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Novel Feature of *Mycobacterium avium* subsp. *paratuberculosis*, Highlighted by Characterization of the Heparin-Binding Hemagglutinin Adhesin

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Mycobacterium avium subsp. *paratuberculosis* comprises two genotypically defined groups, known as the cattle (C) and sheep (S) groups. Recent studies have reported phenotypic differences between *M. avium* subsp. *paratuberculosis* groups C and S, including growth rates, infectivity for macrophages, and iron metabolism. In this study, we investigated the genotypes and biological properties of the virulence factor heparin-binding hemagglutinin adhesin (HBHA) for both groups. In *Mycobacterium tuberculosis*, HBHA is a major adhesin involved in mycobacterium-host interactions and extrapulmonary dissemination of infection. To investigate HBHA in *M. avium* subsp. *paratuberculosis*, we studied *hbhA* polymorphisms by fragment analysis using the GeneMapper technology across a large collection of isolates genotyped by mycobacterial interspersed repetitive-unit-variable-number tandem-repeat (MIRU-VNTR) and IS900 restriction fragment length polymorphism (RFLP-IS900) analyses. Furthermore, we analyzed the structure-function relationships of recombinant HBHA proteins of types C and S by heparin-Sepharose chromatography and surface plasmon resonance (SPR) analyses. *In silico* analysis revealed two forms of HBHA, corresponding to the prototype genomes for the C and S types of *M. avium* subsp. *paratuberculosis*. This observation was confirmed using GeneMapper on 85 *M. avium* subsp. *paratuberculosis* strains, including 67 strains of type C and 18 strains of type S. We found that HBHAs from all type C strains contain a short C-terminal domain, while those of type S present a long C-terminal domain, similar to that produced by *Mycobacterium avium* subsp. *avium*. The purification of recombinant HBHA from *M. avium* subsp. *paratuberculosis* of both types by heparin-Sepharose chromatography highlighted a correlation between their affinities for heparin and the lengths of their C-terminal domains, which was confirmed by SPR analysis. Thus, types C and S of *M. avium* subsp. *paratuberculosis* may be distinguished by the types of HBHA they produce, which differ in size and adherence properties, thereby providing new evidence that strengthens the genotypic differences between the C and S types of *M. avium* subsp. *paratuberculosis*.

Genomic studies have revealed that *Mycobacterium avium* subsp. *paratuberculosis*, the etiologic agent of Johne's disease, or paratuberculosis, has evolved into two distinct lineages related to host specificity. These are referred to as the cattle (C) type and the sheep (S) type (1, 2) and can be consistently distinguished by different genotyping tools available for *M. avium* subsp. *paratuberculosis* (3, 4). In addition to their genomic diversity (5–8), strains belonging to these two lineages exhibit phenotypic differences, including differences in pigmentation, growth rate (9–11), iron metabolic pathways utilized (12), cytokine induction profiles (13), and transcriptional profiles in a human macrophage model (14).

Since the very low growth rate of type S strains is a limiting factor for their characterization, their pathogenicity remains poorly understood. We can assume that the mechanisms of infection for types S and C have common traits, since both can cause paratuberculosis across different ruminant species. Indeed, despite the observed host preferences, bovine paratuberculosis may be due to S strains, and ovine paratuberculosis may be due to C strains (15). By use of a type C strain, it has been established that *M. avium* subsp. *paratuberculosis* crosses the intestinal barrier through the M cells or epithelial cells present in the dome of Peyer's patches (16–18). During these early events in infection,

bacterial adhesins must thus play a crucial role. One of the best-characterized mycobacterial adhesins is the heparin-binding hemagglutinin (HBHA), initially identified in *Mycobacterium tuberculosis* and *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) (19, 20). However, HBHA is also present in many other mycobacteria, both pathogenic and nonpathogenic (21–24).

HBHA is located on the surface of the mycobacterium and has been shown to mediate the binding of the bacillus to epithelial cells and fibroblasts (20). It interacts with sulfated glycoconjugates present on the surfaces of host cells (25). It also plays a role in the dissemination of *M. tuberculosis* from the lungs to deeper tissues (26). Probably due to its cellular location and its role in extrapul-

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TABLE 1 Bacterial strains

Strain(s) ^a	Genotype and/or description	Reference or source
<i>M. avium</i>		
<i>M. avium</i> subsp. <i>paratuberculosis</i> (n = 85)		
K-10 (ATCC BAA-968)	Bovine isolate, C type	45
ATCC 19698 ^T	Bovine isolate, C type	46
85/14	Ovine isolate, S type	2
LN20	Porcine isolate, S type	2
Panel of 65 clinical isolates	C type, including various genotypes	3
Panel of 16 clinical isolates	S type, including various genotypes	3
<i>M. avium</i> subsp. <i>avium</i> (n = 3)		
ATCC 25291 ^T	Bird isolate	46
ST18	Bird isolate	46
1999.05332	Bird isolate	46
<i>M. avium</i> subsp. <i>silvaticum</i> 6409 (ATCC 49884 ^T) (n = 1)	Wood pigeon isolate	47
<i>M. avium</i> subsp. <i>hominissuis</i> (n = 55)		
104	Human isolate	46
Panel of 54 clinical isolates	Various genotypes	34
<i>M. tuberculosis</i> H37Rv	Human isolate	48
<i>M. bovis</i> BCG	Vaccine strain	WHO
<i>M. smegmatis</i> mc ² 155 (ATCC 00084)	Lab strain	
<i>E. coli</i> strains		
TOP10	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>recA1</i> <i>araD139</i> Δ(<i>ara leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> (Str ^r) <i>endA1</i> <i>nupG</i>	Invitrogen
BL21(DE3)	F ⁻ <i>ompT</i> <i>hdsS</i> _B (r _B ⁻ m _B ⁻) <i>gal dcm</i> (DE3)	Invitrogen

^a A superscript "T" indicates a type strain.

monary dissemination, HBHA has shown promise as a diagnostic target for the detection of latent tuberculosis in humans (27, 28).

