Folia Microbiol (2015) 60:21–31
DOI 10.1007/s12223-014-0335-1

The adenylyl cyclase *Rv2212* modifies the proteome and infectivity of *Mycobacterium bovis* BCG

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Received: 13 January 2014 / Accepted: 26 June 2014 / Published online: 21 July 2014

Abstract All organisms have the capacity to sense and respond to environmental changes. These signals often involve the use of second messengers such as cyclic adenosine monophosphate (cAMP). This second messenger is widely distributed among organisms and coordinates gene expression related with pathogenesis, virulence, and environmental adaptation. Genomic analysis in *Mycobacterium tuberculosis* has identified 16 adenylyl cyclases (AC) and one phosphodiesterase, which produce and degrade cAMP, respectively. To date, ten AC have been biochemically characterized and only one (*Rv0386*) has been found to be important during murine infection with *M. tuberculosis*. Here, we investigated the impact of *hsp60*-driven *Rv2212* gene expression in *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG) during growth in vitro, and during macrophage and mice infection. We found that *hsp60*-driven expression of *Rv2212* resulted in an increased capacity of replication in murine macrophages but an attenuated phenotype in lungs and spleen when administered intravenously in mice. Furthermore, this strain displayed an altered proteome mainly affecting proteins associated with stress conditions (*bfrB*, *gro*EL-2, *DnaK)* that could contribute to the attenuated phenotype observed in mice.

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Introduction

Cyclic adenosine 3′5′-monophosphate (cAMP) is one of the most important second messengers used by a diverse array of organisms including bacteria, fungi, and complex eukaryotes (Barba et al. 2010; Bai et al. 2011). In cells, this molecule is responsible in part for the physiological response to environmental changes. Adenylyl cyclases (AC) and phosphodiesterases (PDE) are the enzymes responsible for the synthesis and degradation of cAMP, respectively. In the case of *Mycobacterium tuberculosis*, in 1979, Lowrie and co-workers observed that infection of macrophages with this bacterium increased the levels of cAMP (Lowrie et al. 1979). Analysis of the *M. tuberculosis* H37Rv genome has identified 15 genes and one pseudogene that encode for proteins with predicted AC domains (Shenoy and Visweswariah 2006). Many of the AC present in *M. tuberculosis* have been biochemically characterized and reviews regarding them have been recently published (Shenoy et al. 2004; Barba et al. 2010; Bai et al. 2011). Despite this characterization, only few of the AC have been associated with the physiology of the slow-growing mycobacteria to date.

Mycobacterium bovis BCG Pasteur 1173P2 is an *M. tuberculosis*-complex bacteria, that has accumulated a number of gene deletions, insertions, gene duplications and

single nucleotide polymorphisms compared to the prototype *M. tuberculosis* H37Rv strain, and despite the lack of the major determinants of virulence encoded in region of difference (RD) 1, compared to H37Rv, BCG still conserves other major determinants of virulence such as lipids and posses an overall gene identity around 90 % (Brosch et al. 2007) and is a good model to study interaction with host cells under BSL2 conditions. In silico studies of the genome of *M. tuberculosis* have predicted ten cyclic nucleotide-binding proteins (cNMP) with a wide range of functions. Two of them are encoded by the genes, *Rv3676* and *Rv1675c* and belong to the CRP-FNR family of transcription factors, which binds cAMP, and regulate gene expression (McCue 2000). *Rv3676* (Catabolite repressor protein, cAMP-responsive protein, CRP) is a homolog of the CRP protein from *Escherichia coli* and its deletion in *M. tuberculosis* impairs survival capacity during mice infection (Rickman et al. 2005). Additionally, transcriptional profiling of the mycobacteria identified 16 genes altered in this mutant strain, including *lprQ*, *whiB1*, *ahpC*, and *rpfA* (Rickman et al. 2005). cAMP-macrophage regulator (Cmr, Rv1675c), a CRP-homolog, responds to exogenously added cAMP under low-oxygen conditions. Also, it was shown that cAMP regulates genes such as *mdh*, *gro*EL-2, *Rv1265*, and *PE-PGRS6a* in Bacillus Calmette-Guerin (BCG). However, no significant impairment of growth is observed when murine macrophages are infected with *M. tuberculosis* lacking *cmr* (Gazdik et al. 2009). Another gene, *Rv0998*, is related with the regulation of protein lysine acetylation in a cAMP-responsive manner (Nambi et al. 2010). Recent evidence indicates that Rv0998 acetylated lysine residues in several FadD enzymes in a cAMP-dependent manner (Nambi et al. 2013). All of these data suggest that cAMP plays an important role in global gene regulation and protein function in Mycobacteria. The impact of elevated messenger levels could affect not only mycobacterial physiology, but also impact macrophage responses during infection. Recent data suggest that cAMP produced by *M. tuberculosis* is secreted within the host inducing the production of the pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α) via protein kinase A and the cAMP response-element binding (CREB) protein pathway. In the same study, attenuation and reduced immunopathology was observed in mice infected with *M. tuberculosis* lacking the *Rv0386* AC-related gene. This evidence suggests that cAMP could modulate the host immune response in order to survive (Agarwal et al. 2009).

For the purpose of our study, we analyzed how *hsp60*-driven expression of the biochemically characterized adenylyl cyclase (AC) genes *Rv1318c*, *Rv1359*, *Rv3645*, and *Rv2212*, impacts BCG replication within macrophages. So far, no information on the role of any of these AC in mycobacterial physiology has been reported. All of these AC contain domains that are presumably involved in responding to changes in environmental cues, and have not been tested for their in vivo relevance. We later focused our research on the Rv2212 adenylyl cyclase. Rv2212 is closely related to the pH-sensing AC, Rv1264 (Abdel Motaal et al. 2006). Although the Km for ATP is high for Rv2212, it presents the highest rate of conversion of ATP/cAMP of all ten AC described to date (Abdel Motaal et al. 2006). Guanylyl cyclase activity is absent in comparison with Rv0386 (Shenoy and Visweswariah 2006), and fatty acids significantly enhance the pH sensitivity, converting this AC into a pH sensor. Therefore, it appears likely that adenylyl cyclase Rv2212 might play an important role in the physiology of the mycobacteria (Abdel Motaal et al. 2006). The BCG-Rv2212 was evaluated for its capacity of replicating in cultures during macrophage and mice infection. Also, we determined changes in mycobacterial proteins by proteomic analysis. Our results show that in BCG, *hsp60*-driven *Rv2212* gene expression increases the capacity of replication during macrophage infection but produced an attenuated phenotype in mice, perhaps as a consequence of altered chaperone expression.

