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Involvement of the *Mycobacterium tuberculosis* secreted antigen SA-5K in intracellular survival of recombinant *Mycobacterium smegmatis*

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Abstract

A new protein (SA-5K) secreted in culture filtrates by *Mycobacterium bovis*, *Mycobacterium tuberculosis*, and few other mycobacterial species was previously identified and purified in our laboratory. In order to evaluate the putative role of SA-5K during intracellular mycobacterial growth, in the present study the *SA-5K* gene was cloned and expressed in *Mycobacterium smegmatis*, a rapid growing non-pathogenic mycobacterium which does not contain the gene for the protein. *SA-5K* expression in the THP-1 human macrophage cell line infected with the recombinant strain (*M. smegmatis*-pROL5K) was demonstrated by RT-PCR on RNA extracted from bacterial cells following 24 and 48 h of infection. Intracellular *SA5K* expression was associated with a higher cfu increase of *M. smegmatis*-pROL5K in comparison to the negative control strain (*M. smegmatis* recombinant for the cloning vector) ($P = 0.01$). No significant change in SA-5K synthesis by *M. smegmatis*-pROL5K was observed when the recombinant strain was grown in vitro in different stress conditions such as iron deprivation, pH 4.5, presence of nitric oxide or hydrogen peroxide. The results presented in this study suggest a possible role for SA-5K in intracellular survival of recombinant *M. smegmatis*, though the function of the protein remains unknown.

Keywords: Secreted protein; Intracellular survival; *Mycobacterium tuberculosis*; *Mycobacterium smegmatis*

1. Introduction

Mycobacterium tuberculosis, the causative agent of tuberculosis, is a facultative intracellular pathogen, which survives and replicates within cells of the host immune system, primarily macrophages. The ability of the microorganism to survive during phagocytosis and, subsequently, to multiply within professional phagocytes is critical to the development of the disease.

Although the possible evasion mechanisms employed by the tubercle bacillus to escape killing by activated macrophages have been extensively studied, only few proteins that promote intracellular survival have been identified [1–4]. The study of such proteins may help not only in the development of new tuberculosis vaccines, but also in the identification of targets for the design of new antimycobacterial drugs [3].

Fast growing non-pathogenic mycobacteria such as *Mycobacterium smegmatis* and *Mycobacterium vaccae* have been extensively used as vectors for expression of heterologous genes from virulent mycobacteria. Allowing the evaluation of differences between wild-type and recombinant strains due to the expression of a single protein in the latter, such vectors have allowed identification of proteins involved in survival and multiplication of pathogenic mycobacteria within the macrophages of the infected host [1,3,4].

By means of a recently described monoclonal antibody (mAb) (L8D8) [5] we had previously identified a new protein (SA-5K) with an apparent molecular mass of 5 kDa, secreted in culture filtrates by *M. tuberculosis*, *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) and few other mycobacterial species [6]. No significant homology with other proteins in the databases was found and the physiologic role of SA-5K for mycobacterial cells is unknown.

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The aims of the present study were: (i) to clone the *SA-5K* gene in a shuttle plasmid and express it in *M. smegmatis* which does not contain the gene for the protein [7]; (ii) to evaluate the SA-5K synthesis in intracellular milieu; (iii) to investigate the role of SA-5K during intracellular bacterial growth; and (iv) to establish if different intracellular conditions could modulate SA-5K expression (induction/repression) in vitro.

2. Materials and methods

2.1. Bacterial strains, growth conditions and preparation of bacterial lysates and culture filtrates of recombinant M. smegmatis strains

M. smegmatis mc^2 155 was kindly provided by Dr. D. Young (MRC Tuberculosis and Related Infections Unit, London, UK). Wild-type *M. smegmatis* was grown in standing cultures in Sauton's modified medium, 0.5% sodium pyruvate and 0.5% (w/v) glucose [5]. Recombinant *M. smegmatis* strains were grown in the same medium containing hygromycin (100 μg ml^{-1}) as selection marker. Cfu of recombinant *M. smegmatis* grown intracellularly or in broth were determined by plating 10-fold dilutions on Middlebrook 7H11 agar containing 100 μg hygromycin ml^{-1} and supplemented with oleic acid albumin dextrose complex (OADC). Colonies were counted after incubation at 37°C for 3 days. Bacterial total lysates (TLs) and the corresponding culture filtrates (CFs) were obtained as previously described [8]. *Escherichia coli* XL1 Blue (Stratagene) was grown on Luria–Bertani agar or broth supplemented with hygromycin (100 μg ml^{-1}).

