PARTIAL CHARACTERIZATION OF *Mycobacterium tuberculosis* DosR REGULATED GENES: *Rv2626c, Rv2625 AND Rv2624c*

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SUMMARY

The Rv2626c*,* Rv2625c *and* Rv2624c *genes of the human pathogen* M. tuberculosis *belong to the persistence or dosR regulon and their expression is increased significantly under different persistence-related conditions. In this work the transcription of the* Rv2626c-Rv2624c *region was analyzed using conventional RT-PCR and promoter fusions to gfp. The data suggest that these genes are arranged in an operon that is transcribed from a promoter located in the 178bp upstream of the Rv2626c gene. Using the BACTH system it was found that both* Rv2626 *and* Rv2625 *proteins interact with themselves to form homodimers. Additionally, a* Rv2625c *knock out* M. tuberculosis *strain was constructed, which also showed a significant decrease in* Rv2624c *expression. Contrary to expectations, in vitro growth of the mutant strain was similar to that of wild type strain during vegetative growth, oxidative stress and treatment with low doses of nitric oxide, indicating that these genes are not involved in the protective responses to the tested stress conditions.*

CARACTERIZACIÓN PARCIAL DE LOS GENES REGULADOS POR DosR: *Rv2626c, Rv2625c* Y *Rv2624c* DE *Mycobacterium tuberculosis*

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RESUMEN

Los genes Rv2626c, Rv2625c *y* Rv2624c *del patógeno humano* Mycobacterium tuberculosis *pertenecen al regulón de persistencia o dosR, y su expresión se incrementa de manera significativa en diferentes condiciones asociadas a persistencia. En este trabajo se realizó el análisis transcripcional de la región* Rv2626c-Rv2624c *por RT-PCR convencional y fusiones al gen gfp. Los datos sugieren que estos genes están organizados en un operón que se transcribe a partir de un promotor localizado en las 178pb que preceden a la secuencia codificante del gen* Rv2626c*. Usando el sistema BACTH se observó que las proteínas* Rv2626 *y* Rv2625 *interaccionan con ellas mismas formando homodímeros. Adicionalmente, se construyó una cepa mutante* de M. tuberculosis *que carece del gen* Rv2625c*, la cual también mostró una disminución significativa en la expresión de* Rv2624c*. Contrariamente a lo esperado, el crecimiento in vitro de las cepas mutante y silvestre fue igual durante el crecimiento vegetativo, estrés oxidativo y tratamiento con dosis bajas de óxido nítrico, lo que indica que estos genes no están involucrados en la respuesta protectora a las condiciones de estrés ensayadas.*

Introduction

The latent form of tuberculosis is a major obstacle for the control of the disease (Wayne and Sohaskey, 2001). The mechanisms by which *Mycobacterium tuberculosis* initiates and maintains a persistent state are unknown, but the study of these events and the genes involved in them could open new perspectives to contribute to tuberculosis control in the future.

Persistence *of M. tuberculosis* is presumably induced in the human host by oxygen depletion, exposure to low concentrations of nitric oxide and nutrient deprivation; hence, these conditions are used as *in vitro* models of *M. tuberculosis* persistence. Genes potentially important for establishing or maintaining the persistent state have been identified by studying with microarrays the transcriptional profiles in these models (Sherman *et. al*., 2001; Voskuil *et al*., 2003; Kendall *et al*., 2004; Muttucumaru *et al*., 2004).

The *Rv2626c*, *Rv2625c* and *Rv2624c* genes of *M. tuberculosis* belong to the *dos*R regulon, which is induced during *in vitro* persistence-related conditions, and like most of the genes that constitute this regulon, their functions are un-

KEYWORDS / *Mycobacterium tuberculosis* / Operon / Protein Interaction / *Rv2625c* Deletion / *Rv2626c-Rv2624c* Region /

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CARACTERIZAÇÃO PARCIAL DOS GENES REGULADOS POR DosR: *Rv2626c, Rv2625c* E *Rv2624c* DE *Mycobacterium tuberculosis*

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RESUMO

Os genes Rv2626c*,* Rv2625c *e* Rv2624c *do patógeno humano* Mycobacterium tuberculosis *pertencem ao regulon de persistência ou DosR (DevR regulon), e sua expressão se incrementa de maneira significativa em diferentes condições associadas a persistência. Neste trabalho se realizou a análise transcricional da região* Rv2626c-Rv2624c *por RT-PCR convencional e fusões ao gene gfp. Os dados sugerem que estes genes estão organizados em um operon que se transcreve a partir de um promotor localizado nas 178pb que precedem à sequência codificante do gene* Rv2626c*. Usando o sistema BACTH se observou que as proteínas* Rv2626 *e* Rv2625 *interagem com elas mesmas formando homodímeros. Adicionalmente, foi construída uma cepa mutante de* M. tuberculosis *que carece do gene* Rv2625c*, a qual também mostrou uma diminuição significativa na expressão de* Rv2624c*. Contrariamente ao esperado, o crescimento in vitro das cepas mutante e silvestre foi igual durante o crescimento vegetativo, estresse oxidativo e tratamento com doses baixas de óxido nítrico, o que indica que estes genes não estão envolvidos na resposta protetora às condições de estresse ensaiadas.*

