Biochemical and immunological characterization of a *cpn60.1* knockout mutant of *Mycobacterium bovis* BCG

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Pathogenic mycobacteria possess two homologous chaperones encoded by *cpn60.1* and *cpn60.2*. Cpn60.2 is essential for survival, providing the basic chaperone function, while Cpn60.1 is not. In the present study, we show that inactivation of the *Mycobacterium bovis* BCG *cpn60.1* (*Mb3451c*) gene does not significantly affect bacterial growth in 7H9 broth, but that this knockout mutant (Δ *cpn60.1*) forms smaller colonies on solid 7H11 medium than the parental and complemented strains. When growing on Sauton medium, the Δ *cpn60.1* mutant exhibits a thinner surface pellicle and is associated with higher culture filtrate protein content and, coincidentally, with less protein in its outermost cell envelope in comparison with the parental and complemented strains. Interestingly, in this culture condition, the Δ *cpn60.1* mutant is devoid of phthiocerol dimycocerosates, and its mycolates are two carbon atoms longer than those of the wild-type, a phenotype that is fully reversed by complementation. In addition, Δ *cpn60.1* bacteria are more sensitive to stress induced by H₂O₂ but not by SDS, high temperature or acidic pH. Taken together, these data indicate that the cell wall of the Δ *cpn60.1* mutant is impaired. Analysis by 2D gel electrophoresis and MS reveals the upregulation of a few proteins such as FadA2 and isocitrate lyase in the cell extract of the mutant, whereas more profound differences are found in the composition of the mycobacterial culture filtrate, e.g. the well-known Hsp65 chaperonin Cpn60.2 is particularly abundant and increases about 200-fold in the filtrate of the Δ *cpn60.1* mutant. In mice, the Δ *cpn60.1* mutant is less persistent in lungs and, to a lesser extent, in spleen, but it induces a comparable mycobacteria-specific gamma interferon production and protection against *Mycobacterium tuberculosis* H37Rv challenge as do the parental and complemented BCG strains. Thus, by inactivating the *cpn60.1* gene in *M. bovis* BCG we show that Cpn60.1 is necessary for the integrity of the bacterial cell wall, is involved in resistance to H₂O₂-induced stress but is not essential for its vaccine potential.

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Abbreviations: 2-DE, 2D gel electrophoresis; CE, cell extract; CF, culture filtrate; ICL, isocitrate lyase; IFN- γ , gamma interferon; *Mtb*, *M. tuberculosis*; OCEM, outermost cell envelope material; RLU, relative light units; WT, wild-type.

Supplementary text, describing the proteins whose amount was altered in culture filtrate of the *M. bovis* BCG Δ *cpn60.1* mutant, a supplementary table, indicating the proteins of the three *M. bovis* BCG strains identified by 2-DE/MS, and three supplementary figures are available with the online version of this paper.

INTRODUCTION

Chaperonin 60 (Cpn60), also known as heat-shock protein 60 (Hsp60 or GroEL), performs important functions in protein folding and cell survival in stress conditions (Fisher, 1998; Grimaud & Toussaint, 1998; Hendrix & Tsui, 1978; Horwich *et al.*, 2006, 2007; Sigler *et al.*, 1998; Tang *et al.*, 2006). One of the best-characterized Cpn60 molecular chaperones is the *Escherichia coli* GroE, which is composed of GroEL (60 kDa), a homo-oligomer of 14 subunits arranged into two heptameric rings forming a cylindrical structure with two large cavities, and GroES (10 kDa) forming a heptameric homo-oligomer ring (Braig *et al.*, 1994; Saibil, 2000; Sigler *et al.*, 1998; Xu *et al.*, 1997). Both GroEL and GroES are essential for protein folding under all cell growth conditions (Cheng *et al.*, 1989; Fayet *et al.*, 1989; Horwich *et al.*, 1993).

Most bacteria have only one copy of the *groEL* and *groES* genes in a single operon (Lund, 2001), but mycobacteria are unusual in encoding more than one copy of the Cpn60 protein (Kong *et al.*, 1993; Qamra *et al.*, 2005). Thus, *Mycobacterium tuberculosis* (*Mtb*) and *Mycobacterium bovis* BCG have two Cpn60 (Cpn60.1 and Cpn60.2) and one Cpn10 (Hsp10 or GroES) proteins. The two *cpn60* genes are located distantly on the genomes, and the *cpn60.1* (*Rv3417c*) gene is immediately downstream of *cpn10* (*Rv3418c*) (Kong *et al.*, 1993). The two Cpn60 proteins are upregulated during heat shock (Stewart *et al.*, 2002), oxidative stress response (Dosanjh *et al.*, 2005) and macrophage infection (Monahan *et al.*, 2001). In contrast with Cpn60.1, Cpn60.2 (*Rv0440*, also called Hsp65, Hsp60-2, GroEL-2) and Cpn10 are essential and are required for mycobacterial survival (Hu *et al.*, 2008). The immunological properties of Cpn60.2 have been studied extensively. Mycobacterial Cpn60.2 can facilitate efficient *Mtb* bacterial association with macrophages (Hickey *et al.*, 2009) and is an immunodominant antigen that induces a strong cellular and humoral immune response (De Bruyn *et al.*, 1987; Kaufmann *et al.*, 1987; Kaufmann, 1990; Peetermans *et al.*, 1994; Thole *et al.*, 1988). Immunization with *Mtb* Cpn60.2 can protect against the induction of virtually all forms of experimental arthritis in rats (Prakken *et al.*, 2003; van Eden *et al.*, 1988; van Eden *et al.*, 1989). Moreover, the potential of Cpn60.2 as a possible anticancer vaccine, alone or in combination with some tumour antigens, has been reported in many studies (Cappello *et al.*, 2007, 2008; Huang *et al.*, 2007).

Mtb Cpn60.1 can inhibit allergen-induced pulmonary eosinophilia in mice (Riffo-Vasquez *et al.*, 2004) and it is 100-fold more potent than Cpn60.2 in inducing cytokine release from human monocytes *in vitro* (Lewthwaite *et al.*, 2001). In fact, it has been shown that Cpn60.1 can bind to approximately 90% of circulating monocytes compared with Cpn60.2 that binds less than 50% of these cells (Cehovin *et al.*, 2010). *Mtb* Cpn60.1 also plays a key role in the regulation of the cytokine-dependent granulomatous response in *Mtb* infection in mice and in guinea pigs (Hu

et al., 2008). In this report, we aim to investigate the role of Cpn60.1 on the biochemical and immunological properties of *M. bovis* BCG, in particular on the expression of proteins in this attenuated vaccine strain. By inactivating the *cpn60.1* gene in this strain we show that Cpn60.1 is necessary for the integrity of the bacterial cell wall, which is involved in resistance to H₂O₂-induced stress but is not essential for its vaccine potential.

METHODS

Bacterial strains. *M. bovis* BCG GL2 was used as the parental (wild-type; WT) strain to construct the mutant BCG Δ *cpn60.1*. *E. coli* Dh5 α was used as a host strain for cloning and plasmid propagation and was grown on Luria–Bertani (LB) medium. Antibiotics used were ampicillin (100 μ g ml⁻¹), kanamycin (25 μ g ml⁻¹) and hygromycin (100 μ g ml⁻¹).

***M. bovis* BCG Δ *cpn60.1* mutant construction and complementation.** To construct the *cpn60.1* mutant, a 2628 bp PCR product containing the *cpn60.1* gene and approximately 500 bp of flanking regions of the gene was amplified using the *M. bovis* BCG GL2 genomic DNA as the template and primers Cpn60.1-s (5'-tcgaattccctagctgtctatccttgac-3') and Cpn60.1-as (5'-acgaattcacctggttaattcgacgg-3'). The PCR product was cloned into the *EcoRI* site of pUC18 to generate pUC-*cpn60.1*. A cassette containing the *aph* gene conferring resistance to kanamycin from pYUB53 (Kalpana *et al.*, 1991) was inserted into the *BsaBI* site to disrupt the *cpn60.1* gene. After digesting the plasmid with *EcoRI*, the fragment containing *cpn60.1::aph* was integrated by means of blunt ligation into the *NotI* site of pPR27.XylE (Peirs *et al.*, 2005). The resulting plasmid was transformed into *M. bovis* BCG GL2 and the selection of mutants was performed as described previously (Pelicic *et al.*, 1997). The *M. bovis* BCG knockout mutant was designated Δ *cpn60.1*. The complemented strain was constructed by using plasmid pIPX59 (Berthet *et al.*, 1998), a mycobacteriophage MS6-derived integrative vector containing the *attP* site and a hygromycin resistance marker. A 2168 bp DNA fragment containing the *cpn60.1* gene and its 303 bp upstream sequence was amplified by PCR using primers *cpn60.1c-s* (5'-atgtaccacacgcgaaggagaagccgcag-3') and *cpn60.1c-as* (5'-ctgtgaccgaacaacgcgcgaaggaaatgtgtc-3'), and then cloned into the *KpnI* site of pIPX59 to generate pIPX-*cpn60.1C*. This plasmid was transformed into Δ *cpn60.1*, and hygromycin-resistant transformants (in which *cpn60.1* was introduced at the *attB* site in BCG Δ *cpn60.1*) were selected. The complemented strain was designated Δ *cpn60.1Comp*.