2.2. Cloning of the SA-5K gene in M. smegmatis

A 1048 *Hin*dIII-*Xba*I restriction fragment from the plasmid p-LacZ-H [6], containing the *M. tuberculosis SA5K* gene including the promoter region, was subcloned into the *E. coli*-mycobacteria shuttle vector pROLHYG which encodes hygromycin resistance [9]. The resulting plasmid was propagated in *E. coli* XL1 Blue and introduced, by electroporation, into *M. smegmatis* mc^2 155 to obtain the recombinant strain *M. smegmatis*-pROL5K. The cloning vector pROLHYG was also used to transform *M. smegmatis* wild-type to obtain the recombinant *M. smegmatis*-pROL to be used as negative control. Electroporation of *M. smegmatis* and *E. coli* was performed as described [10].

2.3. RNA extraction and PCR analysis

Total RNA was extracted from recombinant *M. smegmatis* strains by the acid guanidium thiocyanate–phenol–chloroform method [11]. Contaminating DNA was digested by treatment with RNase free DNase I (Sigma) for 1 h at 37°C and DNase was removed by phenol extraction and ethanol precipitation.

For the detection of SA-5K-specific mRNA and of *16S* rRNA, reverse transcription and amplification (RT-PCR) were carried out using the Access RT-PCR System (Promega) following the manufacturer's instructions. After RT reaction, the amplification was carried out as follows: denaturation for 1 min at 94°C; primer annealing for 1 min at 55°C (for *SA-5K*) and at 60°C (for *16S* rRNA); primer extension for 1 min at 72°C. After 30 cycles, a final extension was carried out at 72°C for 10 min. Specific primers were as follows: for *SA-K* gene (reverse primer: CAATGTCGTTGACCGTC; forward primer: ACTCGACAAGGCCGATGTAC); for *16S* rRNA gene (reverse primer: AGAGTTTGATCCTGGCTCAG; forward primer: TGCACACAGGCCACAAGGGA). To exclude the possibility of DNA contamination, control samples were subjected to amplification without prior reverse transcription.

2.4. Infection of the THP-1 human macrophage cell line and evaluation of intracellular growth

Cells of the human monocytic cell line THP-1 (American Type Culture Collection, ATCC) were seeded onto 24-well tissue culture plates at a density of 5×10^4 cells per well. Cells were cultured at 37°C in RPMI medium supplemented with 10% (v/v) FCS, 0.3% (w/v) pen/strep, 2 mM L-glutamine (Sigma). After incubation for 24 h, phorbol 12-myristate 13-acetate (PMA; Sigma) was added to each well at a final concentration of 100 nM. After overnight incubation, cells were washed and fresh complete RPMI, lacking PMA, was added to each well.

For macrophage infection recombinant *M. smegmatis* strains were added to the cells at a multiplicity of infection of 1:1 and/or 1:10 (bacteria:cells). After 1.5 h incubation at 37°C, the medium was removed and cells were incubated for 10 min in RPMI supplemented with 20 μg gentamicin ml^{-1}. Infected monolayers were repetitively washed and incubated in RPMI medium supplemented with 5% (v/v) FCS, 2 mM L-glutamine for 2 days. The incubation with gentamicin was repeated once a day for the duration of the experiment to prevent the multiplication of extracellular bacteria. Infected cells were lysed immediately after, 24 h and 48 h after phagocytosis, by osmotic shock [9]. Cell lysates from multiple wells were pooled, serially diluted and plated on solid medium for cfu counting.

2.5. SDS–PAGE and Western blotting

SDS–PAGE was performed according to Laemmli [12]. Proteins were electrophoretically transferred to nitrocellulose paper by the Transblot apparatus (Hoefer). After blocking, membranes were reacted with mAb L8D8 [5]

directed to SA-5K at 1/50 dilution in TBS, 0.5% Tween 20 and developed as previously described [5].

