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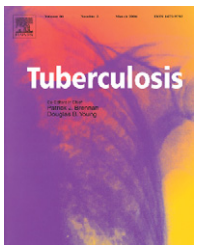

Rv3134c/*devR*/*devS* operon of *Mycobacterium bovis* BCG is differentially transcribed under "*in vitro*" stress conditions

Juan Germán Rodriguez[a], Claudia Sofía Burbano[a], Carmen Nuñez[b], Clara Eugenia González[a], María Mercedes Zambrano[a], María Jesús García[b], Patricia Del Portillo[a,*]

[a]*Corporación Corpogen, Carrera 5 No. 66A-34, Bogotá, Colombia*
[b]*Departamento de Medicina Preventiva y Salud Pública, Facultad de Medicina, Universidad Autónoma de Madrid, st/Arzobispo Morcillo, 4. 28029-Madrid, Spain*

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Summary
DevR is a transcriptional regulator that mediates the genetic response of *Mycobacterium tuberculosis* and *Mycobacterium bovis* to oxygen limitation and nitric oxide exposure. *devR* is part of an operon that includes the genes *devS* and Rv3134c, which encode an oxygen sensor protein and a protein that contains a universal stress protein domain, respectively. Here, we report the transcriptional analysis and quantitative expression of Rv3134c/*devR*/*devS* under *in vitro* stress conditions including oxygen limitation, low nutrients and *ex vivo* macrophage infection. At least three different promoters were found to control Rv3134c/*devR*/*devS* expression under the stresses tested. Two promoters were identified upstream of *devR*, one was active under hypoxia and the other under nutrient starvation. A single promoter was identified upstream of Rv3134c, and transcripts from this promoter were detected only under hypoxia. Rv3134c to *devR* were found to be co-transcribed only under hypoxia, whereas *devR*/*devS* were co-transcribed both in aerobiosis and starvation. RT-qPCR showed an increase in the ratio hypoxia/aerobiosis and in starvation/nutrients in all genes. *devR*/*devS* showed transient expression in the first days of macrophage infection. Our results indicate that Rv3134c/*devR*/*devS* of *M. bovis* BCG constitutes an operon with complex regulation that participates in bacterial response against a wide range of stresses.

*Corresponding author. Tel.: +57 1 3484609; fax: +57 1 3484607.
E-mail address: pdelportillo@corpogen.org (P. Del Portillo).

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Introduction

It is estimated that one-third of the global population is infected with *Mycobacterium tuberculosis* the causative agent of human tuberculosis.[1] Around 90–95% of the infected population is able to control the disease but can nevertheless remain latently infected in a physiological condition characterized by a positive skin test and no demonstrable clinical or radiological signs of active disease.[2] In the latent infection a residual population of viable bacteria can be maintained for long periods of time, constituting an important mycobacterial reservoir.[3,4] A better understanding of the host–pathogen interactions that result in latent infection could therefore provide important insights for efficient tuberculosis control. Recent reports suggest that *M. tuberculosis* coexisted with early hominids and that therefore both the bacillus and its host have developed strategies to coexist.[5] In this context, latency could be considered to be the result of a continuous cross talk between the host immune system and the pathogen.[6]

M. tuberculosis has developed a series of adaptation mechanisms to the numerous environmental conditions that the host offers such as oxidizing agents, low pH, hypoxia and nutrient starvation, among others.[7] Two-component regulatory signal transduction systems (TCS) are one of the major mechanisms by which bacteria can deal with changes in environmental conditions. These signal TCS involve a sensor histidine kinase (HK) that is autophosphorylated in response to an environmental stimulus. The phosphoryl group is then transferred to a response regulator protein (RR) which modulates gene expression in an appropriate bacterial response.[8–10] The genome of *M. tuberculosis* H37Rv has revealed the presence of 11 pairs of TCS including *pho*P–*pho*R, *mpr*A–*mpr*B, *prr*A–*prr*B, *reg*X3–*sen*X3, *trc*R–*trc*S, *dev*R–*dev*S and *kdp*E–*kdp*D[11] and, more recently, P*dta*S–*Pdta*R.[12] The genome also contains 3 isolated regulators and 3 isolated sensor proteins.[11] These TCS are expected to modulate gene expression and direct the adaptation of tubercle bacilli to hostile environmental challenges in order to survive within the human host.[13]