Materials and methods

Plasmid construction and mycobacteria transformation

M. bovis BCG Pasteur 1173P2 was used in this work due to biosafety conditions prevailing at our Center. The genes encoding *M. tuberculosis* H37Rv adenylyl cyclases *Rv2212*, *Rv1318c*, *Rv1359*, and *Rv3645* were amplified from genomic DNA by PCR reaction with the oligonucleotides described in Table 1. The AC open reading frames (ORFs) containing restriction sites were cloned into the integrative mycobacterial vector pMV361 in frame with the strong *hsp60* promoter from BCG (GroEL2, Heat shock protein 65, 65-KDa antigen, Stover et al. 1991). ORF fidelity was confirmed by DNA sequencing. To yield BCG *hsp60*-driven-*Rv2212*, -*Rv1318c*, -*Rv1359*, and -*Rv3645*, *M. bovis* BCG Pasteur 1173P2 was transformed with the plasmid constructions by electroporation. BCG with no plasmid was utilized as a control for all experiments.

Table 1 Oligonucleotides used for PCR amplification and cloning of the adenylyl cyclase-related gene in the integrative vector pMV361

Gene target	Oligonucleotides
Rv2212	Forward 5′-aca cat cga tac atg tat gat tcc ttg gac-3′
	Reverse 5′-gcg tta acc cta atc act ggc gg-3′
Rv1318c	Forward 5′-tga gga att cat gtc ggc caa ga-3′
	Reverse 5-′gtt gat cga tgt tac cta cgc ggc tt-3′
Rv1359	Forward 5-′cgt cag ctg tga tgt tca tgg ctc tt-3′
	Reverse 5-′cat atc gat ttc acc cag caa gga cc-3′
Rv3645	Forward 5-′ccg aat tca tgg atg ccg agg-3′
	Reverse 5-′gcc aat cga tac tag ccg cgt act tc-3′

Quantitative real-time PCR

Pellets of BCG and *hsp*60-driven *Rv2212* BCG (hereafter named BCG-Rv2212) obtained from a culture at A_{600} of 1 were suspended in 1 mL of Trizol (Invitrogen) and disrupted in a bead beater for 30 s for four cycles at highest speed. Then, tubes were centrifuged at 13,000 rpm for 10 min at 4 °C. Supernatant were transferred into Phase Lock Gel Heavy (5′PRIME, USA) with 300 μL of a mixture of chloroform/isoamyl alcohol (24:1), phases were mixed, and the tube was centrifuged at 13,000 rpm for 5 min. Aqueous phases were removed and transferred into tubes containing 270 μL of absolute ethanol and 270 μL citrate solution (sodium citrate 0.5 mol/L and NaCl 1.2 mol/L) and stored at 4 °C for 3 days. Thereafter, tubes were centrifuged at 12,000 rpm for 10 min and the pellets were washed twice with 75 % ethanol. After the pellets were dried, RNA was suspended in 100 μL of sterile water. Prior to cDNA synthesis, 10 μg of RNA were digested with 10 U of RQ1 DNAse (Promega, USA). Reaction was incubated for 1 h at 37 °C and RNA purified using the RNeasy mini kit (Qiagen). cDNA synthesis was performed by using the AffinityScript kit (Agilent) according to the manufacturer's instructions, with 300 ng of total RNA. A control experiment was also conducted for the entire RNA preparation without the addition of the reverse transcriptase to verify the absence of genomic DNA contamination. To evaluate *Rv2212* gene expression levels in the BCG-Rv2212 strain, Real-time (RT)-PCR was performed. The sequences of primers employed for the RT-PCR assay are the following: *Rv2212* (BCG2228-F 5′-aca tcc aaa tga cgc aca cc-3 and BCG2228-R 5′-acg cct tca aag tag gtt cg-3)′ and SigA (SigAF 5′-gcc acg cgc atc gaa-3 and SigAR 5′-gcc acg cgc atc gaa-3). SigA was used as reference gene (Manganelli et al. 1999) and wild-type strain was used for normalization. Specificity of reactions as confirmed by gel electrophoresis and melting analyses. RT-PCR was performed using 7500 Real-Time PCR systems with Fermentas Maxima Syber Green qPCR Master mix (Thermo Scientific, USA) in duplicate for two separate experiments. Ten microliters PCR mix (5 μL Syber Master Mix, 5 ng of cDNA, 500 nmol/L of each primer, and sterile Q water at 10 μL) was used for each gene, and RT-PCR was performed using the following temperature cycle: holding stage, 50ºC for 20 s at 95ºC for 10 min, and 30 cycles of 95ºC for 15 s and 60ºC for 1 min. The raw data were analyzed with 7500 Software ver.2.06 (Applied Biosystems) to obtain the ΔΔCt values.

Growth curves and cAMP quantitation

M. bovis BCG-Rv2212 and BCG as a control were grown in duplicate in 75 cm^2 tissue culture flasks using 50 mL of Middlebrook 7H9 medium supplemented with 10 % oleic acid-albumin-dextrose-catalase (OADC) (Difco Laboratories), 0.05 % Tween 80, and 0.2 % glycerol (Sigma-Aldrich). Cultures were placed in an orbital shaker at 100 rpm until A_{600} of 1 was reached (approximately 5 days). Culture samples were taken at A_{600} of 0.03, 0.3, and 1, serially diluted in PBS with 0.05 % Tween 80 and extensively disaggregated by passing though a hypodermic needle (26 G), and samples from each flask plated by duplicate onto 7H10 agar supplemented with 10 % OADC and 0.5 % glycerol. Plates were incubated for 21 days in a 5 % CO_2 atmosphere at 37 °C and colony-forming units (CFU) were determined. Experiments were carried out on three different dates to validate reproducibility. For cAMP quantitation, in three experiments in duplicated flasks, culture samples were taken at A_{600} of 1. Samples were centrifuged at 12,000 rpm for 10 min at 4ºC and the supernatant separated from the pellet. Pellet and supernatant were treated with 0.1 mol/L HCl, and 0.2 mol/L HCl, respectively; after this, samples were boiled for 5 min and frozen immediately. cAMP levels were estimated using radioimmunoassay as described earlier (Shenoy et al. 2005). CFU were obtained from the same samples in order to determine the cAMP levels in relation with the CFUs in the sample.