known.The Rv2626 protein shows homology to CBS domains (so named because they were first discovered in cystathionine-β-synthase), which are associated with another type of domain in different proteins and can modulate the activity of the catalytic domain (Ignoul and Eggermont, 2005). Also, it has been suggested that CBS domains can mediate the protein oligomerization (Jhee *et al*., 2000; Estévez *et al*., 2004). The putative Rv2625 protein is similar to S2P type proteins (Makinoshima and Glickman, 2006), which promote intramembrane proteolysis and are involved in different pathways. One example is SpoIVFB, a protease involved in the activation cascade of sigma factors during *Bacillus subtilis* sporulation (Rudner *et al*., 1999). The Rv2624 protein shows similarity to universal stress proteins (USP; O´Toole and Williams, 2003), which seem to be important components of bacterial defence against various types of stress (Kvint *et al*., 2003; Siegele, 2005).

DosR is a transcriptional factor required for induction of the dormancy regulon genes. Two putative binding sites for DosR are present in the *Rv2626c-Rv2624c* region, both located upstream of the *Rv2626c* coding sequence (Park *et al*., 2003). The non-coding region upstream of the *Rv2626c* gene has 514 nucleotides, while the intergene regions between *Rv2626c-Rv2625c* and *Rv2625c-Rv2624c* consist of only 59 and 15 nucleotides, respectively, suggesting that these genes may form an operon.

In microarray studies it has been observed that *Rv2625c* and *Rv2624c* are induced *in vitro* by treatment with low concentrations of NO and hypoxia, and in activated macrophages (Schnappinger *et al*., 2003; Voskuil *et al*., 2003). Similar to the other genes that constitute the regulon *dosR*, the induction of *Rv2626c*, *Rv2625c* and *Rv2624c* transcription requires NO in activated macrophages (Schnappinger *et al*., 2003). Thus, these genes appear to be part of the adaptive response of *M. tuberculosis* to the immune defenses of the host.

In this work the co-transcription and promoter activity of *Rv2626c-Rv2624c* region of the *M. tuberculosis* genome was analyzed, and the possible dimerization of Rv2626, Rv2625 and Rv2624 proteins searched for. It was found that *Rv2625c-Rv2624c* and *Rv2626c-Rv2625c* are transcribed in dicistronic messengers, that there is a promoter in the *Rv2626c* upstream region, and that Rv2625 and Rv2626 proteins form homodimers. Also, the effect of the deletion of *Rv2625c* gene and decreased expression of *Rv2624c* were evaluated under various *in vitro* growth conditions. Full expression of both *Rv2625c* and *Rv2624c* were dispensable for growth under the tested conditions.

Materials and Methods

Bacterial strains, media and culture conditions

M. tuberculosis H37Rv (ATCC27294) and *M. tuberculosis* Δ*Rv2625c* (this study) were grown on Middlebrook 7H10 agar supplemented with 10% (v/v) Middlebrook OADC (Difco) or in Middlebrook 7H9 broth (Difco) also supplemented with 0.05% (v/v) Tween 80 and 0.5% (v/v) glycerol. For standing cultures (see below) medium without glycerol was used (Wayne and Sohaskey, 2001). *M. tuberculosis* strains were grown and manipulated inside a BSL3 containment facility. *Escherichia coli* XL1-Blue (Stratagene) was grown in Luria Bertani broth (LB) or on LB agar. *E. coli* BTH101 was grown in minimal medium broth or MacConkey agar (Himedia) containing 1% maltose. The strains were incubated at 37°C, stirring at 100rpm when required. Additionally, the following antibiotics were added to the media when needed: 50μg/ml carbenicillin, 50μg/ml hygromycin and 25μg/ml kanamycin (12 μg/ml for allelic exchange).

In vitro conditions tested in *M. tuberculosis* strains included: vegetative growth to stationary phase, growth without shaking (standing cultures), 0.5 and 5mM diethylenetriamine/nitric oxide adduct (DETA/NO, Sigma) (Voskuil *et al*., 2003), and 10 and 20mM H_2O_2 (Kendall *et al*., 2004). These compounds were added after the cultures had reached the early exponential phase (O.D. 600nm= 0.5-0.6).

