostics) and 0.5 % NP-40 were added to the fixed cells, and cells were ruptured by mild bead beating followed by sonication in a sonifier to obtain fragments <700 bp in length. The cell lysate was centrifuged at 20217 ***g*** for 5 min. The supernatant was transferred to a fresh tube and incubated overnight with anti-Rv0494 serum (immunization sera). A part of the whole-cell lysate was stored, which served as an input sample for the PCR. One part of the extract was incubated with pre-immunization sera (obtained from the mice later on immunized with Rv0494 recombinant protein). After overnight incubation, the samples were incubated with pre-cleared Protein A-agarose beads for 1 h. The samples were again centrifuged at 300 ***g*** for 5 min at 4 °C. The supernatant was discarded and the pellets were washed; once each with low-salt buffer (0.1 % SDS, 0.1 %

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Triton X-100, 2 mM EDTA, 20 mM Tris/HCl, pH 8.0 and 150 mM NaCl), high-salt buffer (0.1 % SDS, 0.1 % Triton X-100, 2 mM EDTA, 20 mM Tris/HCl, pH 8.0 and 500 mM NaCl) and LiCl buffer (250 mM LiCl, 1 % NP-40, 1 % deoxycholate, 1 mM EDTA and 20 mM Tris/HCl, pH 8.0), and twice with Tris-EDTA. Rv0494-bound DNA fragments were finally eluted with 50 μl elution buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA, pH 8.0, 1 % SDS and 0.1 M $NaHCO_3$). Reverse cross-linking was carried out by adding 300 mM NaCl, followed by incubation at 65 °C for 6 h. Then, 1 μg RNase A and 200 μg proteinase K were added and incubation was carried out at 55 °C. The DNA was purified using a Qiagen PCR Purification kit. The purified DNA was analysed for the presence of an upstream region of *Rv0494* using the upstream region-specific primers, ARSEQFOR and ARSEQREV.

Statistical analysis. All the data for relative expression of promoter activities were subjected to Student's *t*-test for analysis of statistical significance. $P<0.05$ was considered significant.

RESULTS

Rv0494 shows enhanced expression during nutrient starvation

To identify the promoter elements of *Rv0494*, 297 bp upstream and 60 bp downstream of the annotated translation start codon were cloned in a promoter-less pEJ414 vector, and named pEJ494up. *M. smegmatis* transformed with pEJ494up was grown in 7H9 complete media and the promoter activity of this fragment was confirmed using *β*-galactosidase assay. To study the conditions affecting the activity of this promoter, *M. smegmatis* harbouring pEJ494up was grown to mid-exponential phase in 7H9 media supplemented with 10 % OADC and 0.1 % Tween 80. At this stage, the cells were harvested by centrifugation, washed twice with sterile PBS and resuspended in Sauton's minimal media, supplemented with different carbon sources as well as subjected to different stress conditions. Comparison of the promoter activities indicated that the major factor contributing to the activity of this promoter was nutrient starvation (Fig. 1a). *M. smegmatis* harbouring pEJ494up, when subjected to nutrient stress by growth in sterile PBS, or Middlebrook 7H9 without glycerol and OADC, showed maximal promoter activity amongst all the conditions tested. The activity of this promoter during nutrient starvation increased steadily with increasing time when monitored over 104 h of growth (Fig. 1b). The promoter activity of pEJ494up was inversely related to the amount of glucose present in the media. As the glucose concentration in the media decreased, promoter activity increased (Fig. 1c). Exogenous addition of glucose at any time during the starvation conditions significantly decreased the promoter activity of pEJ494up (Fig. 1d).

Next, we studied whether the two conditions (rich versus starved) affected the amounts of protein being synthesized. The 240 bp upstream sequence along with the entire ORF was cloned in promoter-less pEJ414 vector to obtain pEJ494up-orf. *M. smegmatis* transformed with this construct was allowed to grow both in rich as well as starvation media for 24 h. The cells were harvested and the whole-cell lysate from each condition was analysed by Western blotting. There was increased protein synthesis under starvation conditions (Fig. 1e, lanes 2 and 3), which was equivalent to the amount of protein being synthesized when the hsp60 promoter was used for Rv0494 expression (Fig. 1e, lane 4). However, during growth under rich conditions, protein synthesis was very low (Fig. 1e, lanes 5 and 6; not detectable in the image). This clearly indicated that increased promoter activity translated to increased protein synthesis under nutrient starvation conditions.

