ELSEVIER

FEMS Microbiology Letters 169 (1998) 117–124

FEMS MICROBIOLOGY LETTERS

Analysis of the *Mycobacterium bovis hsp60* promoter activity in recombinant *Mycobacterium avium*

Giovanna Batoni *, Giuseppantonio Maisetta, Walter Florio, Giulia Freer, Mario Campa, Sonia Senesi

Dipartimento di Patologia Sperimentale, Biotecnologie Mediche, Infettivologia ed Epidemiologia, University of Pisa, Via S. Zeno 35/39, 56127 Pisa, Italy

Received 31 July 1998; received in revised form 6 October 1998; accepted 6 October 1998

Abstract

A clinical isolate of *Mycobacterium avium* was transformed with a new shuttle plasmid containing the *Escherichia coli* β-galactosidase reporter gene under the control of the *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) *hsp60* promoter. β-Galactosidase activity was assayed spectrophotometrically in bacterial homogenates of the recombinant strain (*M. avium::lacZ*) and used for quantification of the *hsp60* promoter strength in different conditions of extra- and intracellular growth. Very low levels of β-galactosidase were recorded during the exponential phase of in vitro growth, while they increased progressively during the late exponential and stationary phases. A significant increase in enzyme activity was also induced in exponentially growing cells by shifting the incubation temperature from 37 to 45°C, but not from 37 to 42°C nor from 30 to 42°C. No induction of the promoter was observed by adding hydrogen peroxide to the cultures. Finally, β-galactosidase levels were quantified during growth of *M. avium::lacZ* in murine macrophages. Soon after phagocytosis and, to a lesser extent at 1, 5 and 7 days after infection, increased levels of bacterial β-galactosidase were observed indicating an increment in transcriptional activity of *hsp60* promoter both at early phases of infection and during the course of intracellular growth.

Keywords: *Mycobacterium avium*; *hsp60* promoter; β-Galactosidase; Intracellular growth

1. Introduction

With the onset of the AIDS epidemic, the *Mycobacterium avium-Mycobacterium intracellulare* complex (MAC) has become of high medical relevance, being the most common cause of disseminated bacterial infection and contributing to the morbidity and mortality of human immunodeficiency virus (HIV)-infected individuals [1]. Although the emerging interest in the microorganisms of the MAC has promoted the development of genetic systems for phage infection, transfection or transformation of such a group of mycobacteria, their genetic manipulation remains very difficult [2]. In fact, only recently has successful electroporation of *M. avium* been reported and in addition only few of the usual genetic markers work efficiently in this species [2]. For these reasons, many aspects of the gene regulation as well as of the physiology and pathogenicity of these my-

* Corresponding author. Tel.: +39 (50) 559429; Fax: +39 (50) 559462; E-mail: batoni@biomed.unipi.it

0378-1097/98/$19.00
PII: S0378-1097(98)00472-8

cobacteria remain to be elucidated. A number of genetic systems developed in mycobacteria use promoters of different mycobacterial genes [3,4]. In particular, heat shock protein (Hsp) promoters have been extensively studied as they are usually upregulated in response to a variety of stress conditions and control the expression of mycobacterial antigens which may represent the major targets of cell-mediated immune responses [5,6]. In mycobacteria, promoter activities have most often been evaluated in vitro, while a limited number of reports have described transcriptional activity during intracellular growth, a condition believed to cause expression of virulence genes [7].

The aims of the present paper were: (i) to develop a genetic system suitable for quantitative analysis of mycobacterial promoter activity; (ii) to test the system both in different condition of extracellular growth and during growth inside macrophages; (iii) to use such a system to transform a clinical isolate of *M. avium* to gain further insight into the genetics and gene regulation of such species of mycobacteria. A new shuttle plasmid was developed containing the hygromycin resistance gene as a selectable marker and the *Escherichia coli* β-galactosidase as a reporter gene. Due to the limited availability of *M. avium* promoters, the *E. coli* β-galactosidase gene was placed under the control of the *M. bovis* bacillus Calmette-Guérin (BCG) *hsp60* promoter. Activity of the heterologous promoter was assessed by direct spectrophotometric determination of β-galactosidase activity in bacterial homogenates of the recombinant strain *M. avium::lacZ*. The system developed proved to be suitable for the rapid and reproducible quantitative analysis of mycobacterial promoter activity in different conditions of extra- and intracellular growth.

