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Rv3852, Uma Nova Proteína *Histone-Like* de *Mycobacterium tuberculosis*

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RESUMO

A tuberculose permanece sendo a principal causa de morte no mundo devido a um único agente infeccioso, *Mycobacterium tuberculosis*. O surgimento de cepas multi- e extensivamente-resistentes tem aumentado a perspectiva de uma futura epidemia de casos de tuberculose sem tratamento. Faz-se cada vez mais urgente a necessidade do desenvolvimento de novas drogas e vacinas, tanto para encurtar o prazo de tratamento, como para combater as cepas resistentes e o processo de latência que é estabelecido pelo bacilo. Os mecanismos moleculares pelos quais esta micobactéria estabelece infecção e latência ainda precisam ser esclarecidos. O estudo de proteínas associadas ao nucleóide tem sido um tema bastante promissor para o entendimento de mecanismos de invasão e persistência em vários microorganismos patogênicos, podendo auxiliar, também, no esclarecimento do metabolismo do bacilo para estas atividades. Neste estudo, descrevemos a caracterização inicial de uma fase de leitura identificada para uma proteína H-NS putativa de *M. tuberculosis*. A H-NS é uma das proteínas associadas ao nucleóide mais bem caracterizadas. O gene foi clonado, expresso, e seu produto foi, então, purificado até sua homogeneidade. Ensaios de ligação a DNA qualitativo, utilizando o EMSA, e quantitativo, por meio de ressonância plasmônica de superfície, foram realizados para a comprovação de sua atividade, tendo sido proposto um mecanismo de ligação ao DNA. Além disso, estudos de complementação realizados com a utilização de uma cepa de *Escherichia coli* mutante para *hns* sugerem que esta proteína pertence a uma nova classe de proteínas associadas ao nucleóide presentes em *Mycobacterium*.

ABSTRACT

Tuberculosis remains the major cause of mortality due to a bacterial pathogen, *Mycobacterium tuberculosis*. The emergence of multidrug- and extensively drug-resistant strains have raised the bleak prospect of a future epidemic of virtually untreatable TB. More effective antimycobacterial agents, besides new vaccines or vaccination strategies, are thus needed to improve the treatment of resistant strains, to shorten the treatment course, and to provide more effective treatment of latent infection. The molecular mechanisms by which the bacillus establishes infection and persistence have not been completely elucidated. Studies involving nucleoid-associated proteins, which have been related to the control and influence of virulence genes in pathogenic bacteria, can help unveil the virulence process of *M. tuberculosis*. In this study, we describe the initial characterization of an open reading frame for the *M. tuberculosis* putative H-NS. This protein is one of the most studied members of the nucleoid-associated proteins family. The gene was cloned, 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. A protein-DNA binding mechanism is proposed. In addition, functional complementation studies of an *E. coli hns* mutant reinforce the likelihood that Rv3852 protein represents a novel nucleoid-associated protein in *M. tuberculosis*.

1. INTRODUÇÃO

1.1. TUBERCULOSE

A tuberculose (TB) é uma doença infecto-contagiosa causada pelo microorganismo *Mycobacterium tuberculosis*, o qual foi identificado por Robert Koch em 1882. Essa doença, cujos processos patológico e inflamatório produzem características como enfraquecimento, febre, perda de peso, sudorese noturna, dor no peito, insuficiência respiratória, tosse e hemoptise, continua sendo a principal causa de morte e padecimento em humanos devido a um único agente infeccioso. O ressurgimento da TB em países desenvolvidos ocorreu no final da década de 80 devido a diversos fatores como a co-infecção pelo HIV, baixas condições sanitárias e de saúde e o surgimento de cepas resistentes (RAVIGLIONE, 2003; DUCATI *et al.*, 2006).

A TB é transmitida por meio de partículas contendo bacilos viáveis que são expelidas por um indivíduo infectado ao tossir e que alcançam a via respiratória de um indivíduo receptor. O desenvolvimento da doença ativa irá depender da capacidade imune do receptor, que quando imunocompetente é capaz de eliminar o bacilo, ou desenvolver um estado latente que poderá ser ativado quando o sistema imune estiver fragilizado; e que quando imunossuprimido tenderá a desenvolver a doença ativa e, dessa forma, irá tornar-se um transmissor (HINGLEY-WILSON *et al.*, 2003; GOMEZ & MCKINNEY, 2004). A doença pode ocorrer em duas formas, a TB pulmonar, que, como o nome diz, atinge os pulmões, e a TB extra-pulmonar, que pode estar localizada em qualquer parte do corpo, sendo mais comuns em adultos e crianças, respectivamente.

O tratamento padrão inclui cinco drogas, isoniazida, rifampicina, pirazinamida, etambutol, e estreptomicina – consideradas de primeira linha, e é dividido em duas fases: a fase inicial, ou de esterilização, onde se utiliza geralmente a combinação das quatro primeiras drogas citadas acima por um período de dois meses, e a fase de continuação, ou de manutenção, que utiliza a combinação das duas drogas mais eficazes, isoniazida e rifampicina, por quatro meses. A escolha das drogas para o tratamento depende do diagnóstico individual do paciente, ou também poderá depender da disponibilidade dessas no sistema de saúde em questão (WHO, 2009).