In this study, we identified and characterized the biochemical features of HBHA proteins produced by *M. avium* subsp. *paratuberculosis* strains of type C and type S. We provide evidence that these major lineages of *M. avium* subsp. *paratuberculosis* produce HBHA forms that differ in structure and activity. These findings add new information on the evolution of *M. avium* subsp. *paratuberculosis* and suggest that this adhesin is an adaptation factor for the mycobacteria.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and DNA manipulations. The bacterial strains and plasmids used in this study are listed in Tables 1 and 2. Mycobacterial strains were grown at 37°C in Sauton medium or Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI) with 0.2% glycerol and albumin-dextrose-catalase enrichment medium (ADC; Becton Dickinson, Le Pont-de-Claix, France). *M. avium* subsp. *paratuberculosis* cultures were supplemented with 2 mg liter⁻¹ mycobactin J (Allied Monitor). Bacteria were harvested at mid-log phase and were kept frozen (−80°C) in aliquots until use. *Escherichia coli* TOP10 (Invitrogen, Carlsbad, CA) and *E. coli* BL21(DE3) (Novagen, Darmstadt, Germany) were grown in LB medium supplemented with 25 μg ml⁻¹ kanamycin or 100 μg ml⁻¹ ampicillin as appropriate. Restriction enzymes, T4 DNA ligase, and other molecular biology reagents were purchased from New England BioLabs, Roche, or Promega. PCRs were performed using a Techne TC-512 thermal cycler, and the PCR products were sequenced by Genome Express (Takeley, United Kingdom).

Fluorescent DNA fragment analysis. The *hbhA* gene polymorphism was investigated using a comprehensive collection of strains of the species *Mycobacterium avium*. The sizes of the PCR products corresponding to the region of the *hbhA* gene coding for the C-terminal domain of the protein were determined after the amplification of genomic DNA from 67

TABLE 2 Plasmids

Plasmid(s)	Description ^a	Reference or source
Original plasmids		
pCR2.1-TOPO	<i>lacZα</i> gene, T7 and Sp6 promoters; Amp ^r Kan ^r	Invitrogen
pET vectors	T7 promoter, His tag coding sequence, <i>lacI</i> gene; Kan ^r [pET-24d(+)] and pET-24a(+)] or Amp ^r [pET-22b(+)]	Novagen
Resulting plasmids		
pET::mapS-HBHA	pET-22b(+): <i>hbhA</i> <i>M. avium</i> subsp. <i>paratuberculosis</i> type S (Amp ^r)	This study
pET::mapS-HBHAΔCter	pET-24a(+): <i>hbhA</i> <i>M. avium</i> subsp. <i>paratuberculosis</i> type S with C-terminal deletion (aa 1–157) and N-terminal His tag (Kan ^r)	This study
pET::mapLN20-HBHA	pET-22b(+): <i>hbhA</i> <i>M. avium</i> subsp. <i>paratuberculosis</i> LN20 (Amp ^r)	This study
pET::BCG HBHA	pET-24d(+): <i>hbhA</i> <i>M. bovis</i> BCG (Kan ^r)	This study
pET::Sm-HBHA	pET-24d(+): <i>hbhA</i> <i>M. smegmatis</i> mc ² 155 (Kan ^r)	21
pET::maa-HBHA	pET-24d(+): <i>hbhA</i> <i>M. avium</i> subsp. <i>avium</i> 25291 (Kan ^r)	This study
pET::mapC-HBHA	pET-24d(+): <i>hbhA</i> <i>M. avium</i> subsp. <i>paratuberculosis</i> type C 19698 (Kan ^r)	This study

^a Antibiotic resistance is shown in parentheses (Kan, kanamycin; Tet, tetracycline; Amp, ampicillin; Str, streptomycin).

TABLE 3 List of primers used in this study^a

Name	Target ^b	Sequence ^c	Restriction site
P1	mapS HBHA-F	ATATACATATGGCGGAAAAACCCG	NdeI
P2	mapS HBHA-R	ATATAAGCTTTTCACTTCTGGGTGACC	HindIII
P3	HBHA-F	TATACATATGACCATGGCGGAAAAACCCGAACATCG	NdeI
P4	HBHA-R	ATATAAGCTTGGTACCCACGAGGTGGTTACGCC	HindIII
P5	HBHA ΔCT-F	ATATCATATGCACCACCACCACCACATGGCGGAAAAACCCG	NdeI
P6	HBHA ΔCT-R	ATATAAGCTTCTAGATGCCACCAGCTTGGC	HindIII
P7	5' FAM-HBHA CT-F	TCGCTCGCAGACCCGCGCGGTGCG	
P8	HBHA CT-R	CTACCTACTTCTGGGTGACCTTCTTGGC	
P9	HBHA CT-F	TATAGAATTCCGCCAAGCTGGTGGGCATCGAGCTGCCG	EcoRI

^a Primers were designed on the basis of conserved *hbhA* sequences of *Mycobacterium avium* subsp. *avium*, *Mycobacterium avium* subsp. *paratuberculosis* (types S and C), *Mycobacterium avium* subsp. *hominissuis*, *Mycobacterium tuberculosis*, and *Mycobacterium bovis* BCG.

^b F, forward; R, reverse; ΔCT, deletion of the C-terminal domain; FAM, the fluorophore 6-carboxyfluorescein.

^c Underlining indicates restriction sites.

M. avium subsp. *paratuberculosis* type C strains, 18 *M. avium* subsp. *paratuberculosis* type S strains, including strain LN20, 55 *Mycobacterium avium* subsp. *hominissuis* strains, and 3 *Mycobacterium avium* subsp. *avium* strains selected according to their genotypes (data not shown). The regions of the *hbhA* genes coding for the C-terminal domains of the proteins were amplified with primer P7, labeled at its 5' end with a 6-carboxy-fluorescein (FAM) fluorophore, and primer P8 (Table 3) according to the manufacturer's recommendations (Applied Biosystems, USA). The fragments were amplified after a denaturation cycle of 5 min at 94°C by using only 23 cycles of 94°C for 30 s, 61°C for 30 s, and 72°C for 30 s, followed by a final elongation cycle at 72°C for 5 min. The PCR products were mixed with Hi-Di formamide and GeneScan 600 LIZ size standard (Applied Biosystems, USA). The results were visualized using ABI GeneMapper software, version 4.0 (Applied Biosystems, USA).

