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Disruption of the gene encoding for secretion antigen SA5K affects growth of *Mycobacterium bovis* bacillus Calmette-Guérin in human macrophages and in mice

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Abstract

An 8.3-kDa secretory antigen of *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), called SA5K, was previously identified and characterized in our laboratory. Sequence analysis of the BCG *sa5k* gene, including the corresponding promoter region, showed that it is identical to the homologous gene in *Mycobacterium tuberculosis* (Rv1174c). No significant homology with other proteins was found and the physiologic role of SA5K for mycobacteria remains unknown. In the present study, a BCG mutant strain (*BCGsa5k*::*aph*) was constructed by allelic exchange involving the replacement of the *sa5k* gene with a kanamycin-inactivated copy. Mutant and parental strains showed similar growth rates in liquid medium, suggesting that the loss of the *sa5k* gene does not affect the in vitro growth of BCG. Nevertheless, *BCGsa5k*::*aph* showed a reduced ability to grow in human macrophages compared with the wild-type BCG, suggesting that SA5K is involved in intracellular survival/multiplication mechanisms. The mutant strain was also attenuated in vivo in a mouse infection model, showing impaired growth/survival in spleen and liver and fewer and smaller granulomatous lesions compared to the parental strain. Complementation of the mutation restored the parental phenotype. Taken together, results presented in this study suggest a role for SA5K in the growth capacity of BCG both in an intracellular milieu and in infected mice.

Keywords: Mycobacterium bovis BCG; *Mycobacterium tuberculosis*; Gene disruption; Mutant strain; Virulence

1. Introduction

Tuberculosis (TB) continues to represent a major threat to humans and is responsible for more deaths than any other single infectious disease [26]. One of the main features of the etiological agents of TB, *Mycobacterium tuberculosis*, *M. bovis* and *M. africanum* is their ability to survive and multiply inside the phagocytic cells of the host, implementing efficient escape systems from the bactericidal mechanisms of these cells. Pathogenic mycobacteria are also able to multiply extracellularly in the open lung cavities which form during the late stages of the disease, or to spread and multiply in a wide variety of tissues and organs, as can be observed in miliary TB. Finally, pathogenic mycobacteria are able to remain quiescent for long periods of time inside the granuloma and to reactivate many years after the primary infection. This ability to adapt to different environments and growth conditions is believed to necessitate differential sets of proteins that sustain survival and multiplication in the different habitats encountered by the microorganism. In recent years, advances in the identification of such proteins have taken place because of the availability of the complete genome sequence of MTB and *M. bovis* [10,19] and the de-

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velopment of methods for mycobacterial gene transfer and disruption [9,34,41]. Several mycobacterial mutant strains carrying an inactivated copy of specific genes have been constructed and analyzed for their phenotypic characteristics under different experimental conditions, thus enabling the identification of many genes, and corresponding proteins, involved in invasion [36,43], survival [6,14,35,37,38,47] or persistence [20,32] of pathogenic mycobacteria.

A live attenuated strain of *M. bovis*, the bacillus Calmette-Guérin (BCG), is the currently available vaccine against TB. The protective efficacy of this vaccine shows a great deal of geographic variability [15,40]. Despite being administered to more than one billion people worldwide, BCG is known to cause severe infections in immunocompromised individuals [1,2,13,46,48], indicating that it is endowed with residual virulence properties. Indeed, BCG is able to replicate inside macrophages in vivo and to survive for prolonged periods in immunocompetent individuals, which is thought to be connected with its protective ability [7]. In mice, BCG does not cause a progressive infection, but is able to grow in target organs such as spleen, liver and lung and to persist in tissues long enough to allow evaluation of the effect of specific gene disruption on in vivo growth.

An 8.3-kDa protein (SA5K), secreted in culture filtrates (CFs) by BCG, MTB and a few other mycobacterial species (*M. microti*, *M. marinum* and *M. kansasii*), was previously identified and purified in our laboratory [17,18]. Sequencing of the BCG *sa5k* gene demonstrated that it is identical in BCG and MTB (gene Rv1174c in the TubercuList gene bank), for both the open reading frame and the promoter region [5]. No significant homology with any other gene product in the database was found and the protein was included in the group of MTB proteins with "unknown" functions [4,10]. Previous studies from our laboratory demonstrated that Rv1174c expression in *Mycobacterium smegmatis*, a fast growing non-pathogenic mycobacterium that does not contain the gene for the protein, was associated with a higher level of intracellular growth of recombinant *M. smegmatis* compared to the control strain, suggesting that SA5K could be involved in intracellular survival mechanisms of mycobacteria [4]. Analysis of *sa5k* gene expression by RT-PCR revealed that the protein is expressed by BCG during growth inside human macrophages, and by MTB in the lungs of TB patients [8].

Recently, it has been reported that peptides derived from the gene Rv1174c were able to stimulate the growth of MTB from a 1-year-old culture which had failed to form any colony on solid medium, suggesting that the protein may be involved in the reactivation of dormant mycobacteria [49].