Devido ao uso incorreto dos medicamentos, ou mesmo ao abandono do tratamento por parte dos pacientes, ocorreu o surgimento de cepas multi-resistentes

(MDR-TB), que apresentam resistência a, no mínimo, isoniazida e rifampicina. O tratamento destas cepas requer a administração adicional de drogas de segunda linha, como as fluoroquinonas (ofloxacino e ciprofloxacino), macrolídeos (amicacina e kanamicina), e etionamida, dentre outras. Estas drogas representam um custo bem maior em relação ao tratamento convencional, além de uma maior toxicidade ao organismo do paciente. O surgimento de casos com cepas extensivamente resistentes (XDR-TB), cuja resistência ocorre à isoniazida e rifampicina, a uma das fluoroquinonas e pelo menos a uma das drogas injetáveis de segunda linha, tem aumentado ainda mais a necessidade do desenvolvimento de novos fármacos antimicobacterianos (CDC, 2007; DORMAN & CHAISSON, 2007).

Desde a segunda metade da década de 90 a Organização Mundial da Saúde (OMS) tem promovido uma estratégia global de controle à TB chamada "DOTS" (*Direct Observed Treatment Short Course*), cujos componentes são o comprometimento político, o acesso aos exames de diagnóstico, a quimioterapia padronizada de curta duração com observação direta do paciente, fornecimento dos medicamentos e garantia de qualidade, e um sistema de registro e análise do programa para a avaliação dos resultados. Os países que adotaram esta estratégia têm verificado importantes avanços no controle da doença e as perspectivas são cada vez mais promissoras (DYE *et al.*, 1998; WHO, 2009). No ano de 2006 iniciou a implementação de um novo programa chamado "*Global Plan to Stop TB*", que tem como objetivos principais a reversão da incidência de TB até o ano de 2015, reforço dos DOTS, tratamento de todos os pacientes com MDR-TB, desenvolvimento de uma vacina efetiva e de uma nova droga anti-TB até o ano de 2015, dentre outros (WHO, 2009).

A atual vacina - BCG (*bacille Calmette-Guérin*), proveniente de uma cepa atenuada de *Mycobacterium bovis*, apresenta uma eficácia variável de zero a 80% (COLDITZ *et al.*, 1994). Ela protege contra formas infantis severas de TB, porém não combate com igual eficiência a forma pulmonar, caracteristicamente encontrada em adultos (YOUNG & DYE, 2006). Assim, vacinas mais eficientes que atuem de forma tanto profilática quanto em indivíduos infectados são de extrema necessidade para o combate desta disseminada doença.

A OMS estimou a incidência de 9,27 milhões de casos de TB no ano de 2007, sendo que, destes, 1,37 milhões eram de indivíduos HIV-positivos e 500.000 eram MDR-TB. Além destes dados ainda alarmantes, no final do ano de 2008, 55 países relataram a ocorrência de pelo menos um caso de XDR-TB (WHO, 2009).

Este preocupante quadro faz com que a descoberta de novas drogas antimicobacterianas, mais eficientes e menos tóxicas, vacinas efetivas pré e pós-exposição ao bacilo, e um maior esclarecimento dos mecanismos de infecção e persistência da micobactéria se tornem um importante alvo de estudo.

1.2. PROTEÍNAS ASSOCIADAS AO NUCLEÓIDE

A pesquisa envolvendo proteínas reguladoras globais vem proporcionando grandes avanços sobre os processos que regulam a infecção e persistência de alguns microorganismos patogênicos, e, dentre essas, estão as proteínas associadas ao nucleóide, também conhecidas como *histone-like*. As proteínas associadas ao nucleóide são uma família de proteínas ligadoras de DNA, pequenas, e geralmente básicas, que exercem importantes funções em mecanismos celulares vitais como recombinação, transcrição e replicação (DORMAN & DEIGHAN, 2003; LUIJSTERBURG *et al.*, 2006). As propriedades funcionais dos principais representantes desta família estão relacionadas à regulação da transcrição de diversos genes, sendo de grande importância a sua relação com genes de virulência e persistência de alguns patógenos, como *Escherichia coli*, *Salmonella* sp. e *Vibrio cholerae*, dentre outros (HARRISON *et al.*, 1994; BERLUTTI *et al.*, 1998; NYE *et al.*, 2000; FALCONI *et al.*, 2001; WILSON *et al.*, 2001; BELOIN & DORMAN, 2003; SCHECHTER *et al.*, 2003; FRANZON & SANTOS, 2004; MANGAN *et al.*, 2006; STONEHOUSE *et al.*, 2008).

Com a publicação da sequência completa do genoma de *Mycobacterium tuberculosis* H37Rv (COLE *et al.*, 1998), surgiram novas possibilidades de estudo tanto para o desenvolvimento de novas drogas ou vacinas, assim como para esclarecer os mecanismos pelos quais esse microorganismo é tão eficiente em infectar o organismo hospedeiro e estabelecer persistência.