Genomic DNA extraction and Southern blot analysis. Genomic DNA extraction was performed as described previously (Galamba *et al.*, 2001). The obtained genomic DNA was digested overnight with *NcoI*, and the fragments were separated by electrophoresis through 1% agarose gels. Southern blot hybridizations were performed using *cpn60.1* and *aph* probes obtained by PCR amplification using the corresponding pair of primers: *cpn60.1-s* (5'-gttgctgtcatcaagggtgg-3') and *cpn60.1-as* (5'-ccgacctagtcaccttgac-3'); *aph-s* (5'-ctcaccgagcagttccatag-3') and *aph-as* (5'-ggtagcgttgccaatgatg-3'). Probes were labelled with [α -³²P]dCTP using megaprime DNA labelling systems (Amersham).

***In vitro* growth without stress.** For cultures in liquid media, the *M. bovis* BCG strains were grown in 7H9 medium containing 0.05% Tween 80 supplemented with 10% albumin–glucose–catalase complex (Difco Laboratories) without agitation, or grown as surface pellicles on Sauton medium using glycerol as a carbon source for 10–14 days at 37 °C. For cultures on solid medium, *M. bovis* BCG was

grown on 7H11 Middlebrook agar supplemented with oleic acid–albumin–glucose–catalase complex (Difco).

In vitro growth under stress conditions. The *M. bovis* BCG strains were grown in 7H9 medium to exponential phase for 7–8 days. A series of 20 ml cultures were used to determine c.f.u. counts after exposure to various stress conditions. For thermal stress, the cultures were incubated at 39 or 42 °C; for hydrogen peroxide stress, a final concentration of 1, 5 or 10 mM was used; for SDS stress, 0.0025 or 0.005 % SDS was applied to the tested samples; and for acidic stress, acidified 7H9 medium (pH 5) was used. Except for the cultures for thermal stress, all other cultures were incubated at 37 °C. At different times, c.f.u. in the control and stressed cultures were determined by plating on 7H11 agar solid medium. Results were expressed as percentage surviving bacteria with respect to the control. Each stress experiment was repeated three times.

Culture filtrate harvesting and pellicle wet-weight estimation.

After 10–14 days of growth on Sauton medium, the culture filtrate was recovered by filtration through a 0.22 µm filter (Nalgene) using a vacuum pump, and the pellicle was harvested and washed three times with a diluted (1/5) Sauton medium, and finally weighed.

Protein quantification. Protein concentration was determined by using the Coomassie Brilliant Blue method (Spector, 1978) with the Bio-Rad reagent using BSA as standard.

Isolation of the crude outermost cell envelope components.

Isolation of the outermost cell envelope material (OCEM) was carried out as described previously (Ortalo-Magné *et al.*, 1995). Briefly, the surface-pellicle-grown cells were harvested by pouring off the medium and then were gently shaken for 1 min with 5 g glass beads (4 mm diameter) per 1 g wet cells. The treated overall bacterial material was collected; the beads were washed three times with 15 ml 20 mM sodium phosphate buffer pH 7.3; and all soluble fractions were pooled. After a 2 h sedimentation, the supernatant was filtered on 0.45 µm filter (Nalgene) and used for different assays.

Isocitrate dehydrogenase assay. Isocitrate dehydrogenase activity in the culture filtrate and in the cellular extract as well as in the OCEM was followed by measuring NADP reduction at 340 nm in 88 mM imidazole buffer, pH 8, containing 3.3 mM MgCl₂ and 0.4 mM NADP. The reaction was initiated by addition of DL-isocitrate to a final concentration of 1 mM.

SDS-PAGE and Western blotting. SDS-PAGE was carried out as described by Laemmli (1970). After transfer of proteins onto nitrocellulose membranes, Western blot analyses were carried out with a rat anti-KasA/KasB antibody raised against *Mtb* KasA (a kind gift from Dr Laurent Kremer, UMR5235, Université Montpellier II, France) or with an anti-Hsp65 monoclonal antibody (mAb) (IA1) (De Bruyn *et al.*, 2000). Protein bands were visualized by using alkaline phosphatase (Sigma).

Affinity chromatography using nickel-chelating agarose resin.

WT *M. bovis* BCG and Δ cpn60.1 strains were grown as a surface pellicle at 37 °C on Sauton medium to stationary phase as described previously (De Bruyn *et al.*, 2000). Bacteria were harvested and suspended in 50 mM Tris, pH 7.5, 150 mM NaCl and disrupted by sonication. The lysates were centrifuged at 13 000 r.p.m. for 30 min and the supernatants were passed through a column containing 1 ml equilibrated Probond Ni²⁺-chelating agarose resin (Invitrogen). After washing with the same buffer, proteins were eluted from the column with the same buffer containing 20 or 75 mM imidazole, as described previously (Ojha *et al.*, 2005). The eluates were analysed by SDS-PAGE and by Western blot using anti-Hsp65 mAb and anti-KasA/B antibody.

Sample preparation for 2D gel electrophoresis (2-DE). WT *M. bovis* BCG, Δ cpn60.1 and Δ cpn60.1Comp strains were grown as a surface pellicle at 37 °C in Sauton medium to stationary phase. Bacteria were separated from the culture filtrate (CF) by filtration through a Whatman filter paper no. 1. Cells were suspended (1 g ml⁻¹) in 50 mM Tris/HCl buffer, pH 7.2, 0.2 % Triton X-100, homogenized with a Dounce homogenizer, and then sonicated for 10 min. This sonicated solution was considered as the cell extract (CE). Proteins in CF were recovered by ammonium sulfate precipitation. Precipitate was extensively dialysed against 50 mM Tris/HCl buffer, pH 7.2. Protein concentrations in the CF and CE were determined by using the Bio-Rad protein Assay kit (Bio-Rad).

2-DE. Proteins from CFs and CEs were diluted in denaturing buffer (7 M urea, 2 M thiourea, 4 % (w/v) CHAPS and 50 mM DTT) containing a complete set of protease inhibitors (Roche) and centrifuged for 20 min at 20 000 g to discard the insoluble components. The supernatant was collected, and the protein concentration was measured using the Bradford method. Iso-electric focusing (100 µg protein) was performed using immobilized pH gradient strips (18 cm; Amersham Pharmacia Biotech). The first-dimension iso-electric focusing was carried out as described previously (Noël-Georis *et al.*, 2004). The second dimension vertical slab SDS-PAGE (12 %) was run for 4 h at 30 mA per gel using the Protean II Xi apparatus (Bio-Rad). The gels were stained with Sypro ruby (Molecular Probes) and imaged using a Typhoon 8600 system (Amersham Biosciences), or the proteins were revealed by silver staining. 2-DE gels were then analysed by using the PDQuest 2D analysis software (V6.2; Bio-Rad).

Sample preparation for MS analysis. Gel spots were excised and separately washed and digested with modified sequencing grade trypsin (Promega), and the resulting peptides were extracted and analysed by nanoLC-MS/MS as described by Leroy *et al.* (2007) and Mastrolo *et al.* (2009).

MALDI-TOF MS analysis. Peptides from digested proteins were solubilized in 2 µl 0.5 % trifluoroacetic acid (TFA). Peptide mass fingerprints (PMF) were obtained by using a MALDI-TOF mass spectrometer (Micromass) working in reflection mode with 15 kV source voltage, 2.5 kV pulse voltage and 2 kV reflecting voltage. A saturated solution of α -cyano-4-hydroxycinnamic acid in 50 % acetonitrile and 0.1 % TFA was used for the matrix. A total of 1 µl of the matrix and sample solution was mixed in a 1:1 (v/v) ratio and applied onto the 96 target wells. Mass accuracy for PMF analysis was 0.1 Da with external calibration. Internal calibration was carried out by using enzyme autolysis peaks, and the resolution was 11 000. The resulting peptide masses were automatically searched for matches, in a local copy of NCBI database release 20080704 using an in-house Mascot 2.2 server (Matrix Science). Searches were carried out in all species with the following parameters: max. missed cleavages=1; mass tolerance=50 p.p.m.; fixed modifications=carbamidomethylation (C); variable modifications=oxidation of methionines. Protein identification results were manually evaluated. Only identification results with a confidence level above 95 % were confirmed as positive hits.

MS/MS analysis. Peptides were analysed using the 'peptide scan' option of the HCT ultra ion Trap (Bruker), consisting of a full-scan MS and MS/MS scan spectrum acquisitions in ultrascan mode (26 000 *m/z* s⁻¹). Peptide fragment mass spectra were acquired in data dependent AutoMS(2) mode with a scan range of 100–2800 *m/z*, three averages and four precursor ions selected from the MS scan 300–1500 *m/z*. Precursors were actively excluded within a 0.5 min window, and all singly charged ions were excluded. Peptide peaks were detected and deconvoluted automatically using Data Analysis 2.4 software (Bruker). Mass lists in the form of Mascot generic files were created automatically and used as the input for Mascot MS/MS ions

searches of the NCBI database release 20080704 using an in-house Mascot 2.2 server (Matrix Science). The default search parameters used were: taxonomy=none; enzyme=trypsin; max. missed cleavages=1; fixed modifications=carbamidomethyl (C); variable modifications=oxidation of methionines; peptide tolerance ± 1.5 Da; MS/MS tolerance ± 0.5 Da; peptide charge=2+ and 3+; instrument=ESI186 TRAP. Only sequences identified with a Mascot score above 50 were considered, and single peptide identification was systematically evaluated manually.

