Identification of Rv3852 as a nucleoid-associated protein in *Mycobacterium tuberculosis*

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Tuberculosis remains the major cause of mortality due to a bacterial pathogen, *Mycobacterium tuberculosis*. The molecular mechanisms of infection and persistence have not been completely elucidated for this pathogen. Studies involving nucleoid-associated proteins (NAPs), which have been related to the control and influence of virulence genes in pathogenic bacteria, can help unveil the virulence process of *M. tuberculosis*. Here, we describe the initial characterization of an ORF for an *M. tuberculosis* putative NAP. The Rv3852 gene was cloned and expressed, and its product purified to homogeneity. A qualitative protein–DNA binding assay was carried out by gel-retardation and the protein affinity for specific DNA sequences was assessed quantitatively by surface plasmon resonance (SPR). A stoichiometry of 10 molecules of monomeric protein per molecule of DNA was determined. The monophasic apparent dissociation rate constant values increased to a saturable level as a function of protein concentration, yielding two limiting values for the molecular recognition of proU2 DNA. A protein–DNA binding mechanism is proposed. In addition, functional complementation studies with an *Escherichia coli* *hns* mutant reinforce the likelihood that the Rv3852 protein represents a novel NAP in *M. tuberculosis*.

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INTRODUCTION

Tuberculosis (TB) is one of the major causes of death worldwide caused by a single infectious agent, *Mycobacterium tuberculosis*. TB resurgence in the late 1980s was caused by a combination of several factors, such as HIV co-infection, increased poverty in urban areas and emergence of *M. tuberculosis* multidrug-resistant strains (MDR-TB) (Raviglione, 2003). According to the 2008 Global TB Control Report of the World Health Organization (WHO, 2008), there were approximately 9.2 million new TB cases in 2006, of which 0.5 million were MDR-TB. Moreover, the emergence of extensively drug-resistant (XDR) TB cases (CDC, 2007), defined as cases in persons with TB whose isolates are MDR-TB as well as resistant to any one of the fluoroquinolone drugs and to at least one of the three injectable second-line drugs, amikacin, kanamycin or capreomycin, and their global

distribution (Dorman & Chaisson, 2007), raise the prospect of virtually incurable TB worldwide. To compound the problem, it has been estimated that of 9.27 million incident TB cases in 2007, 1.37 million (15%) were HIV-positive (WHO, 2009).

M. tuberculosis has been considered the world's most successful pathogen. It is able to resist macrophage killing and persist in body tissues, thereby establishing a latent infection which can be reactivated when the host immune system wanes (Gomez & McKinney, 2004; Hingley-Wilson *et al.*, 2003). The mechanism by which *M. tuberculosis* establishes latency and persistence is largely unknown and efforts have been made to address this and other issues, such as virulence (Andersen, 2007; Gandotra *et al.*, 2007; Saunders & Britton, 2007; Schnappinger *et al.*, 2006).

Nucleoid-associated proteins (NAPs), also known as histone-like proteins, are a diverse group of small, usually basic, DNA-binding proteins that contribute to the organization of bacterial nucleoids and the control of many key cellular processes, such as recombination,

Abbreviations: EMSA, electrophoretic mobility shift assay; NAP, nucleoid-associated protein; SPR, surface plasmon resonance; TB, tuberculosis.

replication and transcription (Dorman & Deighan, 2003; Dame, 2005; Luijsterburg *et al.*, 2006). The best studied NAPs are Fis (factor for inversion stimulation), H-NS (histone-like nucleoid structuring), HU (heat unstable) and IHF (integration host factor), although the existence of other similar proteins is becoming increasingly apparent (Luijsterburg *et al.*, 2008). Their structural and functional properties have been related to chromatin compaction (by bending, bridging and wrapping DNA) and global transcriptional regulation of several genes, including virulence genes of bacterial pathogens (Beloin & Dorman, 2003; Berlutti *et al.*, 1998; Falconi *et al.*, 2001; Franzon & Santos, 2004; Harrison *et al.*, 1994; Mangan *et al.*, 2006; Nye *et al.*, 2000; Schechter *et al.*, 2003; Stonehouse *et al.*, 2008; Wilson *et al.*, 2001). Most of these proteins have been reported to bind to non-specific DNA sequences (Bailly *et al.*, 1995; Krylov *et al.*, 2001; Yamada *et al.*, 1991), to exhibit low sequence or structural conservation (Luijsterburg *et al.*, 2008), and to induce DNA bending and other alterations (Dhavan *et al.*, 2002; Koh *et al.*, 2008; Schneider *et al.*, 2001; Spurio *et al.*, 1997; Swinger & Rice, 2004). Curved DNA is an integral element of promoter architecture and DNA bending is one of the major components of the control of bacterial gene expression (Pérez-Martín *et al.*, 1994).