Duas proteínas associadas ao nucleóide têm sido identificadas em *Mycobacterium tuberculosis*, Hlp (*histone-like protein*) ou MDP1 (*mycobacterial DNA-binding protein-1*) (PRABHAKAR *et al.*, 1998), e Lsr2 (CHEN *et al.*, 2008; GORDON *et al.*, 2008). A função da primeira ainda precisa ser esclarecida, porém estudos mais avançados envolvendo a proteína Lsr2 caracterizam esta como uma proteína HNS-*like*, pois foi capaz de complementar uma série de mutações para o gene *hns*, além de exercer as funções atribuídas às demais proteínas H-NS já caracterizadas. A presença de uma ORF (*Open Reading Frame*) para uma proteína H-NS putativa anotada como Rv3852

no genoma de *M. tuberculosis* e a falta de evidências do papel que esta possa exercer no metabolismo do bacilo, além da comprovada importância desta classe de proteínas em mecanismos de virulência de diversos microorganismos, nos estimulou ao seu estudo e caracterização.

A H-NS é uma das proteínas associadas ao nucleóide mais estudas e caracterizadas em diversos organismos patogênicos, atuando em etapas de regulação global como transcrição, recombinação e transposição, além de participar na estruturação do DNA bacteriano por meio da compactação deste (DORMAN, 2007). Mutações no gene *hns* são altamente pleiotrópicas (HOMMAIS *et al*. 2001), podendo influenciar a patogenicidade dos microorganismos (FALCONI *et al.*, 1998; NYE *et al.*, 2000; NISHINO & YAMAGUCHI, 2004; ONO *et al.*, 2005).

O gene codificado pela anotação Rv3852 contém 405 nucleotídeos, resultando em uma proteína com massa molecular teórica de 13,8 kDa, sendo positivamente carregada. Neste trabalho, descrevemos a clonagem do gene, e a expressão e purificação da proteína Rv3852, além de comprovarmos sua atividade de ligação a DNA por meio de EMSA (Electrophoretic Mobility Shift Assay) e ressonância plasmônica de superfície (SPR – Surface Plasmon Resonance). Testes de complementação foram realizados a fim de obter evidências da função da proteína recombinante como HNS-*like*. Nossos resultados nos levam a propor que a proteína Rv3852 pertence a uma nova classe de proteínas associadas ao nucleóide, cujas funções no metabolismo do bacilo ainda precisam ser esclarecidas.

2. CAPÍTULO 1 – ARTIGO PUBLICADO

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

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ABSTRACT

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*.

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

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, 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'-AACATATGCCAGACCCGCAGGATCGACCC-3') and HNS *Hin*dIII (5'-GTAAGCTTTCAGCGGCGGCGCAGTTGCC-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 *Hin*dIII 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 OD_{600} 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 KH_2PO_4 (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 KH_2PO_4, 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 KH_2PO_4, 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 KH_2PO_4, 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 absorbed 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 KH_2PO_4, 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.

Table 1. DNA sequences for activity assays

DNA fragment	Sequence	Size (bp)	Molecular mass (g mol^{-1})
proU1	-200 to +10 of *proU* promoter region *	221	136,604
proU2	-20 to +189 of *proU* promoter region *	221	136,615
BENT - $(A_6N_4)_{10}$	$(TTTTTTGCCG)_{10}$	112	69,231
NC - $(A_6N_9)_6$	$CCCCGGC(AAAAAACGGCCCGGC)_6$	109	67,400

(* +1 is defined as the point of transcription initiation)

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 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'-CCGGAATTCATGCCAGACCCGCAGGATC-3') and HNS *Hin*dIII (5'-GTAAGCTTTCAGCGGCGGCGCAGTTGCC-3'), which were designed to contain, respectively, *Eco*RI and *Hin*dIII 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* : : Tn*10*) and BSN27 (relevant genotype MC4100 *trp* : : Tn*10* 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 OD_{600} 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

$$R_{eqi} = \frac{R_{max}[\text{Rv3852}]}{K_D + [\text{Rv3852}]}$$

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):

$$n = R_{max}\frac{MW_{DNA}}{R_{DNA}MW_{protein}}$$

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:

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

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):

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

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 *Hin*dIII 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).