In the present study, a BCG mutant strain, lacking a functional copy of the *sa5k* gene, was constructed. The effect of *sa5k* gene disruption was evaluated by comparing the growth characteristics of the mutant strain (BCG*sa5k*::*aph*) with those of the parental strain under different experimental conditions: (i) in vitro, in liquid medium; (ii) ex vivo, in human macrophages; (iii) in vivo, in the mouse model of infection. Data obtained demonstrated that, while *sa5k* gene disruption did not affect the growth of the mutant strain in liquid culture, it correlated with a reduced growth ability of BCG both inside human macrophages and in the mouse model of infection.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Escherichia coli strain JM109, used for cloning procedures, was grown on liquid or solid Luria–Bertani medium (Sigma) supplemented with ampicillin (50 μg ml^{-1}) (Sigma) or gentamicin (20 μg ml^{-1}) (Sigma).

BCG, strain Pasteur, was supplied by Pasteur Merieux. Mutant (BCG*sa5k*::*aph*), parental and complemented (BCG/34) BCG strains were grown in Sauton's modified medium or in Middlebrook 7H9 medium (Difco) as previously described [3]. CFU numbers were determined by plating 10-fold dilutions on Middlebrook 7H11 agar enriched with oleic acid, dextrose, albumin and catalase (OADC, Becton Dickinson). Kanamycin (20 μg ml^{-1}) and hygromycin (50 μg ml^{-1}) (Invitrogen) were added to the medium when required.

Analysis of in vitro growth of mutant, parental and complemented strains was performed using the BACTEC radiometric method [28]. Middlebrook 7H12 BACTEC vials were inoculated with approximately 10^5 CFU of each BCG strain. When the growth index (GI) value reached 300, each sample was diluted 100-fold and 500 μl of the bacterial suspension was re-inoculated into a new BACTEC vial. Bacterial growth was monitored by daily measurement of the GI of each culture.

2.2. PCR

Amplification reactions for cloning, sequencing and preparation of specific probes were carried out as previously described [8]. Primer sequences and the corresponding annealing temperatures are reported in Table 1.

2.3. Disruption of the sa5k gene in BCG

A 1505- and a 1120-bp fragment encompassing respectively the 5′- and 3′-*sa5k* gene termini and the corresponding flanking regions were amplified by PCR on BCG genomic DNA. The amplicons were digested by *Xho*I/*Bam*HI and *Bam*HI/*Xba*I, respectively, and the resulting fragments were cloned into the vector p-BLUESCRIPT to obtain pBLU5K. The *aph* cassette, conferring resistance to kanamycin, was excised by *Bam*HI digestion from pUC4K (Amersham Pharmacia Biotech) and cloned into the unique *Bam*HI site located within the *sa5k* gene in pBLU5K, to obtain pBLU5K-aph. A 3825-bp *Xba*I restriction fragment, containing the *sa5k*::*aph* construct, was excised from pBLU5K-aph and

Table 1
PCR primers and probes used in this study

Gene	Employment	Nucleotidic sequence (5′–3′)	Annealing temp. (°C)
sa5k	Amplification of *sa5k* and corresponding 5′-flanking region for k.o. construction	GTTATTCTAGAGATTCTGGCCGAAACCCAGG CGTTGAACTGTGCGGCAG	60
	Amplification of *sa5k* and corresponding 3′-flanking region for k.o. construction	GTTATGGATCCTCCTATTTGCGCAATTTC GTTATTCTCGAGTCTAGATGACGCCGACGCCTACT CGCT	60
	Screening of BCG transformants	CAATGTCGTTGACCGTCG ACTCGACAAGGCCGATGTAC	60
	Construction of *sa5k* probe	ATTAACACCACCTGC GAAATTGCGCAATA	50
	Amplification of *sa5k* and corresponding 5′-flanking region for sequencing	GTTATTCTAGAGATTCTGGCCGAAACCCAGG CCGACGGTCAACGACATTG	62
	Amplification of *sa5k* and corresponding 3′-flanking region for sequencing	GTTATGGATCCTCCTATTTGCGCAATTTC AACATCTGCTACACCGGTTGC	62
	Sequencing of *sa5k* and corresponding 5′-flanking region	GTTATTCTAGAGATTCTGGCCGAAACCCAGG CCGACGGTCAACGACATTG ATGCTGAGCGACCCGGACTG GAGATCTGGAGAGTGAACTGCG	60
	Sequencing of *sa5k* and corresponding 3′-flanking region	TCCTATTTGCGCAATTTCCTC AACATCTGCTACACCGGTTGC GGCCGGTTCCGAAGACG	60
aph	Construction of *aph* probe	GGAAAACAGCATTCCAGGTATTAG GTCCCGTCAAGTCAGCGTAATGC	60

cloned into *Xba*I site of pPR27 [34], kindly provided by Brigitte Gicquel, Institut Pasteur, Paris. The resulting plasmid, pPR27-5Kaph, was introduced by electroporation into BCG [34]. Transformants were plated onto 7H11 Middlebrook agar supplemented with kanamycin (20 μg ml^{-1}) and incubated at 32 °C. Several kanamycin-resistant clones were grown for 3 weeks in 7H9 Middlebrook broth supplemented with kanamycin (20 μg ml^{-1}) and then plated onto 7H11 Middlebrook agar containing kanamycin (20 μg ml^{-1}) and sucrose (2%, w/v). Plates were incubated at 39 °C for at least 3 weeks to select the kanamycin-resistant clones that were no longer sensitive to sucrose. Such clones were screened by PCR using a set of primers specific to the flanking regions of the *aph* insertion site in the *sa5k* gene (Table 1).