The probable involvement of NAPs in both mycobacterial survival and adaptation to adverse host conditions should provide new insights to elucidate the molecular and cellular mechanisms by which *M. tuberculosis* establishes infection and persistence. In recent years, two NAPs have been identified in mycobacteria: Hlp (histone-like protein) or MDP1 (mycobacterial DNA-binding protein 1), which has been characterized in *M. tuberculosis* (Prabhakar *et al.*, 1998), *Mycobacterium smegmatis* (Lee *et al.*, 1998; Mukherjee *et al.*, 2008; Shires & Steyn, 2001) and *Mycobacterium bovis* (Lewin *et al.*, 2008); and Lsr2, which has been characterized in *M. tuberculosis* and *M. smegmatis* (Chen *et al.*, 2006, 2008; Colangeli *et al.*, 2007). Lsr2 has multiple roles in antibiotic resistance, cell wall biosynthesis and DNA protection against reactive oxygen intermediates, and these have been examined in detail in *M. tuberculosis*, providing evidence that this mycobacterial NAP is functionally equivalent to H-NS from Gram-negative bacteria, despite the lack of significant sequence homology (Colangeli *et al.*, 2009; Gordon *et al.*, 2008).

Another possible histone-like protein (Rv3852) has been proposed to be encoded in the genome of the *M. tuberculosis* H37Rv strain (Cole *et al.*, 1998). The Rv3852 gene sequence consists of 405 nt that encode a small 134 aa protein with a predicted molecular mass of 13.8 kDa and a positive net charge. It is conserved among other mycobacterial species, showing some similarity at the N-terminal region with eukaryotic histones, e.g. histone H1 from *Trypanosoma cruzi* (Cole *et al.*, 1998, 2001; Stinear *et al.*, 2008). Together, these data suggest that *M. tuberculosis* Rv3852 and its homologues are members of a family of DNA-binding proteins. H-NS is a small chromatin-

associated protein which is involved in many different cellular processes, most of them related to environmental changes and adaptation. H-NS modulates these processes through pleiotropic regulation of transcription, recombination and transposition (Atlung & Ingmer, 1997; Dorman, 2004; Hommais *et al.*, 2001; Rimsky *et al.*, 2001; Ward *et al.*, 2007). DNA binding by H-NS is independent of nucleotide sequence, despite its preference for curved sequences usually rich in AT nucleotides (Yamada *et al.*, 1991). It should, however, be pointed out that differential binding of H-NS to defined DNA sequence sites has recently been reported (Bouffartigues *et al.*, 2007). Considering the critical roles played by H-NS and other NAPs in adaptation to environmental changes, and in the growth and virulence mechanisms of pathogens, including facultative intracellular bacteria, attempts to establish/assign the biological function of the putative *M. tuberculosis* histone-like protein Rv3852 are worthwhile.

As a first step towards this goal, biochemical characterization of the putative histone-like protein Rv3852 from *M. tuberculosis* is required. We have thus amplified and cloned the corresponding *M. tuberculosis* ORF, proposed to be a possible histone-like protein, expressed the recombinant protein in *Escherichia coli* cells and purified it to homogeneity. We have also characterized the protein-DNA-binding interactions by a qualitative assay using a gel-retardation technique and verified the protein affinity for specific DNA sequences by surface plasmon resonance (SPR) using BIA-Core equipment. These studies have identified the product of the *M. tuberculosis* Rv3852 DNA sequence as a DNA-binding protein that slightly prefers binding to curved DNA. In addition, we performed complementation studies to clarify whether the *M. tuberculosis* Rv3852 protein acts in a similar or different pattern to the well-characterized *E. coli* H-NS. Our results demonstrate that Rv3852 is a DNA-binding protein with unique structural and functional properties that nevertheless resembles other NAPs. We propose that Rv3852 is a member of a novel class of histone-like proteins present in *M. tuberculosis* and related mycobacteria.

METHODS

Cloning and construction of an Rv3852c expression plasmid.

Synthetic oligonucleotide primers HNS *Nde*I (5'-AACATATGCCAGACCCGCGAGGATCGACCC-3') and HNS *Hind*III (5'-GTAAGCTTTCAGCGCGCGCGCAGTTGCC-3') were designed based on the coding sequence described for the Rv3852 (*hns*) locus from *M. tuberculosis* H37Rv (Cole *et al.*, 1998). *Nde*I and *Hind*III restriction sites are underlined in the respective primers. Primers were used to amplify the target gene (405 bp) from *M. tuberculosis* genomic DNA using 10% DMSO and *Pfu* DNA polymerase (Stratagene), and the PCR product was cloned into the pCR-Blunt vector (Invitrogen) and subcloned into the pET-23a(+) expression vector (Novagen). The nucleotide sequence of the cloned fragment was determined by automated DNA sequencing.

Expression of recombinant Rv3852 in *E. coli*. The recombinant pET-23a(+)::*hns* plasmid was transformed into electrocompetent *E.*

coli Rosetta (DE3) cells, and selected on Luria–Bertani (LB; Difco) agar plates containing 50 µg carbenicillin ml⁻¹. Control experiments were performed under the same experimental conditions, except that transformed *E. coli* cells harboured the expression vector lacking the target gene. A single recombinant colony was used to inoculate 50 ml LB medium containing 50 µg carbenicillin ml⁻¹. Cells were grown with stirring at 180 r.p.m. at 37 °C and, after reaching OD₆₀₀ 0.3–0.5, they were grown for additional 3, 6, 9, 12, 18, 21 and 24 h without addition of IPTG. Cells (1.5 ml) were harvested by centrifugation at 20 800 g for 5 min and stored at –20 °C. The stored cells were suspended in 10 mM KH₂PO₄ (pH 7.2) and disrupted by sonication using three 10 s pulses and cell debris was separated by centrifugation at 20 800 g for 30 min at 4 °C. The soluble protein content was analysed by 15 % SDS-PAGE with Coomassie brilliant blue staining.