Lipid analysis. Mycobacterial cells were treated three times with mixtures of chloroform and methanol according to standard protocols (Constant *et al.*, 2002; Rafidinarivo *et al.*, 2009). The organic phases were pooled, dried and weighed for quantification. In order to distinguish alkali-stable lipids, e.g. phthiocerol-dimycoserates (DIM) and phenol glycolipids (PGL), from alkali-labile lipids, e.g. triacylglycerol, phospholipids and trehalose esters, aliquots of the crude lipid extracts were suspended in chloroform/methanol (2:1, v/v) and incubated at 37 °C for 60 min with an equal volume of 0.2 M NaOH solution in methanol for mild alkaline deacylation. The mixtures were neutralized with glacial acetic acid, concentrated and dissolved in CHCl₃. The organic phases were washed with water and dried. Intact and deacylated lipids were comparatively analysed by thin-layer chromatography (TLC) in various solvent systems, mainly petrol ether/diethyl ether 9:1 (v/v) for DIM and CHCl₃/CH₃OH 9:1 (v/v) for PGL. Mycolic acids were isolated from delipidated cell residues or from extractable lipids by saponification, methylated by diazomethane and their purification as α - and keto-mycolates was achieved by preparative TLC with CH₂Cl₂ as running solvent (Daffé *et al.*, 1983). Purified molecules were analysed by MALDI-TOF MS using the positive mode as described previously (Constant *et al.*, 2002; Laval *et al.*, 2001). Spectra were acquired in reflectron mode with an Applied Biosystems 4700 Analyser mass spectrometer (Applied Biosystems, Voyager DE-STR) equipped with an Nd:YAG laser (wavelength 355 nm; pulse <500 ps; repetition rate 200 Hz). A total of 2500 shots were accumulated, and MS data were acquired with the default calibration for the instrument.

Inoculation of mice. BALB.B10 mice (bred in the animal facilities of WIV-ISP-Site Ukkel) were inoculated intravenously in the tail vein with 10^6 c.f.u. WT *M. bovis* BCG, Δ *cpn60.1* or Δ *cpn60.1*Comp. Bacterial replication in spleen and lungs was monitored for 3 months by plating organ homogenates on Middlebrook 7H11-OADC plates, on days 5, 30 and 90 post-vaccination, as reported previously (Tanghe *et al.*, 2001).

Gamma interferon (IFN- γ) responses induced with WT *M. bovis* BCG, Δ *cpn60.1* and Δ *cpn60.1*Comp. Three months after BCG vaccination, antigen-specific IFN- γ production was analysed in spleen and lung cell cultures from vaccinated mice. Spleens and lungs from three mice per group were removed aseptically and homogenized by gentle disruption in a Dounce homogenizer, and cells were adjusted to 4×10^6 white blood cells ml⁻¹ in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, penicillin, streptomycin, fungizone and indomethacin (1 μ g ml⁻¹; Sigma). Cells were stimulated with BCG CF, purified protein derivative from *M. bovis*, purified recombinant Ag85A (Rv3804c), purified recombinant Ag85B (Rv1886) (all four antigens at 5 μ g ml⁻¹) or polyclonal T cell mitogen Concanavalin A (4 mg ml⁻¹) and incubated at 37 °C in round-bottom, 96-well microtitre plates in a humidified CO₂ incubator. Responses were tested on spleens from individual mice and on lung cells pooled per group. Culture supernatants were harvested after 72 h and stored frozen at -20 °C until testing.

IFN- γ ELISA. IFN- γ activity was quantified by sandwich ELISA using coating antibody R4-6A2 and biotinylated detection antibody XMGI.2 (both BD Pharmingen). The detection limit of the IFN- γ ELISA is 5 pg ml⁻¹.

***Mtb* challenge.** BCG vaccinated and naïve BALB.B10 mice were challenged 3 months after vaccination with 2×10^5 c.f.u. luminescent *Mtb* H37Rv by the intravenous route (Tanghe *et al.*, 2001). Bacterial numbers in spleen and lungs were determined by luminometry at various time points after *Mtb* challenge in a Turner design TD 20/20 luminometer as flash emission (15 s integration time) using 1% *n*-decanal (Sigma) in ethanol as substrate. In this assay, only live bacteria are enumerated because emission of light is dependent on the presence of reduced flavin mononucleotide (FMNH₂), a co-factor that is only found in living cells. For statistical analysis (one-way ANOVA, Tukey's Multiple Comparison Test), results obtained in milli relative light units (RLU) per spleen or lung were converted to log₁₀ values.

RESULTS

Disruption of *cpn60.1* and complementation

To elucidate the function of *cpn60.1* in *M. bovis* BCG, we generated an *M. bovis* BCG Δ *cpn60.1* mutant by the allelic gene exchange method (Pelicic *et al.*, 1997). The *cpn60.1* coding region was disrupted at its unique *Bsa*BI site by insertion of the kanamycin-resistance *aph* gene excised from pYUB53 (Kalpana *et al.*, 1991). The selection of mutants was performed as described previously (Peirs *et al.*, 2005). Restriction digestion of genomic DNA by *Not*I and Southern blot analysis of strain Δ *cpn60.1* revealed a fragment of about 3.2 kb that hybridized to both *cpn60.1* and *aph* probes, indicating that *cpn60.1* was interrupted by insertion of the *aph* gene (Fig. 1). 2-DE combined with MS analysis of the cellular extracts confirmed the absence of Cpn60.1 in this mutant (Fig. 1d). To complement Δ *cpn60.1*, the plasmid pIPX-*cpn60.1*C containing the *cpn60.1* gene together with its 303 bp upstream sequence was transformed into Δ *cpn60.1*, followed by selection for hygromycin resistance. The complemented strain was confirmed by PCR (data not shown) and referred to as Δ *cpn60.1*Comp. Expression of the Cpn60.1 protein by this complemented strain was analysed and confirmed by 2-DE and MS analysis (Fig. 1d).

Growth in liquid 7H9 and on solid 7H11 media

The WT BCG, Δ *cpn60.1* and Δ *cpn60.1*Comp strains were grown in 7H9 broth. Their growth curves were monitored by both measuring the OD₆₀₀ daily and plating a series of culture dilutions on 7H11 agar plates to determine c.f.u. Growth curves in 7H9 medium were similar for the three strains (Supplementary Fig. S1, available with the online version of this paper), but on 7H11 agar plates, colonies of the Δ *cpn60.1* mutant were distinctively smaller than those of the WT and the complemented strains (Fig. 2).

Biochemical characterization of bacterial cultures on Sauton medium

The strains were also cultured as surface pellicles on Sauton medium. All three strains were capable of forming a creased pellicle by 10–14 days, but the pellicle of the mutant was thinner than that of the two other strains (Supplementary Fig. S2, available with the online version of this paper). The

Fig. 1. Verification of the *cpn60.1* inactivation in *M. bovis* BCG GL2 by Southern blotting and 2-DE/MS. Genomic DNA from the WT *M. bovis* BCG and Δ *cpn60.1* strains was digested with *Nco*I, separated by electrophoresis, transferred onto nylon membranes and hybridized to the *aph* probe (a) and the *cpn60.1* probe (b) as illustrated in (c). (d) A partial region of the 2-DE of the total extracts of the three strains to illustrate spots corresponding to the proteins DnaK, Cpn60.2 and Cpn60.1 that were identified by MS.

wild-type, mutant and complemented strains had on average ($\pm$ SD) a wet weight of 5.31 ± 0.22 , 1.44 ± 0.29 and 4.16 ± 0.60 g per pellicle (each pellicle from 120 ml culture; average of nine independent experiments), respectively. Analysis of the culture filtrates revealed a higher protein content for the Δ *cpn60.1* mutant (5.96 ± 0.63 mg per pellicle) than for the WT (1.34 ± 0.09) and the complemented strains (3.01 ± 0.10). The higher protein content in the CF of the mutant was not due to protein release from lysed cells because the homeostasis of bacteria had been verified by testing the isocitrate dehydrogenase activity, an

intracellular marker, in both CF and CE (data not shown). When bacteria were treated mildly with glass beads to isolate the crude outermost cell envelope material (OCEM), the mutant was found to have a lower protein content (about 2.50 ± 0.50 mg per pellicle) in the OCEM than did the WT (4.23 ± 0.70) and the complemented strain (3.30 ± 0.25). The increased protein content in the CF of the mutant coincided with the decrease in the OCEM, suggesting that the cell wall integrity of the mutant was impaired and that some OCEM proteins were unable to properly anchor and were released into the culture media.

Fig. 2. Growth of the WT *M. bovis* BCG, Δ *cpn60.1* and Δ *cpn60.1*Comp strains on solid 7H11-OADC Middlebrook agar plates. Plates were incubated at 37 °C for 3 weeks.