Conventional and quantitative RT-PCR

RNA was isolated as previously described (Salazar *et al*., 2003) from cultures of *M. tuberculosis* H37Rv and the Δ*Rv2625c* mutant strain, in early exponential phase of growth (O.D. 600nm= 0.5-0.6), either treated or not with 0.5mM DETA-NO for 40min, or from 15 days old standing cultures. Total RNA obtained was treated with DNase I (Promega). Each re-

verse transcription reaction used 25 ng·µl^{-1} DNA-free RNA, previously denatured at 65ºC for 60min. The reaction mixture consisting of 1mM each dNTP, 2.5mM specific reverse primer, 1X AMV RT buffer (Promega), 20 U RNase inhibitor RNasin (Promega) and 10 U of AMV RT (Promega), was incubated at 42ºC for 60min. Absence of contaminating DNA was confirmed by a control in which reverse transcriptase was not added. Aliquots of 5µl of RT products were used as template in PCR reactions. At least three separate determinations were made with three different samples of RNA.

RT quantitative PCR (RT-qPCR) was carried out as described by Casart *et al*. (2008) with the following modifications: reverse transcription reaction used 5ng·µl^{-1} DNA -free RNA, 0.05µg/µl random primers (Promega) or 2.5mM specific reverse oligonucleotide, 1X AMV RT buffer (Promega), 20 U RNase inhibitor RNasin (Promega) and 10 U of AMV RT (Promega).

The nucleotide sequence of the primers used in RT-PCR and other methods can be given upon request.

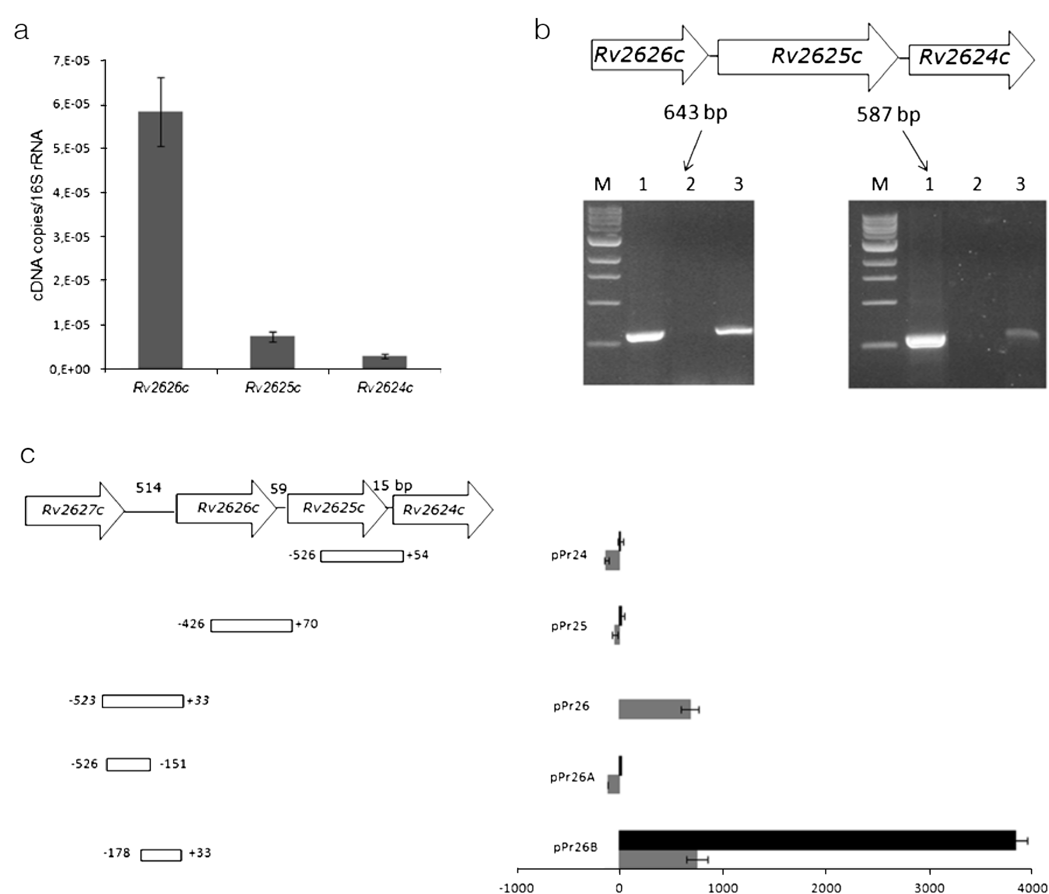

Figure 1. Transcription analysis of the *Rv2626c-Rv2624c* region. The bars show the mean ±SD of three or more separate experiments. a: *In vitro* expression of the *Rv2626c*, *Rv2625* and *Rv2624c* genes in wild type strain under DETA/NO treatment, as measured by RT-qPCR. Data obtained were normalized by 16S ribosomal RNA. b: Conventional RT-PCR. Genetic organization of the *Rv2626c-Rv2624c* region is represented above. The size of the amplified fragments is indicated in pb. M: molecular mass marker, 1Kb DNA ladder. Lanes are 1: *M. tuberculosis* genomic DNA control; 2: control without reverse transcriptase; and 3: RT-PCR (with reverse transcriptase). c: Promoter activity in the *Rv2626c-Rv2624c* region. At left, schematic representation of the *Rv2627c-Rv2625c* region, indicating PCR fragments cloned upstream of the *gfp* gene (white rectangles); at right, fluorescence measurement in *M. smegmatis* (gray bars) and *M. tuberculosis* (black bars) transformants. The plasmids were tested in *M. tuberculosis* in adition to *M. smegmatis*, except for pPr26.