2. Materials and methods

2.1. Bacterial cells and growth conditions

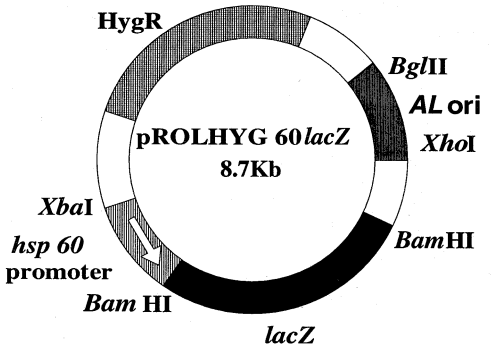
E. coli XL1 blue, grown on LB agar or broth supplemented with the appropriate antibiotic (12.5 μg tetracycline ml^{-1}, 50 μg kanamycin ml^{-1}, 100 μg hygromycin ml^{-1}), was used for selection and propagation of transformants. A clinical isolate of *M. avium* (strain NSN 94) was used throughout the study. For the preparation of standard inocula, bacteria were grown in standing cultures at 37°C in modified Sauton medium enriched with 0.5% sodium pyruvate and 0.5% glucose [8] until late-exponential phase. Recombinant *M. avium::lacZ* was selected on Middlebrook 7H10 agar supplemented with oleic acid albumin dextrose complex (OADC) and with 50 μg X-gal ml^{-1} and grown in the same medium as the parental strain, supplemented with 150 μg hygromycin ml^{-1}.

2.2. Plasmids and DNA cloning procedure

The *Mycobacterium-E. coli* shuttle vector pROLHYG, encoding hygromycin resistance, and the plasmid pLL261, encoding kanamycin resistance and containing the *E. coli lacZ* gene under the control of the *M. bovis* BCG *hsp60* promoter, were kindly provided by D.B. Young. The plasmid pROLHYG was generated by insertion of the polylinker of pBluescript-II-SK (Stratagene) into the *Kpn*I site of p16R1 [9]. PLL261 was digested to generate a *Xba*I-*Bam*HI 398-bp DNA fragment containing the BCG-*hsp60* promoter and a 3078-bp *Bam*HI fragment encoding *E. coli* β-galactosidase, respectively. The two fragments were inserted in pROLHYG by a two-step cloning procedure. The resulting plasmid (pROLHYG 60*lacZ*) (Fig. 1) was propagated in *E. coli* XL1 Blue (Stratagene) after selection on LB agar containing 50 μg X-gal ml^{-1}. *M. avium* and *E. coli* were transformed by electroporation as described [10].

2.3. Evaluation of β-galactosidase activity of *M. avium::lacZ* grown extracellularly

M. avium and *M. avium::lacZ* were grown at 37°C without shaking. At different times, aliquots of 20 ml were centrifuged and the bacterial pellets were resuspended in distilled water to obtain an OD_{598} of 2.0. Bacteria were disrupted by sonication and β-galactosidase activity was assayed in whole cell homogenates of *M. avium* or *M. avium::lacZ*. In induction experiments, the two bacterial strains were grown at 37°C or at 30°C without shaking up to the early-exponential phase. Aliquots of 50 ml were incubated at 45°C or at 42°C or were added with hydrogen



SacI NotI XbaI BamHI SmaI PstI EcoRI
GAGCTCCACCGCGGCCGCTCTAGA----------GGATCCCCCGGGCTGCAGAGGAATTC
hsp60-lacZ

Fig. 1. a: Map of the pROLHYG 60*lacZ* plasmid. The *hsp60* promoter of *M. bovis* BCG, the *E. coli lacZ* reporter gene, the hygromycin resistance gene (HygR) and mycobacterial replication origin (*AL* ori) are shown. Segments from various sources are distinguished by different shading. b: Sequence around the cloning sites (*Xba*I and *Bam*HI).