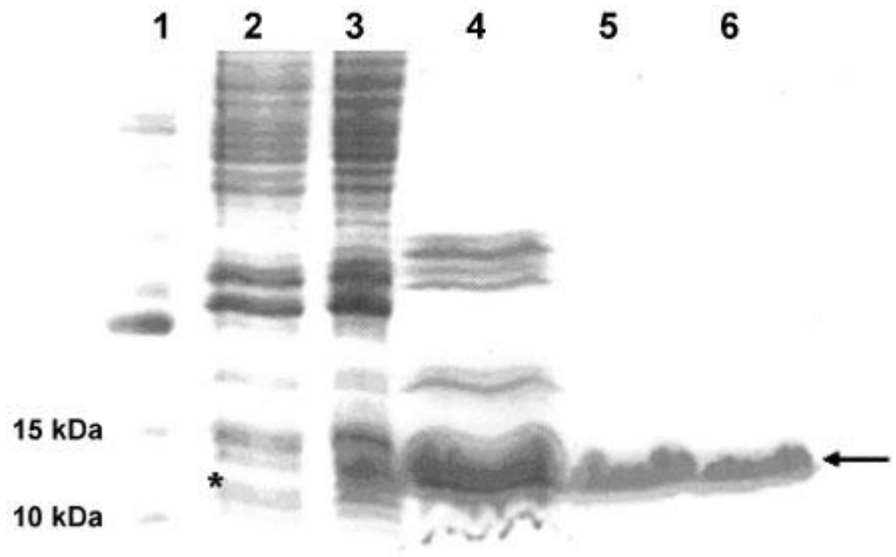

Fig. 1. SDS-PAGE analysis of pooled fractions from the recombinant protein purification protocol. Lanes: 1, BenchMark protein ladder (Invitrogen); 2, crude extract, soluble fraction; 3, crude extract after streptomycin sulfate precipitation and centrifugation; 4, after purification by SP Sepharose FF 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.

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.

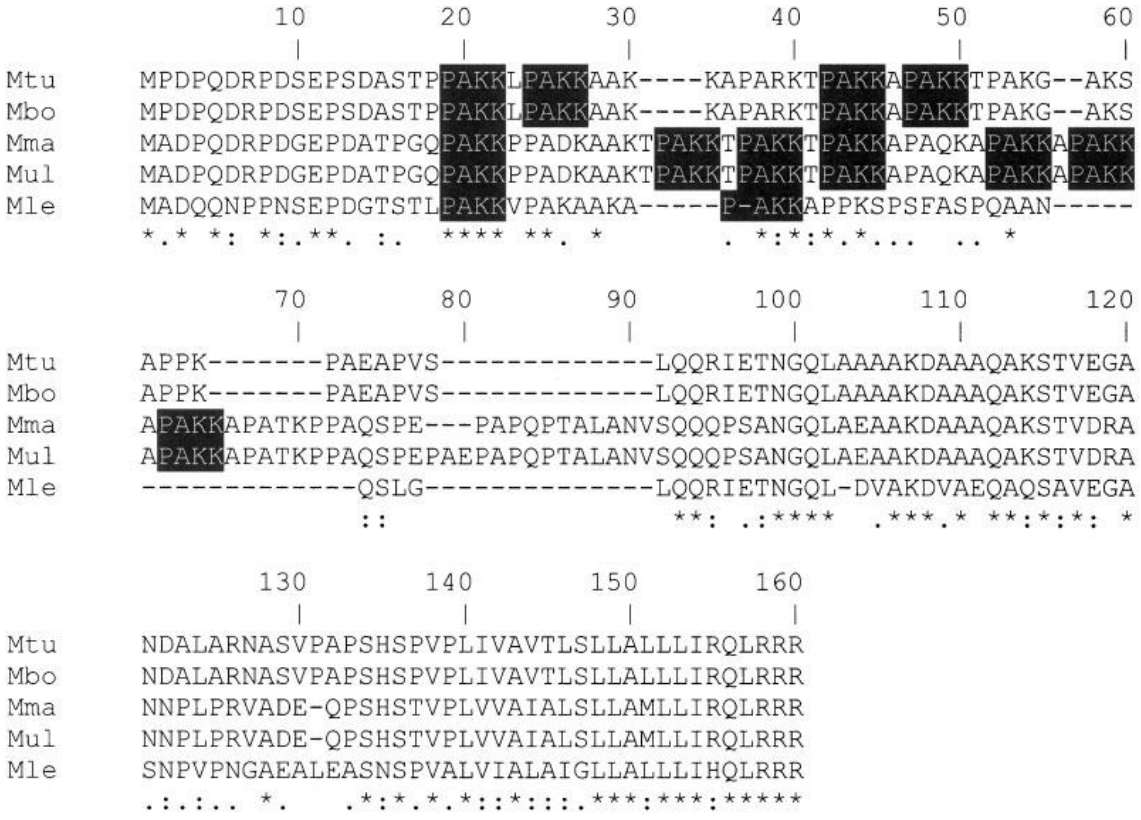

Fig. 2. Multiple sequence alignment of *M. tuberculosis* Rv3852 with its mycobacterial homologues, showing the PAKK repeats in the N-terminal region. *M. tuberculosis* (Mtu) histone-like protein Rv3852 was aligned with other potential mycobacterial homologues from *M. bovis* (Mbo), *M. marinum* (Mma), *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.

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 ~25 200±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 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.