2.6. Densitometric analysis of SA-5K synthesis by M. smegmatis-pROL5K grown extracellularly under stress conditions

M. smegmatis-pROL5K was grown in liquid medium at 37°C, with or without Tween 80, in shaking cultures up to an OD_{600} of 0.5–0.6. Bacterial cultures were divided into several 40-ml aliquots which were incubated for different time intervals (0, 15, 30, 60, 120 min) at 37°C in the presence of: (i) 1 mM GSNO (*S*-nitroso-L-glutathione, a nitric oxide donor; Alexis); (ii) 5 mM H_2O_2 (hydrogen peroxide; Sigma); (iii) 0.05 mM 2,2′-dipyridyl (iron chelator; Sigma); (iv) citric acid (pH 4.5–5). Other aliquots were incubated in standard Sauton's medium and used as controls. At selected times, the cultures without Tween 80 were centrifuged and supernatants recovered. Aliquots of the corresponding cultures, containing Tween 80, were serially diluted and plated on solid medium for cfu counting. After 3 days of incubation at 37°C the number of cfu per ml of culture was estimated for each condition tested and variable volumes of the different supernatants (corresponding to a cfu number of approximately 7×10^8) were desalted and concentrated by filtration on Centriprep 3 (Millipore). The final volumes (about 3 ml) were dried and the proteins dissolved in 0.02 ml of deionized water. Protein samples were separated by SDS–PAGE and analyzed by Western blotting with mAb L8D8 as described above. Recognition bands were quantified densitometrically by Image Master 1D Software (Pharmacia-Biotech).

2.7. Statistical analysis

Results of at least three independent experiments were expressed as the mean ± S.E.M. (standard error of the mean). Student's *t*-test for dependent samples was used to evaluate the results statistically. A P value <0.05 was considered significant.

3. Results and discussion

3.1. Production and localization of SA-5K by recombinant M. smegmatis and protein expression effects on growth in broth

Production and localization of SA-5K protein was evaluated in CFs and TLs of both *M. smegmatis*-pROL5K and the control strain *M. smegmatis*-pROL, by SDS–PAGE and Western blotting with the specific mAb L8D8 [5]. As illustrated in Fig. 1, SA-5K was only detected in CFs of *M. smegmatis*-pROL5K and not in the corresponding lysate. The protein was visible as a recognition band of approximately 5 kDa and was, therefore,

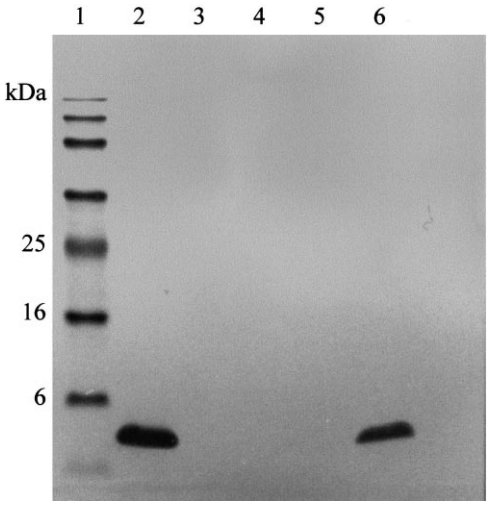

Fig. 1. Expression and localization of SA-5K protein in recombinant *M. smegmatis*. 15 μg of CFs and TLs were analyzed by SDS–PAGE and Western blotting using the specific mAb L8D8. Lanes: 1, molecular mass markers; 2, 3, CF and TL of *M. smegmatis*-pROL5K; 4, 5, CF and TL of *M. smegmatis*-pROL; 6, CF of *M. bovis* BCG.

exhibiting the same electrophoretic mobility as the native protein from BCG CF (Fig. 1). As expected, CF and TL of the control strain *M. smegmatis*-pROL did not show any reactivity with mAb L8D8. Altogether such results suggest that *SA-5K* promoter is also recognized by *M. smegmatis* and that, unlike other secretion proteins expressed in *M. smegmatis* that are retained within the cell envelope with only limited release into culture medium [13], the protein is processed and efficiently translocated in such heterologous host as in *M. tuberculosis*.

In order to evaluate if SA5K expression could influence the growth rate of *M. smegmatis*-pROL5K in comparison to *M. smegmatis*-pROL in broth, both recombinant strains were cultured in Sauton's modified medium and the optical densities were monitored at 600 nm. Both recombinant strains exhibited very similar growth kinetics (data not shown), indicating that SA-5K expression did not seem to influence the replication rate of *M. smegmatis* in broth.