Transcriptional fusions to gfp and fluorescence measurement

Fragments of ~200-600bp, containing the upstream region of the *Rv2626c*, *Rv2625c* and *Rv2624c* genes were obtained by PCR using *M. tuberculosis* H37Rv chromosomal DNA as template and specific primers. These fragments were cloned into the shuttle vector pFPV27 (Valdivia *et al*., 1996) fused to a promotorless *gfp* gene, generating pPr26, pPr25, pPr24, pPr26A and pPr26B plasmids (Figure 1c, left panel). The constructions were confirmed by PCR, digestion with restriction endonucleases and sequencing. All the recombinant plasmids were used to transform *M. smegmatis* mc^{2}155 and *M. tuberculosis* H37Rv, except for pPr26 that was electroporated only into *M. smegmatis*. Bacteria cultures carrying each one of the constructs were grown until stationary phase, and aliquots were taken in order to measure fluorescent emission (Tecan fluorimeter, GENius) at excitation and emission lengths appropriate for GFP. Fluorescence values obtained for the cells carrying the control plasmid (pFPV27) were subtracted from those observed in cells carrying the transcriptional fusions.

Bacterial two hybrid (BACTH) assay

The protein-protein interactions were evaluated using the BACTH system (Euromedex). The complete coding regions of the *Rv2626c*, *Rv2625c* and *Rv2624c* genes, as well as the coding regions of the Rv2625 protein N- and C-terminal domains, were obtained by PCR using *M. tuberculosis* H37Rv chromosomal DNA as template and specific primers. PCR products were ligated in-frame to the coding sequences of the adenylate cyclase T25 and T18 domains. Electroporation, selection of colonies and β-Gal activity measurements were carried out as suggested by the manufacturer.

Construction and confirmation of the mutant strain M. tuberculosis *ΔRv2625c*

A mutant strain of *M. tuberculosis* (Δ*Rv2625c*) was constructed using the method of gene replacement by homologous recombination of Parish and Stocker (2000) to obtain unmarked mutants. To do so, two fragments were amplified by PCR using *M. tuberculosis* H37Rv chromosomal DNA as template: a 1166bp fragment spanning from the 3'-end of

Rv2627c gene to the 5'-end of *Rv2625c* gene; and a 1265bp fragment extending from the 3'-end of *Rv2625c* to the 3' region of the *Rv2623c* gene. These PCR products were sequentially ligated into the p2NIL plasmid, then the *Pac*I cassette from the pGOAL19 plasmid was ligated into the resulting recombinant plasmid and the final suicide plasmid obtained was electroporated into *M. tuberculosis* H37Rv.

Single and double crossover colonies were confirmed by PCR and southern blot. Southern blot was carried out using chromosomal DNA from the wild type parent and the deletion mutant strains, and the 'ECL direct nucleic acid labeling and detection system' kit (GE). The chromosomal DNA was digested with *Nar*I or *EcoR*I/*Not*I, and the probe was obtained by PCR using chromosomal DNA of *M. tuberculosis* H37Rv as template and specific primers.

Western blot assay

Western blot assay was carried out by using standard methods and cell lysates prepared as directed by Parish and Wheeler (1998) from cultures of *M. tuberculosis* H37Rv and the Δ*Rv2625c* mutant strains, treated with 0.5mM DETA-NO and processed after 4h of exposure to the reagent. In immunodetection, anti GST-Rv2624 produced in rabbit (this study; dilution 1:2500) was used as primary antibody and a goat anti-rabbit peroxidase conjugate (Sigma; dilution 1:3000) as secondary antibody.

Results and Discussion

The levels of expression of the *Rv2626c, Rv2625c* and *Rv2624c* genes were determined by RT-qPCR using RNA isolated from cultures either treated or untreated with DETA-NO and random primers in reverse transcription reactions. When the cultures were not treated with NO, there was no mRNA detected for either *Rv2625c* or *Rv2624c* (data not shown). In contrast, different amounts of mRNA were quantified from cultures treated with DETA/NO (Figure 1a), confirming the induction of *Rv2626c*, *Rv2625c* and *Rv2624c* expression by NO, as was previously observed with microarrays (Voskuil *et al*., 2003).

To assess the possibility that each pair of adjacent genes in the *Rv2626c*-*Rv2624c* region were co-transcribed, reverse transcription was performed followed by PCR, to amplify a product containing the 5'-end of *Rv2624c* or *Rv2625c* together with a fragment of the upstream gene. RNA was isolated from cultures in exponential growth phase, treated with 0.5mM DETA-NO. RT-PCR yielded products of the expected size for each region (643 and 583bp; Figure 1b). The presence of these transcripts containing the *Rv2626c*-*Rv2625c* and *Rv2625c*-*Rv2624c* intergene regions suggests that each pair of genes is co-transcribed on a single mRNA.