2-DE/MS analysis of the mutant compared with the WT and the complemented strain

In order to know the effect of Cpn60.1 on expression of proteins in *M. bovis* BCG, we performed 2-DE combined with MS analysis on CEs and CFs of the three strains grown as a surface pellicles on Sauton medium. The 2D pattern was reproducible for all CE and CF preparations, and there was a minimal variation in relative protein amounts from sample to sample. Multiple proteins were identified by using MS (see Supplementary Table S1, available with the online version of this paper, with the respective Mb and Rv

numbers). Figs 3 and 4 show the 2D patterns of whole CEs and CFs for the three strains.

Differences observed in the cellular extract of the three strains. As reported for the *M. smegmatis* Δ cpn60.1 mutant (Ojha *et al.*, 2005), there were few changes in protein expression in the CE. In the Δ cpn60.1 mutant Cpn60.1 was completely absent (Fig. 1d) and two proteins FadA2 and isocitrate lyase (ICL) were found to be upregulated (Fig. 3). Since *M. smegmatis* Cpn60.1 modulates synthesis of mycolates of the cell wall during biofilm formation and

Fig. 3. 2-DE of the total CEs of bacteria recovered from pellicle cultures of the WT *M. bovis* BCG, Δ cpn60.1 and Δ cpn60.1Comp strains. Proteins whose expression was modified are circled. Western blot of the same CE samples (Ext) and their eluates from a Ni^{2+} -affinity chromatography (Elu) separated on SDS-PAGE using an anti-Hsp65 mAb (IA1) and an anti-KasA/KasB antibody is shown in the lower right corner.

Fig. 4. 2-DE of the CFs recovered from pellicle cultures of the WT *M. bovis* BCG, Δ cpn60.1 and Δ cpn60.1Comp strains. Gels were separated from pH 3 to 10 and the circled spots with fraction numbers (F#) were identified and analysed by MS. Amounts of the proteins such as Cpn60.2 (F126, F131), TB16.3 (F15), PPlase A (F16-17), EF-Tu (F11-13), EF-Ts (F127), TB22.2 (F62-63), β -1,3-glucanase (F47), Mpt51 (F57) and APA (F1-2) changed in the CF of the mutant compared with the two other strains.

physically associates with KasA, we analysed the spots corresponding to KasA and KasB using MS and found that there was no significant difference in expression of these proteins between the WT BCG and the Δ cpn60.1 mutant (Fig. 3). This was confirmed by Western blot of the same CE samples separated by SDS-PAGE using an anti-KasA/KasB antibody. The native Cpn60.1 contains a histidine-rich sequence at its C terminus (-DHDHHHGHAAH). If a protein forms a complex with Cpn60.1 by protein-protein

interaction, the complex can be retained onto Ni^{2+} -chelating resin via the Cpn60.1 histidine-rich sequence. When cell extracts of WT BCG were passed through a Ni^{2+} -chelating resin column, Cpn60.1, KasA and KasB proteins were recovered in the imidazole eluate. However, only a small quantity of KasB and neither KasA nor Cpn60.1 was found in the eluate of the Δ cpn60.1 mutant (Fig. 3). This suggests that Cpn60.1 of *M. bovis* BCG can interact physically with KasA, as previously observed in *M. smegmatis* (Ojha *et al.*, 2005).

Differences observed in the culture filtrate of the three strains. In contrast to the cell extracts, major differences were observed in the composition of culture filtrates (Fig. 4, Supplementary Table S1). Five proteins – Cpn60.2 (F126, F131), TB16.3 (F15), PPIase A (F16-17; iron-regulated peptidyl-prolyl *cis-trans* isomerase A), EF-Tu (F11-13; elongation factor thermo unstable) and EF-Ts (F127; elongation factor thermo stable) – were present in higher amounts in the CF of the Δ cpn60.1 mutant. Interestingly, the well-known Hsp65 chaperonin Cpn60.2 was particularly abundant in the CF of the Cpn60.1 mutant and its amount

increased about 200-fold (Fig. 5). Except for EF-Ts, these proteins have been shown to be localized at the bacterial surface (Jungblut *et al.*, 1999; Målen *et al.*, 2007; Rosenkrands *et al.*, 2000a, b). Three proteins TB22.2 (F62-63), β -1,3-glucanase (F47) and Mpt51 (F57; FbpD) were present in significantly reduced amounts while the alanine- and proline-rich protein APA (F1-2; antigen MPT-32) was absent. These four proteins are all secreted. It is noteworthy that the levels of the mycolyl-transferases Ag85A (F30-31, F52, F54), Ag85B (F38) and Ag85C (F50) were comparable in the filtrates of the three strains. Functional descriptions of

Fig. 5. (a) Quantification of Cpn60.2 in filtrates by 2-DE. Black arrows indicate Cpn60.2 spots on 2D gels, and (b) their relative intensity (mean $\pm$ SD) is presented according to the MS number of hits. (c) Identification of Cpn60.2 separated by 2-DE and analysed by peptide mass fingerprinting. Experimental peptide sequences are shown (in red) in the protein sequence and in the MALDI MS spectra. Cpn60.2 was identified by MS with a high protein Mascot score of 235.

these proteins are provided in the Supplementary Material available with the online version of this paper.

Quantification and analysis of bacterial lipids from pellicle culture

For quantification, three independent pellicle culture samples from the WT *M. bovis* BCG, Δ *cpn60.1* mutant and complemented strains were extracted with organic solvents, i.e. chloroform and methanol, to yield extractable lipids (Constant *et al.*, 2002). The comparative analysis of these free lipids by TLC showed no difference concerning the ubiquitous lipids content such as triacylglycerols, phospholipids and trehalose esters. Interestingly, the absence of phthiocerol dimycocerosates in the mutant was repeatedly observed, a phenotype fully restored in the complemented strain. The resulting delipidated cells were weighed and

saponified to liberate the mycolic acids covalently attached to the cell wall arabinogalactan (bound lipids). No significant differences in weight were observed among the three strains; extractable lipids and cell-wall-bound mycolates represented, respectively, 15–20 and 6–8 % of the cell dry masses of the different strains, values commonly found in mycobacteria.

Mycolic acids isolated by saponification from extractable lipids (free lipids) were methylated and compared with those prepared from delipidated cells (bound lipids) by TLC. Wild-type, mutant and complemented strains showed a similar profile, consisting of α - and keto-mycolates, as expected for BCG. After purification by preparative TLC, these mycolates were analysed by MALDI-TOF MS. As shown in Fig. 6 and Table 1, all the strains contained α -mycolates corresponding to mass peaks at m/z of 1146 (C_{76}), 1174 (C_{78}), 1202 (C_{80}) and 1230 (C_{82}), and keto-mycolates corresponding to peaks at m/z 1246 (C_{82}), 1274 (C_{84}), 1302 (C_{86}) and 1330 (C_{88}). Importantly, however, the ratios of major homologues of the mycolates differed according to the strains examined. For the WT strain, the major homologue of α -mycolic acids was the C_{78} -acid and the main representative of keto-mycolic acids was the C_{84} -acid. In contrast, for the Δ *cpn60.1* mutant, the main α -mycolate was the C_{80} -acid (peak at m/z 1202) and the major keto-mycolate was the C_{86} -acid (peak at m/z 1302). Thus, the α - and keto-mycolates of the mutant possessed two carbon atoms more than those of the WT. This difference was repeatedly observed, from mycolates originating from both the bound and the extractable lipids. The phenotype of the mutant was fully restored in the complemented strain. Interestingly, this structural difference in mycolic acids was observed only when bacteria were grown as surface pellicles on Sauton medium, but not in materials from the mutant cultured in 7H9 broth (data not shown).

In vitro growth under stress conditions

In order to determine whether *cpn60.1* plays a role in response to stresses, the WT and the Δ *cpn60.1* mutant strain were grown under different stress conditions. After exposure of the strains to 0.0025 % SDS for 48 h, the viability of both bacterial strains decreased (Fig. 7). The Δ *cpn60.1* mutant seemed slightly more sensitive to SDS than did the WT, but the difference was not significant according to Student's *t*-test. A similar result was obtained when bacteria were stressed with 0.005 % SDS (data not shown). WT BCG was resistant to 10 mM H_2O_2 treatment for 48 h, whereas under the same conditions, about 20 % of the mutant bacteria had died (Fig. 7), indicating that the Δ *cpn60.1* mutant is more sensitive to H_2O_2 . Neither mutant nor WT BCG was affected by the lower concentrations of 1 and 5 mM H_2O_2 . There was no difference in viability between the WT and the mutant strain under other stress conditions, including heat (42 °C) and low pH (pH 5) (data not shown).

Fig. 6. MALDI-TOF mass spectra of mycolic acid methyl esters from bacterial residues of *M. bovis* BCG strains WT, Δ *cpn60.1* and Δ *cpn60.1*Comp. Values indicate the masses of the corresponding sodium adducts $[M+Na]^+$.

In vivo replication of the WT *M. bovis* BCG, Δ cpn60.1 and Δ cpn60.1Comp strains

As shown in Fig. 8(a), the Δ cpn60.1 mutant strain was impaired in its ability to persist in the lungs *in vivo*. At days 5, 30 and 90, bacterial c.f.u. numbers were 100-fold lower than those recovered from lungs of mice vaccinated with the parental WT BCG strain. Mice vaccinated with the complemented BCG strain had similar c.f.u. numbers to mice vaccinated with the WT BCG at days 5 and 30 post-vaccination, whereas at day 90, c.f.u. numbers in lungs from mice vaccinated with the complemented strain were decreased compared with those in lungs from mice vaccinated with WT BCG, but were still higher than c.f.u. numbers found in mice vaccinated with the Δ cpn60.1 mutant strain. In spleen (Fig. 8b), 10-fold higher initial c.f.u. numbers were detected than in lungs following intravenous vaccination. As for the lungs, bacterial numbers of the Δ cpn60.1 mutant were lower than those of the WT or complemented strain, but the differences were less pronounced in spleen.