peroxide at a final concentration of 0.002% (v/v) for different time intervals. After treatment, bacteria were washed with cold PBS and sonicated. β-Galactosidase activity was immediately assayed in bacterial homogenates according to Pardee et al. [11] and expressed as variation of absorbance per min per ml of bacterial homogenate divided by mg of proteins per ml (ΔA min^{-1} mg^{-1}). Protein content was determined according to the method of Lowry et al. [12]. No β-galactosidase activity was detectable in homogenates obtained either from *M. avium* wild-type or from *M. avium* transformed with pROLHYG 60*lacZ* without *hsp60* promoter.

2.4. Infection of macrophages and β-galactosidase activity of M. avium::lacZ grown intracellularly

Macrophages were prepared from 3–6 spleens collected aseptically from 6–9-week-old pathogen-free female BALB/c mice. Spleens were pressed through a stainless steel grid and cells were resuspended in Iscove's modified Dulbecco's medium (IMDM) supplemented with 2 mM L-glutamine, 10% FCS, 0.3% sodium bicarbonate and antibiotics. Spleen macrophages were obtained by seeding spleen cells in 24-well plates at a density of 2×10^6 cells per cm^2. After incubation for 1 week at 37°C in humidified air containing 5% CO_2, non-adherent cells were removed and monolayers were refed with fresh medium devoid of antibiotics until the time of infection. Macrophage infection was carried out as previously described [13]. The presence of mycobacteria within adherent cells, as well as the percentage of infected cells, was evaluated immediately after infection and at 48-h intervals in parallel cultures performed on 12 mm diameter round glass coverslips. To this end, coverslips were removed from the wells and microscopically examined after fixing the cells in acetone-methanol 1:1 and staining for acid-fast bacilli by Kinyoun's method. At selected times after infection, medium was aspirated from the wells and monolayers were repeatedly washed to remove extracellular bacteria. For quantitative analysis of β-galactosidase activity, plates were placed on ice and macrophages lysed by hypotonic shock by adding 0.1 ml of ice-cold distilled water per well. Cells were scraped from the wells with the tip of a plastic pipette and vigorously mixed to allow complete breaking of the macrophages and releasing of intracellular bacteria. Lysates from 12–18 wells were pooled at each time point. The number of live *M.*

avium::lacZ per ml of macrophage lysate was evaluated by plating 10-fold dilutions on Middlebrook 7H10 agar enriched with OADC and supplemented with 150 μg hygromycin ml^{-1}. After incubation for 3 weeks at 37°C, colony-forming units were counted. Mycobacteria in the macrophage lysates were disrupted by sonication and whole homogenates of *M. avium* or *M. avium::lacZ* grown intracellularly were assayed for β-galactosidase activity. Enzyme activity was expressed as variation of absorbance per min per ml divided by 10^9 cfu in macrophage lysates (ΔA min^{-1} $cfu^{-1} \times 10^{-9}$).

2.5. Statistics

All experiments were performed in triplicate; results are expressed as the mean ± S.E.M. One-way analysis of variance and the least significant difference test were used to evaluate the results statistically.