Plasmid constructions, sequencing, and production of recombinant HBHA (rHBHA) in *E. coli*. The *hbhA* genes were amplified by PCR with synthetic oligonucleotides P1, P2, P3, and P4 (Sigma) (Table 3) from chromosomal DNA by using *Pfu* DNA polymerase (Promega). Chromosomal DNA was prepared from cultures of *M. avium* subsp. *avium* strain 25291, *M. avium* subsp. *paratuberculosis* type C strains K-10 and ATCC 19698, *M. avium* subsp. *paratuberculosis* type S strains 19I, 269OV, M71/03, 21I, 85-14, and LN20, *M. bovis* BCG, and *Mycobacterium smegmatis* mc²155 (Table 1; also data not shown).

The fragments were amplified after a short denaturation cycle of 3 min at 95°C by using 35 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 30 s, followed by a final elongation cycle at 72°C for 10 min. The PCR products containing the HBHA-coding sequences, including the stop codons, were digested by NdeI and HindIII and were then inserted into pET-24a(+) (Novagen). These plasmids, listed in Table 2, were used to transform *E. coli* TOP10 for sequencing and *E. coli* BL21(DE3) for recombinant protein production. After transformation, *E. coli* BL21(DE3) cells were grown at 37°C in 250 ml LB broth supplemented with 25 μg ml⁻¹ kanamycin. At an optical density at 600 nm (OD₆₀₀) of 0.5, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and growth was continued for 4 h. The cultures were then centrifuged at 7,000 × g for 15 min at 4°C. The cells were washed twice in 20 ml of 20 mM Tris-HCl (pH 7.2)–0.05% Tween 80 and were centrifuged at 13,000 × g for 20 min at 4°C. The pellets were resuspended with 15 ml of 20 mM Tris-HCl (pH 7.2)–0.05% Tween 80 and were sonicated twice for 5 min at 4°C using a Branson sonifier, model S-250D. The bacterial lysates were then centrifuged at 13,000 × g for 20 min at 4°C, filtered (pore size, 0.45 μm), and subjected to heparin-Sepharose chromatography as described below.

The gene coding for a truncated HBHA molecule, called mapS-HBHAΔCter, was generated by PCR using chromosomal DNA of *M. avium* subsp. *paratuberculosis* type S strain 19I and oligonucleotides P5 and P6 (described in Table 3), designed to produce a hybrid protein carrying a His tag and lacking amino acids 158 to 205. The PCR product was

digested by NdeI and HindIII and was then inserted into the pET expression vector (Novagen), generating pET::mapS-HBHAΔCter. This plasmid was used to transform *E. coli* BL21(DE3) for recombinant protein production. The recombinant mapS-HBHAΔCter was purified by Nitroilotriacetic acid (NTA) agarose chromatography (Sigma) according to the manufacturer's recommendations.

Purification of recombinant HBHA by heparin-Sepharose chromatography. Recombinant HBHA produced in *E. coli* without a His tag was purified by heparin-Sepharose chromatography as described previously (24) using recombinant *E. coli* lysates prepared as described above. All chromatographic steps were carried out on the BioLogic chromatography system (Bio-Rad) at room temperature, and the absorbance at 280 nm was continuously monitored during purification using a HiTrap Heparin HP column (1 ml; 0.7 by 2.5 cm; GE Healthcare) prepacked with heparin-Sepharose. The column was washed with 100 ml of 20 mM Tris-HCl (pH 7.2) until the absorbance at 280 nm was close to zero. The bound material was eluted by a 0 to 1 M NaCl linear gradient in 20 mM Tris-HCl (pH 7.2), using a flow rate of 0.6 ml min⁻¹, and was automatically collected in 1-ml fractions. Whole-cell lysates, flowthrough material, and eluted fractions were analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) performed according to the method of Laemmli (29).

SDS-PAGE and immunoblotting. *Mycobacterium* whole-cell lysates and purified recombinant HBHA were analyzed by SDS-PAGE using 12% running gels and 4% stacking gels. The proteins were then transferred to nitrocellulose membranes (Whatman, Germany), and the membranes were blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS)–0.05% Tween and were washed three times in PBS–0.05% Tween–1% BSA before incubation with the anti-HBHA monoclonal antibody 3921E4 (19, 30). After three washing steps with PBS–0.05% Tween, membranes were incubated with an alkaline phosphatase-conjugated goat anti-mouse antibody (Caltag, Burlingame, CA) at a 1:2,000 dilution. The substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were used to develop the immunoblots.

Protein analysis. Multiple amino acid alignments were performed with ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>).

Biotinylation of heparin. Biotinylated heparin was prepared by following the procedure described by Osmond et al. (31). Briefly, 10 mg of low-molecular-weight heparin (Enoxaparin) was reductively aminated at 70°C under constant stirring, once with 200 mg of NaCNBH₃ and then once with 100 mg of NaCNBH₃ (Sigma), for 48 h for each step. The aminated heparin was then dialyzed against 0.1 M NaHCO₃ (pH 7.4) and was coupled to 0.75 mg of sulfo-*N*-hydroxysuccinimide (NHS)-LC-biotin (Uptima) overnight at 4°C under constant stirring. Biotinylated heparin was dialyzed against H₂O and was lyophilized for storage until use.

Surface plasmon resonance (SPR) analysis. The BIAcore T100 system (GE Healthcare) was used for analysis of the molecular interactions between recombinant HBHA, *M. avium* subsp. *paratuberculosis* type C