Kinyoun stain and microscopy

Mycobacterial samples were taken from A_{600} of 1 and spread onto a glass slide. Then, the stain was performed with the HYCEL Ziehl Neelsen stain kit (HYCEL, Mexico) according to the procedure indicated by the manufacturer. Microscopy was performed at ×1,000 magnification.

Macrophage infection

J774A.1 murine macrophages were maintained and propagated in Dulbecco Modified Eagle Medium plus F12 supplemented with 10 % fetal bovine serum (FBS) (Gibco). Cultures were passaged twice a week and maintained in a 5 % CO_2 atmosphere at 37 °C. Twelve hours prior to infection, 2×10^4 macrophages per well were cultured in a 48-well plate.

Mycobacteria were cultured until an A_{600} of 0.6–0.7 was reached, without shaking at 37 °C, 5 % CO_2. Culture samples were harvested and diluted to a multiplicity of infection of four bacteria per macrophage. To eliminate clumping prior to infection, the suspension was passed through a 28-G syringe. Infection was performed by incubating in 5 % in CO_2 atmosphere at 37 °C for 2 h. After this time, the plates were washed three times with sterile phosphate buffered saline (PBS) ×1; 2 mL of DMEM/F12 supplemented with 10 % FBS were added to each well and incubated for 0, 24, 48, and 72 h. Medium was then removed, the macrophages were incubated for 5 min with 0.025 % SDS, and were lysed by pipetting vigorously. One hundred microliters of each well were removed, serially diluted, and plated onto 7H9 OADC agar with or without kanamycin for determining the CFU as described in growth curves. At least three infection experiments were performed.

Mouse infection

The local Ethics Committee approved all the experiments performed in animals (following NOM-087-SEMARNAT-SSA1-2002, NOM-033-ZOO-1995, and NOM-062-ZOO-1999). Pathogen-free, female, 8–9 weeks-old BALB/c mouse was purchased from Harlan Laboratories (México). Mice were housed and fed a regular rodent diet and water ad libitum. Prior to infection, mycobacteria were grown as described in the previous section until the culture reached A_{600} of 0.7–0.8. Cultures were harvested at 5,000 rpm for 10 min at room temperature. Pellet was suspended in 1 mL of 10 % sterile glycerol and centrifuged. Pellet was suspended in sterile PBS and 30 μL containing approximately 10^6 CFU was administered intravenously (i.v.) in the tail vein as determined by plating samples for CFUs determination. Control group received non-modified BCG. Groups of mice were sacrificed by cervical dislocation at 24 h and 28 days. Lungs and spleen were removed aseptically and homogenized in 1 mL sterile PBS. Homogenized tissues were centrifuged and 100 μL of the supernatant were serially diluted and plated onto 7H10 OADC plates with or without kanamycin for CFU enumeration, depending on the strain having or not the single copy, chromosomally integrated pMV361-derivative. Two infection experiments were performed at different dates to ensure reproducibility of the observations and one of them is shown in the "Results" section. The other experiment showed similar results.

Proteomic analysis

Mycobacterial cultures at A_{600} of 1 were centrifuged at 12,000 rpm for 10 min at 4 °C. Pellets were washed twice with 10 mmol/L NaHEPES (pH 8). To solubilize, denature, and reduce proteins, samples were mixed with 10 mmol/L NaHEPES, 7 % urea, 4 % CHAPS, 2 mol/L thiourea, and 10 mmol/L DTT. Samples were disrupted in a bead beater for 1 min at highest speed. Samples were centrifuged at 12,000 rpm for 10 min and 200 μL were mixed with 800 μL of acetone and incubated for 72 h at −20 °C. After this, samples were centrifuged and pellets were washed twice with absolute ethanol. Pellets were suspended in 50 μL of deionized water and quantified by the Bradford method.

For isoelectric focusing (IEF), 80 μg of protein were mixed with 125 μL DeStreak Rehydration solution (GE; Healthcare Life Sciences), DTT 1 mol/L, and 20 mmol/L IPG buffer pH 3–10. The mixture was added to 12-cm immobilized pH linear dry strips (Bio-Rad) pH 3–6 and incubated at 4 °C overnight. The first dimension separation was performed using an Ettan IPGphor™ 3 IEF system (GE Healthcare Life Sciences) with the four following steps: Step 1, 300 V/200 V h; step 2, gradient 1,000 V/300 V h; step 3, gradient 5,000 V/4,000 V h, and step 4, 5,000 V/1,250 V h. After 1D separation, strips were equilibrated with 1 % DTT and 2.5 % iodoacetamide for 20 min. Immobiline dry strips were washed once with equilibration buffer and transferred onto 10 % SDS-PAGE gel for the second dimension. Separations were carried out in a Mini-protean electrophoresis system (Bio-Rad) at 100 V. Spots were stained with Coomasie blue G-250 for 2 h. Two-dimensional electrophoresis images were captured using a molecular imager (Gel Doc™ XR; Bio-Rad). Gel analysis was performed employing PDQuest v.8.0.1 basic 2D analysis software program (Bio-Rad). Duplicate gels containing proteins from four independent experiments were analyzed and used to create a master gel. For gel comparison, the Student *t* test was performed with a 95 % significance level to determine proteins differentially expressed between BCG and BCG-Rv2212. Spot identification was performed at the Protein Core Facility, Columbia University College of Physicians and Surgeons, New York, USA. Briefly, spots from gels were removed and destained, 100 μL of reducing solution were added (0.01 mol/L DTT/0.1 mol/L Tris, pH 8.5), and tubes were heated at 55 °C for 2 h. After heating, reducing solution was removed and replaced with 100 μL of 0.015 mol/L iodoacetamide (ACN) /0.1 mol/L Tris, pH 8.5; tubes were incubated for 30 min in the dark. Then, the spots were washed twice with 30 % ACN/0.05 mol/L Tris, pH 8.5 for 15 min and dehydrated by soaking for 2 min in 200 μL of acetonitrile. ACN was removed and the gel was completely dried for 30 min in a Vacufuge concentrator (Eppendorf). Gels were rehydrated using 0.020 g modified trypsin and 0.1 g Lys-C (Roche Applied Science) in a minimal amount of 0.025 mol/L Tris, pH 8.5, and the tubes were set at 32 °C overnight. Peptides were extracted with 50 μL of 50 % ACN/2 % TCA and the combined extracts were dried and then suspended in a matrix solution (10 mg/mL solution of CHCA in 50 % ACN/0.1 % TFA); two internal standards, angiotensin, and bovine insulin, were added to the matrix solution. The dried digest was dissolved in 3 μL matrix/standard solution and 0.7 μl were spotted onto the sample plate. MALDI-TOF MS analysis was performed on the digest using a PerSeptive Voyager DE-Pro mass spectrometer in linear mode, extraction delayed, positive polarity, laser internal/default, accelerating voltage 21,000, grid voltage 95 %, guide wire voltage 0.050 %, delay time 200 ns, mass range 500 to 7,000 Da, low mass gate 500 Da, laser power 1900-220, shot spectrum 100. Average peptide masses were entered into search programs such as NCBI and/or GenPept databases. The programs employed were ProFound (http://129.85.19.192/profound-bin/WebProFound.exe) and MS-Fit (http://prospector.ucsf.edu). Error tolerance was set at 0.5 Da for average masses.

