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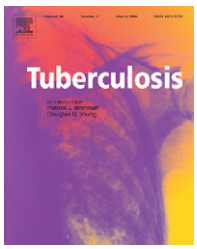

Rv1773 is a transcriptional repressor deleted from BCG-Pasteur

David C. Alexander, Marcel A. Behr*

McGill University Health Centre, McGill University, Montreal, Que., Canada H3G 1A4

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Summary
Mycobacterium bovis Bacille Calmette-Guérin (BCG) is a live attenuated vaccine for the prevention of tuberculosis. Transcriptome comparison reveals dysegulated expression of two genes, *Rv1774* and *Rv1775*, exclusively in the Pasteur strain of BCG. We show that these genes form a bicistronic operon regulated by Rv1773, a transcriptional repressor deleted during the *in vitro* evolution of BCG.

Introduction

Mycobacterium bovis Bacille Calmette-Guérin (BCG) strains are employed as live attenuated vaccines for the prevention of tuberculosis (TB). The original BCG was derived from a virulent strain of *M. bovis* by repeated *in vitro* passaging. Primary attenuation can, in part, be attributed to the loss of the RD1 region,[1] but subsequent propagation of BCG at diverse vaccine laboratories resulted in further *in vitro* evolution, altering the antigenic and metabolic properties of BCG.[2–5] To date, most characterized mutations result in loss-of-function phenotypes and affect processes potentially important to TB pathogenesis.[3,6,7] Unfortunately, roles for the majority of genetic changes, notably, the mutation of several transcriptional regulators, have yet to be established. Loss of a regulator is expected to have pleiotropic effects due to altered expression of its associated regulon.[3,6] In previous work, transcriptome comparison of *M. tuberculosis* complex organisms suggested elevated expression of two genes, *Rv1774* and *Rv1775* exclusively in the Pasteur 1173 strain of BCG.[8,9] Here, we confirm that transcription of these genes in BCG-Russia and BCG-Pasteur is different. Moreover, we examine regulation of this dysregulated dyad by its flanking genes, which encode the transcriptional regulators Rv1773 and Rv1776.

Materials and methods

Mycobacterial strains and growth conditions

BCG strains Pasteur 1173 and Russia (ATCC 35740) were routinely cultured in Middlebrook 7H9 broth (Becton Dickinson) supplemented with 0.5% glycerol, 10% albumin–dextrose–catalase (Becton Dickinson) and 0.05% Tween 80 (Sigma-Aldrich). *M. smegmatis* strain MC^2155 was routinely cultured in LB broth (10 g tryptone, 10 g NaCl, and 5 g yeast

*Corresponding author. Division of Infectious Diseases and Medical Microbiology, A5–156, Montreal General Hospital, 1650 Cedar Avenue, Montreal, Que., Canada H3G 1A4.
Tel.: +1 514 934 1934x42815; fax: +1 514 934 8423.
E-mail address: marcel.behr@mcgill.ca (M.A. Behr).

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extract/L) containing 0.05% Tween 80 or LB agar (LB broth plus 15 g agar/L) and supplemented with antibiotics (kanamycin 30 μg/mL and/or hygromycin 100 μg/mL; Sigma-Aldrich) as necessary. Liquid cultures were incubated at 37 °C on a rolling platform.

RNA analysis and quantitative real time RT-PCR (qRT-PCR)

Protocols for RNA isolation and analysis have been described previously.[3] Enzymatic reactions were performed using methods recommended by the manufacturers. Briefly, total RNA was extracted from mid-log phase cultures of BCG-Russia and BCG-Pasteur. RNA integrity and concentration were determined by spectrophotometry and gel electrophoresis. The effectiveness of DNase treatment (Turbo DNA-free, Ambion) was monitored by PCR. For qRT-PCR, cDNA was synthesized from 1 μg of total RNA with MMLV reverse transcriptase (Fermentas). Expression levels of *Rv1774* and *Rv1775* were measured with SYBR green (Invitrogen) using the oligonucleotide primers RT–Rv1774F (5′-ATACGACCCCGACGGTTT-3′) and RT–Rv1775R (5′-ATAGCCCTCATCAACGATGC-3′). Oligonucleotide primers SIGA-L (5′-TGCAGTCGGTGCTGGACA-3′) and SIGA-R (5′-CGCGCAGGACCTGTGAGC-3′) were used to determine levels of *sigA*, the standard for normalization of gene expression.