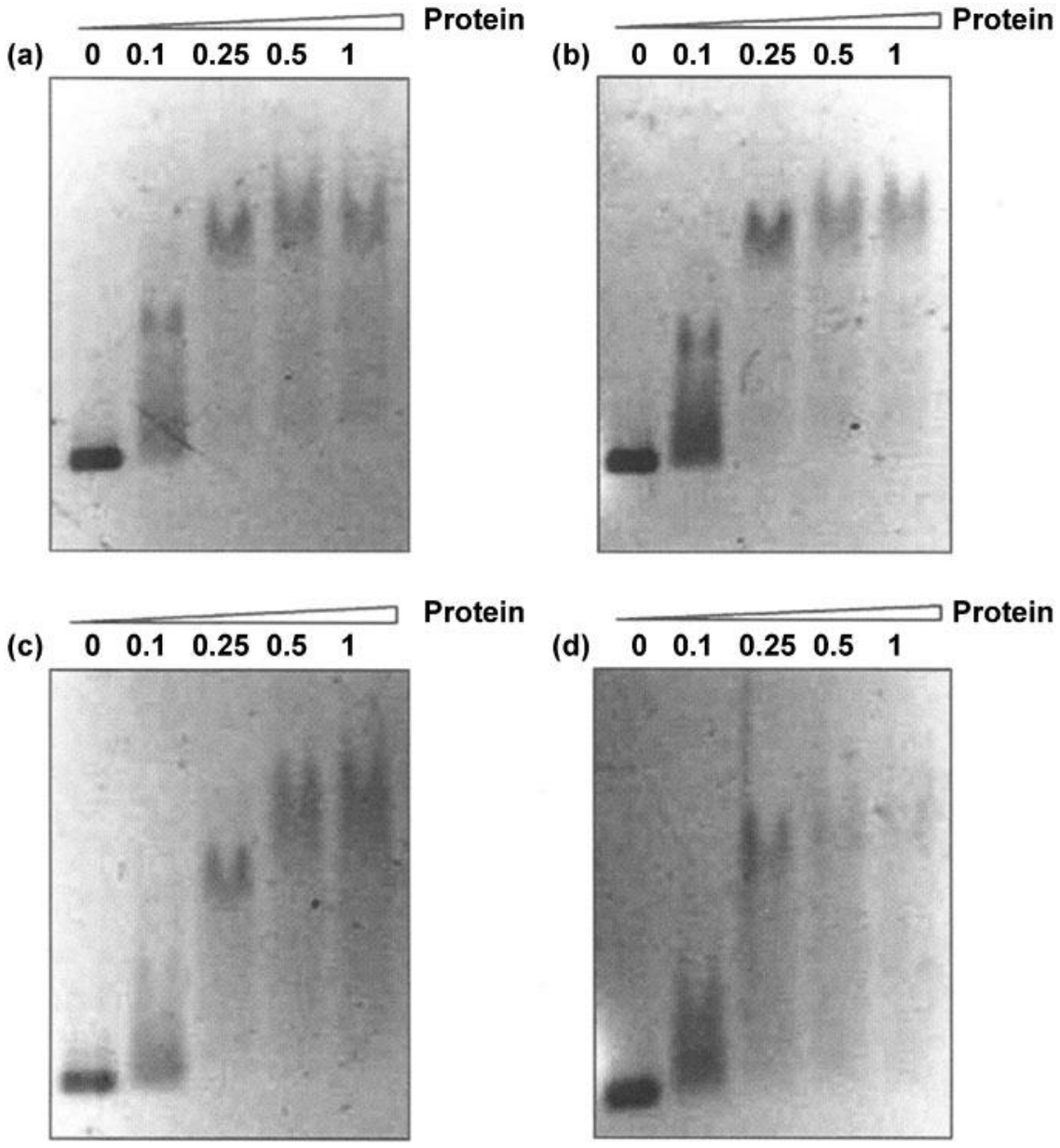

Fig. 3. Activity assay by EMSA. (a) Rv3852 protein–proU1 binding assay, (b) Rv3852 protein–proU2 binding assay, (c) Rv3852 protein–BENT binding assay, (d) Rv3852 protein–NC binding assay. All DNA sequences were tested with the following protein amounts: 0, 0.1, 0.25, 0.5 and 1 µg.

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 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).