Purification of recombinant protein. For protein purification under native conditions, 15 g cells was resuspended in 75 ml lysis buffer [10 mM KH₂PO₄, pH 7.2, 500 mM NaCl, Complete Protease Inhibitor Cocktail (Roche)] and sonicated (15 times for 20 s at an amplitude of 60 % in a Vibra-Cell ultrasonic processor). Cell debris was pelleted by centrifugation (51 900 g for 30 min). The supernatant containing soluble protein was incubated with 1 % (w/v) streptomycin sulfate for 30 min and centrifuged at 51 900 g for 30 min. The supernatant was dialysed six times against 2 l 10 mM KH₂PO₄, pH 7.2 (buffer A), using dialysis tubing with a molecular mass exclusion limit of 3500 Da. This sample was clarified by centrifugation (51 900 g for 30 min) and loaded on an SP-Sepharose Fast Flow column (GE Healthcare) pre-equilibrated with buffer A. The column was washed with six column volumes of buffer A and the adsorbed material was eluted with a linear gradient (0–100 %) of 20 column volumes 10 mM KH₂PO₄, pH 7.2, 0.6 M KCl (buffer B) at 1 ml min⁻¹. The fractions containing recombinant protein were pooled (75 ml) and concentrated to 8 ml using an Amicon ultrafiltration membrane (molecular weight cut-off 3000 Da). The sample was loaded on a Sephacryl S-100 column (GE Healthcare) pre-equilibrated with buffer A. The Rv3852 recombinant protein was eluted in a total volume of 155 ml at a flow rate of 0.25 ml min⁻¹ and loaded on a cation-exchange Mono-S column (GE Healthcare) pre-equilibrated with buffer A. The column was washed with six column volumes of the same buffer and the adsorbed material was eluted with 20 column volumes of a linear gradient (0–100 %) of buffer B at 1 ml min⁻¹. Elution profiles were followed at 280 and 215 nm. Homogeneous Rv3852 protein was eluted in a total volume of 7 ml and stored at –80 °C. The recombinant protein purity was assessed by 15 % SDS-PAGE with Coomassie brilliant blue staining, and protein concentration was measured spectrophotometrically at 215 nm as described by Scopes (1994).

N-terminal amino acid sequencing. The N-terminal amino acid residues of the homogeneous recombinant Rv3852 protein preparation were identified by automated Edman degradation sequencing using a PPSQ 21A gas-phase sequencer (Shimadzu).

Determination of native Rv3852 molecular mass. The molecular mass of native recombinant protein was estimated by gel-permeation chromatography on a Superdex 200 HR column (GE Healthcare). The column was eluted with 10 mM KH₂PO₄, pH 7.2, at a flow rate of 0.5 ml min⁻¹. The eluate was monitored at 215 and 280 nm, and the column was calibrated with the following protein standards (GE Healthcare): RNase A (13 700 Da), chymotrypsinogen (25 000 Da), ovalbumin (43 000 Da) and albumin (67 000 Da). Blue Dextran 2000 was used to determine the void volume (*V*₀). The *K*_{av} value was calculated for each protein using the equation (*V*_e – *V*₀)/(*V*_t – *V*₀), where *V*_e is the elution volume for the protein and *V*_t is the total bed volume, and *K*_{av} was plotted against the logarithm of standard molecular masses.

Electrophoretic mobility shift assay (EMSA). Four DNA sequences were constructed for DNA–protein binding assay. proU1 (–200/+10) and proU2 (–20/+189) are sequences of the *proU* promoter region from *M. tuberculosis* H37Rv (<http://genolist.pasteur.fr/TubercuList/index.html>). Primers were designed, synthesized and used for amplifying each fragment by PCR using *Pfu* DNA polymerase (Stratagene). BENT (curved) and NC (noncurved) sequences were synthesized by annealing oligodeoxynucleotides (Table 1) of ~100 bp (Shimizu *et al.*, 1995). All fragments were cloned into the *Eco*RI restriction site of the pCR-Blunt vector (Invitrogen). Plasmid DNAs were cleaved with *Eco*RI (Invitrogen) and the inserts were purified from agarose gels. An equal amount (300 ng) of the DNA fragments was mixed with purified recombinant protein (0–1 µg) in binding buffer (10 mM Tris/HCl, pH 7.5, 80 mM NaCl, 1 mM DTT, 1 mM EDTA, 5 %, v/v, glycerol) in a total volume of 40 µl. After 15 min incubation at room temperature, these mixtures were loaded on a 1.5 % agarose gel and run in Tris-borate-EDTA buffer for 5 h. The gel was stained with 0.05 µg ethidium bromide ml⁻¹ and visualized under UV light.

