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Examining the role of Rv2895c (*viuB*) in iron acquisition in *Mycobacterium tuberculosis*

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Abstract

Iron acquisition is essential for *Mycobacterium tuberculosis* (*Mtb*) virulence. Understanding the molecular mechanisms used by *Mtb* to scavenge iron during infection might reveal new targets for antimicrobial development. Rv2895c, a homolog of ViuB from *Vibrio cholerae* has been postulated to be involved in iron-siderophore uptake and utilization in *Mtb*. This study examines the requirement of Rv2895c for adaptation of *Mtb* to iron limitation. We show that Rv2895c is dispensable for normal replication of *Mtb* in iron deficient conditions and in human macrophages. Thus, contrary to the predictions of sequence analysis and in vitro studies the genetic evidence indicates that in normal conditions Rv2895c is not required for iron acquisition in *Mtb*.

Keywords

siderophore; iron transport; mycobactin; FAD

Iron is an essential nutrient for most living organisms including *Mycobacterium tuberculosis* (*Mtb*). Due to the poor solubility of ferric iron (Fe^{+3}) in the presence of oxygen and at neutral pH, free iron is not found in the mammalian host, but sequestered in complexes with proteins such as transferrin, lactoferrin and ferritin.[1] Acquiring iron during infection poses a challenge for most pathogens. In bacteria, a common mechanism to acquire iron is the synthesis and secretion of high affinity iron chelators (siderophores) that can solubilize iron and efficiently compete with iron-binding proteins of the host. Fe^{+3}-siderophore complexes are transported into the cell by specific importers. In the cytosol, Fe^{+3} is dissociated from the siderophore via cleavage of the siderophore or by the action of a ferric reductase. Reduction of Fe^{+3} results in a weaker binding of Fe^{+2} to the siderophore, allowing release of iron.[2]

Iron uptake systems are attractive targets for the development of antimicrobial agents that interfere with iron acquisition. *Mtb* synthesizes siderophores named mycobactins. One form of mycobactin is very hydrophobic and remains cell associated while carboxymycobactin (also known as exomycobactin) is more hydrophilic, and is secreted.[3] The operon encoding

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the ABC permeases IrtA and IrtB is required for uptake of iron from Fe^{+3}-carboxymycobactin and growth of *Mtb* in iron deficient conditions.[4] The cytosolic, amino terminal domain of IrtA (IrtA-NTD) binds one molecule of flavin adenine dinucleotide (FAD) and this property is essential for iron acquisition through IrtAB.[5] Since most bacterial ferric reductases are flavin reductases[6], IrtA-NTD might be a ferric-reductase of Fe^{+3}-carboxymycobactin imported by IrtAB. IrtA-NTD is highly similar to *Vibrio cholerae*'s ViuB, a cytosolic protein required for the utilization of ferric-vibriobactin [5,7] ViuB homologs are present in several bacteria and are annotated as siderophore interacting proteins. Structural studies of the ViuB homologs of *Shewanella putrefaciens* and *E. coli* (YqjH) support a role of these proteins as NAD(P)H:flavin oxidoreductases [8].

In addition to the IrtA-NTD, the cytosolic protein encoded by Rv2895c shows 31% similarity to ViuB, and it has been annotated as the *Mtb* ViuB possibly functioning as a siderophore utilization protein (genolist.pasteur.fr/TubercuList). Rv2895c is induced in *Mtb* growing in low iron[9] and in BCG replicating in mouse macrophages.[10] Based on physical interactions detected in-vitro between recombinant Rv2895c and IrtB as well as Rv2895c and Fe^{+3} -carboxymycobactin, it was suggested that Rv2895c acted as siderophore binding protein for iron transport by IrtB.[9,11] Given the potential of iron transporters as drug targets, identification of the proteins involved in iron acquisition in *Mtb* is important. In this study we sought a genetic proof of the role of Rv2895c in iron acquisition. An Rv2895c mutant in *Mtb* H37Rv was generated and the effect of the deletion on the adaptation of *Mtb* to low iron was evaluated. A 0.7 kb region encoding 249 amino acids of the 283 constituting Rv2895c was deleted and replaced with a hygromycin resistant cassette by specialized transduction and homologous recombination.[12] The mutant (ST212) was confirmed using Southern blot (figure 1) and PCR analysis (data not shown). Growth of the Rv2895c mutant (ST212) strain was equivalent to the wild type in standard 7H9, which is an iron rich medium containing 100–165uM iron depending upon the source of ferric ammonium citrate. We next examined the effect of Rv2895c deletion on the ability of *Mtb* to replicate under iron deficient conditions. To assess replication under iron deficient conditions the strains were grown in a defined low iron (~2uM $FeCl_3$) medium (MM).[4] A mutant of the iron transporter IrtAB (ST73)[4] was used as control. Growth was assessed as a function of increase in optical density at 540nm. ST212 grew as well as the wild type strain under iron deficiency (figure 2A), whereas the same conditions limited the growth of the iron transport mutant ST73 (figure 2B). Further reduction of iron in the cells was achieved by addition of the permeable ferrous iron chelator 2,2′-dipyridyl (DPI) to the growth medium. DPI drastically reduced growth of ST73 (figure 2D) but did not affect growth of ST212 (figure 2C). These results show that disruption of Rv2895c does not alter the ability of *Mtb* to replicate under iron deficient conditions and indicate that Rv2895c is dispensable for iron acquisition in *Mtb*. To examine whether deletion of Rv2895c could have a minor effect in iron acquisition, not detected in the growth assay, we determined the MIC of streptonigrin for the wild type and the mutant strains. Streptonigrin is used as an indirect indicator of intracellular iron levels since this antibiotic requires iron for its bactericidal activity.[13] Indeed, iron deficient mutants exhibit increased resistance to streptonigrin than their parental strains.[5] This experiment was conducted in MM and the MIC was determined using the microplate Alamar Blue Assay.[14] MIC was defined as the concentration that inhibit at least 90% growth. Wild type and ST212 strains exhibit the same MIC for streptonigrin (2.5 ug/ml) indicating that there is no detectable difference in the intracellular levels of iron in the wild type and the Rv2895c mutant.