Among the TCS, the *dev*R (Rv3133c; BCG 3156c)/*dev*S (Rv3132c; BCG 3155c) genetic system (also known as *dos*R/*dos*S) has been amply studied. It was first identified by its differential expression in the virulent *M. tuberculosis* H37Rv and the avirulent *M. tuberculosis* H37Ra strains.[14] The *dev*S gene that encodes a sensor HK protein of 578 amino acids is preceded by the *dev*R gene which encodes an RR protein of 217 amino acids.[14] DevR is a transcriptional regulator that mediates the response of *M. tuberculosis* to oxygen limitation and which, under hypoxic conditions, regulates the expression of around 48 genes.[15–17] This transcriptional factor is also induced by nitric oxide.[18] Twenty-seven base pairs upstream of *dev*R is the gene Rv3134c (BCG 3157c), that is presumably in the same operon and transcribed in the same direction as the TCS. It is predicted to encode an Ala-Val rich protein of 268 amino acids containing a universal stress protein domain.[14] Studies of Rv3134c/*dev*R/*dev*S operon in *M. tuberculosis* under aerobic culture conditions have demonstrated multiple transcriptional start points, thus indicating a complex regulation of these genes.[19]

In this paper, we report the transcriptional analysis and the quantitative expression of the Rv3134c/*dev*R/*dev*S operon in *Mycobacterium bovis* BCG under stress conditions that included *in vitro* oxygen depletion and low nutrients, as well as *ex vivo* macrophage infection. *M. bovis* BCG was used in this study because the structural organization of the Rv3134c/*dev*R/*dev*S region is identical, to that of *M. tuberculosis* H37Rv and it has been previously shown that oxygen depletion also induces a dormancy response in *M. bovis* BCG.[13,20]

Materials and methods

M. bovis BCG culture conditions

For all experiments *M. bovis* BCG Pasteur strain 1137P2 was grown in Middlebrook 7H9 broth (Difco) plus 10% OADC supplement (Becton Dickinson) and 0.05% v/v Tween 80 (JT Baker), unless otherwise specified. The aerobic cultures were grown to exponential phase (OD_{600} of 0.5) in 500 cm flasks at 37 °C and 90 rpm. The *in vitro* low oxygen stress condition was performed as described previously,[13] with minor modifications. The primary culture was diluted to an OD_{600} of 0.02–0.03 in 400 ml of fresh medium in a 1 l flask, grown to an OD_{600} of 0.4–0.5 (exponential phase) and then divided in halves. One half was centrifuged at 4600*g*, and cells were resuspended in fresh medium and grown with agitation as described above (aerobic culture). The other half was divided into 13 ml cultures that were dispensed in 5×15 ml screw-capped polypropylene tubes and maintained static throughout the experiment (hypoxic culture). A parallel culture with methylene blue, which was monitored daily by spectrophotometric reading at 665 nm, was used as an indicator of oxygen consumption. Hypoxic cultures were processed at 0, 10 and 20 days to extract RNA.

Low nutrient experiments were performed according to Betts et al.[21] The primary culture was diluted to an OD_{600} of 0.02–0.03 in 250 ml fresh medium in a 1 l flask, grown to exponential phase and then divided in halves. Cells were pelleted by centrifugation and resuspended in either 7H9 medium or PBS. RNA isolation was carried out at 0, 4 and 15 days.

Macrophage culture and mycobacterial infection

M. bovis BCG was grown to exponential phase ($OD_{600} =$ 0.4–0.6) and resuspended in cell culture medium (DMEM, Gibco BRL®), supplemented with inactivated 10% fetal calf serum (Gibco BRL®) without adding antibiotics. Aliquots were stored at −70 °C. The J774 murine macrophage cell line was prepared and infected as previously described.[22] Before infection the bacterial clumps were dispersed by shaking in a bead beater (BioSpec Products) in the presence of 0.7–1 mm glass beads for few seconds. For each time point, 6×10^7 macrophages were infected using 1:5 (macrophage:bacilli) as multiplicity of infection (MOI). Uptake of bacteria by macrophages was analyzed by acid-fast staining of the monolayer. After 6 h of phagocytosis, extracellular bacteria were eliminated by washes with fresh warmed medium. Infected monolayers were recovered by adding guanidium isothiocyanate, and the macrophages were lysed at 0, 24 and 72 h after infection. The bacteria were