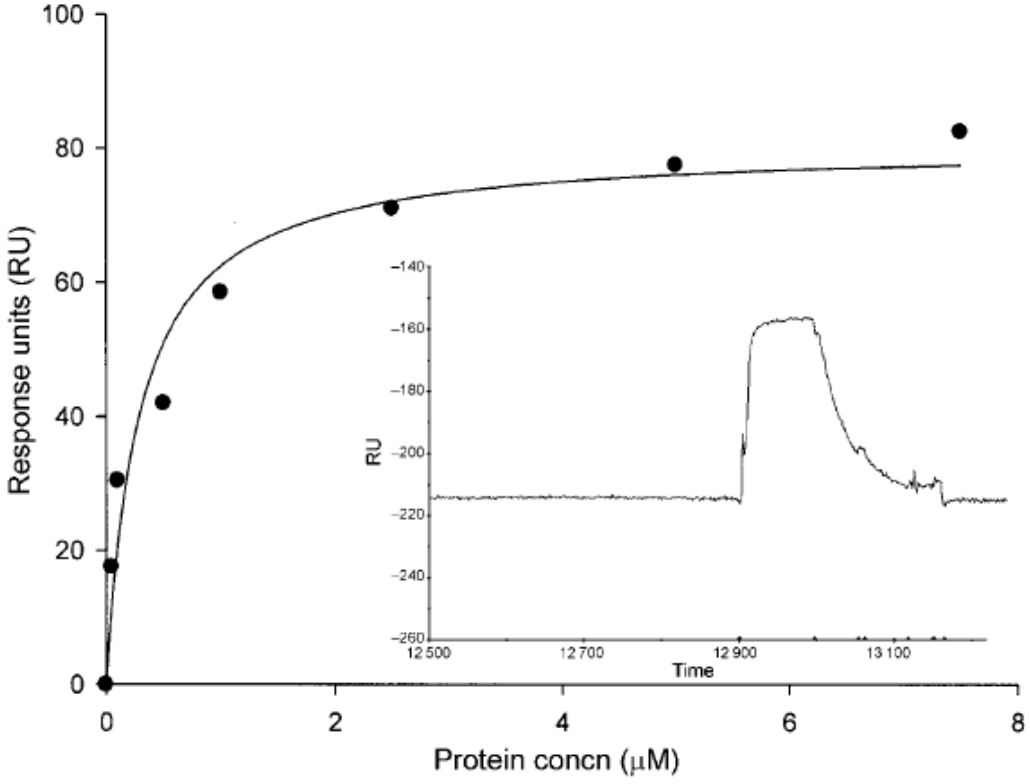

Fig. 4. Saturation curve of the Rv3852 protein–proU2 complex. Mean RU values were plotted against the corresponding protein concentrations and the analysis was performed using the SigmaPlot program. Fitting the data to a rectangular hyperbola (Equation 1) yielded values of 289 (±84) nM for the equilibrium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

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 saturating 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.

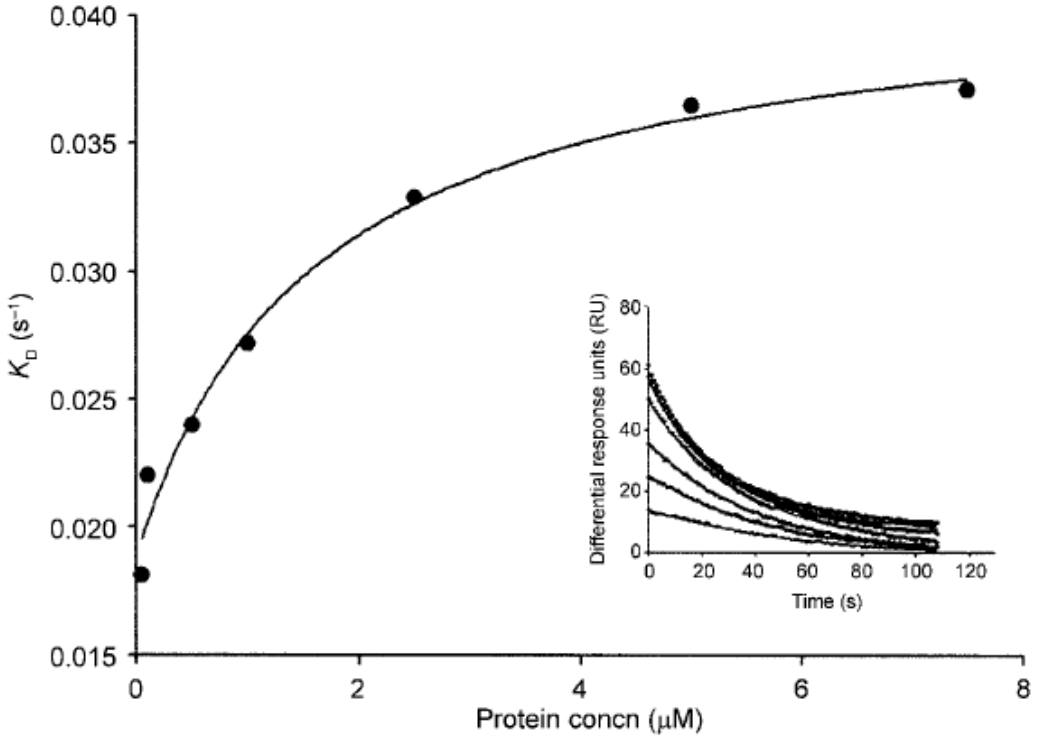

Fig. 5. Rv3852 protein dissociation rate constants for the proU2-immobilized DNA. The data were obtained by SPR from a delay of 120 s after the end of sample injection. Each apparent dissociation rate constant was plotted against its corresponding protein concentration. The data were fitted to a rectangular 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 (0.05–7.5 μM).

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 the surface gives a response of 1000 RU (Bouffartigues *et al.*, 2007), we can expect ~0.086 ng or $6.3x10^{-16}$ mol of proU2 DNA (136 615 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.