In order to identify the promoter regions of the *Rv2626c*, *Rv2625c* and *Rv2624c* genes, fragments of ~600bp containing the upstream region of each gene were cloned into the pFPV27 vector, upstream of the promoterless *gfp* gene. The generated plasmids, named pPr26, pPr25 and pPr24 (Figure 1c), were then electroporated into *M. smegmatis* mc^{2}155. GFP protein expression was determined by measuring the fluorescence emission from stationary phase cultures of *M. smegmatis* cells bearing these plasmids. As shown in Figure 1c, *M. smegmatis* mc^{2}155 cells carrying the pPr26 plasmid were fluorescent, while those carrying pPr25 and pPr24 were not, suggesting the presence of a promoter sequence in the upstream region of *Rv2626c*,

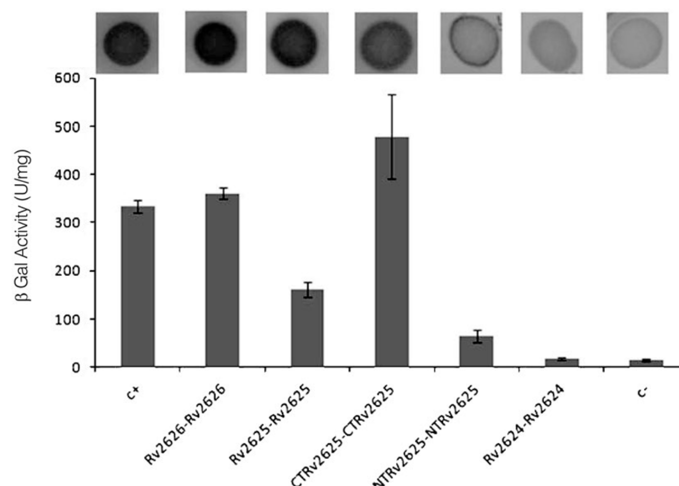

Figure 2. Evaluation of homodimerization of Rv2626, Rv2625 and Rv2624 proteins, using the BACTH system. The graph shows quantification by β-galactosidase assay. Above is shown the growth of co-transformed colonies on MacConkey-maltose agar. NTRv2625: Rv2625 N-terminal domain, corresponding to S2P-type domain (aminoacids 1 to 230); CTRv2625: Rv2625 C-terminal domain, corresponding to CBS domain (aminoacids 233 to the last); c+: positive control (cell carrying pKT25-zip and pUT18c-zip plasmids); c-: negative control (cells carrying pKNT25 and pUT18 plasmids). The bars show the mean ±SD of three or more separate experiments.

and absence of a promoter in the other regions analyzed.

To determine more precisely the location of the potential promoter sequence (s) upstream of *Rv2626c*, two additional fragments, corresponding to 391 nucleotides at the 5'-end and 211 nucleotides at the 3'-end of *Rv2627c*-*Rv2626c* intergene region, were amplified by PCR and ligated into pFPV27. The fluorescence of *M. smegmatis* cells bearing the resultant constructs, named pPr26A and pPr26B, suggests that the 211bp fragment cloned in pPr26B contains at least one promoter which drives the transcription of the *Rv2626c* gene, and possibly also that of *Rv2625c* and *Rv2624c* genes (Figure 1c, right). Since the fluorescence intensity emitted by cells carrying pPr26 was very similar to the fluorescence of those carrying pPr26B, it was deduced that the 211bp fragment cloned in pPr26 contains all cis-acting elements required for *Rv2626c* transcription. Promoter activity found at pPr26B, and the lack of it in pPr24 and pPr25, was confirmed in *M. tuberculosis*. As expected, pPr26B promoter activity was significantly higher in *M. tuberculosis.*

Additionally, using primer extension a single sharp signal was identified with an oligonucleotide complementary to the first nucleotides of the *Rv2626c* coding sequence (data not shown), and no transcriptional start sites were detected with primers complementary to the 5′end the coding sequences of either *Rv2625c* and *Rv2624c*. Although it was not possible to map the precise 5'termini of the mRNA, this result supports the suggestion from the transcriptional *gfp* fusions that the only promoter is upstream of *Rv2626c*.