Induction of antigen-specific IFN- γ responses with the WT *M. bovis* BCG, Δ cpn60.1 and Δ cpn60.1Comp strains

Comparable mycobacteria-specific IFN- γ responses were detected in 72 h culture supernatant of spleen cells from mice vaccinated with the three BCG strains 3 months previously (Table 2). IFN- γ responses in lungs were undetectable at this time point (data not shown).

Protective efficacy of the WT *M. bovis* BCG, Δ cpn60.1 and Δ cpn60.1Comp strains against *Mtb*

The protective efficacy of the Δ cpn60.1 mutant strain was compared with that of WT BCG in mice challenged with luminescent *Mtb* H37Rv 3 months after vaccination. As shown in Fig. 9, the Δ cpn60.1 mutant BCG conferred equal protection to parental BCG at day 20, 30, 58 and 98 post-infection both in spleen (Fig. 9a) and in lungs (Fig. 9b). In

another experiment, the Δ cpn60.1 mutant conferred equal protection against *Mtb* as did the complemented and the WT BCG, as assessed at weeks 4 and 12 after *Mtb* challenge (Supplementary Fig. S3, available with the online version of this paper). A long-term survival study did not show any significant difference between mice vaccinated with the WT or Δ cpn60.1 strain either. Thus, the median survival time (MST) of unvaccinated, *Mtb*-challenged BALB.B10 mice was 20 weeks, the MST of mice vaccinated with WT BCG was 32 weeks, and the MST of mice vaccinated with Δ cpn60.1 BCG was 29 weeks.

DISCUSSION

In this study, we analysed in more detail the biochemical and immunological properties of *M. bovis* BCG Cpn60.1 (GroEL-1). Genetic inactivation of *cpn60.1* of *M. bovis* BCG did not significantly influence bacterial growth in 7H9 broth, but on solid 7H11 agar plates, its colonies were smaller than those of the WT parental BCG. Furthermore, mutant BCG formed a thinner surface pellicle on Sauton medium than did the WT or complemented BCG. Interestingly, the Δ cpn60.1 mutant was found to be devoid of phthiocerol dimycocerosates, and its mycolates were two carbon atoms longer than those of the wild-type strain, a phenotype that was fully reversed by complementation. The different lengths of mycolates were not observed in the mutant grown in 7H9 broth, suggesting that this phenotype is directly linked to the formation of a surface pellicle. Ojha *et al.* (2005) have shown that in *M. smegmatis*, Cpn60.1 modulates the synthesis of mycolates specifically during biofilm formation and physically associates with KasA, a key component of the type II fatty acid synthase involved in mycolate synthesis (Veyron-Churlet *et al.*, 2004). Affinity chromatography and Western blot analyses revealed that Cpn60.1 of BCG also physically associated with KasA. In addition, we observed that KasB also interacted with Cpn60.1, a finding not reported by Ojha *et al.* (2005). The phenomenon and mechanism(s) associated with the two

Table 1. MALDI-TOF MS data of individual types of methyl mycolates from *M. bovis* BCG strains

Values in bold represent the major homologue.

No. of C atoms of free acids*	α -Mycolate			Keto-mycolate		
	WT BCG	Δ cpn60.1	Δ cpn60.1Comp	WT BCG	Δ cpn60.1	Δ cpn60.1Comp
76	1146		1146			
78	1174	1174	1174			
80	1202	1202	1202			
82		1230		1246		1246
84				1274	1274	1274
86				1302	1302	1302
88					1330	

*Values represent the pseudomolecular masses of mycolic acid methyl esters [M + Na]⁺.

Fig. 7. Sensitivity of the Δ *cpn60.1* mutant to SDS and H₂O₂. Bacteria were grown in 7H9 Middlebrook medium to exponential phase for 7–8 days. SDS and H₂O₂ were added to the cultures at final concentrations of 0.0025% and 10 mM, respectively. c.f.u. counts were determined after 48 h of stress exposure. Results are expressed as the percent survival of the strain exposed to the stress compared to the same strain without treatment. The values reported are the mean $\pm$ SD of three independent experiments. Statistical significance is indicated.

carbon extension of the mycolates in the knock-out strain remain to be elucidated. The mycolate extension event may be linked to an association with KasB since this enzyme is responsible for the extension of mycolates to full-length (Bhatt *et al.*, 2007; Gao *et al.*, 2003). For the *Mtb* H37Rv Δ *cpn60.1* mutant, Hu *et al.* (2008) did not use Sauton medium but liquid 7H9 and solid 7H11 Middlebrook media to culture *Mtb*, and they found no differences in mycolic acid composition between the WT and the mutant, which is in accordance with our observation for the BCG Δ *cpn60.1* mutant cultured in 7H9 medium.

The BCG Δ *cpn60.1* mutant had a higher protein content in its culture filtrate, and at the same time it had fewer proteins in its OMCE compared with the two other strains, suggesting that the cell wall of the mutant is probably impaired, resulting in a less stable anchoring of certain surface proteins that are consequently released into the culture medium. This release did not result in/from the cell lysis because all the tested bacteria had similar homeostasis, and enzymic assays showed no leakage of isocitrate dehydrogenase, an intracellular enzyme. 2-DE/MS analysis revealed a number of changes in protein composition of the mutant culture filtrate (Supplementary Material). For example, Cpn60.2 (GroEL-2), which is localized on the mycobacterial surface where it facilitates efficient bacterial association with macrophages (Hickey *et al.*, 2009), was found to be particularly abundant in the CF of the Δ *cpn60.1* mutant, while it was almost absent in the CF of the WT BCG. Two elongation factors EF-Tu and EF-Ts were also present in increased amounts in the CF of the mutant. These proteins are often observed in the CF when bacteria are stressed (Jungblut *et al.*, 1999; Mollenkopf *et al.*, 1999). Our data could therefore suggest that lack of Cpn60.1 resulted in a sustained stress-like state of the BCG bacteria. In line with this hypothesis, ICL, a key enzyme of the glyoxylate pathway, was slightly more abundant in the CE of the Δ *cpn60.1* mutant. In anaerobic conditions and/or when encountering difficult environments, *Mtb* usually switch the TCA cycle to the glyoxylate metabolic pathway to conserve energy and to cope better with difficult situations (Gengenbacher *et al.*, 2010). An increase of ICL has been considered as one of the markers for the non-replicating persistence of *Mtb* (McKinney *et al.*, 2000; Muñoz-Eliás & McKinney, 2005). Our results suggest that Cpn60.1 might have a regulatory role in the expression of ICL in BCG.

Mtb is exposed to various environmental and/or physiological stresses during growth in the host. When *Mtb* resides within a granulomatous lesion during persistent stages of

Fig. 8. Bacterial numbers in lungs (a) and spleen (b) of BALB.B10 mice vaccinated intravenously with 10^6 c.f.u. WT *M. bovis* BCG (■), Δ *cpn60.1* (○) and Δ *cpn60.1*Comp (●) and analysed at days 5, 30 and 90 post-vaccination. Results were reported as log₁₀ c.f.u. per organ (mean $\pm$ SD of three mice tested per group per time point).

Table 2. IFN- γ in spleen cell cultures from BALB.B10 mice 3 months after vaccination with WT, Δ *cpn60.1* and Δ *cpn60.1Comp* BCG strains

Data are expressed as IFN- γ levels (mean $\pm$ SD) detected in 72 h culture supernatants of spleen cells of BCG-vaccinated mice ($n=3$ per group), either unstimulated (control) or stimulated with the respective mycobacterial antigens or the polyclonal T-cell mitogen Concanavalin A (ConA). Results are expressed in pg ml $^{-1}$.