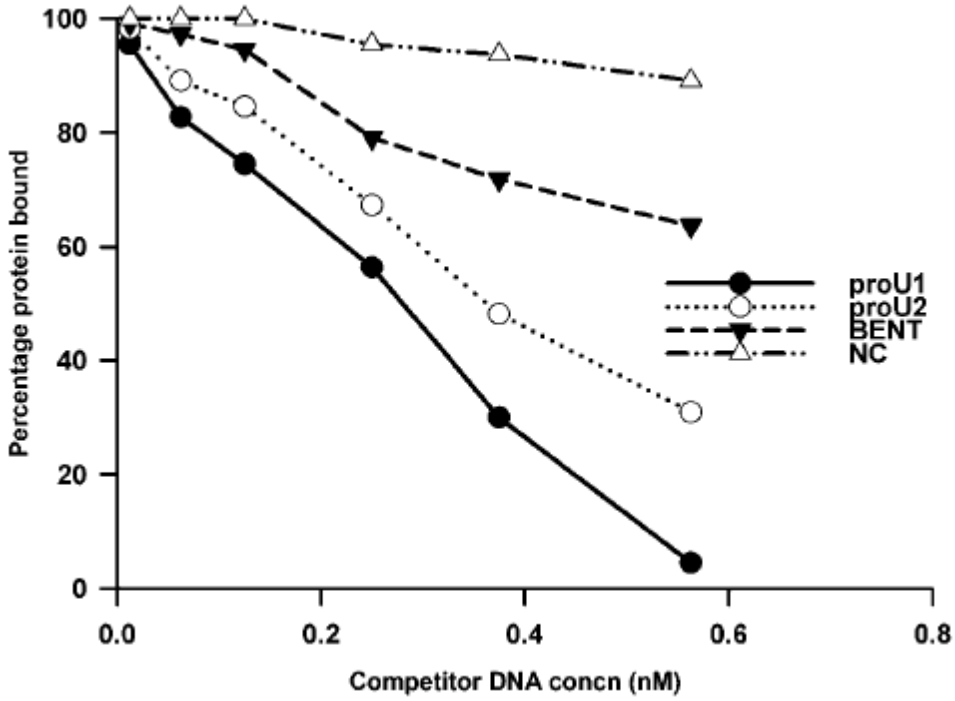

Fig. 6. Competition profile of the Rv3852 protein binding to the proU2-immobilized DNA in the presence of competitor DNAs. The protein binding signal in the absence of competitor DNAs was set as 100 %. Protein binding was tested at a 1-, 5-, 10-, 20-, 30- or 45-fold excess of competitor DNA.

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✦). 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 (Δ*hns*) 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 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 proU2 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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3. DISCUSSÃO

O fato da TB ainda permanecer uma urgência global em saúde, principalmente devido à crescente infecção pelo vírus HIV, que provoca uma imunossupressão nos indivíduos portadores e facilita o estabelecimento da infecção pelo bacilo, e ao surgimento das cepas resistentes ao tratamento, traz a grande necessidade do desenvolvimento de novas drogas anti-TB e vacinas efetivas para o controle da doença (WHO, 2009). Seguindo esta necessidade, é de extrema importância o estudo dos mecanismos celulares e moleculares pelos quais esta micobactéria invade o organismo e estabelece persistência, pois, desta forma, novos alvos poderão surgir.

Neste trabalho descrevemos a caracterização inicial de uma ORF que codifica uma proteína associada ao nucleóide até então não estudada neste organismo. O estudo de proteínas pertencentes a essa família tem auxiliado no esclarecimento dos processos pelos quais diversos patógenos infectam e são capazes de enganar o sistema imune do hospedeiro (HARRISON *et al.*, 1994; NYE *et al.*, 2000; FALCONI *et al.*, 2001; WILSON *et al*., 2001; BELOIN & DORMAN, 2003; SCHECHTER *et al.*, 2003; FRANZON & SANTOS, 2004; MANGAN *et al.*, 2006; STONEHOUSE *et al.*, 2008).

A proteína Rv3852 não apresenta uma identidade estrutural significante com quaisquer das outras proteínas associadas ao nucleóide que têm sido caracterizadas, porém sua região N-terminal exibe uma importante homologia às histonas H1 (KASINSKY *et al.*, 2001), devido à presença de repetições PAKK, assim como pôde ser verificado para a proteína Hlp de *Mycobacterium tuberculosis* (PRABHAKAR *et al.*, 1998). A ocorrência destas repetições indica que a proteína recombinante exerce uma forte atividade de ligação a DNA e sugere uma possível função na estruturação e/ou regulação do cromossomo micobacteriano. A proteína recombinante, em virtude de ser carregada positivamente devido à presença de diversos resíduos de arginina e lisina, também é sugerida formar pontes salinas com a contraparte negativa da estrutura do DNA (CHEN *et al.*, 2008), reforçando sua afinidade por estas moléculas.

Quatro sequências de DNA foram selecionadas para a realização dos testes de atividade de ligação. As sequências proU1 e proU2, provenientes do operon *proU* de *M. tuberculosis*, foram escolhidas por pertencerem a um operon que têm sido amplamente utilizado para os ensaios de atividade da proteína H-NS em outros organismos (OWEN-HUGHES *et al.*, 1992; LUCHT *et al.*, 1994; TUPPER *et al.*, 1994), e, devido a anotação

Rv3852 estar relacionada a uma proteína H-NS putativa, decidimos investigar a afinidade da proteína recombinante para esta região da micobactéria. As outras sequências utilizadas, BENT e NC, que correspondem a sequências padrões curvada e não-curvada, respectivamente, foram selecionadas para verificarmos se existe a preferência da proteína Rv3852 por ligantes com estrutura intrínseca definida, como ocorre para alguns representantes da família de proteínas associadas ao nucleóide (YAMADA *et al.*, 1991; BAILLY *et al.*, 1995).