Macrophages provide an iron-limiting environment for *Mtb* and siderophore mediated iron acquisition is required for normal replication of *Mtb* in these cells.[13,4] To assess the effect of Rv2895c deletion on *Mtb*'s replication in human macrophages, THP-1 cells were differentiated into macrophages and infected with ST212 and H37Rv as described before.[4]

As shown in figure 3, ST212 replicated similarly to the wild type in THP-1 cells indicating that Rv2895c is not necessary to overcome iron deficiency in macrophages. Could IrtA-NTD and Rv2895c have a redundant function? This is unlikely considering that even a single amino amino-acid substitution in the FAD binding domain of IrtA-NTD practically abolishes growth in low iron despite the presence of Rv2895c.[5] Rv2895c may not be able to function as IrtA-NTD because of its localization, inability to bind FAD or other reasons. To examine the ability of Rv2895c to bind FAD, the protein was over expressed and purified in *E. coli*. The expressed protein was mostly insoluble. The protein was purified from inclusion bodies under denaturing conditions and then solubilised by step-wise dialysis and removal of the denaturant. FAD was included in the final step of dialysis and during an additional overnight incubation at a protein:FAD ratio of 1:3. Upon removal of unbound FAD the UV/visible absorbance spectrum of the solubilised protein indicated no binding of FAD (data not shown). It is possible that the protein did not refold properly into the native conformation or alternatively it does not bind FAD. Since one of the critical FAD binding residues Tyr 72 in IrtA-NTD, which is conserved in ViuB[5], has been replaced by a Met in Rv2895c, it is possible that this change resulted in loss of FAD binding activity.

Taken together, the results of this study indicate that despite the similarity to ViuB and IrtA-NTD, under normal conditions, Rv2895c is dispensable for iron acquisition in *Mtb*. Regarding the biochemical interaction observed in a previous study between Rv2895c and IrtB[9] it is not unusual that physical interactions observed with cell lysates do not actually represent functional interactions in vivo.

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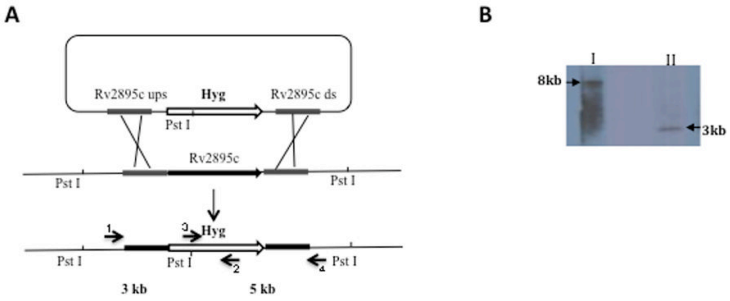

Figure 1.

Strategy for generation of a knock out mutant of Rv2895c in H37Rv. The Rv2895c gene was replaced with a hygromycin (Hyg) resistance cassette by phage transduction and homologous recombination. A) Scheme of the genome region containing Rv2895c and the substrate used for homologous recombination. Position of the *PstI* restriction sites used for chromosomal DNA digestion and the primers used for PCR amplification. B) Southern blot to confirm the ST212 mutant. The genomic DNA of H37Rv and ST212 was completely digested with *PstI* enzyme and the blot was probed with a fragment containing the 500 bp upstream Rv2895c. The *PstI* restriction sites in the vicinity of Rv2895c in wild type H37Rv are 8 kb apart (**I**) whereas restriction of ST212 DNA generates a 3 kb band (**II**), since the Hyg cassette has an internal *PstI* site. Gene replacement was also confirmed by PCR and sequencing. Primers 1 and 4 were designed in the region upstream and downstream of Rv2895c. Primers 2 and 3 are internal primers of the Hyg cassette. PCR was done using primers 1 and 2 and primers 3 and 4 with DNA from both wild type and mutant. No product was obtained using the wild type DNA whereas products of 2kb and 1.4kb were obtained using DNA isolated from the mutant as template. Sequencing of the PCR products confirmed the homologous recombination and gene replacement.