Statistical analysis

Data were analyzed by two-sided unpaired *t* test and one-way analysis of variance (ANOVA) with Tukey's or Dunn's post-

hoc test. $P<0.05$ was considered statistically significant. Statistical analyses were performed utilizing GraphPad Prism v.5.03 software.

Results

Rv2212 augments BCG replication in murine macrophages

We started our study by comparing the infective and replicating capacities of BCG, BCG-Rv2212, BCG-Rv1318c, BCG-Rv1359, and BCG-Rv3645 during J774A.1 murine macrophages infection. Every 24 h, infected macrophages were lysed and the supernatant was plated to determine mycobacterial CFU (Fig. 1). We observed that at time zero (refers to mycobacterial CFU after 2 h of infection), and at 24 h, CFU counts did not demonstrate a statistical difference between any recombinant strain compared to parental, wild-type BCG. However, at 48 h, an increase of 0.8 log in BCG-Rv2212 compared with BCG was observed. Similarly, at 72 h an increase of ca. 1-log was found; in both cases, the differences were significant ($p<0.05$). Other BCG expressing AC did not demonstrate significant effects during macrophage infection (Fig. 1). Next, we determined if there was a correlation between *Rv2212* gene transcript levels, which could be associated with this enhanced capacity of intracellular survival in BCG-Rv2212. The levels of the *Rv2212* transcript were determined by RT-PCR and found to vary between 1.8 and 2.8 times more than non-transformed BCG (data not shown). In vitro-grown BCG-Rv2212 showed a significant increase in intracellular cAMP in comparison to wild-type strains (Fig. 2a, $P<0.05$), and extracellular levels were almost two-fold higher in *Rv2212*c expressing strains (Fig. 2a, $P<0.005$), therefore strengthening the idea of cAMP increase being the consequence of *Rv2212* expression in recombinant BCG. These results suggest that *hsp*60-driven *Rv2212* may be responsible of the enhanced capacity of replication of BCG-Rv2212 in macrophages

Rv2212 impacts BCG growth and clumping in vitro

To determine the effects of *Rv2212* in BCG during in vitro growth, BCG and BCG-Rv2212 were cultured in standard 7H9 medium. The cultures were started at A_{600} of 0.03 and followed for 5 days until they reached A_{600} of 1. As shown in Fig. 2b, based on absorbance reading, BCG-Rv2212 apparently grew faster than BCG. However, as shown in Fig. 2c, a 0.8 log (1.75×10^8) and 0.7 log (1.6×10^7) reduction in CFUs were observed in BCG-Rv2212 in comparison to BCG at A_{600} of 0.3 and 1 ($p<0.05$). Next, we asked if this discordant result was due to bacterial clumping during culture; we performed staining of bacterial cells with Kinyoun stain (Fig. 2d) and found that BCG-Rv2212 bacteria showed slightly diminished aggregation when compared with BCG. No apparent difference in bacterial width or length was found (Fig. 2d); and no difference in colony morphology between strains was observed (data not shown). These results suggest that *Rv2212* diminish bacterial clumping in BCG; however; more experiments are needed to confirm this finding.

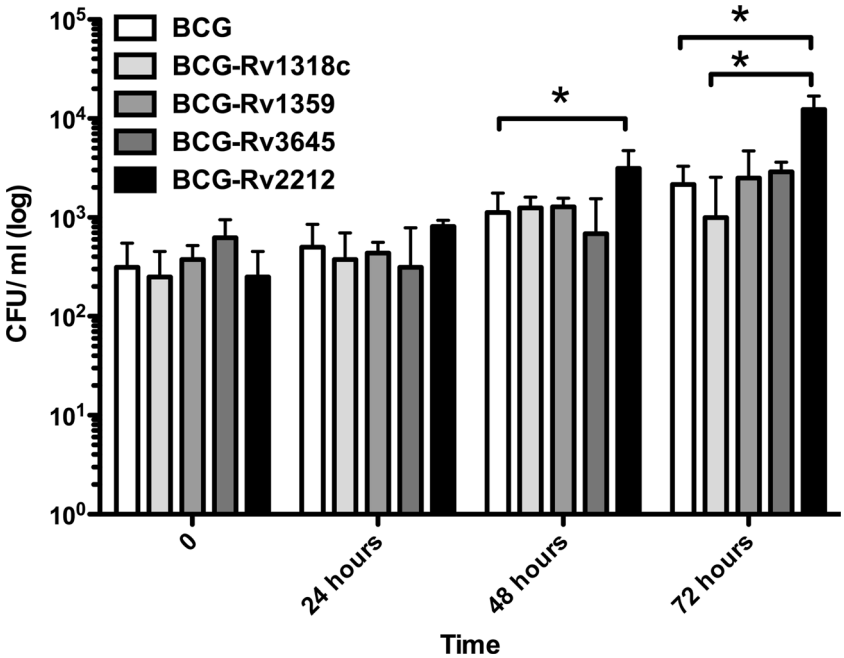

Fig. 1 *Rv2212* augments BCG survival and replication in J774A.1 murine macrophages. BCG, BCG-Rv2212, BCG-Rv1318c, BCG-Rv1359, and BCG-Rv3645 were used to infect macrophages. Every 24 h, cells were lysed and bacteria were plated for CFU determination. A representative experiment is shown with experiments repeated four times and duplicate determination for each plating; *$p<0.05$. *Error bars* represent standard deviation (SD)