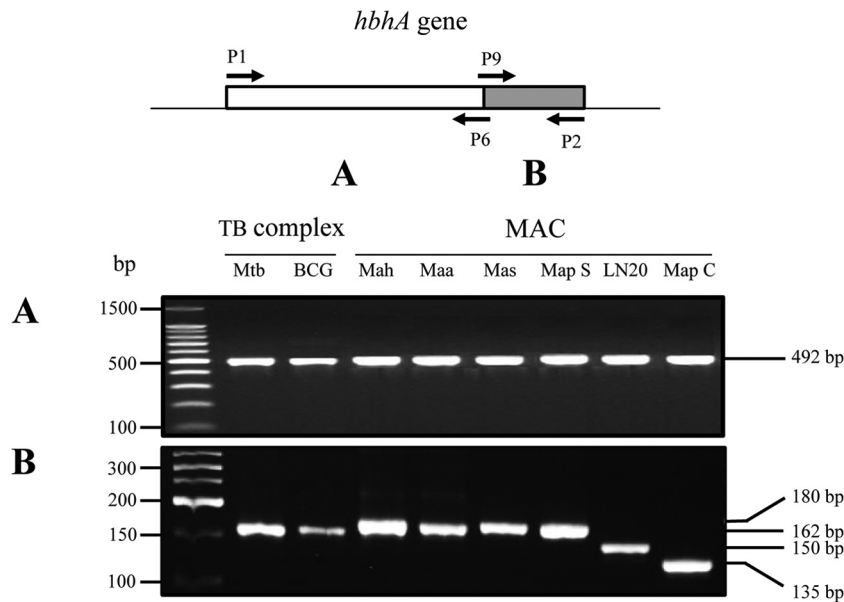


FIG 1 Interspecies and intraspecies variability of the *hbaA* gene. Shown are PCR amplification products of the DNA coding for the N-terminal domain (A) and the C-terminal domain (B) of HBHA, obtained by using genomic DNA of *M. tuberculosis* (Mtb), BCG, *M. avium* subsp. *hominissuis* (Mah) (1 sample representative of 28 strains analyzed), *M. avium* subsp. *avium* (Maa), *M. avium* subsp. *silvaticum* (Mas), *M. avium* subsp. *paratuberculosis* type S (Map S) (1 sample representative of 17 strains analyzed), *M. avium* subsp. *paratuberculosis* strain LN20, and *M. avium* subsp. *paratuberculosis* type C (Map C) (1 sample representative of 34 strains analyzed). The arrows in the diagram at the top indicate the positions and directions of primers P1, P2, P6, and P9, the sequences of which were deduced from the HBHA-encoding gene of *M. tuberculosis*. Positions on a 100-bp ladder (Promega) (A) and a low-molecular-weight DNA ladder (New England BioLabs) (B), used as molecular weight markers, are indicated on the left. The sizes of the PCR products are indicated on the right.

HBHA (mapC-HBHA), *M. avium* subsp. *paratuberculosis* type S HBHA (mapS-HBHA), *M. avium* subsp. *paratuberculosis* L20 HBHA (mapLN20-HBHA), *M. avium* subsp. *avium* HBHA (maa-HBHA), BCG HBHA, *M. smegmatis* HBHA (ms-HBHA), or mapS-HBHAΔCter and heparin. Recombinant streptavidin (Pierce) was covalently bound on two flow cells (Fc) of a CM4 sensor chip (GE Healthcare) with HBS (10 mM HEPES, 150 mM NaCl, 0.05% Tween 20 [pH 7.4]) as the running buffer. Standard amine coupling was performed according to the manufacturer's instructions, generating an immobilization signal of 1,000 resonance units (RU). After three 60-s injections of 1 M NaCl–50 mM NaOH, 5 $\mu\text{g ml}^{-1}$ of biotinylated heparin was injected at a flow rate of 2 $\mu\text{l min}^{-1}$ for 90 s onto a streptavidin-coated Fc to obtain 30 RU. The other streptavidin-coated Fc was not modified and was used as a control surface. Binding was performed at 25°C, with 50 mM Tris-HCl–50 mM NaCl–0.05% Tween 20 (pH 7.2) as the running buffer. The protein solutions were injected over the two flow cells for 180 s at 30 $\mu\text{l min}^{-1}$ at concentrations ranging from 1.2 to 300 or 900 nM. Dissociation was measured over 180 s. The surfaces were regenerated with a 1 M NaCl solution for 60 s, followed by a 100-IU/ml heparin solution for 60 s and a 120-s buffer wash. The signals obtained on the control surface and with the buffer injection were subtracted from the signal responses with the test samples. Constant affinities were calculated at equilibrium by the steady-state method and with BIAevaluation software, version 2.03.

ELISA. Enzyme-linked immunosorbent assays (ELISA) were performed on 96-well Maxisorp microtiter plates (Nunc, Roskilde, Denmark) coated with 50 μl of a purified protein derivative from *M. avium* subsp. *paratuberculosis* (Johnin PPD [PPDj]) (National Veterinary Institute, Oslo, Norway) at 25 mg liter $^{-1}$ in PBS or with recombinant BCG HBHA, mapS-HBHA, mapC-HBHA, or mapS-HBHAΔCter at 0.1 mg ml $^{-1}$ in PBS at 4°C overnight. Plates were then washed three times with PBS–0.05% Tween 20 (PBS/T) and were blocked for 1 h at 37°C with PBS/T containing 0.5% (wt/vol) gelatin (PBS/T/G). The sera tested have been described previously (32) and include a positive control from *M. avium* subsp. *paratuberculosis*-infected cattle, a negative control, and a set

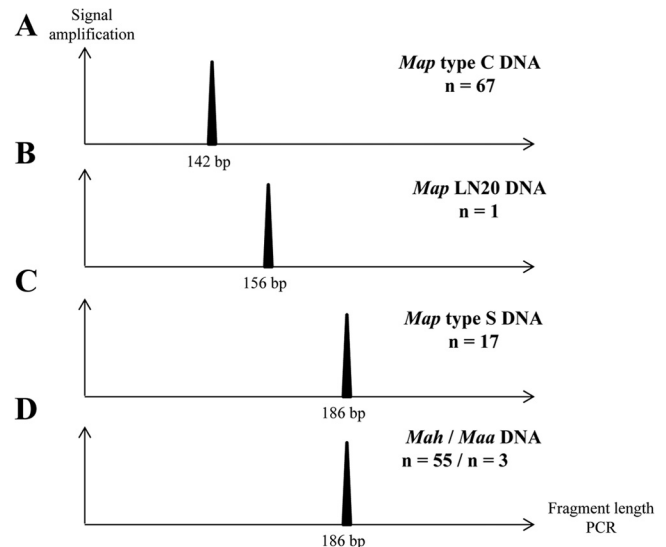
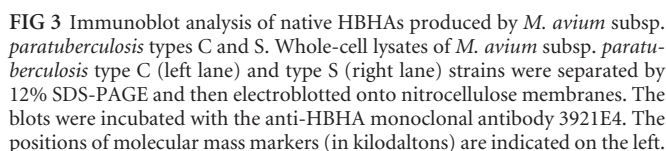