Mapping of the TSS and identification of the −10 element of the promoter

Having shown that the 240 bp upstream sequence of *Rv0494* contained the transcription regulatory elements, we used primer extension analysis to map the TSS and thereby identify the −10 element of the promoter. Primer extension analysis (with primer AR494pextn2) was carried out using total cellular RNA isolated from *M. bovis* BCG grown under nutrient-rich conditions. The top band, E, marked by an arrow in lane PE1, was considered to represent the actual 5′ end of the transcript and all the lower bands were assumed to be truncated transcripts (Fig. 2a). The 5′ end of the transcript corresponded to a base 'G', six nucleotides downstream of the annotated translational start codon. Upstream of TSS, a −10-like element 'TATATT' is present, similar to the −10-like sequences found in *E. coli* promoters (Fig. 2b). To establish the role of this −10-like sequence in promoter activity, the first, second and sixth bases of the native promoter were mutated. As a result of these mutations, an approximately fivefold decrease in promoter activity was observed with all the mutant constructs (Fig. 2c). The −10 element of the promoter P1 (the mutant version is henceforth referred to as P1-M) was comparable to the −10 element of the *E. coli* sigma70 promoter sequences. However, the −35 hexamer sequence showed little conservation although this promoter construct was still able to demonstrate activity in *E. coli* (data not shown).

A second promoter is utilized for enhanced expression under nutrient stress conditions

M. smegmatis strains harbouring empty pEJ414 vector, WT promoter (pEJ494up) and mutant promoter (pEJP1-M) constructs were subjected to nutrient stress. During nutrient starvation, the WT promoter construct yielded the highest *β*-galactosidase units (60–80 MU) compared with the activity of the same promoter under nutrient-rich conditions (15–20 MU). The construct with a mutation in promoter P1 (pEJP1-M) also showed significant activity under starvation conditions (~30 MU), which was lacking during growth in rich media; hence, the possibility of a second promoter being active during nutrient starvation condition was evident (Fig. 3a). Primer extension analysis was again carried out with RNA isolated from bacteria subjected to nutrient starvation. Two prominent bands, one

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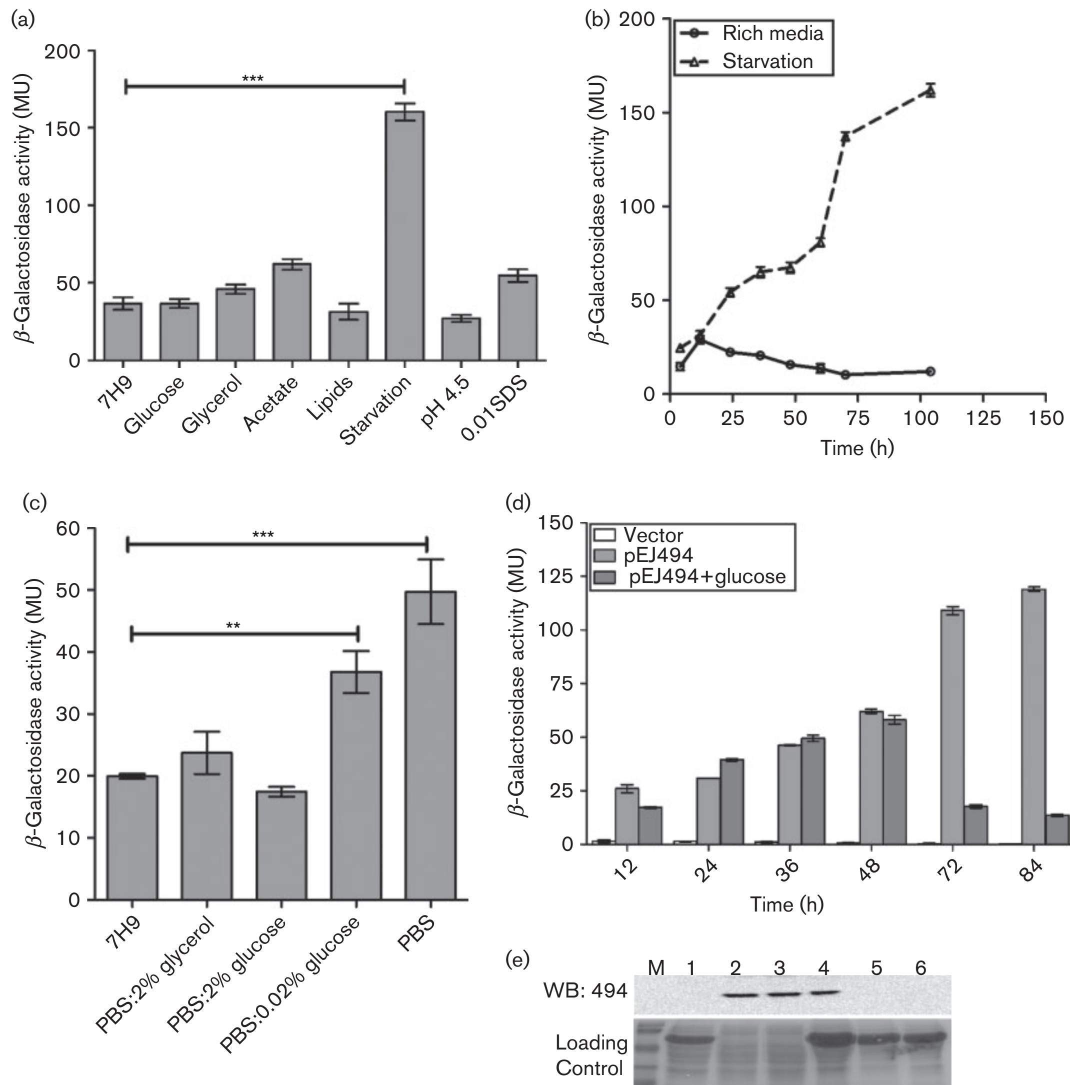