2.4. Southern blot analysis

One microgram of genomic DNA from wild-type and mutant BCG strains was digested with *Pvu*II, electrophoresed in a 0.8% agarose gel and transferred by capillarity onto a nylon membrane following standard procedures [39]. A 114-bp *sa5k* fragment or a 400-bp *aph* fragment were obtained by PCR on BCG genomic DNA or pUC4K plasmid, and were used as specific probes for *sa5k* and *aph* genes, respectively. Hybridization of the probes and signal detection were performed using the ECL System (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

2.5. DNA sequencing

DNA sequencing of 5′- and 3′-*sa5k* flanking regions in mutant and wild-type BCG strains was carried out in both directions using the Thermo Sequenase^{TM} Cy5 Dye Terminator Sequencing kit (Amersham Pharmacia Biotech), following the manufacturer's instruction. The sequence reactions were run on an ALFexpress automatic sequencer (Amersham Pharmacia Biotech) and sequence analysis was performed using ALFwin Sequence Analyser 2.00 program (Amersham Pharmacia Biotech).

2.6. Complementation of *BCG*sa5k::aph

An 870-bp fragment containing the *sa5k* gene, including the promoter region [18], was amplified by PCR and cloned into the *Eco*RV site of the plasmid pYUB413 [30], kindly provided by Riccardo Manganelli, University of Padova. The construct obtained, named pYUB5K, was introduced into BCG*sa5k*::*aph* by electroporation following standard procedures [25]. Transformants were selected on 7H11 Middlebrook agar supplemented with hygromycin (50 μg ml^{-1}) and hygromycin-resistant clones were analyzed for SA5K production in culture supernatants by immunoblotting.

2.7. Preparation of bacterial culture filtrates

Cultures of mutant, wild-type or complemented BCG strains were grown until mid-log growth phase in Sau-

ton's modified medium and were harvested by centrifugation. Culture supernatants were concentrated by ultrafiltration through an Amicon YM3 membrane (Amicon), dialyzed with PBS and further concentrated by low speed centrifugation under vacuum to obtain the corresponding culture filtrates (CFs) [16]. Protein concentration was determined by the method of Lowry [29].

2.8. SDS–PAGE and western blot

SDS–PAGE was performed using gels at 15% acrylamide–bisacrylamide under reducing conditions. The immunoblot was carried out using the monoclonal antibody (mAb) L8D8, specific for SA5K, as previously described [17].

2.9. Infection of human macrophages and evaluation of intracellular growth

Mononuclear cells were isolated from peripheral blood of healthy donors by centrifugation on standard density gradient (Lympholyte H, Euroclone). Cells were resuspended in RPMI 1640 supplemented with 2 mM L-glutamine and seeded in 48-well plates at a density of 1.5×10^6 cells cm^{-2}. After 1 h incubation at 37 °C, non-adherent cells were removed by repetitive washes with prewarmed RPMI. Adherent cells were cultured for 5 days at 37 °C in RPMI medium supplemented with 10% (v/v) autologous serum, 20% (v/v) FCS (Euroclone) and 2 mM L-glutamine (Invitrogen) (complete medium). For infection, wild-type, mutant and complemented BCG strains from broth cultures in logarithmic growth phase were resuspended in complete medium and added to the monolayers. Pilot experiments were carried out in order to determine the bacteria:cell ratio able to ensure the integrity of the monolayers for all the duration of the incubation period. A 1:10 multiplicity of infection (bacteria:cells) was considered optimal. Phagocytosis was allowed to occur for 3 h at 37 °C. Infected monolayers were washed repeatedly with PBS to remove uningested mycobacteria and incubated in complete medium for 6 days. Infected macrophages were lyzed by osmotic shock after 3 h of phagocytosis and 2, 4 and 6 days post-infection as previously described [4]. Cell lysates from multiple wells were pooled, serially diluted and plated on solid medium for CFU counting.

2.10. Mouse infection

Eight-week-old female BALB/c mice (Harlan-Nossan) were infected intravenously with approximately 10^6 CFU of parental, mutant or complemented BCG strains in 0.3 ml of PBS. At each time point (1, 7, 14, 21, 28, 35 days after infection), four mice per group were killed. Spleens and livers were removed aseptically and homogenized in 5 ml of PBS containing 0.05% (v/v) tween 80 in a Stomacher homogenizer (Lab-Blender 80, P.B.I. International). Serial 10-fold dilutions of organ homogenates were plated on solid medium for CFU counting.

For histological analysis, spleens and livers were recovered from animals killed at day 21 post-infection, fixed in 10% formalin and embedded in paraffin. Four micrometer sections were cut, stained with hematoxylin–eosin and examined under a light microscope with 10×, 20× and 40× magnification objective. Two pathologists, blinded to the strain being examined, separately evaluated the number and the size of the granuloma on each slide; a collection of more than 10 mononuclear cells was considered to be a granuloma [33].

2.11. Statistical analysis

Student's *t*-test was used to evaluate the results statistically. A P value <0.05 was considered significant.

3. Results

*3.1. Construction of the BCG*sa5k::aph *strain*

A mutant BCG strain, carrying a disrupted copy of the *sa5k* gene, was constructed using ts-sacB technology [34]. The *sa5k* gene was interrupted with a kanamycin-resistance selectable marker and cloned into the plasmid pPR27 [34]. The construct obtained, named pPR5K, was introduced into BCG and transformants were selected for kanamycin and sucrose resistance at 39 °C. To identify BCG clones with *sa5k*::*aph* gene disruption, kanamycin and sucrose-resistant transformants were screened by PCR with one set of primers specific for 5′- and 3′-flanking regions of the *aph* cassette insertion site in the *sa5k* gene. This set of primers would generate a 1458-bp PCR product, in the case of homologous recombination by double cross-over, or 1458- and 258-bp PCR products in the case of non-homologous recombination. PCR screening of 30 transformants revealed five mutants exhibiting the correct PCR pattern for an allelic exchange mutant. One of the mutant strains obtained (Fig. 1A) was chosen for further study and was referred to as BCG*sa5k*::*aph*.