De acordo com a análise do estado oligomérico, a proteína Rv3852 apresentou-se como um dímero em solução, o que está de acordo com a maioria das demais proteínas associadas ao nucleóide (DORMAN & DEIGHAN, 2003). Quando associados ao DNA, estes dímeros são capazes de formar estruturas maiores, ou complexos, como pôde ser verificado por meio dos experimentos de EMSA, onde os complexos proteína-DNA aumentam gradualmente de tamanho de acordo com o aumento da concentração protéica. Assim, por meio dos ensaios de atividade utilizando SPR, verificamos a ocorrência de duas velocidades de dissociação distintas, que atribuímos às velocidades de dissociação dos dímeros e dos multímeros formados pela proteína ao longo do DNA testado, sendo que a dissociação ocorre de maneira mais rápida quando a proteína está em complexos maiores.

O ensaio de competição realizado por meio de ressonância plasmônica de superfície pôde comprovar uma maior afinidade da proteína Rv3852 pelas sequências proU1 e proU2, indicando que a proteína de ligação apresenta uma afinidade por este operon de *M. tuberculosis*, podendo exercer atividades de regulação em genes relacionados com adaptações a estímulos ambientais, que é o caso do operon *proU*, que codifica um sistema de transporte para os osmoprotetores glicina e betaína (HIGGINS et al., 1987). Uma regulação da expressão da proteína Rv3852 tem sido verificada por mecanismos de estresse ambiental, indicando sua participação nestes mecanismos no bacilo (BETTS *et al.*, 2002; STEWART *et al.*, 2002). Ainda podemos sugerir uma sutil preferência da proteína recombinante por sequências intrinsicamente curvadas, como foi verificado em relação à afinidade para as sequências BENT e NC. Esta característica pode ser atribuída ao fato de que a maioria das proteínas associadas ao nucleóide realizam *bending* do DNA previamente à sua ligação (SPURIO *et al.*, 1997; SCHNEIDER *et al.*, 2001; DHAVAN *et al.*, 2002; SWINGER & RICE, 2004; KOH *et al.*, 2008), e que a ligação à sequência já curvada proporcionaria um gasto menor de energia para a proteína, aumentando sua afinidade.

Por fim, a realização de estudos de complementação comprovou que a proteína Rv3852 pertence a uma nova classe de proteínas associadas ao nucleóide, já que não foi capaz de complementar a cepa de *E. coli* mutante para *hns* por meio de testes de sensibilidade à serina, um método que tem sido amplamente utilizado para a identificação de proteínas H-NS-*like* (TENDENG & BERTIN, 2003; RODRIGUEZ et al., 2004). Além disso, a publicação dos estudos com a proteína Lsr2 de *M. tuberculosis* (GORDON *et al.*, 2008) identificou esta como sendo uma proteína H-NS-*like*, cujas funções exercidas comprovaram se tratar de uma proteína H-NS. Assim, propomos que possa ter ocorrido um erro de anotação no genoma de *M. tuberculosis* em relação à Rv3852, pois, segundo nossos resultados, esta não representa uma proteína H-NS, mas uma nova proteína associada ao nucleóide com funções a serem ainda esclarecidas para este organismo e demais relacionados.

4. PERSPECTIVAS

Este trabalho apresenta a caracterização inicial de uma nova proteína associada ao nucleóide de *Mycobacterium tuberculosis* H37Rv anotada como Rv3852. Uma continuação do trabalho deverá focar na elucidação de seus papéis no bacilo, já que o estudo dos membros dessa família de proteínas tem possibilitado diversos esclarecimentos sobre os mecanismos de infecção de patógenos importantes.

O estudo do perfil de expressão gênica por meio da superexpressão da proteína Rv3852 *in vitro* em *M. tuberculosis* H37Rv informará quais os genes que podem estar sob o controle desta proteína, tanto aqueles *downregulated*, como aqueles *upregulated*. A partir destes dados poderemos verificar em quais mecanismos a proteína Rv3852 está envolvida no bacilo, em adição à construção de cepas mutantes na tentativa de obter amostras menos invasivas ou virulentas, caso seja comprovada a participação da proteína Rv3852 em mecanismos relacionados à infecção e persistência da micobactéria.

Também será realizado o estudo da estrutura tri-dimensional da proteína por meio de Cristalografia, o que poderá trazer importantes informações sobre seu mecanismo de atividade.

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6. APÊNDICE

6.1 CURRÍCULO RESUMIDO

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3. ESTÁGIOS

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4. ARTIGO

* Werlang I.C.R., Schneider C.Z., Mendonça J.D., Palma M.S., Basso L.A. & Santos D.S. 2009. **Identification of Rv3852 as a nucleoid-associated protein in *Mycobacterium tuberculosis***. *Microbiology,* 155: 2652-2663.

5. RESUMOS EM CONGRESSOS

* 32° Reunião da SBBq – Caxambú, MG – "Cloning, sequencing and expression of von Willebrand factor." Werlang I.C.R., Basso L.A. & Santos D.S.

* 34° Reunião da SBBq – Águas de Lindóia, SP – "Histone-like protein (H-NS) from *Mycobacterium tuberculosis* H37Rv: cloning and expression." Werlang I.C.R., Basso L.A. & Santos D.S.