Antigen	WT BCG	Δ <i>cpn60.1</i>	Δ <i>cpn60.1Comp</i>
Control	116 $\pm$ 43	64 $\pm$ 21	62 $\pm$ 26
CF-BCG	1614 $\pm$ 466	1034 $\pm$ 1215	2281 $\pm$ 1279
Purified protein derivative	27 473 $\pm$ 14 891	18 863 $\pm$ 14 776	24 309 $\pm$ 24 621
recAg85A	2722 $\pm$ 973	3440 $\pm$ 3562	5431 $\pm$ 699
recAg85B	3493 $\pm$ 2770	5250 $\pm$ 3433	7513 $\pm$ 307
ConA	49 169 $\pm$ 6460	53 242 $\pm$ 20 090	66 629 $\pm$ 3002

infection, it may encounter stresses such as hypoxia, nutrient limitation, reactive oxygen and nitrogen intermediates, low pH, alveolar surfactants etc. (Manganelli *et al.*, 2004). Here, we have shown that inactivation of *cpn60.1* rendered *M. bovis* BCG slightly more sensitive to SDS and particularly more susceptible to H₂O₂ exposure, but not to high temperature or acidic pH stress. This suggests that Cpn60.1 may have a role in resistance of BCG to bacterial surface stress and a role in intracellular survival. A number of stress regulons have been identified in mycobacteria, such as the DosR regulon in response to hypoxia, nitric oxide and dormancy (Kendall *et al.*, 2004; Roberts *et al.*, 2004; Voskuil *et al.*, 2003), the SigE/SigB regulon in response to cell surface stress (Fontán *et al.*, 2009; Manganelli *et al.*, 2001), the SigF regulon in adaptation to stationary phase, heat and oxidative stress (Hümpel *et al.*, 2010), and *sigJ* that controls sensitivity of *Mtb* to hydrogen peroxide (Hu *et al.*, 2004). Thus, *Mtb* uses a variety of two-component regulatory systems and stress-responsive sigma factors in regulating bacterial interactions with the extracellular environment to adapt to stresses (Missiakas & Raina, 1998). Another two-component regulatory system MprAB

also participates in surface stress response by regulating expression of sigma factors SigB and SigE in *Mtb* (Donà *et al.*, 2008; He *et al.*, 2006). In *Corynebacterium glutamicum*, the promoters of heat-shock protein genes *cpn60.1*, *cpn10* and *cpn60.2* are all recognized by the stress-responsive sigma factor SigH (Barreiro *et al.*, 2004). Although not formally proven, it is possible that the role BCG Cpn60.1 has in the response to H₂O₂ stress could also be SigH-dependent. Hu *et al.* (2008) found that Cpn60.1 has a role in thermotolerance in *Mtb* when tested at 55 °C. We have not tested the BCG strains at this temperature since it is not really a physiological condition. At 42 °C, we could not demonstrate a protective role of Cpn60.1 against heat stress.

The Δ *cpn60.1* mutant of *Mtb* was previously reported to grow slightly slower than the WT strain in mouse lungs and spleen in early infection, but by 12 weeks the number of organisms in these tissues were equivalent (Hu *et al.*, 2008). In our experiments, the BCG Δ *cpn60.1*, more susceptible to H₂O₂ killing, showed a reduced *in vivo* persistence, particularly in lungs when compared with the WT and

Fig. 9. Bacterial replication of luminescent *Mtb* H37Rv in spleen (a) and lungs (b) of unvaccinated BALB.B10 mice (■) or BALB.B10 mice vaccinated 3 months prior to sampling with WT *M. bovis* BCG (▲) or Δ *cpn60.1* (●) and tested on days 20, 30, 58 and 98 after intravenous infection with 2×10^5 milliRLU (corresponding to 2×10^5 c.f.u.) *Mtb*, as monitored by luminometry. Data were reported as log₁₀ milliRLU per total organ (mean $\pm$ SD of five mice tested per group per time point, except for day 98 when only two mice survived in the *Mtb* control group). Statistically significant results are indicated: *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

complemented strains. In spleen, bacteria persisted at a higher level, and differences between the mutant and WT strain were less pronounced. Although the mutant in *M. bovis* BCG persisted less effectively, the actual number of mutant bacteria present in the animals was apparently sufficient to induce a Th1 response comparable with that induced by WT BCG and mycobacteria-specific IFN- γ responses were similar following vaccination with the two BCG strains. The Δ cpn60.1 mutant also conferred equal protection against a challenge with *Mtb* as WT and complemented BCG, as indicated by similar reductions in the number of luminescent *Mtb* bacilli in spleen and lungs compared with unvaccinated mice. The comparable overall mycobacteria-specific IFN- γ response induced with the Δ cpn60.1 mutant, directed among others against the mycolyl-transferases Ag85A and Ag85B with well-known protective potential (Huygen *et al.*, 1988, 1996; Lozes *et al.*, 1997; Munk *et al.*, 1994), may explain this observation. It should also be noted that the expression of antigens Ag85A and Ag85B was comparable for both the WT BCG and the Δ cpn60.1 mutant, but that the Cpn60.2 was found remarkably more abundant in the CF of the mutant. Cpn60.2 (Hsp65) is another well-known immunodominant and protective antigen (Bonato *et al.*, 1998; Thole *et al.*, 1988). Although we did not examine Cpn60.2-specific immune responses, it is possible that they were increased in mice vaccinated with the Δ cpn60.1 mutant. This could be an additional reason why the protective potential of the mutant was not affected.

In conclusion, our results show that Cpn60.1, despite being a nonessential protein and lacking obvious vaccine potential, is necessary for the integrity of the bacterial cell wall of BCG, is involved in resistance to H₂O₂-induced stress and might have a regulatory role in the expression of ICL.

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REFERENCES

- Barreiro, C., Gonzalez-Lavado, E., Patek, M. & Martin, J. F. (2004). Transcriptional analysis of the *groES*–*groEL1*, *groEL2*, and *dnaK* genes in *Corynebacterium glutamicum*: characterization of heat-shock-induced promoters. *J Bacteriol* **186**, 4813–4817.
- Berthet, F. X., Lagranderie, M., Gounon, P., Laurent-Winter, C., Ensergueix, D., Chavarot, P., Thouron, F., Maranghi, E., Pelicic, V. & other authors (1998). Attenuation of virulence by disruption of the *Mycobacterium tuberculosis* *erp* gene. *Science* **282**, 759–762.
- Bhatt, A., Fujiwara, N., Bhatt, K., Gurucha, S. S., Kremer, L., Chen, B., Chan, J., Porcelli, S. A., Kobayashi, K. & other authors (2007). Deletion of *kasB* in *Mycobacterium tuberculosis* causes loss of acid-fastness and subclinical latent tuberculosis in immunocompetent mice. *Proc Natl Acad Sci U S A* **104**, 5157–5162.
- Bonato, V. L., Lima, V. M., Tascon, R. E., Lowrie, D. B. & Silva, C. L. (1998). Identification and characterization of protective T cells in *hsp65* DNA-vaccinated and *Mycobacterium tuberculosis*-infected mice. *Infect Immun* **66**, 169–175.
- Braig, K., Otwinowski, Z., Hegde, R., Boisvert, D. C., Joachimiak, A., Horwich, A. L. & Sigler, P. B. (1994). The crystal structure of the bacterial chaperonin GroEL at 2.8 Å. *Nature* **371**, 578–586.
- Cappello, F., Czarnecka, A. M., La Rocca, G., Di Stefano, A., Zummo, G. & Macario, A. J. (2007). Hsp60 and Hsp10 as antitumor molecular agents. *Cancer Biol Ther* **6**, 487–489.
- Cappello, F., de Macario, E. C., Marasa, L., Zummo, G. & Macario, A. J. (2008). Hsp60 expression, new locations, functions, and perspectives for cancer diagnosis and therapy. *Cancer Biol Ther* **7**, 801–809.
- Cehovin, A., Coates, A. R., Hu, Y., Riffo-Vasquez, Y., Tormay, P., Botanch, C., Altare, F. & Henderson, B. (2010). Comparison of the moonlighting actions of the two highly homologous chaperonin 60 proteins of *Mycobacterium tuberculosis*. *Infect Immun* **78**, 3196–3206.
- Cheng, M. Y., Hartl, F. U., Martin, J., Pollock, R. A., Kalousek, F., Neupert, W., Hallberg, E. M., Hallberg, R. L. & Horwich, A. L. (1989). Mitochondrial heat-shock protein Hsp60 is essential for assembly of proteins imported into yeast mitochondria. *Nature* **337**, 620–625.
- Constant, P., Perez, E., Malaga, W., Laneelle, M. A., Saurel, O., Daffé, M. & Guilhot, C. (2002). Role of the *pks15/1* gene in the biosynthesis of phenolglycolipids in the *Mycobacterium tuberculosis* complex. Evidence that all strains synthesize glycosylated p-hydroxybenzoic methyl esters and that strains devoid of phenolglycolipids harbor a frameshift mutation in the *pks15/1* gene. *J Biol Chem* **277**, 38148–38158.
- Daffé, M., Laneelle, M. A., Asselineau, C., Levy-Frebault, V. & David, H. (1983). Taxonomic value of mycobacterial fatty acids: proposal for a method of analysis. *Ann Microbiol (Paris)* **134B**, 241–256.
- De Bruyn, J., Bosmans, R., Turneer, M., Weckx, M., Nyabenda, J., Van Vooren, J. P., Falmagne, P., Wiker, H. G. & Harboe, M. (1987). Purification, partial characterization, and identification of a skin-reactive protein antigen of *Mycobacterium bovis* BCG. *Infect Immun* **55**, 245–252.
- De Bruyn, J., Soetaert, K., Buysens, P., Calonne, I., De Coene, J. L., Gallet, X., Brasseur, R., Wattiez, R., Falmagne, P. & other authors (2000). Evidence for specific and non-covalent binding of lipids to natural and recombinant *Mycobacterium bovis* BCG hsp60 proteins, and to the *Escherichia coli* homologue GroEL. *Microbiology* **146**, 1513–1524.
- Donà, V., Rodrigue, S., Dainese, E., Palu, G., Gaudreau, L., Manganelli, R. & Provvedi, R. (2008). Evidence of complex transcriptional, translational, and posttranslational regulation of the extracytoplasmic function sigma factor σ E in *Mycobacterium tuberculosis*. *J Bacteriol* **190**, 5963–5971.
- Dosanji, N. S., Rawat, M., Chung, J. H. & Av-Gay, Y. (2005). Thiol specific oxidative stress response in *Mycobacteria*. *FEMS Microbiol Lett* **249**, 87–94.
- Fayet, O., Ziegelhoffer, T. & Georgopoulos, C. (1989). The *groES* and *groEL* heat shock gene products of *Escherichia coli* are essential for bacterial growth at all temperatures. *J Bacteriol* **171**, 1379–1385.
- Fisher, M. T. (1998). GroE chaperonin-assisted folding and assembly of dodecameric glutamine synthetase. *Biochemistry* **63**, 382–398.
- Fontán, P. A., Voskuil, M. I., Gomez, M., Tan, D., Pardini, M., Manganelli, R., Fattorini, L., Schoolnik, G. K. & Smith, I. (2009). The *Mycobacterium tuberculosis* sigma factor σ B is required for full response to cell envelope stress and hypoxia in vitro, but it is dispensable for in vivo growth. *J Bacteriol* **191**, 5628–5633.
- Galamba, A., Soetaert, K., Wang, X. M., De Bruyn, J., Jacobs, P. & Content, J. (2001). Disruption of *adhC* reveals a large duplication in the *Mycobacterium smegmatis* mc(2)155 genome. *Microbiology* **147**, 3281–3294.
- Gao, L. Y., Laval, F., Lawson, E. H., Groger, R. K., Woodruff, A., Morisaki, J. H., Cox, J. S., Daffé, M. & Brown, E. J. (2003).