Vector construction

All genes were amplified from genomic *M. tuberculosis* H37Rv DNA template by PCR and the products ligated to PCR-cloning vector pCR2.1 (Invitrogen). Oligonucleotide primers Rv1773F (5′-TGACAACGAATCAACGAACC-3′) and Rv1773R (5′-TCAGCACTGACAGCCTGAAC-3′) were used for amplification of *Rv1773* while Rv1776F (5′-GATGGAAATGCGCCTGAC-3′) and Rv1776R (5′-TTCGGTGACGAGTGAGGTTT-3′) were used for *Rv1776*. For each, the regulatory gene was excised from pCR2.1 with *Eco*RI and ligated to *Eco*RI-linearized pJEB402 (provided by Dr. D.R. Sherman, University of Washington). The kanamycin-resistant pJEB402 shuttle vector is a multicopy episomal plasmid in *E. coli* but integrates as a single copy into the mycobacterial chromosome. It contains the mycobacterial optimized promoter (Pmop).[10] The regulatory genes were cloned in both orientations relative to this strong promoter such that *Rv1773* and *Rv1776* are under Pmop control in vectors pJ73.1 and pJ76.1, but in vectors pJ73.2 and pJ76.2 expression is not Pmop mediated.

To monitor gene expression, a series of transcriptional *luxAB* fusions was constructed in pMIND-lx (provided by Dr. B.D. Robertson, Imperial College, London).[11] For plasmid pM74.1, the product of primers PRv1774F (5′-GGATCCTCAGCACTGACAGCCTGAAC-3′) and PRv1774R (5′-CTCTAGATCGACTAGGCGCAGGT-3′) was digested with *Xba*I and *Bam*HI and ligated to pMIND-lx linearized with the same endonucleases. For plasmid pM74.2, a *Bam*HI fragment derived from the Rv1773R and PRv1774R product was used. Both constructs include the 5′ end of *Rv1774* plus DNA upstream of the start codon: 187 bp in pM74.1 and 680 bp pM74.2. A third reporter construct, pM76, was created with primers PRv1776F (5′-CTCTAGAACGTGTTGGCATCCTTG-3′) and PRv1776R (5′-GGATCCGTAGCGCGTAATGAGGTC-3′). The *Xba*I–*Bam*HI fragment encompasses 315 bp, including the 5′ end of *Rv1776* and the putative promoter. All constructs were verified by DNA sequencing.

Luciferase assays

Luciferase reporter strains were generated in two steps. First, *M. smegmatis* strain mc^{2}155 was electroporated with the pJEB402 control vector or a derivative containing *Rv1773* (pJ73.1, or pJ73.2), or *Rv1776* (pJ76.1 or pJ76.2) using standard protocols.[12] Kanamycin-resistant clones were examined, via PCR, to confirm successful transformation and then electroporated with one of the hygromycin-resistant reporter constructs: pMIND-lx, pM74.1, pM74.2, or pM76 *luxAB*.

For qualitative assessment of luciferase activity, *M. smegmatis* reporter strains were streaked on to solid media and incubated for 48 h at 37 °C. Decanal (Sigma-Aldrich) was added to the plate lid, and inverted plates were photographed with a digital imaging system (Perkin-Elmer, ChemiGenius^2). A short (50 ms) exposure under white light was used to visualize all colonies. A longer (5 min) exposure in the dark revealed autoluminescent colonies.

For quantitative measurement, clones were incubated overnight in liquid media. Cultures were adjusted to an optical density of $A_{600} = 0.7$ and multiple 90 μL samples were transferred to opaque 96-well plates. Then, 10 μL of decanal solution (1:100, v/v, decanol diluted in LB) was added to the cells and relative light units (RLUs) were measured with a spectrophotometer (Perkin-Elmer, Victor^3) over 10 s.

Results

RNA analysis and qRT-PCR

Total RNA isolated from BCG-Pasteur and BCG-Russia was free of contaminating DNA; when used as template for PCR, only RNA pre-treated with reverse transcriptase generated a product (Fig. 1A). In the *M. tuberculosis* H37Rv genome annotation, the stop codon of *Rv1774* overlaps with the start codon of *Rv1775*.[13] This suggests that the two genes form an operon. PCR of cDNA using primers RT–Rv1774F and RT–Rv1775R generates a single 228 bp amplification product (Fig. 1A). This product bridges *Rv1774* and *Rv1775*, confirming co-transcription of the gene dyad.

Gene expression was measured with qRT-PCR. When normalized against expression of a control gene, *sigA*, levels of the *Rv1774–Rv1775* transcript were determined to be eight-fold higher in BCG-Pasteur than BCG-Russia (Fig. 1B).

Luciferase assays

When co-transformed with the parental vectors, pJEB402 and pMIND-lx, *M. smegmatis* is weakly luminescent. In contrast, strains co-transformed with pJEB402 and the *Rv1774::luxAB* reporter constructs are strongly luminescent (Fig. 2B). When quantified, RLU values of the pM74.1 and pM74.2 strains were 16.9 (±1.1) and 39.2 (±6.8) fold