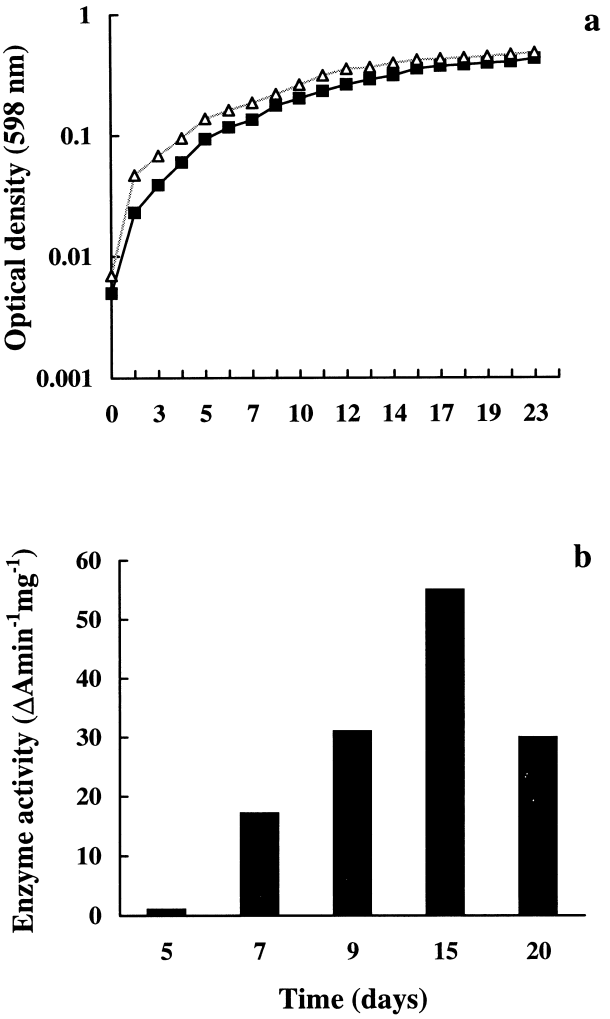


Fig. 2. Growth in broth of *M. avium* and *M. avium::lacZ* (a) and β-galactosidase activity of the recombinant strain at different times of growth in vitro (b). a: Modified Sauton medium was inoculated with *M. avium* (△) or *M. avium::lacZ* (■) and the bacilli were grown at 37°C without shaking. Mycobacterial growth was monitored by measuring optical density. b: β-Galactosidase activity was measured in bacterial extracts obtained by sonication of bacteria collected at different times of culture. Results from a representative experiment are shown.

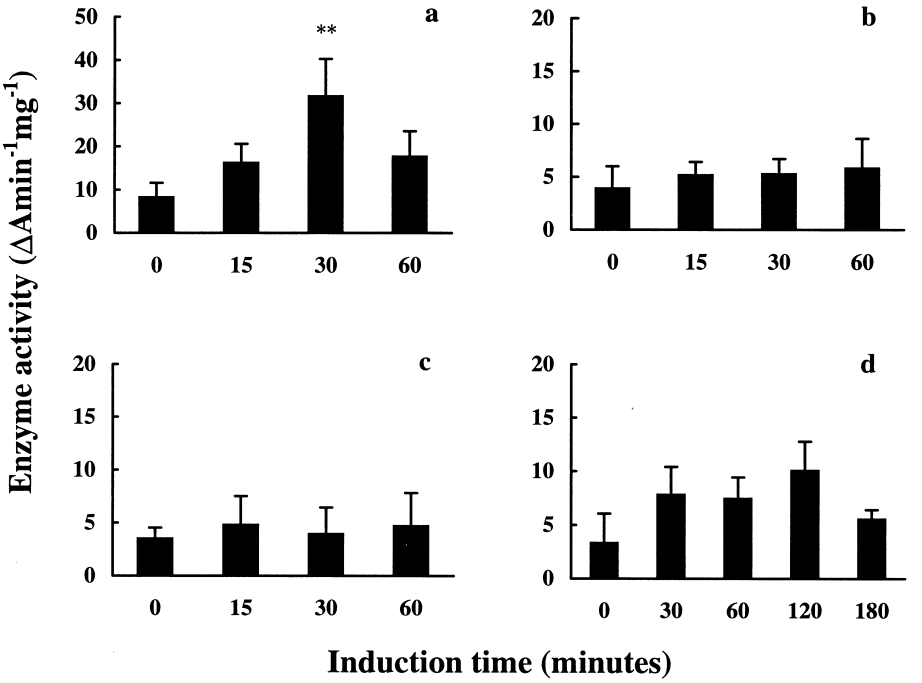


Fig. 3. β-Galactosidase activity in *M. avium::lacZ* cells in response to heat or oxidative stress. Bacilli were collected at different times after shifting the culture temperature from 37 to 45°C (a), from 37 to 42°C (b), from 30 to 42°C (c) or after adding hydrogen peroxide (0.002%, v/v) to the growth medium (d). Cells were sonicated and enzyme activity measured in bacterial homogenates. In each panel, the enzyme activity of non-induced cultures corresponds to the value indicated by the bar at time 0. $^{**}P < 0.01$, one-way analysis of variance.