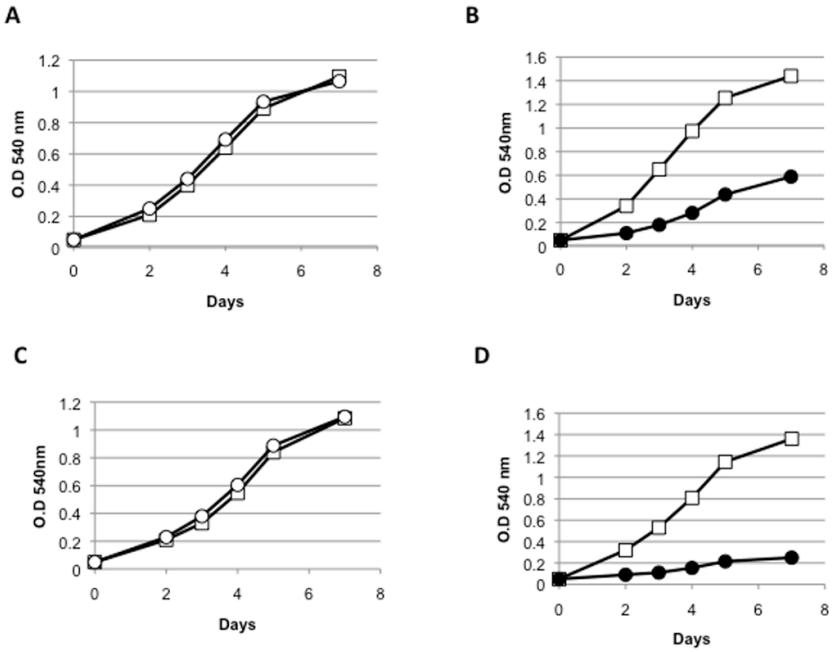

Figure 2.

Comparison of the growth of *M. tuberculosis* strains ST212 (○) and ST73 (●) with wild type H37Rv (□). (A and B). **Growth in iron deficient medium (MM) (0.5 % w/v asparagine, 0.5% w/v KH_2P0_4, 2% glycerol, 0.05% Tween-80 and 10% albumin-dextrose-NaCl complex (ADN), 0.5 mg $ZnCl_2$/L, 0.1 mg/L $MnS0_4$, and 40 mg/L $MgS0_4$). Strains were passed two times in MM to generate iron starvation and then the growth was monitored by measuring change in optical density. Strains were grown at 37° C with agitation.** (C and D) Growth in iron-deficient medium containing 75μM DPI. One representative experiment is shown. The experiments were repeated three times.

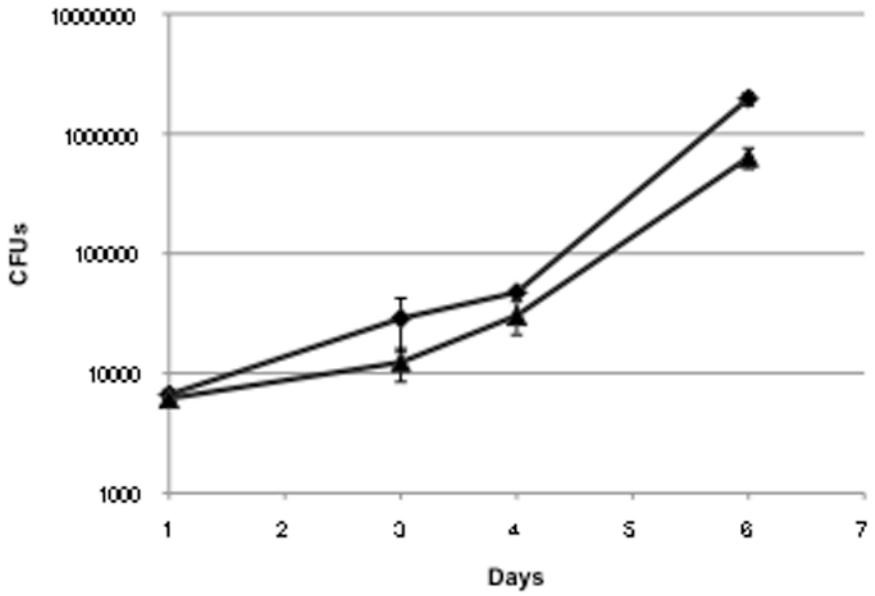

Figure 3.
Replication of *M. tuberculosis* strains in THP-1 cells. THP-1 cells were induced to differentiate into macrophages[5] and infected with H37Rv and ST212. The cells were lysed to release mycobacteria on select days and were plated onto 7H10 to determine CFU. The reported values represent the averages and standard deviations of three parallel independent infections. Strains: H37Rv (◆); ST212 (▲).