Requirement for *kasB* in *Mycobacterium* mycolic acid biosynthesis, cell wall impermeability and intracellular survival: implications for therapy. *Mol Microbiol* **49**, 1547–1563.

Gengenbacher, M., Rao, S. P., Pethe, K. & Dick, T. (2010). Nutrient-starved, non-replicating *Mycobacterium tuberculosis* requires respiration, ATP synthase and isocitrate lyase for maintenance of ATP homeostasis and viability. *Microbiology* **156**, 81–87.

Grimaud, R. & Toussaint, A. (1998). Assembly of both the head and tail of bacteriophage Mu is blocked in *Escherichia coli* *groEL* and *groES* mutants. *J Bacteriol* **180**, 1148–1153.

He, H., Hovey, R., Kane, J., Singh, V. & Zahrt, T. C. (2006). MprAB is a stress-responsive two-component system that directly regulates expression of sigma factors SigB and SigE in *Mycobacterium tuberculosis*. *J Bacteriol* **188**, 2134–2143.

Hendrix, R. W. & Tsui, L. (1978). Role of the host in virus assembly: cloning of the *Escherichia coli* *groE* gene and identification of its protein product. *Proc Natl Acad Sci U S A* **75**, 136–139.

Hickey, T. B., Thorson, L. M., Speert, D. P., Daffé, M. & Stokes, R. W. (2009). *Mycobacterium tuberculosis* Cpn60.2 and DnaK are located on the bacterial surface, where Cpn60.2 facilitates efficient bacterial association with macrophages. *Infect Immun* **77**, 3389–3401.

Horwich, A. L., Low, K. B., Fenton, W. A., Hirshfield, I. N. & Furtak, K. (1993). Folding in vivo of bacterial cytoplasmic proteins: role of GroEL. *Cell* **74**, 909–917.

Horwich, A. L., Farr, G. W. & Fenton, W. A. (2006). GroEL–GroES-mediated protein folding. *Chem Rev* **106**, 1917–1930.

Horwich, A. L., Fenton, W. A., Chapman, E. & Farr, G. W. (2007). Two families of chaperonin: physiology and mechanism. *Annu Rev Cell Dev Biol* **23**, 115–145.

Hu, Y., Kendall, S., Stoker, N. G. & Coates, A. R. (2004). The *Mycobacterium tuberculosis* *sigJ* gene controls sensitivity of the bacterium to hydrogen peroxide. *FEMS Microbiol Lett* **237**, 415–423.

Hu, Y., Henderson, B., Lund, P. A., Tormay, P., Ahmed, M. T., Gurcha, S. S., Besra, G. S. & Coates, A. R. (2008). A *Mycobacterium tuberculosis* mutant lacking the *groEL* homologue *cpn60.1* is viable but fails to induce an inflammatory response in animal models of infection. *Infect Immun* **76**, 1535–1546.

Huang, C. Y., Chen, C. A., Lee, C. N., Chang, M. C., Su, Y. N., Lin, Y. C., Hsieh, C. Y. & Cheng, W. F. (2007). DNA vaccine encoding heat shock protein 60 co-linked to HPV16 E6 and E7 tumor antigens generates more potent immunotherapeutic effects than respective E6 or E7 tumor antigens. *Gynecol Oncol* **107**, 404–412.

Hümpel, A., Gebhard, S., Cook, G. M. & Berney, M. (2010). The SigF regulon in *Mycobacterium smegmatis* reveals roles in adaptation to stationary phase, heat, and oxidative stress. *J Bacteriol* **192**, 2491–2502.

Huygen, K., Van Vooren, J. P., Turneer, M., Bosmans, R., Dierckx, P. & De Bruyn, J. (1988). Specific lymphoproliferation, gamma interferon production, and serum immunoglobulin G directed against a purified 32 kDa mycobacterial protein antigen (P32) in patients with active tuberculosis. *Scand J Immunol* **27**, 187–194.

Huygen, K., Content, J., Denis, O., Montgomery, D. L., Yawman, A. M., Deck, R. R., DeWitt, C. M., Orme, I. M., Baldwin, S. & other authors (1996). Immunogenicity and protective efficacy of a tuberculosis DNA vaccine. *Nat Med* **2**, 893–898.

Jungblut, P. R., Schaible, U. E., Mollenkopf, H. J., Zimny-Arndt, U., Raupach, B., Mattow, J., Halada, P., Lamer, S., Hagens, K. & Kaufmann, S. H. (1999). Comparative proteome analysis of *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG strains: towards functional genomics of microbial pathogens. *Mol Microbiol* **33**, 1103–1117.

Kalpana, G. V., Bloom, B. R. & Jacobs, W. R., Jr (1991). Insertional mutagenesis and illegitimate recombination in mycobacteria. *Proc Natl Acad Sci U S A* **88**, 5433–5437.

Kaufmann, S. H. (1990). Heat shock proteins and the immune response. *Immunol Today* **11**, 129–136.

Kaufmann, S. H., Vath, U., Thole, J. E., Van Embden, J. D. & Emmrich, F. (1987). Enumeration of T cells reactive with *Mycobacterium tuberculosis* organisms and specific for the recombinant mycobacterial 64-kDa protein. *Eur J Immunol* **17**, 351–357.

Kendall, S. L., Movahedzadeh, F., Rison, S. C., Wernisch, L., Parish, T., Duncan, K., Betts, J. C. & Stoker, N. G. (2004). The *Mycobacterium tuberculosis* *dosRS* two-component system is induced by multiple stresses. *Tuberculosis (Edinb)* **84**, 247–255.

Kong, T. H., Coates, A. R., Butcher, P. D., Hickman, C. J. & Shinnick, T. M. (1993). *Mycobacterium tuberculosis* expresses two chaperonin-60 homologs. *Proc Natl Acad Sci U S A* **90**, 2608–2612.

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

Laval, F., Laneelle, M. A., Deon, C., Monsarrat, B. & Daffé, M. (2001). Accurate molecular mass determination of mycolic acids by MALDI-TOF mass spectrometry. *Anal Chem* **73**, 4537–4544.

Leroy, B., Roupie, V., Noël-Georis, I., Rosseels, V., Walravens, K., Govaerts, M., Huygen, K. & Wattiez, R. (2007). Antigen discovery: a postgenomic approach to paratuberculosis diagnosis. *Proteomics* **7**, 1164–1176.

Lewthwaite, J. C., Coates, A. R., Tormay, P., Singh, M., Mascagni, P., Poole, S., Roberts, M., Sharp, L. & Henderson, B. (2001). *Mycobacterium tuberculosis* chaperonin 60.1 is a more potent cytokine stimulator than chaperonin 60.2 (Hsp 65) and contains a CD14-binding domain. *Infect Immun* **69**, 7349–7355.

Lozes, E., Huygen, K., Content, J., Denis, O., Montgomery, D. L., Yawman, A. M., Vandenbussche, P., Van Vooren, J. P., Drowart, A. & other authors (1997). Immunogenicity and efficacy of a tuberculosis DNA vaccine encoding the components of the secreted antigen 85 complex. *Vaccine* **15**, 830–833.

Lund, P. A. (2001). Microbial molecular chaperones. *Adv Microb Physiol* **44**, 93–140.

Mälen, H., Berven, F. S., Fladmark, K. E. & Wiker, H. G. (2007). Comprehensive analysis of exported proteins from *Mycobacterium tuberculosis* H37Rv. *Proteomics* **7**, 1702–1718.

Manganelli, R., Voskuil, M. I., Schoolnik, G. K. & Smith, I. (2001). The *Mycobacterium tuberculosis* ECF sigma factor σE : role in global gene expression and survival in macrophages. *Mol Microbiol* **41**, 423–437.