3. Results and discussion

3.1. Hsp60 promoter activity during in vitro growth of M. avium::lacZ

Parental (*M. avium*) and recombinant (*M. avium::lacZ*) strains exhibited similar growth kinetics in liquid culture (Fig. 2a). β-Galactosidase activity measured in bacterial homogenates of *M. avium::lacZ* at different time intervals during growth (Fig. 2b) clearly showed that exponentially growing bacilli (4–5 days of culture) expressed very low levels of β-galactosidase, while enzyme activity increased considerably in the late exponential phase (day 7–8) and became maximal during the late stationary growth phase (day 15). These findings suggest that the *hsp60* promoter is responsive to starvation stimuli. Because nutrient deprivation within phagosomes is one of the oxygen-independent antimicrobial activities of phagocytic cells [14], the ability to survive and promptly respond to nutrient limitation may represent an important virulence factor during mycobacterial infection.

3.2. Hsp60 promoter activity in response to heat or oxidative stress

In order to evaluate induction of the *hsp60* promoter to heat or oxidative stress exponentially growing *M. avium::lacZ* was used to minimize basal transcriptional activity. Temperature shifting of the liquid cultures from 37 to 45°C caused an increase in enzyme activity as early as 15 min after heat shock, with a maximum after 30 min, when the levels of β-galactosidase were 4 times higher ($P < 0.01$) than those measured before temperature shift (Fig. 3a). The increment in enzyme activity obtained after 30 min of heat shock paralleled an increased synthesis of β-galactosidase protein, as visualized by SDS-PAGE of bacterial homogenates obtained from *M. avium::lacZ* before and after temperature shifting (data not shown). No increase in enzyme activity

was observed following temperature shift from 37 to 42°C (Fig. 3b) and from 30 to 42°C (Fig. 3c). Increased levels of β-galactosidase were also induced by hydrogen peroxide after 30 min of incubation with a peak after 120 min; however, enzyme activity was not statistically different in induced and non-induced bacterial cells (Fig. 3d). Disparate responses to oxidative stress have been described in saprophytic and pathogenic mycobacteria [15]. Young and Garbe [16] failed to demonstrate induction of Hsps in *M. tuberculosis* after addition of hydrogen peroxide to liquid cultures. By using extrachromosomal and integrative expression vectors carrying the regulatory sequences for major BCG Hsps (i.e. Hsp60, Hsp70), Stover et al. [3] were able to demonstrate *hsp60* promoter induction only in BCG recombinant for the integrative vector and not for the extrachromosomal one. The modest response to peroxide observed in this study in recombinant *M. avium* only after prolonged times of exposure may suggest that *M. avium*, as well as *M. tuberculosis* or *M. bovis*, has adapted to the intracellular environment by constitutively synthesizing enzymes inactivating toxic radicals which make them less sensitive to oxidative stress.