Considering that *Rv2626c* and *Rv2625c* genes form part of an operon, which may also include *Rv2624c*, their encoded proteins may have a coordinated function that could be reflected as interaction between these proteins. Using the BACTH system no interaction between Rv2626, Rv2625 and Rv2624 proteins was found

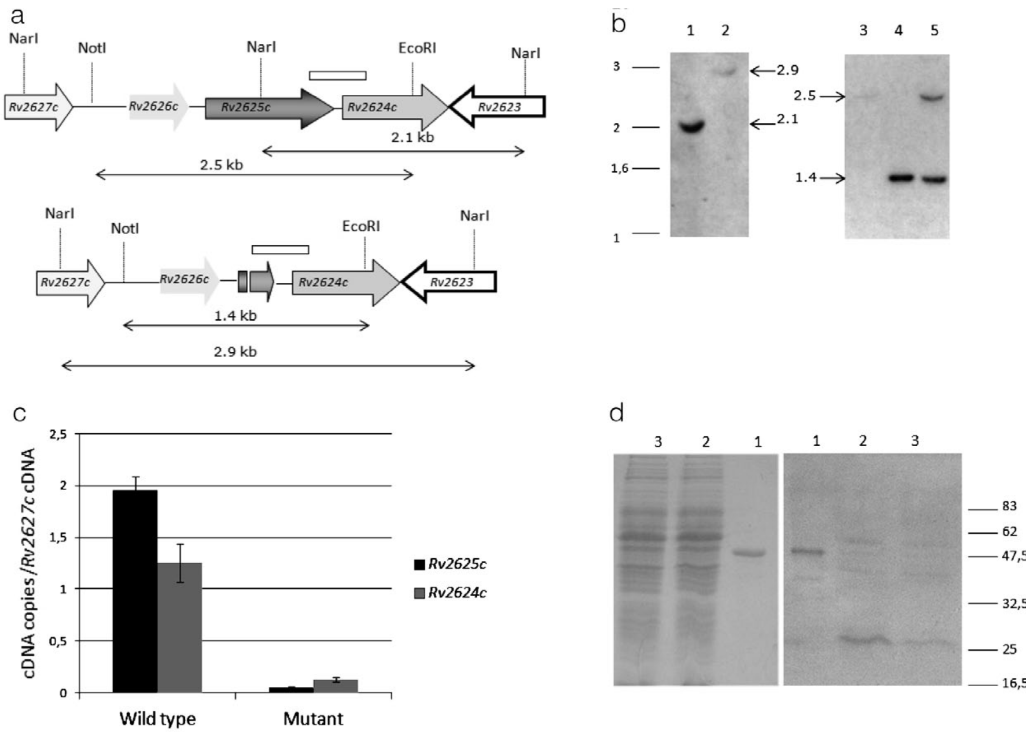

Figure 3. Construction and confirmation of the mutant strain *M. tuberculosis* Δ*Rv2625*. a: Squematic representation of the *Rv2627c*-*Rv2623* region, indicating restriction sites for enzymes used and size of fragments generated by digestion. The probe is represented by a white bar. b: Southern blot analysis. Lanes 1 and 3: genomic DNA from wild type strain, 2 and 4: genomic DNA from Δ*Rv2625c* strain, 5: genomic DNA from single cross over strain. Samples of genomic DNA were digested with *Nar*I (lanes 1, 2), or with *EcoR*I/*Not*I (lanes 3-5). c and d: Analysis of *Rv2624c* expression in *Rv2625c* knockout strain. c: qRT-PCR. *Rv2625c* expression was measured as control. The total RNA was isolated from standing cultures. Error bars show the SD of at least three separate determinations made with three different samples of RNA. d: Western blot analysis using antibody against GST-Rv2624 fusion protein. The cell lysates were obtained from cultures at exponential phase after treatment with 0.5mM DETA-NO for 4h. Lanes are 1: GST-Rv2624 protein, 2: cell lysate of wild type strain, 3: cell lysate of Δ*Rv2625c* strain. Equal protein loading was confirmed by Coomassie blue staining (left panel).

(data not shown), but other approaches must be tested to conclusively rule out that possibility. However, using the same system, Rv2626 and Rv2625 proteins were observed to interact with themselves to form homodimers. Additionally, the interaction of the Rv2625 N-terminal domain with itself was weaker than the interaction of the full-length protein, while that of the C-terminal region was stronger (Figure 2), suggesting that the dimerization of Rv2625 occurs through the interaction of the C-terminal region.

The C-terminal regíon of the Rv2625 protein (Makinoshima and Glickman, 2006) and the complete sequence of Rv2626 are similar to CBS domains. Rv2625 and Rv2626 dimerization through their CBS domains is consistent with the proposal that this type of domain can mediate protein oligomerization, based on the finding that deletion of CBS domains from cystathionine-β-synthase avoids the formation of tetramers and octamers seen with the full length enzyme (Jhee *et al*., 2000; Estéves *et al*., 2004).

In the *M. tuberculosis Rv2625c* knock out strain a region of 3517 nucleotides spanning from the 3' region of the *Rv2627c* gene to the 3' region of the *Rv2623* gene was replaced by a fragment of 2431 nucleotides, corresponding to the same region but lacking most of the coding sequence of *Rv2625c* gene (Figure 3a). The deletion of *Rv2625c* gene was confirmed by Southern blot analysis (Figure 3b) and sequencing of PCR products, thus demonstrating that the *Rv2625c* gene is not essential in *M. tuberculosis*.