Sa5k gene disruption in the mutant strain was verified by Southern blot analysis on genomic DNA using *aph*- and *sa5k*-specific DNA probes, respectively. DNA from the mutant strain showed a single hybridization band with the *aph*-specific probe, while no signal was detected after hybridization with the *sa5k*-specific probe, demonstrating the effective interruption of the *sa5k* gene (Fig. 1B).

In order to characterize the recombination event that occurred in the mutant strain, the 5′- and 3′-*sa5k* gene termini and the corresponding flanking regions of both BCG*sa5k*::*aph* and wild-type BCG strains were subjected to sequence analysis. The sequences obtained were identical in mutant and parental strains (data not shown). These results were consistent with a homologous recombination event by double cross-over within the *sa5k* locus.

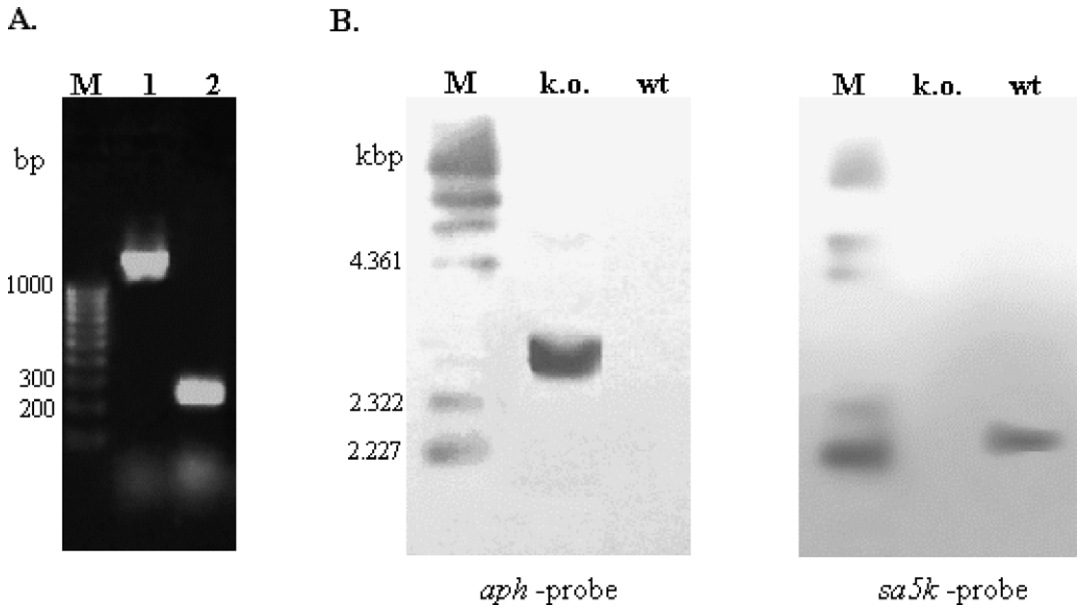

Fig. 1. Genotypic characterization of mutant strain BCG*sa5k*::*aph*. (A) PCR profiles of genomic DNA from BCG*sa5k*::*aph* (lane 1) and wild-type BCG (lane 2). Primers used for amplification are indicated in Table 1. (B) Southern blot analysis of chromosomal DNA from mutant (k.o.) and parental (wt) strains probed with *aph*- or *sa5k*-specific probes. M: molecular weight markers.

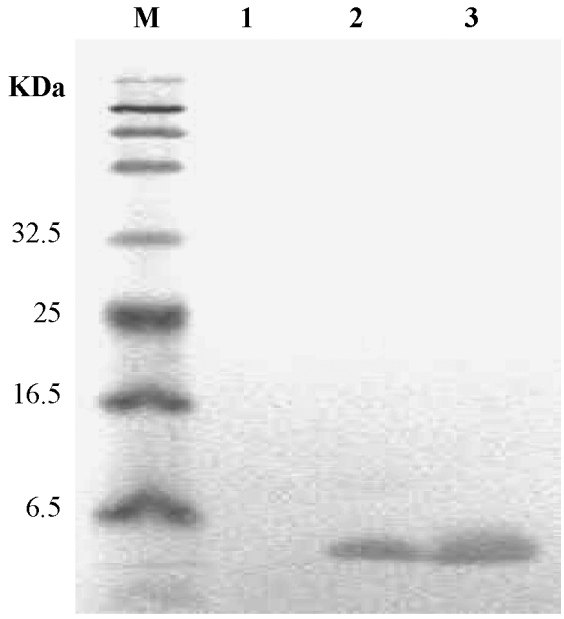

Fig. 2. Evaluation of SA5K production by mutant, complemented, and parental BCG strains. Fifteen micrograms of culture filtrates (CFs) from BCG*sa5k*::*aph* (lane 1), BCG/34 (lane 2) or wild-type BCG (lane 3) were analyzed by SDS–PAGE and western blot using SA5K-specific monoclonal antibody L8D8. M: molecular weight markers.