3.2. Expression of SA-5K in infected THP-1 cell line

To evaluate whether the *SA-5K* gene was expressed during growth inside human macrophages, total RNA was extracted from intracellularly growing recombinant *M. smegmatis* strains, at 24 and 48 h after infection, and subjected to RT-PCR with gene-specific primers. Following both 24 and 48 h of infection, a single 258-bp fragment was detected after RT-PCR of RNA extracted from macrophage-grown *M. smegmatis*-pROL5K (Fig. 2A, lines 2, 3), but not after PCR of the control samples (Fig. 2A, lines 4, 5). The length of the amplified fragment was in agreement with that deduced by the *SA-5K* nucleotide sequence. Following RT-PCR of RNA extracted from macrophage-grown *M. smegmatis*-pROL no amplification signal was observed using *SA-5K*-specific primers (Fig. 2A,

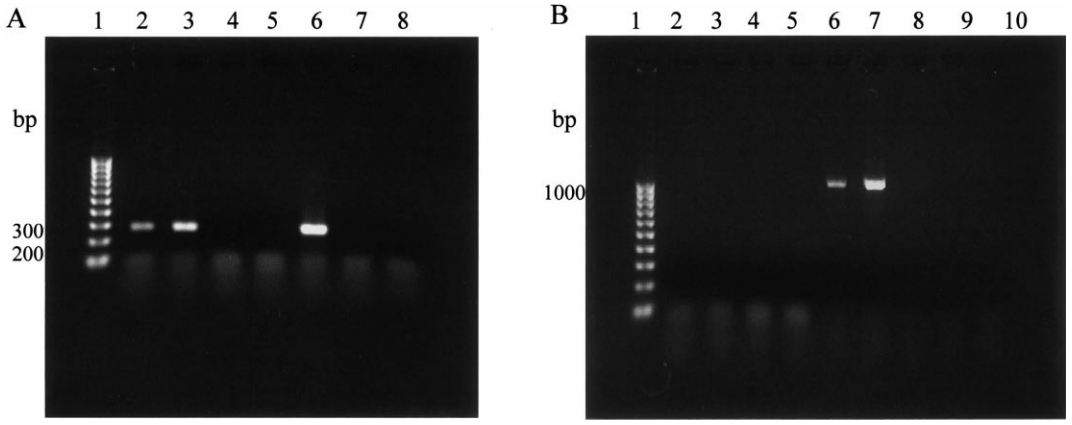

Fig. 2. Intracellular expression of *SA-5K* gene evaluated by RT-PCR. A: RT-PCR (lanes 2, 3) and PCR (lanes 4, 5) on total RNA extracted from *M. smegmatis*-pROL5K grown in THP-1 cells for 24 h and 48 h; RT-PCR (lane 6) and PCR (lane 7) on total RNA extracted from *M. smegmatis*-pROL5K grown in broth; H_2O (negative control; lane 8). B: RT-PCR (lanes 2, 3) and PCR (lanes 4, 5) on total RNA extracted from *M. smegmatis*-pROL grown in THP-1 cells for 24 h and 48 h; effectiveness of the RNA extraction procedure was demonstrated, at 24 h and 48 h, by RT-PCR and PCR with *16S* rRNA-specific primers (lanes 6, 7 and 8, 9); H_2O (lane 10).

lines 2, 3), while a band of approximately 1000 bp was visible after amplification with *16S* rRNA-specific primers, indicating that RNA extraction had occurred efficiently (Fig. 2B, lines 6, 7).