Fig. 1. Rv0494 shows increased promoter activity during nutrient starvation. (a) β-Galactosidase activity of *Rv0494* under different conditions. 7H9, 7H9 broth with 10 % OADC and 0.1 % Tween 80. Glucose, Sauton's minimal medium with 2 % glucose. Glycerol, Sauton's minimal medium with 2 % glycerol. Acetate, Sauton's minimal medium with 5 mM acetate. Lipids, Sauton's minimal medium with 50 μM palmitic acid in tyloxopol. Starvation, growth in sterile PBS. pH 4.5, 7H9 complete medium pH 4.5. 0.01SDS, 7H9 complete medium with 0.01 % SDS. ***$P<0.0001$ between promoter activities in 7H9 medium and PBS (starvation). (b) β-Galactosidase activity of *Rv0494* promoter in liquid culture of *M. smegmatis.* Mid-exponential-phase bacteria growing in 7H9 complete medium were harvested, washed twice with sterile PBS and one part was resuspended in PBS containing 0.05 % tyloxopol and the other was resuspended in 7H9 complete medium. Aliquots of the culture were collected at different time points as indicated and activity of the promoter was measured. (c) Relative expression of *Rv0494* promoter at different concentrations of glucose. ***$P<0.0001$ between promoter activities in 7H9 complete medium and PBS (starvation); **$P<0.001$ between the promoter activities in 7H9 and PBS with 0.02 % glucose. (d) Exogenous addition of glucose (2 %) after 48 h of starvation decreased promoter activity, whilst the other aliquot of culture which was left untreated showed an increase in activity. (e) Western blot analysis of Rv0494 ('WB: 494') expression under different conditions. Lane M, marker lane. Lane 1, whole-cell lysate from the strain transformed with pEJ414 vector backbone. Lanes 2 and 3, whole-cell lysate from strain expressing Rv0494 through its native promoter grown under starvation conditions. Lane 4, Rv0494 protein expressed via hsp60 promoter. Lanes 5 and 6, whole-cell lysate from strain expressing Rv0494 through its native promoter grown in rich media.

corresponding to TSS1 (proximal promoter) and the other corresponding to a base 'G', 33 bp upstream of the annotated translational start codon, were observed (Fig. 3b). Whilst the promoter activity under growth in rich conditions was contributed entirely by proximal promoter P1, activity in starved media was contributed by

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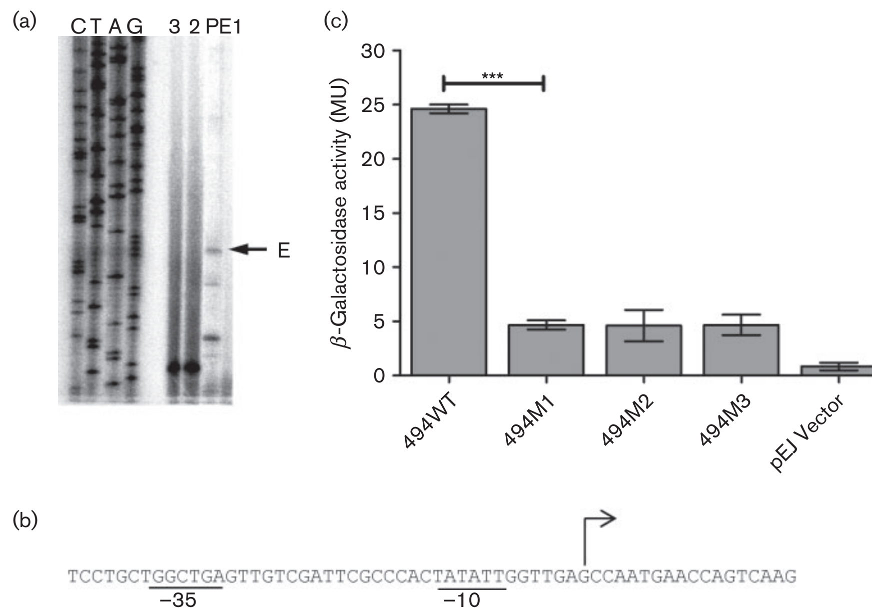