Kinetic analysis using a BIA-Core X system. Another 221 bp fragment of the proU2 sequence was amplified from the pCR-Blunt-proU2 recombinant plasmid using the forward primer labelled with biotin at the 5' terminal end. The labelled fragment was immobilized (0.15 µg ml⁻¹) onto the second channel of a streptavidin sensor chip (SA Chip; GE Healthcare) and the first channel was used for reference. Unconjugated streptavidin was removed by three consecutive injections of 1 M NaCl, 50 mM NaOH (10 µl; 5 µl min⁻¹), and then 60 µl proU2-biotin was passed at 5 µl min⁻¹ in HBS-EP buffer (BIA-Core; GE Healthcare) with 0.5 M NaCl. Several concentrations of protein (0, 0.05, 0.1, 0.5, 1, 2.5, 5 and 7.5 µM) were injected in HBS-EP buffer (80 µl; 50 µl min⁻¹) with a dissociation phase of 120 s. At the end of each interaction, the chip surface was regenerated by injection of 1 M NaCl (10 µl). The experiment was carried out in duplicate.

Competition assay. A new SA Chip was immobilized with the proU2-labelled fragment for the competition assay as described above. Protein concentration was fixed at 200 nM and this was

Table 1. DNA sequences for activity assays

DNA fragment	Sequence	Size (bp)	Molecular mass (Da)
proU1	–200 to +10 of <i>proU</i> promoter region*	221	136 604
proU2	–20 to +189 of <i>proU</i> promoter region*	221	136 615
BENT – (A ₆ N ₄) ₁₀	(TTTTTTGCCG) ₁₀	112	69 231
NC – (A ₆ N ₉) ₆	CCCCGGC(AAAAAACGGCCCCGGC) ₆	109	67 400

* +1 is defined as the point of transcription initiation.

followed by preincubation with a 1-, 5-, 10-, 20-, 30- or 45-fold excess of non-biotinylated competitor DNAs (proU1, proU2, BENT, NC) in HBS-EP buffer for 15 min at room temperature prior to injection to the proU2-immobilized surface. Preincubated protein–DNA mixtures were injected in HBS-EP buffer (50 µl; 50 µl min⁻¹) with a dissociation phase of 120 s, and carried out in duplicate.

Complementation studies. A complementation plasmid based on the *M. tuberculosis* Rv3852 gene was constructed by PCR amplification of the target sequence with primers HNS *Eco*RI (5'-CCGGAATTCATGCCAGACCCGAGGATC-3') and HNS *Hind*III (5'-GTAAGCTTTCAGCGCGCGCAGTTGCC-3'), which were designed to contain, respectively, *Eco*RI and *Hind*III restriction sites (underlined). The previously generated pET-23a(+):*hns* expression vector was used as a template, and 10 % DMSO and native *Pfu* DNA polymerase (Stratagene) were employed under standard PCR conditions (cycling parameters were: an initial denaturation step at 96 °C for 5 min, followed by 30 cycles at 96 °C for 45 s, 55 °C for 45 s and 72 °C for 1 min, followed by a final extension step at 72 °C for 10 min). The 405 bp PCR product was gel-purified, cloned into the pCR-Blunt vector (Invitrogen) and subcloned into the pKK223-3 expression vector (Pharmacia) under the control of the strong *tac* promoter, resulting in pKH2 (pKK223-3::Rv3852_{Mtb}).

For the complementation assay, *E. coli* BSN26 (relevant genotype MC4100 *trp*::Tn10) and BSN27 (relevant genotype MC4100 *trp*::Tn10 Δ *hns*) strains (Johansson *et al.*, 1998) were grown in LB medium or M63 minimal medium (MM) supplemented with 0.2 % glucose as a carbon source (Miller, 1992) and 40 µg L-serine ml⁻¹. Both strains were used as experimental controls. Plasmids pKK223-3 and pKH2 were separately electroporated into competent BSN27 cells, and transformants were isolated on LB agar plates containing 50 µg ampicillin ml⁻¹. Strains BSN26, BSN27, BSN27 (pKK223-3) and BSN27 (pKH2) were grown at 37 °C in 50 ml MM supplemented with serine (and 50 µg ampicillin ml⁻¹, where appropriate) in 250 ml flasks. Growth was monitored for serine sensitivity (from starting cultures that had reached OD₆₀₀ 0.01) for up to 8 h. Three independent experiments were conducted for each strain under the above-mentioned conditions.

Data analysis. The dissociation constant for Rv3852 protein binding to immobilized proU2 DNA sequence was obtained by fitting the data to equation 1:

$$R_{eqi} = \frac{R_{max}[Rv3852]}{K_D + [Rv3852]} \quad (1)$$

where R_{eqi} is the equilibrium (maximum) resonance response unit for *i* concentration of Rv3852 protein (*i*=0, 0.05, 0.1, 0.5, 1, 2.5, 5 or 7.5 µM), R_{max} is the maximum resonance response unit at saturating protein concentrations, and K_D is the dissociation constant at equilibrium.