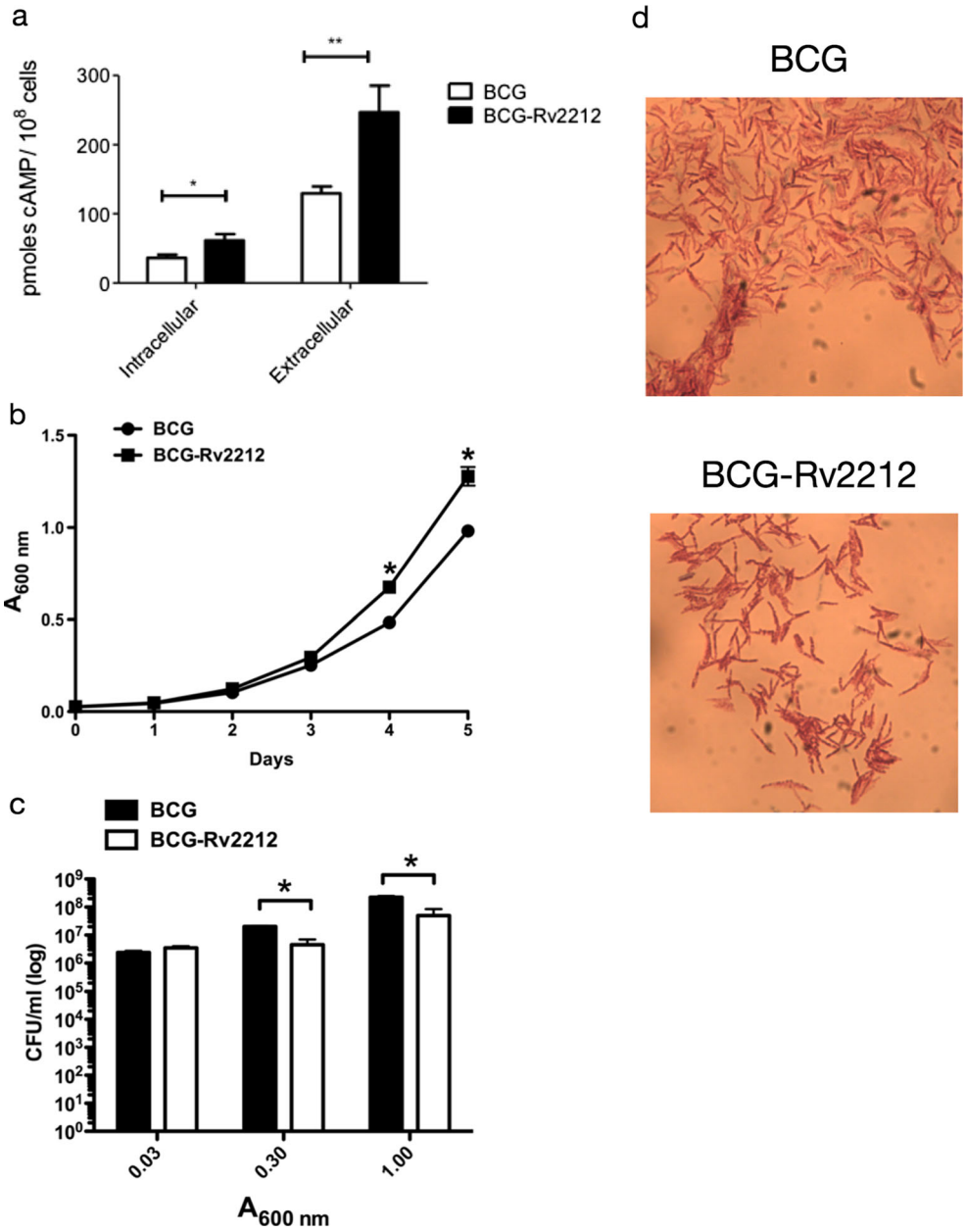

Fig. 2 **a** *Rv2212* expression increased levels of cAMP. Control BCG and BCG-Rv2212 were cultured until A_{600} of 1 and aliquots were taken for CFU and cAMP estimations. Data represent mean±SD of three experiments in duplicated and each sample was evaluated in twice. Statistical significance was evaluated using Student's *t* test. *$p<0.05$; **$p<0.005$. **b** Effect of *hsp60*-driven *Rv2212* expression in BCG in vitro replication. *Mycobacterium bovis* BCG and BCG-Rv2212 were grown in Middlebrook 7H9 medium with 10 % supplemented OADC, as described in "Material and methods". **b** Absorbance was measured repeatedly until A_{600} of 1 was reached. **c** Culture samples were taken at A_{600} of 0.03, 0.3, and 1 and plated for CFU determination. A representative experiment is shown; the *$p<$ 0.05. Using biological duplicates, three experiments were performed. **d** *Rv2212* induced a decrease in clumping. Samples from cultures of BCG and BCG-Rv2212 at A_{600} of 1 were stained by Kinyoun procedure. ×1,000 total magnification

Rv2212 induces an attenuated phenotype in infected mice

Given the enhanced replication observed in macrophages (Fig. 1), we hypothesized that BCG-Rv2212 may show an increased capacity to survive and replicate in mice. To assess this, we performed an intravenous (i.v.) infection of groups of immunocompetent BALB/c mice with BCG and BCG-Rv2212. CFU in lungs and spleens were similar with no statistical difference between BCG and BCG-Rv2212 after 24 h of infection (Fig. 3). However, after 28 days of infection, a significant reduction in BCG-Rv2212 CFU was determined when compared with BCG. In lungs, a 0.7 log (7.1×10^4) reduction in CFUs in BCG-Rv2212 was determined ($p<0.05$) (Fig. 3a). From 24 to 28 days, BCG increased 0.15 log whereas BCG-Rv2212 showed 0.65 log decrease (Fig. 3a). Similar results were found in the spleen after 28 days of infection, where a 0.8 log (5×10^5) reduction in CFU of BCG-Rv2212 was found ($p<0.05$) (Fig. 3b). Here, wild-type BCG increased 0.45 log from 24 to 28 days, and BCG-Rv2212 decreased 0.2 log during the same time (Fig. 3b). This shows how *hsp60*-driven *Rv2212* affects in vivo replication, leading the transformed bacteria to be less able to replicate in immunocompetent mice.