Fig. 2. Mapping of the TSS and identification of promoter element. (a) TSS mapping of the *Rv0494* gene using primer extension analysis. Total cellular RNA was isolated from *M. bovis* BCG grown in 7H9 complete medium. The uppermost band (E) indicated by an arrow in lane PE1 is the TSS. Lane 2 contains primer extension reaction product, which did not work. Lane 3 contains only labelled primer. Dideoxy sequencing ladder marked as G, A, T and C was generated using pUC19 plasmid as template and M13 universal forward primer. (b) The DNA sequence of the *Rv0494* upstream region. The identified TSS is marked by an arrow, and probable −10 and −35 regions are highlighted. (c) β-Galactosidase activity of Rv0494 WT and mutant promoters. 494WT, WT promoter; 494M1, TATATT→CATATT mutation; 494M2, TATATT→TCTATT mutation; 494M3, TATATT→CCTATC mutation. ***$P<0.0001$ between WT promoter (494WT) and mutant promoter (494M1) activities.

both promoters, with the distal promoter the more active. A promoter-like sequence resembling a sigC-like consensus was present upstream of this second TSS (TSS2) (Fig. 3c). The second promoter, referred to as P2, was conditionally active during nutrient starvation, as promoter-down mutations around P1 were sufficient to significantly reduce the activity comparable to that of an empty vector during growth under rich conditions. To further confirm the presence of an upstream second promoter, we cloned the upstream sequence of *Rv0494* in the promoter-less vector, pEJ414, without the proximal promoter, P1, and named the construct pEJ494ΔP1. The promoter activity of pEJ494ΔP1was measured under both rich and starvation conditions. As shown in Fig. 3(d), pEJ494ΔP1 showed activity under both conditions studied hence confirming the presence of the upstream second promoter.

Rv0494 promoter region interacts with both sigA- and sigC-bound RNA polymerase

Rv0494 transcription utilizes two promoters in a condition-dependent manner. Such a type of differential expression pattern requires the use of multiple sigma factors. In order to identify the sigma factors initiating the transcription of *Rv0494*, we followed the approach reported by Bharati *et al.* (2013). Briefly, biotin-labelled reverse primer was used to amplify the 350 bp fragment containing the promoter elements. Biotin-labelled, PCR-amplified product was mixed with streptavidin-agarose beads and incubated overnight. The DNA-binding protein fraction was prepared from *M. bovis* BCG, mixed with promoter fragment immobilized on beads and the mixture was then incubated for 2–3 h at room temperature. Following incubation, the beads were washed twice and the protein was eluted. RNA polymerase bound to sigma factors which interacted with *Rv0494* upstream elements were identified by Western blot using different anti-sigma antibodies (AstraZeneca). Blotting of the membranes with different anti-sigma antibodies indicated that the upstream fragment could interact with RNA polymerases bound by two different sigma factors, i.e. sigA and sigC (Fig. 4a). In our previous set of results, we showed that *Rv0494* transcription was initiated at two different locations. The promoter P1 resembled a sigA consensus, whereas promoter P2 resembled a sigC consensus sequence (Fig. 4b). Based on the promoter sequence conservation and sigma factor pull-down experiments, we concluded that *Rv0494* expression was possibly driven by both sigA and sigC in a condition-dependent manner.