The stoichiometry for Rv3852 protein–proU2 complex formation was determined from fitting the data to equation 2 (Majka & Speck, 2007):

$$n = R_{max} \frac{MW_{DNA}}{R_{DNA} MW_{protein}} \quad (2)$$

where *n* is the number of protein molecules bound to DNA or the number of protein binding sites on DNA (assuming that a single protein molecule binds a single binding site), R_{max} is the resonance response for a saturating concentration of protein, R_{DNA} is the amount of immobilized DNA (RU), MW_{DNA} is the molecular mass of DNA (proU2=136 615 Da), and $MW_{protein}$ is the molecular mass of the Rv3852 protein from *M. tuberculosis* (13 822 Da).

Values for the monophasic apparent dissociation rate constants (single exponentials) were obtained by fitting the data to equation 3:

$$R(t) = R_0 e^{-k_{off} t} \quad (3)$$

where $R(t)$ is the resonance response at time *t*, R_0 is the resonance response at the analysis start point (which is not necessarily at the beginning of the dissociation phase), and k_{off} is the apparent dissociation rate constant.

The competition assay analysis was carried out as described by Teh *et al.* (2007). Briefly, the level of protein binding to immobilized proU2 was set at 100 %. The competition at a certain concentration of non-biotinylated competitor DNA was calculated in relation to the percentage binding value (% binding) as follows (equation 4):

$$\% \text{ binding} = \left[\frac{R_i}{R_0} \right] 100 \quad (4)$$

where R_i is the signal obtained at a certain concentration of competitor, and R_0 is the signal obtained in the absence of competitor DNA.

RESULTS AND DISCUSSION

Cloning, expression and purification of Rv3852

PCR amplification of the 405 bp corresponding to the Rv3852 coding sequence required the presence of 10 % DMSO, a co-solvent that improves denaturation of GC-rich DNA sequences (Pomp & Medrano, 1991), which is consistent with the high GC content of the *M. tuberculosis* H37Rv genome (Cole *et al.*, 1998). The amplified product was cloned into the pCR-Blunt cloning vector and subcloned into the pET-23a(+) expression vector between *Nde*I and *Hind*III restriction sites. Rv3852 recombinant protein was heterologously expressed in *E. coli* Rosetta (DE3) cells in the soluble fraction with no IPTG induction (data not shown). This unusual feature of the pET system has been verified in stationary phase for cells growing in the absence of appropriate inducer (Magalhães *et al.*, 2002; Mendonça *et al.*, 2007; Rizzi *et al.*, 2005; Silva *et al.*, 2003). It has been proposed that leaky protein expression in the pET system is part of a general cellular response to nutrition limitation when cells approach stationary phase, and that cyclic AMP, acetate and low pH are involved in derepression of the *lac* operon (Grossman *et al.*, 1998). However, it has more recently been shown that unintended induction of the pET system is due to the presence of as little as 0.0001 % lactose in the medium (Studier, 2005).

Rv3852 recombinant protein was purified to homogeneity by liquid chromatography. The purification protocol included 1 % streptomycin precipitation to reduce contaminant DNA. To obtain electrophoretically homogeneous protein, three chromatographic steps were required: SP-Sepharose Fast Flow, Sephacryl S-100 and Mono-S HR. The use of a cation-exchange column was based on the theoretical isoelectric point calculated for Rv3852, which is 11.35. The purification protocol for *M. tuberculosis* Rv3852 protein yielded ~3 mg homogeneous protein with an

apparent molecular mass of ~14 kDa from 15 g wet weight of cells (Fig. 1). The relatively low yield was probably due to the small amount of recombinant protein expressed in soluble form. Several *E. coli* strains were tested (data not shown) and the best result was obtained with *E. coli* Rosetta (DE3) cells. Even though we have no experimental evidence, it is tempting to propose that the pET vector suffers transcriptional repression by the expressed recombinant protein, since an inhibitory effect of H-NS-related proteins on the *lacUV5* promoter has been documented (Goransson *et al.*, 1990), as well as a likely deleterious effect on cell growth (Spurio *et al.*, 1997).

Protein characterization and amino acid sequence analysis of *M. tuberculosis* Rv3852

N-terminal sequencing of the homogeneous protein preparation unambiguously confirmed the identity of the first 23 aa and removal of the N-terminal methionine residue. The *M. tuberculosis* Rv3852 N-terminal region exhibits significant homology to eukaryotic H1 linker histones (Kasinsky *et al.*, 2001) due to the presence of proline, alanine and lysine residues clustered in tetrapeptide repeats (PAKK, KAAK). As seen in the multiple sequence alignment (Fig. 2), four PAKK sequences occur in both *M. tuberculosis* and *Mycobacterium bovis* (the causative agent of bovine TB), whereas the *Mycobacterium marinum* (a pathogen of fish and amphibia) and *Mycobacterium ulcerans* (the causative agent of Buruli ulcer, a severe skin disease) homologues possess seven PAKK sequences in their proteins. These repetitive amino acid sequences are known to bind DNA with high affinity, and it is interesting to note that a very similar protein

organization has been described for the Hlp protein from *M. tuberculosis*, which has been characterized as a histone-like protein (Prabhakar *et al.*, 1998). However, the typical tetrapeptide repeats occur at the C-terminal domain of *M. tuberculosis* Hlp, with the N-terminal domain showing considerable sequence homology to HU proteins. *M. smegmatis* Hlp is organized according to the same pattern (Shires & Steyn, 2001). It has also been demonstrated that *M. smegmatis* Hlp lacking the entire C-terminal domain binds poorly to DNA and that the C-terminal repeats are responsible for DNA end-joining (Mukherjee *et al.*, 2008). Therefore, these important similarities point to a probable involvement of the *M. tuberculosis* Rv3852 protein in DNA binding and a structural and/or regulatory role in the mycobacterial chromosome.