3.2. SDS–PAGE and western blot analysis of culture filtrates from BCG::sa5k::aph

In order to verify whether disruption of the *sa5k* gene resulted in a lack of synthesis of the corresponding protein, CFs from the mutant strain were analyzed for the presence of SA5K by SDS–PAGE and western blot with the specific mAb L8D8 [17]. As shown in Fig. 2 (lane 1), CFs from the mutant strain did not exhibit any reaction with the SA5K-specific mAb, demonstrating that the *aph* insertion had suppressed SA5K production in the culture supernatant. Also, no reaction with L8D8 was detected after SDS–PAGE and western blot analysis of total lysate of the mutant strain (data not shown), demonstrating that the *sa5k* interruption did not determine the production of an immature or truncated form of the protein. As expected, a single recognition band of approximately 5 kDa, corresponding to SA5K, was observed in CFs of wild-type BCG (Fig. 2, lane 3). Indeed, although the molecular mass of mature SA5K, calculated after signal cutting from the estimated amino acidic sequence, is 8.3 kDa, following western blotting the protein is visible as a band of 5 kDa. This seems to be due to abnormal electrophoretic mobility of the protein in SDS–PAGE that makes it appear lighter than it is [5].

3.3. Construction of the complemented strain

In order to restore SA5K production by BCG*sa5k*::*aph* a complemented strain was constructed. To this aim a wild-type copy of the *sa5k* gene, including the corresponding promoter region, was cloned into the integrative vector pYUB413. This plasmid carries the *int* gene and the *attP* site from the L5 mycobacteriophage and integrates into the mycobacterial chromosome in a single copy by site-specific recombination at the *attB* site [30]. The pYUB413 derived plasmid, pYUB5K, carrying the *sa5k* gene, was introduced in BCG*sa5k*::*aph* by electroporation and the complemented strain obtained was referred to as BCG/34. Analysis of CFs from BCG/34 by SDS–PAGE and western blot with the specific mAb L8D8 revealed a single recognition band of approximately 5 kDa (Fig. 2, lane 2), corresponding to SA5K.

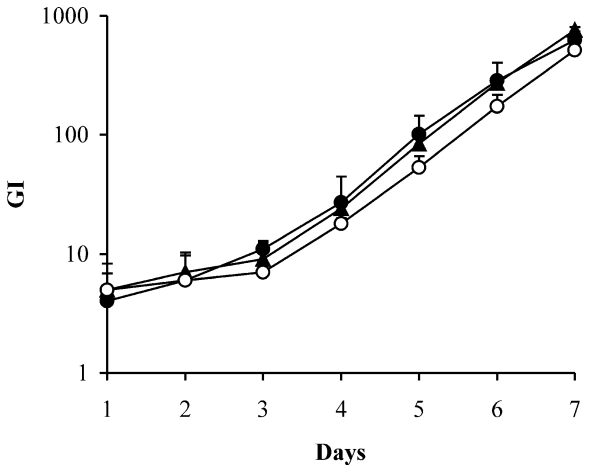

Fig. 3. Growth curves of mutant, parental and complemented strains in liquid medium. Cultures were grown in Middlebrook 7H12 B medium and the growth index (GI) was measured daily by the radiometric BACTEC system. Each point represents the means ± standard error of the means (SEM) of values obtained in three independent experiments. (●): BCG*sa5k*::*aph* mutant strain; (▲): wild-type BCG strain; (○) BCG/34 complemented strain.

Such results indicate that the integration of the *sa5k* gene at the *attB* site of the mutant strain restored the production of the corresponding protein.

3.4. Effect of sa5k *gene disruption on growth of BCG in liquid medium*

The growth characteristics of mutant, parental and complemented strains were compared using the BACTEC radiometric system. Each of the three strains was harvested from exponentially growing cultures and inoculated into 7H12 BACTEC vials as described in Section 2. Bacterial growth was monitored by daily measurements of GI of the cultures. As depicted in Fig. 3, the three strains exhibited very similar growth kinetics in vitro, indicating that disruption of the *sa5k* gene did not affect the growth of BCG in liquid medium. Similar results were obtained when the growth of mutant and parental strains in liquid medium was compared by daily measurement of the OD of the cultures (data not shown).

3.5. Effect of sa5k *gene disruption on growth of BCG in monocyte-derived human macrophages*

In order to evaluate whether the *sa5k* gene was involved in growth within phagocytic cells, the growth of BCG*sa5k*::*aph* in human macrophages was compared to that of wild-type and complemented strains, respectively. Human monocyte-derived macrophages were obtained from peripheral blood of healthy donors and infected with each of the three strains at a multiplicity of infection of 1:10 (bacteria:cells). After 3 h of phagocytosis and at days 2, 4, and 6 post-infection the number of viable intracellular bacteria was evaluated by plating macrophage lysates onto solid medium and by counting CFUs after 3 weeks of incubation at 37 °C. After 3 h phagocytosis, similar CFU numbers of mutant, wild-type and complemented BCG strains were recovered from infected cells ($4.06 \times 10^3 \pm 7.6 \times 10^2$, $4.44 \times 10^3 \pm 8.3 \times 10^2$ and $3.4 \times 10^3 \pm 1 \times 10^2$, respectively; $P > 0.05$), indicating that *sa5k* disruption did not influence the ability of BCG to invade human macrophages. In contrast, the CFU number of intracellular BCG*sa5k*::*aph* was statistically lower than that of wild-type BCG at each time point tested post-infection, demonstrating impaired ability of the mutant strain to grow/survive inside human macrophages (Fig. 4). The intracellular growth kinetics of the complemented strain was similar to that observed for wild-type BCG (Fig. 4), indicating that the mutant phenotype was indeed dependent on the mutation in the *sa5k* gene and was not due to a polar effect on the expression of neighboring genes.