3.3. Hsp60 promoter activity in *M. avium::lacZ* during growth in murine macrophages

Parental and recombinant *M. avium* were used to infect monolayers of murine macrophages. Both strains grew mostly intracellularly at a bacteria:macrophage ratio of approximately 10:1, as assessed by microscopical examination of infected monolayers stained for acid-fast bacilli. At such a ratio, about 20–30% of the infectious dose was phagocytosed within 3 h of infection. The number of cfu per well kept stable during the first day of culture and progressively increased, reaching a maximum after 6–7 days of culture, when about 50–60% of the macrophages were infected (data not shown). In order to quantify *hsp60* promoter activity during intracellular growth of *M. avium::lacZ*, β-galactosidase was assayed in homogenates of bacteria recovered from lysed macrophages and expressed as ΔA min^{-1} cfu^{-1}. Enzyme activity was significantly increased at 3 h ($P < 0.025$) and persisted at levels higher than those recorded for *M. avium::lacZ* before macrophage infection (time 0), up to 7 days after infection (Fig. 4). *M. avium::lacZ* bacilli incubated in cell culture medium without macrophages exhibited levels β-galactosidase similar to those recorded at time 0 (data not shown). The intracellular environment may represent one of the most hostile conditions encountered by invading microorganisms and efforts have been made during the last few years to identify genes preferentially or exclusively expressed during intracellular growth of several bacterial species. Although the immunology, production and regulation of Hsps have been studied extensively, their role in macrophage-pathogen interaction is still poorly understood. The very high levels of β-galactosidase expression detected soon after phagocytosis and, to a lesser extent, at later times after infection may indicate that phagocytosis itself, as well as conditions encountered during intracellular growth, may represent a stress stimulus able to promote an in-

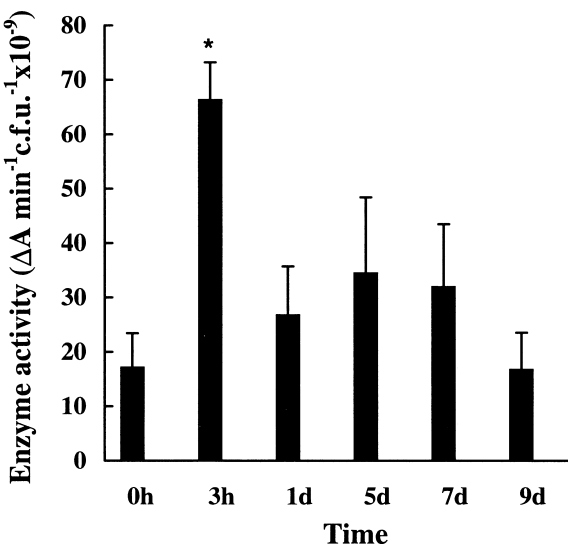


Fig. 4. β-Galactosidase activity in recombinant *M. avium::lacZ* during growth in murine macrophages. Bacterial cells grown in broth at 37°C for 5 days were used to infect macrophage monolayers at a bacteria:macrophage ratio of approximately 10:1. Enzyme activity was assayed in sonicates of the bacterial suspension used for infection (time 0 h), as well as in sonicates of macrophage lysates obtained at different times after infection. At each time point the number of cfu was assessed by plating the infectious dose or the macrophages lysates on solid medium. Enzyme activity (ΔA min^{-1} ml^{-1}) was expressed as specific activity (ΔA min^{-1} $cfu^{-1} \times 10^{-9}$) after dividing by the cfu ml^{-1}. *$P < 0.025$, one-way analysis of variance.

crease in transcriptional activity of the *hsp60* promoter. Therefore, the rapid induction of Hsps during intracellular growth as well as in response to different kinds of stimuli during extracellular growth may be regarded as a prompt adaptive response to hostile environments. The activity of mycobacterial *hsp* promoters in macrophages infected with recombinant BCG expressing the *lacZ* gene was investigated by Dellagostin et al. [7]. In that study, however, β-galactosidase activity was assessed by fluorescence microscopy using a fluorescein-conjugated substrate. In addition, in an attempt to quantify β-galactosidase activity, fluorescence of infected macrophages was measured by flow cytometry and expressed as arbitrary units. The novelty of the present study is the development of a system which allows rapid and reproducible quantification of mycobacterial promoter expression by the direct assessment of β-galactosidase activity as a function of the number of bacteria grown intracellularly. By using this method, the inducibility of *hsp60* promoter during intracellular growth could be accurately compared with that obtained during in vitro growth.

3.4. Conclusions

In conclusion, beside contributing to the understanding of some basic aspects of the physiology and gene regulation of *M. avium*, an area of mycobacterial research still very little explored, the genetic system described in the present paper proved to be very useful for the study of extracellular and, more importantly, intracellular expression of mycobacterial genes.