Rv3852 from *M. tuberculosis* exhibits 30 alanine, 8 arginine, 16 lysine and 19 proline residues in its amino acid sequence (Cole *et al.*, 1998), with a calculated isoelectric point of 11.34. As recently described for another *M. tuberculosis* NAP, Lsr2, Rv3852 could make use of this high proportion of positively charged arginine and lysine residues to form salt bridges with the negative phosphate backbone of DNA (Chen *et al.*, 2008). Moreover, it is tempting to hypothesize that the lysine residues present in Rv3852 serve as acetylation sites, leading to potential mechanisms of differential gene regulation, since lysine acetylation has been considered an important, though underestimated, regulatory post-translational modification in bacteria (Zhang *et al.*, 2009).

To evaluate the native oligomeric state of Rv3852, we performed gel filtration on a calibrated Superdex 200 HR column. Elution of a single peak corresponding to a protein of $\sim 25\,200 \pm 2520$ Da indicated that the *M. tuberculosis* Rv3852 protein is a dimer in solution (subunit molecular mass is 13 822 Da), resembling other histone-like proteins (Dorman & Deighan, 2003).

DNA sequences selected for Rv3852 binding assays

Four DNA sequences were constructed to determine the DNA-binding activity of homogeneous Rv3852 protein (Table 1). We performed activity assays using two sequences of the *proU* operon from *M. tuberculosis* H37Rv. This operon, which encodes a transport system for the osmoprotectant glycine betaine and is induced by high osmolarity (Higgins *et al.*, 1987), is one of the most studied systems whose transcription is affected by H-NS in other pathogenic micro-organisms. Several reports have demonstrated that a sequence element (DRE), located downstream of the *proU* promoter (usually between +24 and +202), is preferred for binding by H-NS (Lucht *et al.*, 1994; Owen-Hughes *et al.*, 1992; Tupper *et al.*, 1994). Based upon these studies, we constructed two sequences, *proU1* and *proU2*, that correspond to -200 to +10 and -20 to +189 of the *proU* promoter from *M. tuberculosis*, respectively, in order to confirm binding activity to these

Fig. 1. SDS-PAGE analysis of pooled fractions from the recombinant protein purification protocol. Lanes: 1, BenchMark protein ladder (Invitrogen); 2, crude extract, soluble fraction; 3, crude extract after streptomycin sulfate precipitation and centrifugation; 4, after purification by SP Sepharose FF column; 5, after purification by Sephacryl S-100 column; 6, after purification by Mono S HR column. Homogeneous Rv3852 protein was visualized at 14 kDa (arrow). The asterisk marks the Rv3852 protein in soluble form in the crude extract.

Fig. 2. Multiple sequence alignment of *M. tuberculosis* Rv3852 with its mycobacterial homologues, showing the PAKK repeats in the N-terminal region. *M. tuberculosis* (Mtu) histone-like protein Rv3852 was aligned with other potential mycobacterial homologues from *M. bovis* (Mbo), *M. marinum* (Mma), *M. ulcerans* (Mul) and *Mycobacterium leprae* (Mle) using the program CLUSTAL W (Thompson *et al.*, 1994). Tetrapeptide PAKK repeats (which are present in eukaryotic H1 linker histones and are known to bind DNA) are highlighted in white type on a black background. Identical conserved residues are indicated by asterisks below the alignment. Strongly similar and weakly similar residues are identified by colons and full stops, respectively.

regions by Rv3852 recombinant protein. The latter sequence is located at the beginning of the *proX* structural gene, the first gene of the *proU* operon (*proXVWZ*). Two other sequences were employed in DNA-binding assays: BENT, which is a curved sequence containing six contiguous A–T residues in phase (10 bp period), and NC, a noncurved sequence in which the adenine tracts are out of phase (15 bp period) (Koo *et al.*, 1986; Shimizu *et al.*, 1995). All sequences were cloned, excised from the agarose gel and purified prior to activity assays.

Activity of Rv3852 homogeneous protein by EMSAs

EMSA measurements at varying protein amounts (0–1 µg) showed that the Rv3852 protein had a similar relative affinity for all the sequences tested (Fig. 3). In spite of the preference of Rv3852 for curved DNA, some histone-like proteins can bind and bend noncurved DNA (Dame & Goosen, 2002; Dorman & Deighan, 2003; Schneider *et al.*, 2001; Spurio *et al.*, 1997; Swinger & Rice, 2004). We also verified that the protein is able to form multiple protein–DNA complexes, and that this process is concentration-dependent, suggesting the formation of higher forms of the

dimeric protein when bound to DNA. To confirm that DNA binding was a particular property of the recombinant *M. tuberculosis* Rv3852 protein, two other *M. tuberculosis* homogeneous proteins were tested, InhA (Oliveira *et al.*, 2006) and PNP (Silva *et al.*, 2003). They showed no DNA-binding interaction under the same experimental conditions described for the Rv3852 protein assay (data not shown), thereby showing that this protein is indeed a DNA-binding protein.