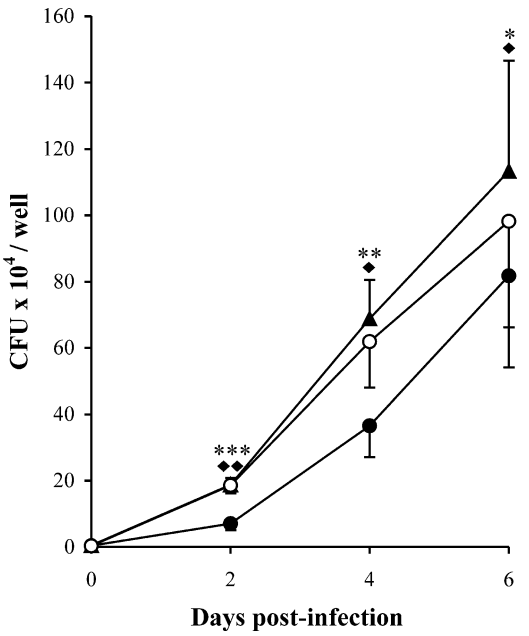

Fig. 4. Intracellular growth of BCG strains in monocyte-derived human macrophages. Macrophages were infected at a multiplicity of infection of 1:10 (bacteria:cells). After 3 h phagocytosis (time 0) and at 2, 4 and 6 days post-infection monolayers were lysed and plated for mycobacterial CFU counts on solid medium. Intracellular growth kinetics of wild-type BCG (▲), BCG*sa5k*::*aph* (●) and BCG/34 (○). Data represent the means ± standard error of the means (SEM) of values obtained from five different donors in five independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, BCG wild-type versus BCG*sa5k*::*aph*; ♦ $P < 0.05$; ♦♦ $P < 0.01$, BCG/34 versus BCG*sa5k*::*aph*, Student's *t*-test for paired samples.

3.6. Effect of sa5k *gene disruption on growth of BCG in mice*

In order to investigate the effect of *sa5k* gene disruption on the growth of BCG in a high-dose animal infection model, BALB/c mice were infected intravenously with approximately 10^6 CFUs of mutant, parental and complemented strains, respectively. Mice were killed at different time points and CFU numbers of each strain were deter-

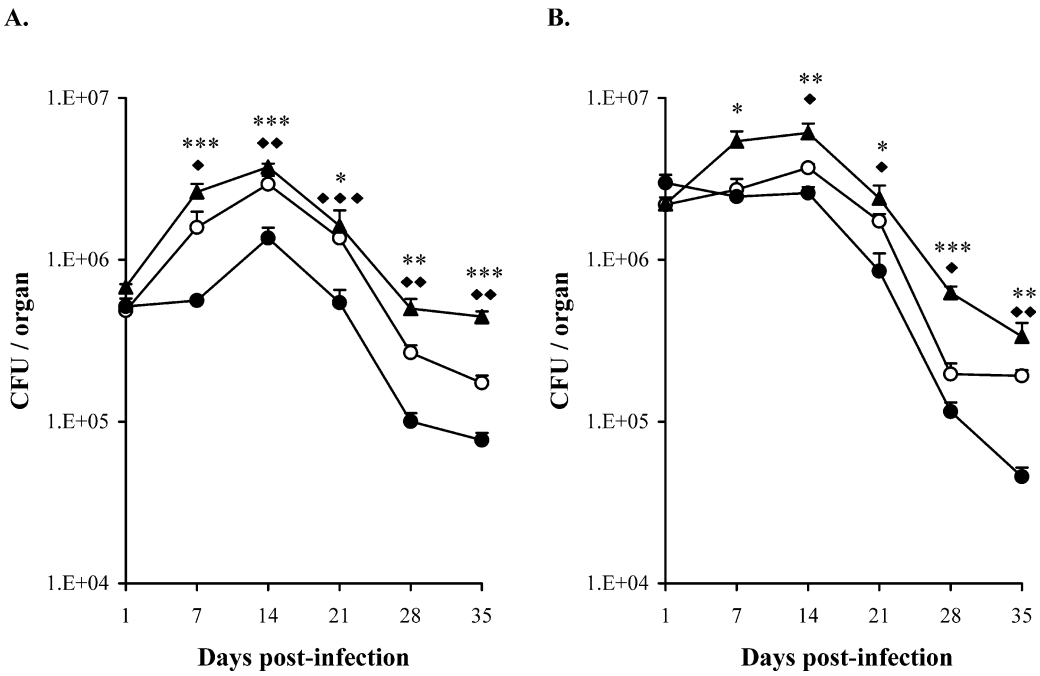

Fig. 5. Survival of BCG strains in mice. BALB/c mice were infected intravenously with approximately 10^6 CFUs of mutant (●), parental (▲) and complemented (○) BCG strains and the CFU numbers of bacteria in spleen (A) and liver (B) were determined over a period of 5 weeks. Each point represents the means + standard error of the means (SEM) of CFU values obtained from four different mice. CFU numbers were compared by Student's *t*-test for independent samples; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, BCG wild-type versus BCG*sa5k*::*aph*; $^{\blacklozenge}P < 0.05$; $^{\blacklozenge\blacklozenge}P < 0.01$; $^{\blacklozenge\blacklozenge\blacklozenge}P < 0.001$, BCG/34 versus BCG*sa5k*::*aph*. The experiment was carried out twice on different occasions and the results obtained were similar.

mined in spleen and liver (Fig. 5), as these are the organs predominantly colonized after an intravenous infection. On day 1 post-infection, similar CFU numbers of mutant, parental and complemented strains were recovered from each of the two organs analyzed.