In order to evaluate a possible decrease in the expression of *Rv2624c* as a result of a polar effect due to the elimination of the *Rv2625c* gene, the expression of the *Rv2624c* gene in both wild type and mutant strains was determined. RNA was isolated from 15 day old standing cultures of both wild type and mutant strains and subjected to RT-qPCR using specific reverse oligonucleotides. When *M. tuberculosis* cultures are left standing, hypoxia is generated and the DosR regulon is induced (Wayne and Sohaskey, 2001; Kendall *et al*., 2004). The *Rv2627c* gene expression was used for normalization because its level was constant between the two strains. As shown in Figure 3c, the expression of *Rv2624c* was very low in the mutant strain, whereas significant levels of expression were found in the parent wild type *M. tuberculosis* H37Rv. These results indicate that in the *M. tuberculosis* Δ*Rv2625c* strain, apart from eliminating the expression of *Rv2625c*, there is a significant decrease of *Rv2624c* expression.

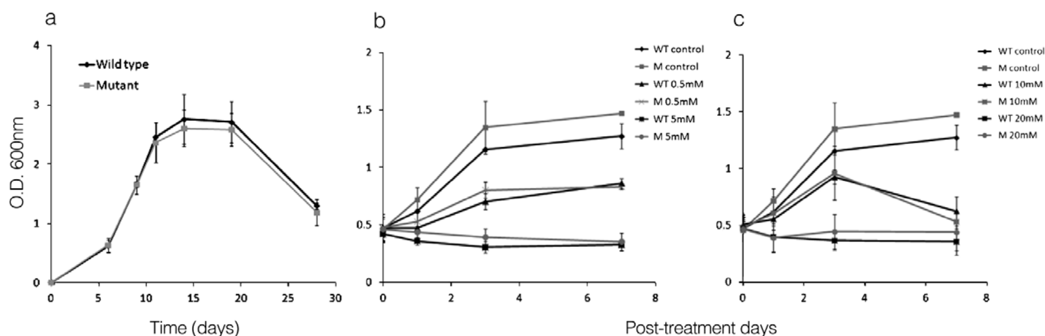

Figure 4. *In vitro* growth of mutant strain *M. tuberculosis* Δ*Rv2625*. a: Vegetative growth. b and c: Growth following exposure to different conditions. b: Treatment with DETA-NO. c: Treatment with H_2O_2. M: mutant strain, WT: wild type strain. Each graph represents the average of three individual experiments.

To confirm the reduction in the expression of *Rv2624c* in the mutant strain, a western blot assay was performed using cell lysates obtained from cultures treated with DETA-NO. Compared to the *M. tuberculosis* H37Rv parent strain, less Rv2624 protein was observed in the Δ*Rv2625c* mutant (Figure 3d), confirming that its expression was reduced.

Finally, we tried to find clues to the possible function of *Rv2625c* by looking for the effects of its elimination and decreased expression of *Rv2624c* on the vegetative growth of the bacteria, as well as growth under low concentrations of NO or oxidative stress. As shown in Figure 4, the growth of mutant strain *M. tuberculosis* Δ*Rv2625c* is similar to that of the wild type during both exponential and stationary growth phases, and also when the cultures were treated with DETA-NO (0.5 and 5mM) or H_2O_2 (10 and 20mM). These results show that the mutant is not more sensitive to these treatments with respect to the wild type strain, suggesting that the function of the *Rv2625c* and *Rv2624c* genes is not involved in or is not essential for the survival of bacteria under these conditions. This was unexpected, as it has been shown that the expression of both *Rv2625c* and *Rv2624c* is induced in several of these conditions (Voskuil *et al*., 2003; Voskuil *et al.,* 2004).

However, the expression of *Rv2624c* was not completely eliminated in the Δ*Rv2625c* mutant strain, so it is possible that the small amount of Rv2624 protein in the mutant strain could be sufficient to carry out its possible role in growth under the tested conditions. On the other hand, the Rv2624 protein shows similarity to USP proteins which are involved in resistance of *E. coli* to multiple stresses, and other eight putative USP type proteins exist in *M. tuberculosis*, five of which are also regulated by DosR (Park *et al*., 2003; O´Toole and Williams, 2009). Hence, the lack of requirement for full expression of *Rv2624c* under the stress conditions tested in the present study could be explained by a functional redundancy amongst the USP type proteins of *M. tuberculosis*, so that the decrease in the Rv2624 protein could be compensated by some of these other putative proteins.

In summary, the results suggest that the *Rv2626c*, *Rv2625c* and *Rv2624c* genes are expressed as a single operon whose promoter is contained in the region upstream of the *Rv2626c* gene. We also observed homodimerization of both Rv2626 and Rv2625 proteins, perhaps through their CBS domains, which has been proposed to mediate protein oligomerization. Finally, we demonstrated that *Rv2625c* of *M. tuberculosis* is not essential and that neither it nor full expression of *Rv2624c* are required for growth under various *in vitro* conditions, some of which induce its expression; including one related to an *in vitro* persistence model.