Manganelli, R., Provvedi, R., Rodrigue, S., Beaucher, J., Gaudreau, L. & Smith, I. (2004). Sigma factors and global gene regulation in *Mycobacterium tuberculosis*. *J Bacteriol* **186**, 895–902.

Mastroleo, F., Leroy, B., Van Houdt, R., s'Heeren, C., Mergeay, M., Hendrickx, L. & Wattiez, R. (2009). Shotgun proteome analysis of *Rhodospirillum rubrum* S1H: integrating data from gel-free and gel-based peptides fractionation methods. *J Proteome Res* **8**, 2530–2541.

McKinney, J. D., Höner zu Bentrop, K., Muñoz-Elías, E. J., Miczak, A., Chen, B., Chan, W. T., Swenson, D., Sacchetti, J. C., Jacobs, W. R., Jr & Russell, D. G. (2000). Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature* **406**, 735–738.

Missiakas, D. & Raina, S. (1998). The extracytoplasmic function sigma factors: role and regulation. *Mol Microbiol* **28**, 1059–1066.

Mollenkopf, H. J., Jungblut, P. R., Raupach, B., Mattow, J., Lamer, S., Zimny-Arndt, U., Schaible, U. E. & Kaufmann, S. H. (1999). A dynamic two-dimensional polyacrylamide gel electrophoresis database: the mycobacterial proteome via Internet. *Electrophoresis* **20**, 2172–2180.

- Monahan, I. M., Betts, J., Banerjee, D. K. & Butcher, P. D. (2001). Differential expression of mycobacterial proteins following phagocytosis by macrophages. *Microbiology* **147**, 459–471.
- Munk, M. E., De Bruyn, J., Gras, H. & Kaufmann, S. H. (1994). The *Mycobacterium bovis* 32-kilodalton protein antigen induces human cytotoxic T-cell responses. *Infect Immun* **62**, 726–728.
- Muñoz-Elías, E. J. & McKinney, J. D. (2005). *Mycobacterium tuberculosis* isocitrate lyases 1 and 2 are jointly required for in vivo growth and virulence. *Nat Med* **11**, 638–644.
- Noël-Georis, I., Vallaëys, T., Chauvaux, R., Monchy, S., Falmagne, P., Mergeay, M. & Wattiez, R. (2004). Global analysis of the *Ralstonia metallidurans* proteome: prelude for the large-scale study of heavy metal response. *Proteomics* **4**, 151–179.
- Ojha, A., Anand, M., Bhatt, A., Kremer, L., Jacobs, W. R., Jr & Hatfull, G. F. (2005). GroEL1: a dedicated chaperone involved in mycolic acid biosynthesis during biofilm formation in mycobacteria. *Cell* **123**, 861–873.
- Ortalo-Magné, A., Dupont, M. A., Lemassu, A., Andersen, A. B., Gounon, P. & Daffé, M. (1995). Molecular composition of the outermost capsular material of the tubercle bacillus. *Microbiology* **141**, 1609–1620.
- Peetermans, W. E., Raats, C. J., Langermans, J. A. & van Furth, R. (1994). Mycobacterial heat-shock protein 65 induces proinflammatory cytokines but does not activate human mononuclear phagocytes. *Scand J Immunol* **39**, 613–617.
- Peirs, P., Lefèvre, P., Boarbi, S., Wang, X. M., Denis, O., Braibant, M., Pethe, K., Locht, C., Huygen, K. & Content, J. (2005). *Mycobacterium tuberculosis* with disruption in genes encoding the phosphate binding proteins PstS1 and PstS2 is deficient in phosphate uptake and demonstrates reduced in vivo virulence. *Infect Immun* **73**, 1898–1902.
- Pellicic, V., Jackson, M., Reyat, J. M., Jacobs, W. R., Jr, Gicquel, B. & Guilhot, C. (1997). Efficient allelic exchange and transposon mutagenesis in *Mycobacterium tuberculosis*. *Proc Natl Acad Sci U S A* **94**, 10955–10960.
- Prakken, B. J., Roord, S., Ronaghy, A., Wauben, M., Albani, S. & van Eden, W. (2003). Heat shock protein 60 and adjuvant arthritis: a model for T cell regulation in human arthritis. *Springer Semin Immunopathol* **25**, 47–63.
- Qamra, R., Mande, S. C., Coates, A. R. & Henderson, B. (2005). The unusual chaperonins of *Mycobacterium tuberculosis*. *Tuberculosis (Edinb)* **85**, 385–394.
- Rafidinarivo, E., Laneelle, M. A., Montrozier, H., Valero-Guillen, P., Astola, J., Luquin, M., Prome, J. C. & Daffé, M. (2009). Trafficking pathways of mycolic acids: structures, origin, mechanism of formation, and storage form of mycobacteric acids. *J Lipid Res* **50**, 477–490.
- Riffo-Vasquez, Y., Spina, D., Page, C., Tormay, P., Singh, M., Henderson, B. & Coates, A. (2004). Effect of *Mycobacterium tuberculosis* chaperonins on bronchial eosinophilia and hyper-responsiveness in a murine model of allergic inflammation. *Clin Exp Allergy* **34**, 712–719.
- Roberts, D. M., Liao, R. P., Wisedchaisri, G., Hol, W. G. & Sherman, D. R. (2004). Two sensor kinases contribute to the hypoxic response of *Mycobacterium tuberculosis*. *J Biol Chem* **279**, 23082–23087.
- Rosenkrands, I., King, A., Weldingh, K., Moniatte, M., Moertz, E. & Andersen, P. (2000a). Towards the proteome of *Mycobacterium tuberculosis*. *Electrophoresis* **21**, 3740–3756.
- Rosenkrands, I., Weldingh, K., Jacobsen, S., Hansen, C. V., Florio, W., Gianetri, I. & Andersen, P. (2000b). Mapping and identification of *Mycobacterium tuberculosis* proteins by two-dimensional gel electrophoresis, microsequencing and immunodetection. *Electrophoresis* **21**, 935–948.
- Saibil, H. R. (2000). Conformational changes studied by cryo-electron microscopy. *Nat Struct Biol* **7**, 711–714.
- Sigler, P. B., Xu, Z., Rye, H. S., Burston, S. G., Fenton, W. A. & Horwich, A. L. (1998). Structure and function in GroEL-mediated protein folding. *Annu Rev Biochem* **67**, 581–608.
- Spector, T. (1978). Refinement of the coomassie blue method of protein quantitation. A simple and linear spectrophotometric assay for less than or equal to 0.5 to 50 microgram of protein. *Anal Biochem* **86**, 142–146.
- Stewart, G. R., Wernisch, L., Stabler, R., Mangan, J. A., Hinds, J., Laing, K. G., Young, D. B. & Butcher, P. D. (2002). Dissection of the heat-shock response in *Mycobacterium tuberculosis* using mutants and microarrays. *Microbiology* **148**, 3129–3138.
- Tang, Y. C., Chang, H. C., Roeben, A., Wischnewski, D., Wischnewski, N., Kerner, M. J., Hartl, F. U. & Hayer-Hartl, M. (2006). Structural features of the GroEL-GroES nano-cage required for rapid folding of encapsulated protein. *Cell* **125**, 903–914.
- Tanghe, A., D'Souza, S., Rosseels, V., Denis, O., Ottenhoff, T. H., Dalemans, W., Wheeler, C. & Huygen, K. (2001). Improved immunogenicity and protective efficacy of a tuberculosis DNA vaccine encoding Ag85 by protein boosting. *Infect Immun* **69**, 3041–3047.
- Thole, J. E., van Schooten, W. C., Keulen, W. J., Hermans, P. W., Janson, A. A., de Vries, R. R., Kolk, A. H. & van Embden, J. D. (1988). Use of recombinant antigens expressed in *Escherichia coli* K-12 to map B-cell and T-cell epitopes on the immunodominant 65-kilodalton protein of *Mycobacterium bovis* BCG. *Infect Immun* **56**, 1633–1640.
- van Eden, W., Thole, J. E., van der Zee, R., Noordzij, A., van Embden, J. D., Hensen, E. J. & Cohen, I. R. (1988). Cloning of the mycobacterial epitope recognized by T lymphocytes in adjuvant arthritis. *Nature* **331**, 171–173.
- van Eden, W., Hogervorst, E. J., van der Zee, R., van Embden, J. D., Hensen, E. J. & Cohen, I. R. (1989). The mycobacterial 65 kD heat-shock protein and autoimmune arthritis. *Rheumatol Int* **9**, 187–191.
- Veyron-Churlet, R., Guerrini, O., Mourey, L., Daffé, M. & Zerbib, D. (2004). Protein–protein interactions within the fatty acid synthase-II system of *Mycobacterium tuberculosis* are essential for mycobacterial viability. *Mol Microbiol* **54**, 1161–1172.
- Voskuil, M. I., Schnappinger, D., Visconti, K. C., Harrell, M. I., Dolganov, G. M., Sherman, D. R. & Schoolnik, G. K. (2003). Inhibition of respiration by nitric oxide induces a *Mycobacterium tuberculosis* dormancy program. *J Exp Med* **198**, 705–713.
- Xu, Z., Horwich, A. L. & Sigler, P. B. (1997). The crystal structure of the asymmetric GroEL–GroES–(ADP)₇ chaperonin complex. *Nature* **388**, 741–750.

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