3.3. Influence of SA-5K expression on mycobacterial growth within human macrophages

To evaluate if SA-5K could contribute to the survival of mycobacteria within macrophages, the THP-1 human macrophage cell line was infected with *M. smegmatis*-pROL5K or *M. smegmatis*-pROL at a multiplicity of infection of 1:10 (bacteria:cells). After 1.5 h of phagocytosis similar cfu numbers of both strains were recovered from infected cells, indicating that *SA-5K* expression does not seem to influence the efficiency of phagocytosis of recombinant *M. smegmatis* (data not shown). In contrast, when intracellular growth was evaluated by dividing the number of cfu recovered after 24 and 48 h of infection by that obtained after phagocytosis, a statistically significant increase in the cfu number of intracellular *SA-5K*-expressing *M. smegmatis* was observed in comparison to that of *M. smegmatis*-pROL, after 48 h of infection (712 ± 46.51 times versus 463 ± 126.9 times, Student's *t*-test for dependent samples: $P = 0.01$) (Fig. 3). Thus, while SA-5K did not seem to affect the mechanisms of entry in phagocytic cells of recombinant *M. smegmatis*, expression of the protein in the intracellular milieu was associated with a higher growth level than in the control strain, indicating that the protein could be involved in intracellular survival mechanisms.

3.4. Quantitative analysis of SA-5K synthesis in response to different stress conditions

In order to establish if the environment encountered by the microorganism within host cells could modulate (induce or repress) SA-5K synthesis, SA-5K-expressing *M. smegmatis* was grown in broth, miming some known killing mechanisms of phagocytes such as iron deprivation, pH 4.5, presence of nitric oxide or hydrogen peroxide. At selected time intervals, SA-5K synthesis was densitometrically evaluated after Western blotting of CFs from *M. smegmatis*-pROL5K grown in different stress conditions and compared to that of the recombinant strain grown in standard Sauton's medium. As illustrated in Fig. 4 for a representative experiment, none of the above mentioned conditions seemed to induce any significant change in SA-5K synthesis. These findings may suggest that the *SA-5K* gene is constitutively expressed within macrophages or,

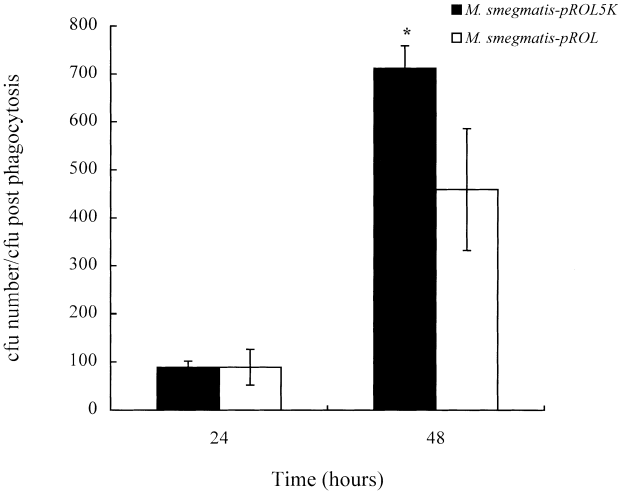

Fig. 3. Increase in the cfu number of recombinant *M. smegmatis* strains during growth in the THP-1 cell line. Macrophages were infected with *M. smegmatis*-pROL5K or *M. smegmatis*-pROL as described in Section 2. The increase in the cfu number was calculated by dividing the number of cfu recorded at 24 h and 48 h by the number of cfu recovered after phagocytosis. Results are expressed as mean values of seven independent experiments ± S.E.M. $*P = 0.01$; Student's *t*-test for dependent samples.

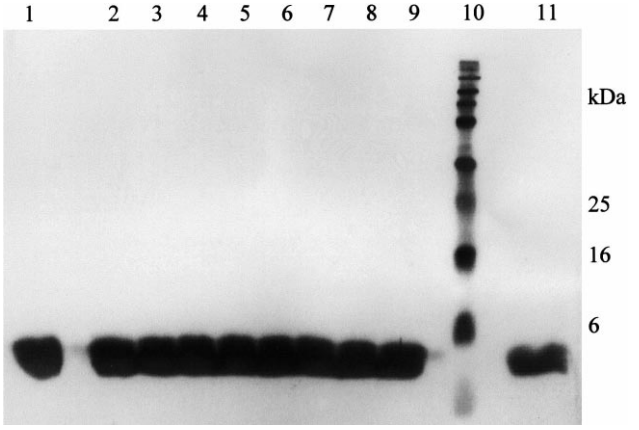

Fig. 4. Synthesis of SA-5K protein by *M. smegmatis*-pROL5K grown in broth at pH 4.5. CFs were analyzed by SDS–PAGE and Western blotting using the specific mAb L8D8. Lanes: 1, CF from exponentially growing *M. smegmatis*-pROL5K in standard Sauton's medium at pH 7.2; 3, 5, 7, 9, CF at 15, 30, 60, 120 min of growth at pH 4.5; 2, 4, 6, 8, CF at 15, 30, 60, 120 min of growth without treatment; 10, molecular mass markers; 11, CF from BCG.

alternatively, that intracellular conditions other than the ones tested could regulate expression of the *SA-5K* gene.