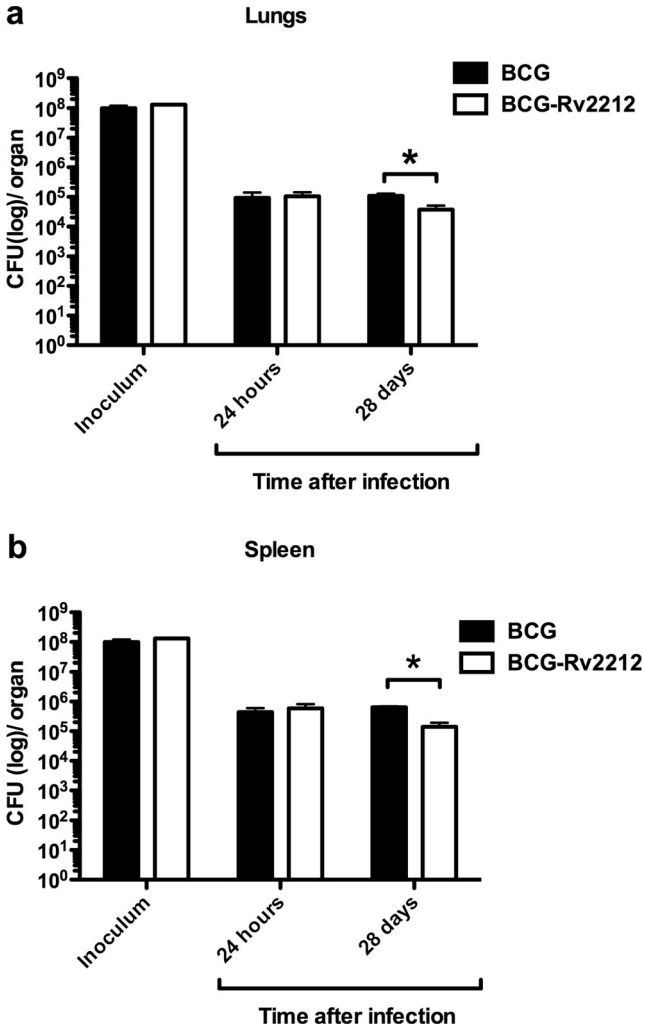

Fig. 3 BCG-Rv2212 is attenuated in mice. BCG and BCG-Rv2212 were used to infect mice intravenously. At 24 h and 28 days after infection, groups of mice were sacrificed and CFU were determined from **a** lung, and **b** spleen. A representative experiment is shown; the *asterisk* indicates $p<0.05$. At least three experiments were performed ($n=3$ per group at each time)

Rv2212 induced an altered proteome in BCG

Two-dimensional proteome analysis of Coomasie-stained gels and peptide identification were performed to determine possible changes associated with *hsp60*-driven *Rv2212* expression in comparison with parental BCG. Preliminary 2D and peptide mapping using silver stained gels showed more spots but MS results were of poor quality on these samples and were therefore not used for further experiments. Proteins extracted from both strains were separated in two-dimensional gels and analyzed for differentially expressed proteins (Fig. 4). Of the 124 spots that could be automatically detected by the software, eight significantly differed in their expression ($p<0.05$). From these, five were selected and identified by peptide mapping (Tables 2 and 3). The proteins Ferritin B (BCG_3904) and the heat shock protein GroEL-2 (BCG_0479) were found only in BCG-Rv2212. Two more, DnaK (BCG_0389) and NAD (P) transhydrogenase subunit alpha, PntAa (BCG_0191), are increased in BCG-Rv2212 (ratios BCG-Rv2212/BCG of 1.62 and 1.17, respectively), and only one, the serine protease PepA (BCG_0159), was increased in BCG. Three of these (Ferritin B, PntAa, and PepA) were related with intermediary metabolism and respiration, and the two remaining ones (GroEL-2 and DnaK) prevent misfolding and promote the refolding and proper assembly of polypeptides generated under stress conditions (http://genolist.pasteur.fr/TubercuList/).

Discussion

The adenylyl cyclase (AC) Rv2212 has been reported to have the highest cAMP-synthesizing activity in vitro and respond to fatty acids, converting the AC to a pH sensor (Abdel Motaal

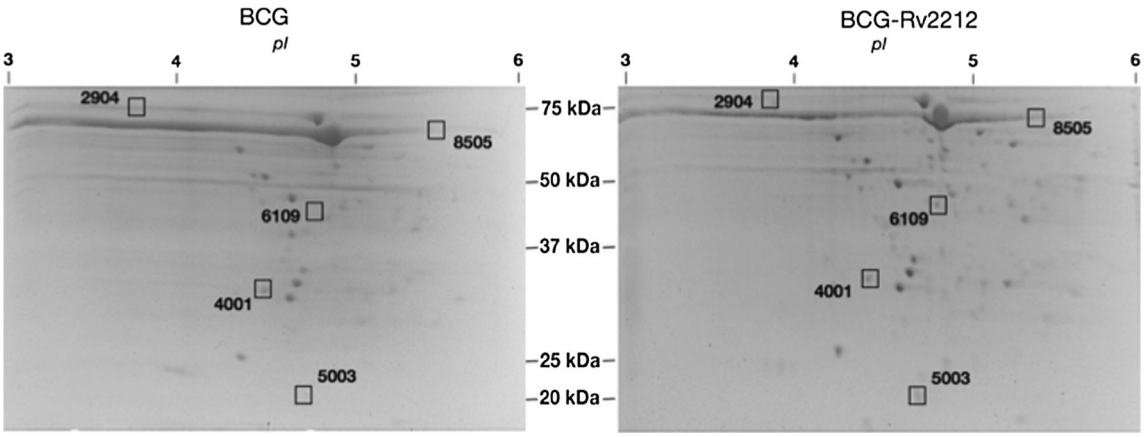

Fig. 4 *Rv2212* induced an altered proteome profile in BCG. Protein extracts from A_{600} of 1 were analyzed by 2D-PAGE. Representative images from BCG and BCG-Rv2212 proteomes. Four independent experiments were performed to render a total of eight gels, which were analyzed with PDQuest v.8.0.1 basic 2D analysis software. *Squares* represent spots that were different by *t* test ($p<0.05$)

Table 2 Spots identified by peptide mapping

Spot	Accession number	Name (s)	Gene name in BCG	Mtb H37Rv orthologue	Ratio BCG-Rv2212/BCG	Hypothetical function[a]	Number of peptides	Sequence coverage (%)
5003	gi:167969958	Ferritin B, bfrB	*BCG_3904*	*Rv3841*	Only present in BCG-Rv2212	Intermediary metabolism and respiration	6	41
8505	gi:15607581	60 kDa chaperonin 1, groEL-2 groEL-2 hsp65	*BCG_0479*	*Rv0440*	Only present in BCG-Rv2212	Virulence, detoxification and adaptation	15	42
2904	gi:289542014	Chaperone protein DnaK, Hsp70	*BCG_0389*	*Rv0350*	1.62	Virulence, detoxification and adaptation	16	35
6109	gi:15607297	NAD (P) transhydrogenase subunit alpha, pntAA	*BCG_0191*	*Rv0155*	1.17	Intermediary metabolism and respiration	6	22
4001	gi:289414416	Serine protease pepA	BCG_0159	Rv0125	0.29	Intermediary metabolism and respiration	4	31