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REFERENCES

Casart Y, Gamero E, Rivera-Gutiérrez S, González-y-Merchand JA, Salazar L (2008) par genes in *Mycobacterium bovis* and *Mycobacterium smegmatis* are arranged in an operon transcribed from "SigGC" promoters. *BMC Microbiol.* www.biomedcentral.com/1471-2180/8/51.

Estévez R, Pusch M, Ferrer-Costa C, Orozco M, Jentsch TJ (2004) Functional and structural conservation of CBS domains from CLC chloride channels. *J. Physiol. 557*: 363-378.

Ignoul S, Eggermont J (2005) CBS domains: structure, function, and pathology in human proteins. *Am. J. Physiol. Cell Physiol. 289*: 1369-1378.

Jhee KH, McPhie P, Miles EW (2000) Yeast cystathionine beta-synthase is a pyridoxal phosphate enzyme but, unlike the human enzyme, is not a heme protein. *J. Biol. Chem. 275*: 11541-11544.

Kendall S, Movahedzadeh F, Rison S, Wernisch L, Parish T, Duncan K, Betts JC, Stoker NG (2004) The *Mycobacterium tuberculosis* dosRS two-component system is induced by multiple stresses. *Tuberculosis 84*: 247-255.

Kvint K, Nachin L, Diez A, Nyström T (2003) The bacterial universal stress protein: function and regulation. *Curr. Opin. Microbiol. 6*: 140-145.

Makinoshima H, Glickman M (2006) Site-2 proteases in prokaryotes: regulated intramembrane proteolysis expands to microbial pathogenesis. *Microb. Infect. 8*: 1882-1888.

Muttucumaru D, Roberts G, Hinds J, Stabler R, Parish T (2004) Gene expression profile of *Mycobacterium tuberculosis* in a non-replicating state. *Tuberculosis 84*: 239-246.

O'Toole R, Williams H (2003) Universal stress proteins and *Mycobacterium tuberculosis*. *Res. Microbiol. 154*: 387-392.

Parish T, Stoker NG (2000) Use of a flexible cassette method to generate a double unmarked *Mycobacterium tuberculosis* tlyA plcABC mutant by gene replacement. *Microbiology 146*: 1969-1975.

Parish T, Wheeler P (1998) Preparation of cell-free extracts from mycobacteria. In Parish T, Stoker N (Eds.) *Mycobacteria Protocols, Methods in Molecular Biology*. Humana. New York, USA. pp. 77-89.

Park H, Guinn KM, Harrell MI, Liao R, Voskuil MI, Tompa M, Schoolnik G, Sherman D (2003) Rv3133c/dosR is a transcription factor that mediates the hypoxic response of *Mycobacterium tuberculosis*. *Mol. Microbiol. 48*: 833-843.

Rudner D, Fawcet P, Losick R (1999) A family of membrane-embedded metalloproteases involved in regulated proteolysis of membrane-associated transcription factors. *Proc. Nat. Acad. Sci. USA. 96*: 14765-14770.

Salazar L, Guerrero E, Casart Y, Turcios L, Bartoli F (2003) Transcription analysis of the dnaA gene and *oriC* region of the chromosome of *Mycobacterium smegmatis* and *Mycobacterium bovis* BCG, and its regulation by the DnaA protein. *Microbiology 149*: 773-784.

Schnappinger D, Ehrt S, Voskuil M, Liu Y, Mangan J, Monahan I, Dolganov G, Efron B, Butcher P, Nathan C, Schoolnik G (2003) Transcriptional adaptation of *Mycobacterium tuberculosis* within macrophages: Insights into the phagosomal environment. *J. Exp. Med. 198*: 693-704.

Sherman D, Voksuil M, Schnappinger D, Lia R, Harrell M, Schoolnik G (2001) Regulation of the *Mycobacterium tuberculosis* hypoxic response gene encoding α-crystallin. *Proc. Nat. Acad. Sci. USA*. 98: 7534-7539.

Siegele D (2005) Universal stress proteins in *Escherichia coli*. *J. Bacteriol. 187*: 6253-6254.

Valdivia R, Hromockyj A, Monack D, Ramakrishnan L, Falkow S (1996) Applications for green fluorescent protein (GFP) in the study of host-pathogen interactions. *Gene 173*: 47-52.

Voskuil MT, Schnappinger D, Visconti, KC, Harrell MI, Dolganov GM, Sherman DR, Schoolnik GK (2003) Inhibition of respiration by nitric oxide induces a *Mycobacterium tuberculosis* dormancy program. *J. Exp. Med. 198*: 705-713.

Voskuil MI, Visconti KC and Schoolnik GK (2004) *Mycobacterium tuberculosis* gene expression during adaptation to stationary phase and low-oxygen dormancy. *Tuberculosis 84*: 218-227.

Wayne L, Sohaskey Ch (2001) Nonreplicating persistence of *Mycobacterium tuberculosis*. *Annu. Rev. Microbiol. 55*: 139-163.