In conclusion, the results obtained in the present study indicate that SA-5K may contribute to the intracellular survival of mycobacteria, although the mechanism(s) by which the protein acts to increase the survival rate of *M. smegmatis* and possibly of *M. tuberculosis* in macrophages remains unexplained.

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References

[1] Wei, J., Dahl, J.L., Moulder, J.W., Roberts, E.A., O'Gaora, P., Young, D.B. and Friedman, R.L. (2000) Identification of a *Mycobacterium tuberculosis* gene that enhances mycobacterial survival in macrophages. J. Bacteriol. 182, 377–384.

[2] Miller, B.H. and Shinnick, T.M. (2000) Evaluation of *Mycobacterium tuberculosis* genes involved in resistance to killing by human macrophages. Infect. Immun. 68, 387–390.

[3] Harth, G. and Horwitz, M.A. (1999) An inhibitor of exported *Mycobacterium tuberculosis* glutamine synthetase selectively blocks the growth of pathogenic mycobacteria in axenic culture and in human monocytes: extracellular proteins as potential novel drug targets. J. Exp. Med. 189, 1425–1436.

[4] Wieles, B., Ottenhoff, T.H.M., Steenwijk, T.M., Fraken, K.L.M., Devries, R.R.P. and Langermars, J.A.M. (1997) Increased intracellular survival of *Mycobacterium smegmatis* containing the *Mycobacterium leprae* thioredoxin-thioredoxin reductase gene. Infect. Immun. 65, 2537–2541.

[5] Freer, G., Florio, W., Dalla Casa, B., Castagna, B., Maisetta, G., Batoni, G., Corsini, V., Senesi, S. and Campa, M. (1998) Subcellular localization of antigens recognized by new monoclonal antibodies against proteins of *Mycobacterium bovis* bacillus Calmette-Guèrin. FEMS Immunol. Med. Microbiol. 20, 129–138.

[6] Freer, G., Florio, W., Dalla Casa, B., Bottai, D., Batoni, G., Maisetta, G., Senesi, S. and Campa, M. (1998) Identification and molecular cloning of a novel secretion antigen from *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG. Res. Microbiol. 149, 265–275.

[7] Coler, R.N., Skeiky, Y.A., Vedvick, T., Bement, T., Ovendale, P., Campos-Neto, A., Alderson, M.A. and Reed, S.G. (1998) Molecular cloning and immunologic reactivity of a novel low molecular mass antigen of *Mycobacterium tuberculosis*. J. Immunol. 161, 2356–2364.

[8] Florio, W., Freer, G., Dalla Casa, B., Batoni, G., Maisetta, G., Senesi, S. and Campa, M. (1997) Comparative analysis of subcellular distribution of protein antigens in *Mycobacterium bovis* bacillus Calmette-Guèrin. Can. J. Microbiol. 43, 744–750.

[9] Batoni, G., Maisetta, G., Florio, W., Freer, G., Campa, M. and Senesi, S. (1998) Analysis of the *Mycobacterium bovis hsp60* promoter activity in recombinant *Mycobacterium avium*. FEMS Microbiol. Lett. 169, 117–124.

[10] Jacobs Jr., W.R., Granjam, K.V., Cirillo, J.D., Pascopella, L., Snapper, S.B., Udani, R.A., Jones, W., Barletta, R.G. and Bloom, B.R. (1991) Genetic systems for the mycobacteria. Methods Enzymol. 204, 537–555.

[11] Chomczynski, P. and Sacchi, M. (1987) Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156–159.

[12] Laemmli, U.K. (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227, 680–685.

[13] Wiker, H.G., Michell, S.L., Hewinson, R.G., Spierings, E., Nagai, S. and Harboe, M. (1999) Cloning, expression and significance of MPT53 for identification of secreted proteins of *Mycobacterium tuberculosis*. Microb. Pathog. 26, 207–219.