From the 2D gel analysis, five spots were identified as differentially expressed compared with parental BCG. Two of these were only present in BCG-Rv2212, two more were upregulated, and one was downregulated

[a] Hypothetical functions according to Tuberculist web site. http://tuberculist.epfl.ch/index.html

et al. 2006), therefore, it might play a key role during mycobacterial adaptation and growth. As a first step in evaluating this hypothesis, we compared the capacity of recombinant BCG strains expressing from the strong *hsp60* promoter one out of several AC genes, in order to have transcription of these genes independent of their specific stimulatory signal, and found that BCG-Rv2212 was more capable of surviving and replicating in comparison to BCG and other BCG expressing

Table 3 Peptide coverage of the full-length proteins identified after 2D and MS analyses

Protein	Sequence and identified peptides highlighted in bold
Ferritin b, bfrb	MQEQIHNEFTAAQQYVAIAVYFDSEDLPQLA**KHFYSQAVEERN**HAMMLVQHLLDRDLR**VEIPGVDTVRNQ FDRPREALALALDQERT**VTDQVGRLTAVARDEGDFLGEQFMQWFLQEQIEEVALMATLVR**VADRAGA NLFELENFVAREVDVAPAASGAPHAAGGRL**
60 kda chaperonin 1, groel-2	MA**KTIAYDEEARRG**LERGLNALADAVKVTLGPKGRNVVLEKK**WGAPTITNDGVSIAKE**IELEDPYEKIGALVKE VA**KKTDDVAGDGTTTATVLAQALVRE**GL**RNVAAGANPLGLKRG**IEKAVEKVTETLLKGAKEVETKEQIAATA AISAGDQSIGDLIAEAMDKVGNEGVITVEESNTFGLQLELTEGMRFDKGYISGYFVTDPERQEAVLEDPYILLVSS KVSTVKDLLPLLEKVIGAGKPLLIIAEDVEGEALSTLVVNKIRGTFKSVAVKAPGFGDRRKAMLQDMAILTGGQ VISEEVGLTLENADLSLLGKA**RKVVVTKDETTIVEGAGDTDAIAGRV**AQI**RQEIENSDSDYDREKLQERL**AKL AGGVAVIKAGAATEVELKER**KHRIEDAVRN**A**KAAVEEGIVAGGGVTLLQAAPTLDELKLEGDEATGANIVKV** ALEAPL**KQIAFNSGLEPGVVAEKVRNLPAGHGLNAQTGVYEDLLAAGVADPVKVTRSALQNAASIAGLFL TTEAVVADKPEKEKA**SVPGGGDMGGMDF
Chaperone protein dnak, hsp70	MTAHRRRGKLERGALIIVQERSSTYSGGFTMARAVGIDLGTTNSVVSVLEGGDPVVVANSEGS**RTTPSIVAFARN** GEVLVGQPAKNQAVTNVDRTVRSVK**RHMGSDWSIEIDGKKYTAPEISARI**LMKLKRDAEAYLGEDITDAVI TTPAYFNDAQRQAT**KDAGQIAGLNVLRIVNEPTAAALAYGLDKGEKE**QRILVFDLGGGTFDVSLLEIGEGVVE VCATSGDNHLGGDDWDQ**RVVDWLVDKFKG**TSGIDLTKDKMAMQRLREAAEKAKIELSSSQSTSINLPYITV DADKNPLFLDEQLTRAEFQRITQDLLDRTRKPFQSVIADTGISVSEIDHVVLVGGST**RMPAVTDLVKE**L TGGKEPN**KGVNPDEVVAVGAALQAGVLKG**EV**KDVLLLDVTPLSLGIETKGGVMTRLIERNTTIPTKRS**ETFT TADDNQPSVQIQVYQGEREIAAHN**KLLGSFELTGIPPAPRGIPQIEVTFDIDANGIVHVTAKD**KGT GKENTI**RIQEGSGLSKEDIDRM**IKDAEAHAEEDR**KRREEADVRN**QAETLVYQT
NAD (P) transhydrogenase subunit alpha, pntAA	MTDPQTQSTRVGVVAESGPDERRVALVPKAVASLVN**RGVAVVVEAGAGERA**LLPDELYTAVGASIGDAW AADVVV**KVAPPTAAEVGRLRGGQTLIGFLAPRNA**DNSIGALTQAGVQAFALEAIPRISRAQVMD ALSSQANVSGY**KAVLLAASESTRF**FPMLTTAAGTVKPATVLVLGVGVAGLQALATAKRLGA**RTTG YDVRPEVADQVR**SVGAQWLDLGISASGEGGYARELTDDERAQQQKALEEAISGFDVVITTALVPGRPAP TLVTAAAVEAMKPGSVVVDLAGETGGNCELTEPGRTVVKHDVTIAAPLNLPATMPEHASELYSKNITA LLDLLIKDG**RLAPDFDDEVIAQSCVTRG**KDS
Serine protease pepA	MVGYD**RTQDVAVLQLRGAGGLPSAAIGGGVAVGEPVVAMGNSGGQGGTPRA**VPGRVVALGQTVQASDS LTGAEETLNGLIQFDAAIQPGDSGGPVVNGLGQVVGMNTAASDNFQLSQGGQGFAIPIGQAMAIAGQI **RSGGGSPTVHIGPTAFLGLGVVDNNGNGARV**QRVVGSAPAASLGISTGDVITAVDGAPINSATAMADA LNGHHPGDVISVTWQTKSGGTRTGNVTLAEGPPA

AC in murine macrophages (Fig. 1). This suggests that only the AC encoded by *Rv2212* gene is relevant for intracellular replication, at least under the experimental conditions employed here, and therefore we selected this strain to further conduct cAMP assessment, 2D profile and in vivo phenotype evaluation.