From day 1 to day 14 post-infection the parental BCG strain showed a small increase in CFUs in spleen and liver (0.6-log and 0.3-log, respectively), while starting from the third week of infection it began to decrease gradually in both organs. The mutant strain showed impaired growth ability in the spleen and no replication in the liver during the first 2 weeks of infection. At each time point tested, the bacterial burden was statistically lower in the spleens and livers of mice infected with BCG*sa5k*::*aph* compared with the wild-type BCG (Fig. 5). Integration of a single copy of *sa5k* gene in the mutant strain restored its growth ability in infected organs almost completely, and the differences in the CFU number obtained from mice infected with the complemented strain and the mutant strain remained significant both in the spleen (at all the time point tested) and in liver (at days 14, 21, 28, 35).

3.7. Histological analyses of infected organs

Histological analyses were carried out to evaluate the lesions (number and size of granulomas) produced by mutant and parental strains in the organs following infection. Sections from spleens and livers of infected mice were stained with hematoxylin-eosin and examined under a light microscope. As depicted in Fig. 6 for the liver, many enlarging and coalescing granulomatous lesions were observed in organs of mice infected with parental strain. In contrast, smaller, less abundant granulomas and reduced tissue damage were observed in animals infected by the mutant strain. Thus, the differential bacterial burden was reflected in the pathology of the mice.

4. Discussion

At present, the construction of mutant strains is one of the most rational approaches to determining whether a particular bacterial gene is involved or not in virulence. In order to investigate the role of SA5K in growth of BCG, in the present study, a BCG mutant strain (BCG*sa5k*::*aph*) was constructed by allelic exchange involving the replacement of the *sa5k* gene with a kanamycin-inactivated copy.

Analysis of the growth characteristics of mutant and parental BCG strains in liquid culture revealed that they exhibit similar growth kinetics, demonstrating that *sa5k* gene disruption does not affect the in vitro growth of BCG.

The ability of pathogenic mycobacteria to enter and grow in phagocytic cells (or other host cell types) is considered a virulence factor that enables the microorganism to reach the intracellular environment where it has evolved efficient mechanisms to survive and replicate despite the microbicidal effector functions of macrophages. A number of proteins promoting invasion and/or multiplication of

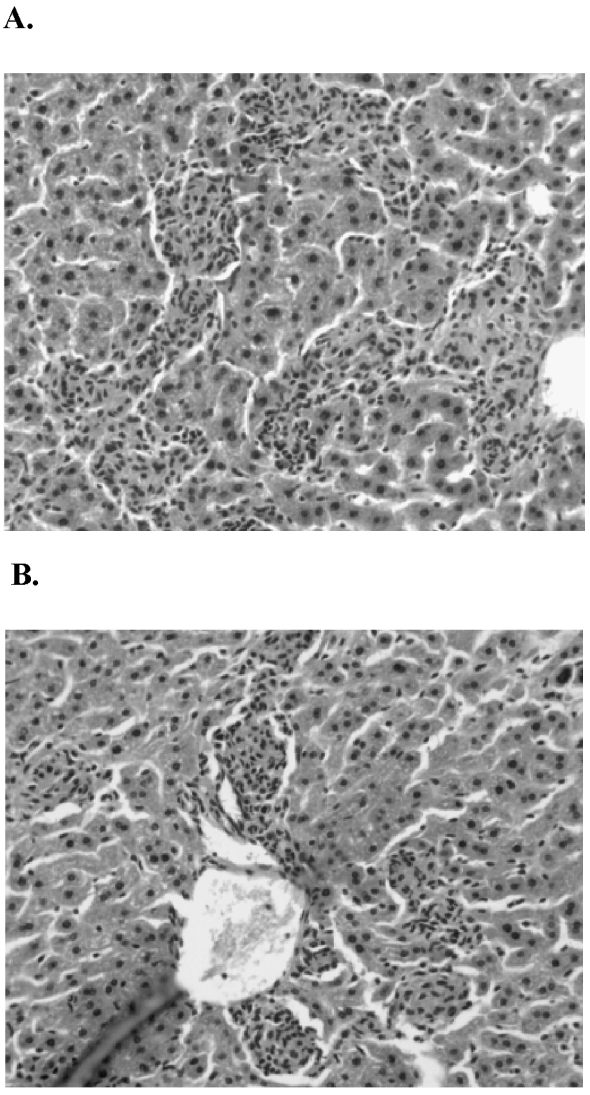

Fig. 6. Tissue sections from livers harvested on day 21 from mice infected with wild-type BCG (A) or BCG*sa5k*::*aph* (B). The sections were stained with hematoxylin–eosin and observed under a light microscope (×20).