Activity assay of Rv3852 homogeneous protein by SPR

Rv3852 protein affinity for proU1, proU2, BENT and NC DNA sequences was assessed by SPR using BIA-Core equipment. We immobilized 80 RU (resonance response units) of the proU2 DNA sequence onto an SA Sensor Chip (GE Healthcare). A low RU value (80) and high flow rate (50 µl min^{−1}) were employed to reduce mass transport effects that can limit the assay (Majka & Speck, 2007). Sensorgrams for varying protein concentrations (0, 0.05, 0.1, 0.5, 1, 2.5, 5 and 7.5 µM) in HBS-EP buffer were obtained in duplicate (Fig. 4, inset shows a typical sensorgram). Average values for equilibrium resonance

Fig. 3. Activity assay by EMSA. (a) Rv3852 protein–proU1 binding assay, (b) Rv3852 protein–proU2 binding assay, (c) Rv3852 protein–BENT binding assay, (d) Rv3852 protein–NC binding assay. All DNA sequences were tested with the following protein amounts: 0, 0.1, 0.25, 0.5 and 1 µg.

responses were fitted to Equation 1, yielding a dissociation constant (K_D) value of 289 (± 84) nM and a saturation response ($R_{\max}$) value of 80.43 (± 4.67) RU for Rv3852 protein–proU2 complex formation (Fig. 4). The DNA–protein complex stoichiometry could also be estimated as ~ 10 molecules of protein monomers per molecule of DNA by fitting the data to Equation 2 (Majka & Speck, 2007).

The binding process was characterized by a fast association phase that did not allow reliable estimates to be obtained for the association rate constants. However, the dissociation phase was assessed using a delay of 120 s after the end of sample injection. The monophasic apparent dissociation rate constant (Fig. 5, inset) values were obtained by fitting data to Equation 3. These values were plotted against their respective protein concentrations and, fitting the data to a rectangular hyperbola that intersects at a finite value on the y axis, two limiting values could be determined: 0.0189 (± 0.0010) s^{-1} at very low protein concentrations (approaching zero), and 0.0415 (± 0.0020) s^{-1} at saturat-

ing protein concentrations (Fig. 5). It is thus tempting to propose that the value of 0.0189 s^{-1} is for the dissociation rate constant of the dimeric protein and the value of 0.0415 s^{-1} is for the dissociation rate constant of the oligomeric protein. We propose that the dimers of the recombinant Rv3852 protein are able to self-associate in higher oligomers along the DNA molecules and that this property is concentration-dependent, as we could verify by EMSA. We thus propose that higher oligomerization of the Rv3852 protein results in faster dissociation rate constant values.

Competition assay

To investigate the relative affinity for the DNA sequences evaluated in our studies, we immobilized a new SA Sensor Chip and carried out a competition assay. A value of 110 RU was obtained for the 221 bp proU2 DNA. Considering that 0.78 ng of a DNA molecule bound at

Fig. 4. Saturation curve of the Rv3852 protein–proU2 complex. Mean RU values were plotted against the corresponding protein concentrations and the analysis was performed using the SigmaPlot program. Fitting the data to a rectangular hyperbola (Equation 1) yielded values of $289 (\pm 84)$ nM for the equilibrium dissociation constant (K_D) and of $80.43 (\pm 4.67)$ RU for the maximum resonance at saturating protein concentrations (R_{max}). The inset shows an illustrative sensorgram obtained with 1 μ M protein.

the surface gives a response of 1000 RU (Bouffartigues *et al.*, 2007), we can expect ~ 0.086 ng or 6.3×10^{-16} mol of proU2 DNA ($136\,615 \text{ g mol}^{-1}$) distributed within the dextran layer. Protein concentration was fixed at 200 nM and was incubated with a 1-, 5-, 10-, 20-, 30- or 45-fold excess of non-biotinylated competitor DNAs (proU1, proU2, BENT and NC). This competition assay approach allows assessment of the relative affinity of a large number of DNA sequences with one surface preparation (Teh *et al.*, 2007).

Among the tested DNA sequences, fitting the data to Equation 4, proU1 showed the most pronounced reduction in Rv3852 protein binding to immobilized proU2, indicating that this sequence has the strongest affinity for the recombinant protein as compared with the other sequences tested (Fig. 6). We found the following affinity order: proU1 > proU2 > BENT > NC. The competition assay showed that proU1 has a slightly higher affinity than proU2 for recombinant *M. tuberculosis* Rv3852 protein. It is likely that this observed preference for proU1 is because it corresponds to the promoter region of the *proU* operon, in contrast to proU2, which corresponds to a sequence of the first gene of the operon. The preference of the protein for these two sequences as compared with BENT and NC can be explained by additional structural characteristics,

besides DNA curvature, that may favour DNA–protein interactions. This experiment also demonstrated that the recombinant protein shows a slight preference for the curved sequence rather than the noncurved. This can be explained by the fact that the DNA-binding protein expends less energy to bind a curved sequence, since to exert its activity it first needs to bend a noncurved sequence. *E. coli* H-NS protein binds with relatively high affinity to DNA of any sequence (Dorman, 2007), although, as mentioned above, a well-defined preferential DNA-binding site has been reported (Bouffartigues *et al.*, 2007). The competition results presented here are in agreement with this.