Extension of this system to the study of other promoters will allow a better understanding of the genes specifically or preferentially required for growth within host cells and, hence, most probably involved in virulence of the MAC group of mycobacteria.

Acknowledgments

This investigation received support from Istituto Superiore di Sanità, Progetto Tubercolosi, Rome and EU Biomed II Programme, Contract BMH4-CT97-2671. We are grateful to D.B. Young, Department of Medical Microbiology, Imperial College School of Medicine at St. Mary's Hospital, London, for providing pROLHYG and pLL261 plasmids.

References

[1] Nightingale, S.D., Byrd, L.T., Southern, P.M., Jockusch, J.D., Cal, S.X. and Wynne, B.A. (1991) Incidence of *M. avium-intracellulare* complex bacteraemia in human immunodeficiency virus-positive patients. J. Infect. Dis. 165, 1082–1085.

[2] Foley-Thomas, E.M., Whipple, D.L., Bermudez, E. and Barletta, R.G. (1995) Phage infection, transfection and transformation of *Mycobacterium avium* complex and *Mycobacterium paratuberculosis*. Microbiology 141, 1173–1181.

[3] Stover, C.K., De la Cruz, V.F., Fuerst, T.R., Burlein, J.E., Benson, L.A., Bennett, L.T., Bansal, G.P., Young, J.F., Lee, M.H., Hatfull, G.F., Snapper, S.B., Barletta, R.G., Jacobs Jr., W.R. and Bloom, B.R. (1991) New use of BCG for recombinant vaccines. Nature 351, 456–460.

[4] Das Gupta, S.K., Bashyam, M.D. and Tyagi, A.K. (1993) Cloning and assessment of mycobacterial promoters using a plasmid shuttle vector. J. Bacteriol. 175, 5186–5192.

[5] Kaufmann, S.H.E. (1990) Heat shock proteins and the immune response. Immunol. Today 2, 129–136.

[6] Lindquist, S. and Craig, E.A. (1989) The heat-shock proteins. Annu. Rev. Genet. 22, 631–637.

[7] Dellagostin, O.A., Esposito, G., Eales, L.J., Dale, J.W. and McFadden, J.J. (1995) Activity of mycobacterial promoters during intracellular and extracellular growth. Microbiology 141, 1785–1792.

[8] Andersen, P., Askgaard, D., Ljungqvist, L., Bennedsen, J. and Heron, I. (1991) Proteins released from *Mycobacterium tuberculosis* during growth. Infect. Immun. 59, 1905–1910.

[9] Garbe, T.R., Barathi, J., Barnini, S., Zhang, Y., Abou-Zeid, C., Tang, D., Mukherjee, R. and Young, D.B. (1994) Transformation of mycobacterial species using hygromycin resistance as selectable marker. Microbiology 140, 133–138.

[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] Pardee, A.P., Jacob, F. and Monod, J. (1959) The genetic control and cytoplasmic expression of 'inductibility' in the synthesis of β-galactosidase and tryptophanase induction in *E. coli*. J. Mol. Biol. 1, 656–664.

[12] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

[13] Esin, S., Batoni, G., Källenius, G., Gaines, H., Campa, M., Svenson, S.B., Andersson, R. and Wigzell, H. (1996) Proliferation of distinct human T cell subsets in response to live, killed or soluble extracts of *Mycobacterium tuberculosis* and *Mycobacterium avium*. Clin. Exp. Immunol. 104, 419–425.

[14] Rainer, N.E. (1994) Altered cell signaling and mononuclear

phagocyte deactivation during intracellular infection. Immunol. Today 15, 374–381.

[15] Sherman, D.R., Sabo, P.J., Hickey, M.J., Arain, T.M., Mahairas, G.G., Yuan, Y., Barry III, C.E. and Stover, C.K. (1995) Disparate responses to oxidative stress in saprophytic and pathogenic mycobacteria. Proc. Natl. Acad. Sci. USA 92, 6625–6629.

[16] Young, D.B. and Garbe, R.T. (1991) Heat shock proteins and antigens of *Mycobacterium tuberculosis*. Infect. Immun. 59, 3086–3093.