We think enhanced mycobacterial replication and survival is likely the direct consequence of *Rv2212* expression concomitantly with increased cAMP production (Fig. 2). However, *Rv2212* gene deletion in virulent *M. tuberculosis* is necessary to shed light on the relevance of this AC during macrophage infection, considering that in BCG Pasteur CRP (BCG3734) has a E178K substitution affecting the DNA-binding domain (Brosch et al. 2007), as well as several gene deletions that affect overall virulence of this bacterium. A caveat in our work is that we did not determine cAMP levels or gene transcripts in the other BCG strains harboring different AC genes; therefore not being able to completely rule out other AC might influence survival in macrophages without significantly altering cAMP levels. We should also mention we did not use BCG transformed with pMV361 empty vector as control in all experiments, basically because other studies have shown it has no effects in gene expression (Flores-Valdez and Schoolnik 2010). In a previous report, deletion of the AC gene *Rv0386* in *M. tuberculosis* caused a diminished survival capacity in lungs and spleen during mouse infection (Agarwal et al. 2009). During our study, we found that BCG-Rv2212 produced more cAMP both intracellular and extracellular (Fig. 2) than wild-type BCG. Nevertheless, we observed attenuation rather than enhanced growth in mice (Fig. 3). Even though we did not monitor signal transduction pathways in the macrophage, we would have expected that the enhanced replication of BCG-Rv2212 observed in J774A.1 (the same cell line used in *Rv0386* characterization) would have resulted in more growth in mice. This might be the consequence of different species used (CDC1551 and isogenic derivatives vs BCG Pasteur 1173P2 and isogenic derivatives), or variable infection routes used (aerosol vs intravenous) between these studies.

Proteomic analysis on BCG-Rv2212 identified three proteins expressed with a statistically significant difference with respect to parental BCG, related with environmental stress: bfrB, GroEL-2 and DnaK. Ferritin B is a protein responsible for iron storage and function as supplier to the bacteria when required (Reddy et al. 2012). The lack of *bfrB* in conjunction with *bfrA* in *M. tuberculosis* impairs survival, pathogenesis, and establishment of chronic infection of *M. tuberculosis* in mouse model (Pandey and Rodriguez 2012; Reddy et al. 2012). Furthermore, Rohde et al. found that *bfrB* gene expression was increased during long-term macrophage infection (Rohde et al. 2012). On the other hand, GroEL-2 and DnaK are chaperones necessary for correct protein folding and are involved in the survival of cells during stress conditions such as changes in temperature, hypoxia, nutrient deprivation, etc.

In agreement with our findings, Gazdik and Mcdonough showed that exogenously added cAMP to *M. tuberculosis* culture upregulated GroEL-2 (Gazdik and Mcdonough 2005). Subsequently, the same group showed that *gro*EL-2 gene expression is dependent of cAMP, which binds to the macrophage regulator (Cmr) transcription factor (Rv1675c) (Gazdik et al. 2009).

GroEL-2 and DnaK, which were more abundantly produced by BCG-Rv2212 than BCG (Fig. 4 and Tables 2 and 3), are highly immunogenic proteins. These two proteins have been extensively used in the formulation of adjuvants (Li et al. 2010; Yang et al. 2012). Interestingly, the sole use of a plasmid encoding *gro*EL-2 gene can confer the same degree of protection as live BCG against tuberculosis (Silva et al. 1999). This natural immunogenicity could have many implications in our study; first, as we have discussed, heat shock proteins are overexpressed under environmental stress conditions and this protein overexpression help cells to survive under such conditions. It is likely that *hsp60*-driven *Rv2212* may improve protein stability in BCG by means of overexpression of GroEL-2 and DnaK, which in turn improves mycobacterial survival during macrophage infection in vitro (Fig. 1). However, attenuation of BCG-Rv2212 strain in vivo (Fig. 3) could be partly explained by the high levels of immunogenic proteins present in the strain and the result of multiple interactions between macrophages and other immune cells that only take place in the whole organism. Preliminary evaluation of TNF-α, IL-5, and IL-10 secreted from infected macrophages suggested a pro-inflammatory profile of BCG-Rv2212, with slightly increased TNF-α and IL-5 levels with lower IL-10 values, although none of them reached statistical significance (data not shown), and will be the subject of future investigation. Stewart and colleagues demonstrated that overexpression of the Hsp70 protein by knocking-down the HspR repressor induced an attenuated phenotype in *M. tuberculosis* when mice infection was performed (Stewart et al. 2001). It is worth noting that more experiments are needed to clarify these findings. If the expression of *Rv2212* produces a metabolic burden in BCG that results in the observed reduced replication (attenuation) in mice could be explored, for instance, by performing infection of immunodeficient mice or even by using BCG-Rv2212 with expression of Hsp70 restored to wild-type levels.

In conclusion, our results demonstrate the first direct evidence of the influence of the adenylyl cyclase Rv2212 in the physiology of *M. bovis* BCG. Our results suggest that *hsp60-driven Rv2212* induces a phenotype resembling stressed mycobacteria, where gene regulation of chaperones might induce the capacity of adaptation to macrophage infection, mediated by proteins related with correct folding and metabolic pathways. In agreement with this proposal, it has just

been shown that *dnaK*, *grpE*, *dnaJ*, and *Rv2025c* are regulated by cAMP in *M. tuberculosis* (Choudhary et al. 2014). We acknowledge that other proteins we found differentially expressed in response to Rv2212 in BCG have not previously been shown to respond directly to CRP, therefore indicating either cAMP produced by Rv2212 activity might be modifying primary targets to then affect BfrB, GroEL2, BCG0191, and PepA, or that these proteins respond to general stress. In fact, we have found PepA differentially expressed between strains with different alleles of the second messenger c-di-GMP phosphodiesterase encoded by *Rv1357c/BCG1419c* (Flores-Valdez et al. submitted). So, we think BCG-Rv2212 is somehow going through adaptations due to some mimicking of the first steps of infection, in which bacteria need to sense and adapt to the adverse environment in the host, and then modify its genetic program to enter into a chronic phase. Whole genome transcriptional profiling or directed qPCR would have allowed us further confirm expression value changes, and might be pursued in future works. Finally, it appears likely that a tight regulation of cAMP is necessary during the infection where some virulence factors are required for survival but at the same time these factors could be detected by the immune system in the host. Our evidence, along with others (Agarwal et al. 2009), suggests that modifying the AC gene content could have a detrimental impact on the capacity of mycobacteria to adapt to varying niches.

Acknowledgments The authors gratefully acknowledge Dr. Jorge Bravo for technical help. CPR was the recipient of a postdoctoral fellowship from CONACYT-México (290616-CIATEJ. A.C.). SSV acknowledges support from the Department of Biotechnology, Government of India.

Conflict of interest The authors declare that no competing financial interests exist.

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