MTB and/or BCG in host cells have been identified by using mutant strains with specific gene disruption [31,38,44]. To investigate whether SA5K had a role during intracellular growth, the abilities of mutant, parental and complemented strains to invade and replicate in phagocytic cells were compared after infection of macrophages derived from peripheral blood monocytes of healthy donors. In fact, although usually harder to obtain and more variable than monocytic cell lines, primary macrophages are considered more representative of the actual in vivo condition [44]. Mutant, parental and complemented strains showed a similar ability to enter human macrophages, indicating that SA5K is not involved in macrophage invasion mechanisms of BCG. In contrast, BCG*sa5k*::*aph* showed an impaired intracellular growth ability compared to the parental and complemented strains as demonstrated by the significantly lower number of viable bacteria recovered by macrophage lysates at all the time points tested. Major differences, attributable to different genetic backgrounds, have been observed among human donors in the ability of macrophages to contain the intracellular growth of MTB/BCG [21,23]. Although a variability in the CFU number of the intracellular BCG parental strain among different donors was also observed in the present study, the reduced ability of the mutant strain to grow/survive inside human macrophages was a consistent finding in all the subjects analyzed. Such results support previous data from our laboratory obtained using the fast-growing non-pathogenic *M. smegmatis* as a model [4]. In that study it was demonstrated that insertion of a multicopy plasmid expressing *sa5k* gene from its own promoter in *M. smegmatis* resulted in enhanced growth of the microorganism in the human monocytic cell line THP-1 as compared to the control strain (*M. smegmatis* recombinant for the cloning vector alone). Altogether, the results obtained herein and previously demonstrate a role for SA5K in intracellular growth/survival mechanisms of BCG.

The bacterial load in the organs during an experimental/natural infection represents another important parameter that is usually associated with virulence, as it reflects the ability of different bacterial strains to survive host responses [22]. In the present study, the BCG*sa5k*::*aph* strain showed an attenuated phenotype also in BALB/c mice, although disruption of the *sa5k* gene did not completely abolish its ability to replicate, at least, in the spleen. Attenuation of the mutant strain was particularly evident during the first 2 weeks of infection when it exhibited impaired replication compared to wild-type BCG in the spleen and no multiplication in the liver. From day 14, the number of viable bacilli decreased gradually in the two organs analyzed and mutant and parental BCG strains were eliminated with similar rate efficiency. These results suggest a role for SA5K during the early stages of the interaction of the infectious microorganism with phagocytic cells of the host.

To identify genes essential for growth of MTB in vivo, Sassetti and Rubin recently used a library of 100 000 transposon mutants in MTB H37Rv to infect mice intravenously [41,42]. The surviving bacteria were recovered from the spleens (in vivo pools), and mutants that were underrepresented, relative to an in vitro-grown pool, were identified by a microarray-based technique called “transposon site hybridization (TraSH)”. Using this method, the Rv1174c gene was not found to be essential for growth of MTB in the spleen [42]. However, highly stringent criteria were used in the study to avoid “false-positives” (i.e., genes underrepresented, but not truly involved in growth of MTB in vivo). As suggested by the authors, adoption of such criteria may have excluded mutants with subtle growth defects in vivo, as may be the case for the clone carrying a mutated copy of the Rv1174c gene. In fact, according to the data obtained in the present study, interruption of the Rv1174c-homologous in BCG did not completely abolish the ability of the mutant strain to replicate in vivo and in hu-

man macrophages, indicating that inactivation of this gene results in a subtle, although measurable, defect in growth. In addition, in a mouse infection model, several genes (e.g., *purC*, *sigE*, *phoP*, and *ompA*) previously demonstrated to be involved in the virulence of MTB, when comparing CFUs of the wild-type and the corresponding mutant strain [24,31, 35,37,44], were found not to be essential for growth in vivo according to criteria adopted in TraSH screening [42].

It has been proposed that evaluation of bacterial load in the organs during infection is not the only indicator of mycobacterial virulence in vivo [22] and that, for comprehensive characterization of a mutant strain, histological analyses should also be performed. Indeed, MTB mutant strains have been described that showed no difference in the bacterial burden in infected mice compared with that of the wild-type strain, yet they exhibited an attenuated phenotype evaluated as tissue damage in the organs [27,45]. In the present study, histological analyses revealed smaller and less abundant granulomatous lesions in organs from mice infected with BCG*sa5k*::*aph* compared with those obtained by wild-type BCG, further confirming the attenuation of virulence of the mutant strain.

Previous studies by ourselves and others have demonstrated that a recombinant form of the SA5K protein is able to induce proliferation and cytokine production by peripheral blood lymphocytes of healthy sensitized donors [5,11]. In addition, immunization of mice with the same antigen, encoded by plasmid DNA or formulated with IFA, elicits strong $CD4^+$ T cell and CD8 CTL responses and induces protection on challenge with virulent MTB [12]. Besides being immunologically important, the results presented here demonstrate that SA5K may also contribute to the virulence properties of BCG. Although, at present, the mechanism of action of SA5K remains unknown, it might be argued that the protein is involved in promoting growth in an intracellular environment. The observation that peptides derived from the MTB Rv1174c gene product (identical to BCG SA5K) are involved in reactivation of aged cultures [49] may support the hypothesis that the protein promotes mycobacterial growth under stress conditions. Alternatively, SA5K could have a role in conferring resistance to intracellular killing. Experiments are in progress to compare the survival of mutant and wild-type BCG strains following exposure to different stress conditions encountered during growth inside macrophages.

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