Lack of functional complementation of an *E. coli* *hns* mutant by *M. tuberculosis* Rv3852

Since we initially did not know whether Rv3852 would represent an authentic H-NS-like homologue or another type of NAP, we performed a complementation assay to test the ability of the *M. tuberculosis* protein to restore an *hns* mutant phenotype. *In vivo* complementation of serine susceptibility of *E. coli* *hns* mutants has been commonly employed to identify H-NS-like proteins in a number of bacteria (Rodríguez *et al.*, 2005; Tendeng & Bertin, 2003).

Fig. 5. Rv3852 protein dissociation rate constants for the proU2-immobilized DNA. The data were obtained by SPR from a delay of 120 s after the end of sample injection. Each apparent dissociation rate constant was plotted against its corresponding protein concentration. The data were fitted to a rectangular hyperbola that intersects at a finite value on the y axis by SigmaPlot, from which we can determine two limiting values for the dissociation rate constants: 0.0189 s^{-1} for dimeric protein at concentrations approaching zero, and 0.0415 s^{-1} for oligomeric protein at saturating concentrations. Inset: traces showing the monophasic apparent dissociation rate constants for varying protein concentrations ($0.05\text{--}7.5 \text{ }\mu\text{M}$).

Accordingly, a pKK223-3-based complementation plasmid containing the Rv3852-coding sequence (pKH2) cloned under control of the strong *tac* promoter was constructed. Plasmids pKK223-3 (control) and pKH2 were separately

electroporated into BSN27 (Δhns) cells. *E. coli* BSN26 (wild-type) and BSN27 not transformed with any plasmid were included as controls. As expected, BSN26 cells grew at a higher rate than the BSN27 mutant (which exhibits a

Fig. 6. Competition profile of the Rv3852 protein binding to the proU2-immobilized DNA in the presence of competitor DNAs. The protein binding signal in the absence of competitor DNAs was set as 100%. Protein binding was tested at a 1-, 5-, 10-, 20-, 30- or 45-fold excess of competitor DNA.

severe growth defect), but no difference between the latter and BSN27 (pKK223-3) or BSN27 (pKH2) transformants could be observed under the conditions tested (data not shown), demonstrating that *M. tuberculosis* Rv3852 does not correspond to a 'classical' H-NS-like protein, and thus reinforcing the probability that it represents a novel NAP of unknown function.

Expression of Rv3852 has been shown to be altered under some stress conditions. In a nutrient starvation model in *M. tuberculosis*, Rv3852 mRNA levels have been shown to be upregulated after 4 h of starvation (Betts *et al.*, 2002). In another study, in which the heat-shock response of *M. tuberculosis* was analysed using an *hspR* (which encodes the transcriptional repressor HspR) deletion mutant, Rv3852 expression was also upregulated compared with the wild-type strain (Stewart *et al.*, 2002). These data suggest that, as exemplified by other NAPs, transcription of Rv3852 is most probably regulated by environmental stresses, such as nutrient availability, heat- and cold-shock responses, and oxidative damage.

Conclusion

In this work, we present the initial characterization of the putative ORF for an NAP (Rv3852) from *M. tuberculosis*. Its amino acid sequence in the N-terminal region shows the presence of tetrapeptide repeats that are found in H1 histones, and this feature, which is similar to that of another histone-like protein from *M. tuberculosis*, MDP1, suggests that the protein encoded by the ORF Rv3852 is involved in the structuring of the mycobacterial DNA and similar functions, contributing to the molecular machinery of the TB bacillus. We have shown that the Rv3852 recombinant protein binds to the *proU* promoter region, an important environmentally controlled system that has been shown to be regulated by H-NS in other pathogenic bacteria. The dimeric structure of the protein is suggested to self-associate to form higher oligomers, as indicated by the multiple protein-DNA complexes observed by EMSA. A stoichiometry of 10 molecules monomeric protein per molecule of DNA was determined by SPR. Analysis by SPR of dissociation rate constant values as a function of Rv3852 concentration suggests that the dimeric protein dissociates from the *proU* DNA sequence more slowly than the oligomeric protein. Complementation studies confirmed that the ORF annotated as a possible H-NS protein from *M. tuberculosis* does not correspond to this protein. Recent reports have demonstrated that the Lrs2 protein has H-NS activity in *M. tuberculosis*, based on its ability to complement several phenotypes from an *E. coli* *hns* mutant (Gordon *et al.*, 2008). We therefore propose that the recombinant protein characterized here has regulatory activity(ies) related to adaptation or even virulence genes, as have several other described NAPs. Notwithstanding these findings, these issues will need to be clarified in future research.

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