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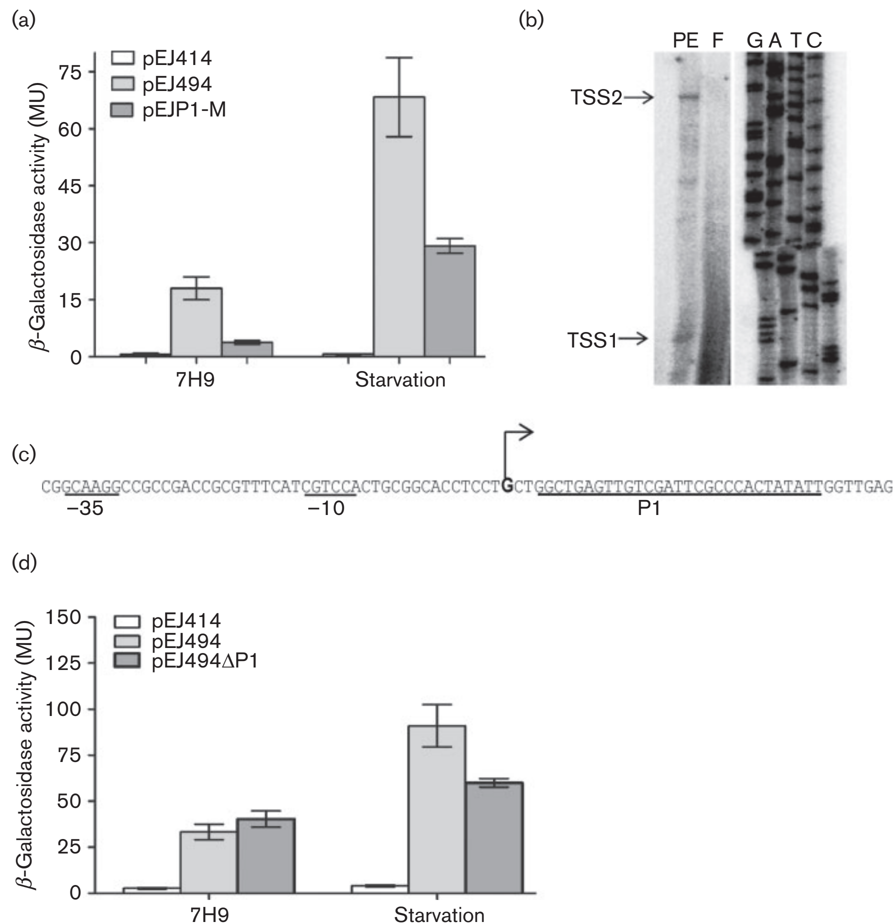

Fig. 3. A second promoter drives increased expression during starvation. (a) β-Galactosidase activity of *Rv0494* WT and mutant promoter. pEJ494up, pEJP1-M and pEJ414 were used to transform *M. smegmatis*, and the promoter activity of each construct was measured during growth in rich media and under starvation conditions. (b) A primer extension reaction was carried out with RNA isolated from *M. bovis* BCG grown under starvation conditions. Lane PE contains the primer extension product. Two bands, TSS1 and TSS2, marked by an arrow are the two TSSs. TSS1 is rich media-specific as described earlier; TSS2 is starvation-specific and observed only during starvation. Lane F is the control lane with RNA and labelled primer, but no reverse transcription was carried out. A dideoxy sequencing ladder was generated with pUC19 plasmid as template and M13 universal forward primer. (c) DNA sequence of the *Rv0494* gene with both promoters highlighted. P1 refers to the proximal promoter characterized earlier. The +1 site of the second promoter is marked and the direction of transcription is indicated by an arrow. The −10 and −35 regions of the second promoter are underlined and marked. (d) β- Galactosidase activity of pEJ414, pEJ494 and pEJ494ΔP1 during growth in rich and starvation media.

Translation of the Rv0494 transcript initiates at a translational start codon nine bases downstream of the annotated position

Primer extension results, as described in Fig. 2(a, b), indicated that the TSS for the proximal promoter was six bases downstream of the annotated translational start codon; hence, the possibility of a different translational start codon downstream of the annotated ORF was evident. Sequence analysis of the coding frame revealed the presence of another possible start codon, 'ATG', nine bases downstream from the annotated start codon, 'TTG'. To verify the role of 'ATG' in translation initiation, the Rv0494 protein-coding sequence along with the upstream 240 bp region was cloned in pEJ414 to obtain pEJ494up-orf. Two deletion mutations, one rendering the coding sequence with respect to TTG out of frame and the other rendering the coding sequence with respect to ATG out of frame, named 'pEJ494TTGout' and 'pEJ494ATGout', respectively, were created (Fig. 5a). *M. smegmatis* was transformed with WT as well as mutant constructs, and whole-cell lysates were prepared. Total protein from all three different strains was resolved on 12 % SDS-PAGE, followed by Western blotting using anti-Rv0494 serum. As shown in Fig. 5(b), a 26 kDa band was observed in the positive control lane (lane 1: pEJ494up-orf) along with a lane containing lysate from the strain harbouring the pEJ494TTGout construct

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