FIG 2 Distinct *hbaA* genes within the subspecies *M. avium* subsp. *paratuberculosis* were examined with a large collection of isolates. The gene coding for the C-terminal domain of HBHA was amplified by PCR on genomic DNA using primers labeled at their 5' ends with the fluorophore 6-carboxyfluorescein. Results were visualized using ABI GeneMapper software, version 4.0, with 600 LIZ size standards (Applied Biosystems, USA). The expected sizes of the PCR products are 142 bp with *M. avium* subsp. *paratuberculosis* type C genomic DNA (A), 156 bp with *M. avium* subsp. *paratuberculosis* LN20 genomic DNA (B), and 186 bp with *M. avium* subsp. *paratuberculosis* type S (C), *M. avium* subsp. *avium*, or *M. avium* subsp. *hominissuis* (D) genomic DNA. This analysis was performed on 67 strains of *M. avium* subsp. *paratuberculosis* type C, 18 strains of *M. avium* subsp. *paratuberculosis* type S, including strain LN20, 55 *M. avium* subsp. *hominissuis* strains, and 3 strains of *M. avium* subsp. *avium*. n, number of strains analyzed.



37°C with 50 µl peroxidase-conjugated anti-ruminant IgG (Chekit; Bommeli AG, Bern, Switzerland), diluted at 1:600 in PBS/T/G. Plates were washed five times with PBS/T, and 50 µl peroxidase substrate was added. After a 15-min incubation at 37°C, the plates were read photometrically at 414 nm. Statistical analysis was performed using GraphPad Prism, version 5.00 for Windows (GraphPad Software, San Diego, CA, USA). Data were analyzed by one-way analysis of variance (ANOVA) with Tukey's multiple comparison test.

Nucleotide sequence accession numbers. The sequences of *hbbA* have been deposited in the GenBank databases under the following accession numbers: for *M. tuberculosis*, [AF074390](#); for *M. bovis* BCG, [4696475](#); for *M. smegmatis*, [DQ059383](#); for *M. avium* subsp. *avium*, [JN129485](#); for *M. avium* subsp. *paratuberculosis* type C strain ATCC 19698, [JX536267](#); for *M. avium* subsp. *paratuberculosis* type C strain K-10, [JX536266](#); for *M. avium* subsp. *paratuberculosis* type S strain 19I, [KC920678](#); for *M. avium* subsp. *paratuberculosis* type S strain 269OV, [KC920679](#); for *M. avium* subsp. *paratuberculosis* type S strain M71/03, [KC920680](#); for *M. avium* subsp. *paratuberculosis* type S strain 21I, [KC920681](#); for *M. avium* subsp. *paratuberculosis* type S strain 85/14, [KC920682](#); and for *M. avium* subsp. *paratuberculosis* type S strain LN20, [KC920683](#).

Variability of the C-terminal domain of HBHA. Comparative genomic data show that *hbhA* is highly conserved among mycobacterial species. Alignment of HBHA protein sequences reveals that the large N-terminal domains are highly conserved, while the C-terminal domains are heterogeneous between mycobacterial species (24, 33). To identify the *hbhA* genes in *M. avium* subsp. *paratuberculosis* type S strains, separate PCR analyses were performed on the 5' and 3' portions of the gene. The results indicate that the *hbhA* gene is present in S strains of *M. avium* subsp. *paratuberculosis* and that the sizes of the 5' ends, coding for the

[illegible]

			N Charges	n
Mtb	160	P-----K KAAAPAKKAAPAKKAAPAK KAAAKKAPAK KAAAK KVTQK--	15	9.6
Mah	160	PGKAEAAAGKKAQKAIA IAPAKKAPAKKAPAKKAPAKAAAA KVTQK-	16	10.2
Maa	160	PGKAEAAAGKKAQKAIA KAPAKKAPAKKAPAKKAPAKAAAA KVTQK-	16	10.2
MapS	160	PGKAEAAAGKKAQKAIA KAPAKKASAKKAPAKKAPAKAAAA KVTQK-	16	9.9
MapLN20	160	PGKAEAAAGKKAQKAIA----- KAPAKKAPAKAAAA KVTQK-	12	8.9
MapC	160	PGKAEAAAGKKAQKAIA----- KAPAKKAPAK KVTQK-	10	7
Ms	191	PVKKAAAAPAAK--- KAAPAKKAAPAK -KATPAKSAAKAPAKKVTQK-	13	8.8

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N-terminal moieties of HBHA, are invariable among the samples analyzed (Fig. 1A). In contrast, the sizes of the PCR products targeting the 3' end of the gene, coding for the C-terminal domain of HBHA, were variable; PCR products from type S strains were longer than those from type C strains. The *hbhA* gene amplified from type S strains was the same size as the *M. avium* subsp. *avium* and *M. avium* subsp. *hominissuis* *hbhA* genes. The shorter *hbhA* gene was observed only with *M. avium* subsp. *paratuberculosis* type C strains. Isolate LN20, a strain intermediate between the S and C lineages according to deletion analysis (1), had an *hbhA* gene of a size intermediate between those for types C and S (Fig. 1B).

Specificity of the *hbhA* gene according to the *M. avium* subsp. *paratuberculosis* lineage. To extend the analysis of *hbhA* variability across *M. avium* subsp. *paratuberculosis* lineages, we performed fluorescent PCR fragment analyses on the 3' portions of the genes from a representative panel of isolates, selected from a comprehensive collection of strains at the French National Institute for Agricultural Research (INRA). This panel (Table 1; also data not shown) consists of strains isolated from various animals (cattle, sheep, goats, red deer, rabbits) and humans of various geographical origins (data not shown). Furthermore, the isolates were selected based on distinct mycobacterial interspersed repetitive-unit-variable-number tandem-repeat (MIRU-VNTR) and IS900 restriction fragment length polymorphism (RFLP) profiles as described previously (3). This panel was completed by 3 strains of *M. avium* subsp. *avium* isolated from birds and 55 human isolates of *M. avium* subsp. *hominissuis* described previously (34).

