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

Elba Guerrero, Rossana Jaspe and Leiria Salazar

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 transcricional 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 interactuan 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; Mut-tucumaru et al., 2004).

The Rv2626c, Rv2625c and Rv2624c genes of M. tuberculosis belong to the dosR regulon, which is induced during in vitro persistence-related conditions, and like most of the genes that constitute this regulon, their functions are un-
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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 à 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 enzaiadas.
verse transcription reaction used 25 ng·µl⁻¹ DNA-free RNA, previously denatured at 65°C for 60 min. The reaction mixture consisting of 1 mM each dNTP, 2.5 mM specific reverse primer, 1X AMV RT buffer (Promega), 20 U RNase inhibitor RNasin (Promega) and 10 U of AMV RT (Promega), was incubated at 42°C for 60 min. Absence of contaminating DNA was confirmed by a control in which reverse transcriptase was not added. Aliquots of 5 µl 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 5 ng·µl⁻¹ DNA-free RNA, 0.05 µg/µl random primers (Promega) or 2.5 mM 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.

Transcriptional fusions to gfp and fluorescence measurement

Fragments of ~200-600 bp, 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 mc²155 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 (ΔRv2625c) 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 1166 bp 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 Pacl 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 NaeI or EcoRI/NotI, 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 ΔRv2625c 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 was 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 mc²155. 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 mc²155 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, 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 Rv2626c-Rv2624c 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 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 of 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.
(data not shown), but other approaches must be tested to conclusively rule out that possibility. However, using the same system, Rv2625 and Rv2626 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 region of the Rv2625 protein (Makushima 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 Rv2625 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 ΔRv2625c strain, apart from eliminating the expression of Rv2625c, there is a significant decrease of Rv2624c expression.
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 ΔRv2625c 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 ΔRv2625c 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 H2O2 (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 ΔRv2625c 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


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.