All strains of *M. avium* subsp. *avium* and *M. avium* subsp. *hominissuis* gave PCR products of 186 bp. All strains of *M. avium* subsp. *paratuberculosis* type S (except for LN20) yielded products of the same size, 186 bp (Fig. 2C and D). *M. avium* subsp. *paratuberculosis* LN20 was the only strain to yield a PCR product of 156 bp (Fig. 2B). For all 67 *M. avium* subsp. *paratuberculosis* strains of type C, fragments of 142 bp were amplified (Fig. 2A). These results confirm that within the subspecies *M. avium* subsp. *paratuberculosis*, lineages S and C can be distinguished by the sizes of their *hbhA* genes.

Size of native HBHA in *M. avium* subsp. *paratuberculosis*. To determine whether the longer *hbhA* genes of *M. avium* subsp. *paratuberculosis* type S strains result in HBHA proteins longer than those of type C strains, immunoblot analyses were performed on the different strains. Whole-cell lysates of *M. avium* subsp. *paratuberculosis* type C (strain K-10) and *M. avium* subsp. *paratuberculosis* type S (strain 85/14) were analyzed using monoclonal antibody 3921E4 (35). These analyses showed the different forms of HBHA produced in each lineage of *M. avium* subsp. *paratuberculosis*, with apparent molecular masses of 22 kDa for HBHA from type C and 28 kDa for HBHA from type S (Fig. 3).

Sequence features of the HBHA homologues. To determine the basis for the genetic variability of HBHAs from the different lineages, the *hbhA* gene of each lineage was sequenced, and deduced amino acid sequences were aligned with those of sequences already known for the *M. tuberculosis* complex and *M. smegmatis* (Fig. 4). The sequence data confirmed the findings from the PCR and immunoblot analyses. The HBHA of *M. avium* subsp. *paratuberculosis* type S (mapS-HBHA) consists of 205 amino acids, whereas that of type C (mapC-HBHA) has 190 amino acids. *M. avium* subsp. *paratuberculosis* strain LN20 produces a 195-amino-acid HBHA molecule (mapLN20-HBHA). The sequence similarities among these HBHA proteins are high (89% similarity), as

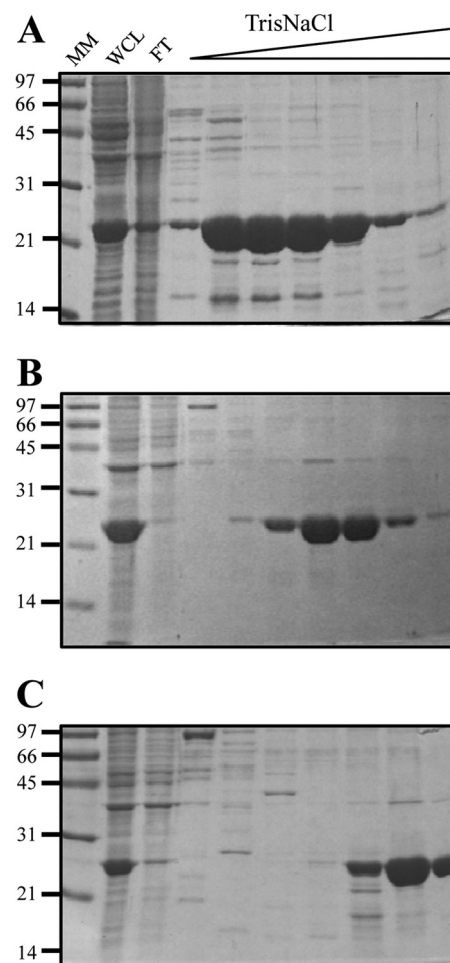


FIG 5 Differences between rHBHA proteins from *M. avium* subsp. *paratuberculosis* type C, LN20, and type S in the strength of binding to heparin-Sepharose. Lysates of *E. coli* expressing recombinant HBHA of *M. avium* subsp. *paratuberculosis* type C (A), *M. avium* subsp. *paratuberculosis* LN20 (B), and *M. avium* subsp. *paratuberculosis* type S (C) were sonicated. The soluble material was subjected to heparin-Sepharose chromatography and was eluted using a 0 to 1 M NaCl gradient. SDS-PAGE analyses were performed on whole-cell lysates (WCL), flowthrough (FT), and elution fractions of rHBHA. The positions of molecular mass markers (MM), expressed in kilodaltons, are indicated on the left.

expected, up to residue 162, after which the sequences are more divergent. The divergences observed in the C-terminal regions of HBHA correspond mainly to deletions or differences in the lysine-rich repeats. In Fig. 4B, the sequences are presented as a succession of the repeats defined by Lebrun et al. (25). Interestingly, the divergences of HBHA correspond to different numbers of these repeats (Fig. 4B). Pethe et al. (36) and, recently, Lebrun et al. (25) have shown a direct correlation between the number of lysine-rich repeats (R2) and the strength of heparin binding of HBHA. Since the HBHA produced by *M. avium* subsp. *paratuberculosis* type S contains 5 repeats, we hypothesized that its heparin-binding activity may be similar to that of HBHA produced by *M. avium* subsp. *avium* but greater than that for *M. avium* subsp. *paratuberculosis* type C, which contains only 2 repeats.

Capacity of map-HBHA to bind to heparin-Sepharose. Recombinant HBHA proteins produced in *E. coli* were analyzed by

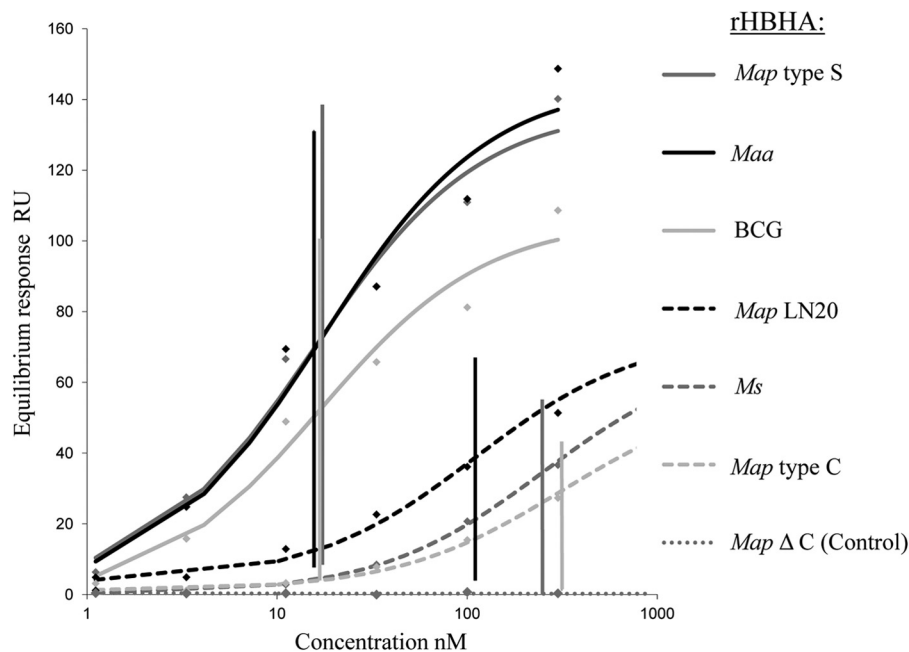


FIG 6 Quantification by SPR analysis of the heparin-binding activities of the rHBHA proteins of *M. avium* subsp. *paratuberculosis* type C, LN20, and type S. SPR analysis of the binding of recombinant HBHA to heparin was performed on a BIAcore T100 system (GE Healthcare). Biotinylated heparin was immobilized on a streptavidin-coated CM4 sensor chip. Recombinant HBHA proteins purified by heparin-Sepharose chromatography were injected over a range of concentrations at a flow rate of 30 $\mu\text{l min}^{-1}$. Recombinant mapS-HBHA Δ Cter was used as a negative control. K_D were determined by the equilibrium method. The K_D results (nM) are indicated at the intersections of the vertical lines with the concentration axis.

heparin-Sepharose chromatography. As described previously (24), mapC-HBHA bound weakly to heparin-Sepharose and was eluted at 200 mM NaCl in a NaCl gradient from 0 to 1 M (Fig. 5A). The presence of a third R2 motif in mapLN20-HBHA strengthened the interaction with heparin, as demonstrated by delayed elution at 410 mM NaCl (Fig. 5B). The highest affinity was observed for mapS-HBHA, which contains 5 R2 repeats. This HBHA variant was eluted only when the NaCl concentration was increased to 600 mM (Fig. 5C). Together, these results indicate that a genetic difference between the lineages of *M. avium* subsp. *paratuberculosis* translates into different lengths of the HBHA protein and into differences in binding activity with sulfated glycoconjugates.

SPR analysis of the HBHA-heparin interactions. To quantify the specific interactions between the different recombinant HBHA forms and heparin, we employed the surface plasmon resonance (SPR) method. In addition to mapC-HBHA, mapS-HBHA, and mapLN20-HBHA, produced in *E. coli* and purified by heparin-Sepharose chromatography, HBHA proteins from *M. smegmatis* (ms-HBHA), *M. avium* subsp. *avium* (maa-HBHA), and *M. bovis* BCG (BCG HBHA) were used as controls for low and high affinity for heparin, as described previously (21, 25, 36). Additionally, recombinant mapS-HBHA Δ Cter, lacking the C-terminal domain, was used as a negative control. As shown in Fig. 6, and as expected, recombinant mapS-HBHA Δ Cter did not interact with the heparin sensor chip, demonstrating that the N-terminal domain of HBHA is not involved in heparin binding. In contrast, the other HBHA molecules bound to the sensor chip with various affinities. These proteins could be classified into two groups: (i) HBHA proteins with low heparin-binding affinity, including ms-HBHA, mapC-HBHA, and mapLN20-HBHA, with K_D (equilib-

rium dissociation constants) around 249, 314, and 110 nM, respectively, and (ii) HBHA proteins with high heparin-binding affinity, comprising BCG HBHA, maa-HBHA, and mapS-HBHA, for which the K_D were close to 20 nM. These results concurred with the qualitative results obtained by affinity chromatography and strengthen the relationship between the length of the C-terminal domain and the binding function of HBHA with sulfated glycoconjugates.

Sera from animals with Johne's disease react with HBHA produced by *M. avium* subsp. *paratuberculosis*. To investigate whether the antigenic properties of HBHA differed according to the *M. avium* subsp. *paratuberculosis* lineage, we performed ELISA based on mapC-HBHA versus mapS-HBHA by using sera from animals with clinical infections by *M. avium* subsp. *paratuberculosis* type C or type S strains. As shown in Fig. 7, the sera from animals infected with a type C or a type S strain reacted similarly with both forms of HBHA. In addition, all the serum samples in this panel responded similarly to BCG HBHA and mapS-HBHA Δ Cter, as well as to PPDj. These results indicate that the differences in HBHA sequences did not affect the antigenic properties of HBHA and that this B-cell response is not specific with respect to mycobacterial species and subspecies.

DISCUSSION

During the early stages of infection, pathogenic microorganisms interact with cell surface receptors of eukaryotic target cells that are normally required for their physiological processes, such as signal transduction, cell-cell interactions, and cell-matrix interactions. In mycobacteria, two major adhesins, named HBHA and laminin binding protein (LBP), have been reported (20, 21, 37–39) to mediate these interactions with sulfated glycosaminoglycan

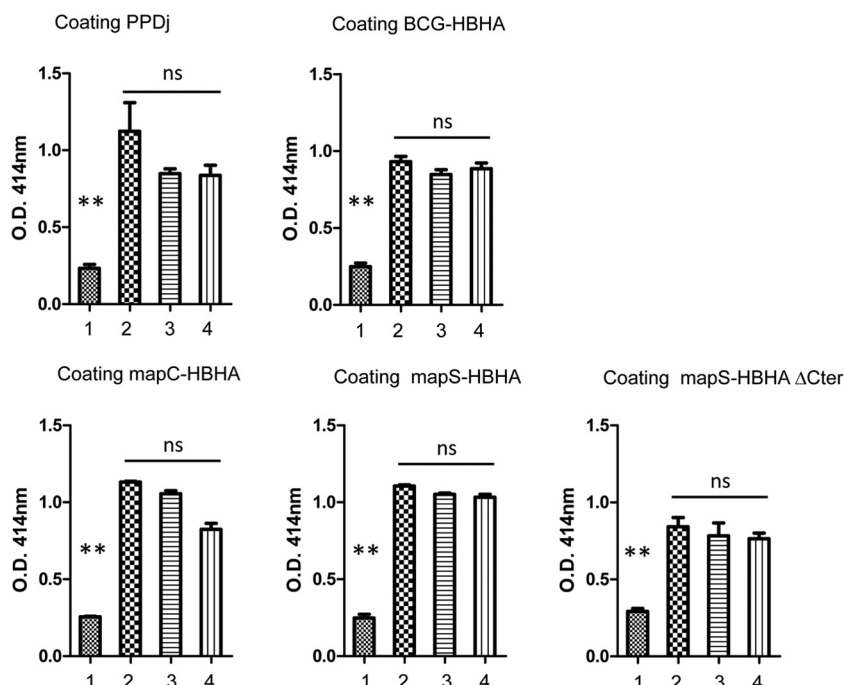


FIG 7 Reactivity of a serum panel with the rHBHA proteins from *M. avium* subsp. *paratuberculosis* strains of types C and S. ELISA were performed with plates coated with either PPDj, recombinant BCG HBHA, mapS-HBHA, mapS-HBHAΔCter, or mapC-HBHA. The panel of sera tested included a *M. avium* subsp. *paratuberculosis*-negative commercial antiserum (lane 1), a *M. avium* subsp. *paratuberculosis*-positive commercial serum (lane 2), and sera obtained from ruminants infected by *M. avium* subsp. *paratuberculosis* type C (lane 3) or type S (lane 4). Each serum sample was diluted at 1:100 in 50 μ l PBS/T/G and was incubated for 2.5 h at 37°C. The plates were then washed and were incubated for 90 min at 37°C with 50 μ l peroxidase-conjugated anti-ruminant IgG diluted at 1:600 in PBS/T/G. After washing, the plates were developed with 50 μ l of a peroxidase substrate for 30 min at 37°C. The reaction was stopped with 50 μ l 1 N HCl, and the plates were read photometrically at 490 nm. The results shown are averages for triplicate samples from one experiment representative of three independent experiments. Statistical analysis was performed with Tukey's multiple comparison tests. **, $P < 0.001$; ns, not significant ($P > 0.05$).

(GAG) molecules, such as heparan sulfate (HS-GAG), present on the surfaces of various types of eukaryotic cells (40). This study focused on the HBHA expressed in *M. avium* subsp. *paratuberculosis*, and we identified HBHA expressed by *M. avium* subsp. *paratuberculosis* type S strains and compared its biochemical properties to those for type C strains.

Surprisingly, *in silico* analysis showed that the C-terminal part of HBHA identified in *M. avium* subsp. *paratuberculosis* diverged according to the lineage (type C versus type S). Until now, the HBHA molecules reported for the subspecies *M. avium* subsp. *paratuberculosis* belonged to type C (23, 24). The variability between mapC-HBHA and mapS-HBHA is restricted to the C-terminal domain of the protein. However, this difference in structure may be important for the function of these adhesins, because this domain is responsible for the binding of HBHA to HS-GAG (25, 36). These observations add new evidence to the distinctness of type S versus type C strains of *M. avium* subsp. *paratuberculosis* and are consistent with the previously described biphasic scenario for *M. avium* subsp. *paratuberculosis* evolution (2).

One of the major outcomes of this study is the discovery that, in contrast to mapC-HBHA, mapS-HBHA was 98.6% similar to the HBHA proteins expressed by *M. avium* subsp. *avium* and *M. avium* subsp. *hominissuis*. These results suggest that for this locus, *M. avium* subsp. *paratuberculosis* type S more closely resembles other subspecies of *M. avium* than *M. avium* subsp. *paratuberculosis* type C, as has also been observed for some other markers used to study *Mycobacterium avium* complex (MAC) evolution (2, 41).

Furthermore, while typing methods such as *gyrA* and *gyrB* single nucleotide polymorphism (SNP) typing have suggested that type S strains can be subdivided into subtypes called I and III (3), this study showed no difference between the *hbhA* genes of subtype I and III strains. Additionally, a single strain of *M. avium* subsp. *paratuberculosis* (LN20), originally isolated from a pig in Quebec, Canada, presented an HBHA molecule of intermediate size. Interestingly, this observation correlates with the analysis of Alexander et al. (1) showing certain intermediate features of strain LN20.

This study also revealed that *M. avium* subsp. *paratuberculosis* HBHA molecules also differ in biochemical properties according to lineage (S versus C). Further studies should be undertaken to decipher the consequences of these two forms of HBHA expressed in *M. avium* subsp. *paratuberculosis* in terms of attachment to epithelial cells (21) and host and/or organ specificity (42). Indeed, the particularity of *M. avium* subsp. *paratuberculosis* is to have a tropism for the intestine and, within the subspecies *M. avium* subsp. *paratuberculosis*, a preferred host range, such as sheep for S strains and cattle for C strains. Since HS-GAG structures differ according to both hosts and organs (42), it is possible that mycobacterial pathogens use heparin-binding domain variability to define their host preference or anatomic tropism.

Despite the structural and biochemical differences between mapC-HBHA and mapS-HBHA, B-cell responses to the two different forms were identical. However, T-cell responses to these different HBHA forms were not investigated. It has been shown that in humans, the native HBHA purified from BCG can be used

to detect individuals latently infected by *M. tuberculosis* (27, 43), and that important effector functions of the HBHA-specific T cells require the protein to be methylated. Furthermore, protective immunogenicity induced by HBHA has been shown to depend also on a precise methylation pattern (28, 44). Investigation of the T-cell responses in animals infected by strains of each type thus requires establishment of the exact methylation patterns of the HBHA produced by each lineage and the availability of mapC-HBHA and mapS-HBHA in their correctly methylated forms. We have observed recently that lysine residues 168 and 153 of native mapC-HBHA are methylated (24), but the precise methylation patterns and other translational modifications remain to be determined.

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