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Identification and Characterization of a Major Cell Wall-Associated Iron-Regulated Envelope Protein (Irep-28) in *Mycobacterium tuberculosis*

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Iron limitation and the expression of mycobactin and carboxymycobactin by *Mycobacterium tuberculosis* are known. Here, we report how iron regulated the coordinate expression of these two siderophores and a 28-kDa cell wall-associated iron-regulated protein (Irep-28). Irep-28 is identified as the DNA-binding HU homologue HupB protein (*hupB* [Rv2986c]). Antibodies to this protein were detected in sera from tuberculosis patients. The location of the protein in the cell wall makes it a potential drug target.

Iron limitation in mycobacteria results in the expression of two siderophores: the intracellular hydrophobic mycobactin and the extracellular water-soluble carboxymycobactin/exochelin (18, 26). In *Mycobacterium smegmatis*, a 29-kDa iron-regulated envelope protein (IREP) was shown to associate directly in vitro with ferri-exochelin, and the addition of a polyclonal antiserum generated against it significantly inhibits ferri-exochelin-mediated iron uptake by live organisms (9). This was the first evidence of a ferric-siderophore receptor in mycobacteria. In *Mycobacterium neoaurum*, iron coordinately regulated the levels of mycobactin, exochelin, and a 21-kDa envelope protein (24).

Calder and Horwitz showed the iron-regulated expression of Irp10 and Mta72 in *Mycobacterium tuberculosis* by single-dimension electrophoresis (3) and later, using two-dimensional gel electrophoresis combined with mass spectrometry and sequence information (27), demonstrated the upregulation of a putative cation transporting ATPase, a mycobacterial homologue of phosphoenolpyruvate carboxykinase, and an NADP-dependent dehydrogenase in bacteria grown in low-iron medium, whereas these authors observed increased levels of FurA, a homologue of EF-Tu, and an aconitase in iron-rich medium.

Microarray analysis revealed a large number (n = 155) of iron-regulated genes (20). Studies on the role of the iron regulator IdeR, in both iron acquisition and oxidative stress (8, 20), and the presence of homologues of both Fur (FurA and FurB) and DtxR (IdeR and SirR) in the *M. tuberculosis* genome (5) show that iron-dependent regulation in mycobacteria is complex and much remains to be understood.

Although there is no direct evidence of mycobactin/carboxymycobactin in in vivo derived mycobacteria, mycobactin was shown to be a virulence determinant by De Voss et al., who demonstrated that mutants defective in mycobactin synthesis failed to infect and multiply within macrophages (6, 7). Iron-regulated proteins are expressed by *Mycobacterium avium* and *Mycobacterium leprae* isolated from experimentally infected animals (25). Since the availability of iron is one of the contributing factors in determining the outcome of a bacterial infection, it is likely that they adapt with a great deal of fineness to changes in iron levels in their immediate environment.

In the present study, we studied the expression of mycobactin, carboxymycobactin, and envelope proteins in *M. tuberculosis* grown in a range of iron concentrations. With due emphasis on the importance of sample preparation and effective solubilization of wall and membrane proteins, we modified the Triton X-114 protocol of Sinha et al. (22, 23) and identified a 28-kDa cell wall-associated protein expressed under conditions of iron restriction. The identification of this protein as HupB and its clinical significance in tuberculosis patients are presented.

MATERIALS AND METHODS

Growth of *M. tuberculosis* (ATCC 27294). The organism was grown in liquid Proskauer and Beck medium using iron-free glassware (9, 27). Known concentrations of iron, ranging from 0.02 to 12 μg of Fe/ml, were added as required. The cells were grown with continuous shaking at 180 rpm in an orbital shaker and harvested after 2 weeks of growth.

Estimation of siderophores. The extracellular carboxymycobactin was assayed by the Universal Chrome Azurol assay (CAS) (21). In brief, 2 ml of culture filtrate was added to 2 ml of CAS solution, and the absorbance at 630 nm was measured after 30 min of incubation at room temperature. The concentration of the siderophore was calculated as follows: one siderophore unit = $(A_C - A_S/A_C) \times 100$, where A_C represents the absorbance of CAS solution plus medium and A_S is the absorbance of the CAS solution plus culture filtrate of the respective sample. Carboxymycobactin is expressed as siderophore units/100 mg of cell dry weight.

The cell wall-associated mycobactin was extracted by overnight incubation of the cell pellet with ethanol-chloroform (1:1 [vol/vol]), followed by the addition of a drop of saturated ferric chloride in ethanol. The optical density at 450 nm (OD_{450}) of the ferric-MB was determined after the chloroform layer was washed with water to remove excess ferric chloride and is expressed as the OD_{450} units/g of cell dry weight.

Cell wall and cell membrane proteins. The procedure followed for isolating the cell wall and membrane fractions was essentially that described by Hall et al. (9). Modification of the Triton X-114 extraction protocol of Sinha et al. (22) was used for the solubilization of the wall and membrane proteins. The wall and

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membrane pellets were resuspended in 5% Triton X-114 (Sigma, equilibrated with 0.05 M Tris-HCl [pH 7.8]) and left overnight with rocking at 4°C. The Triton X-114 layer was removed by centrifugation and warmed to 37°C for 1 h to separate the detergent and aqueous layers, followed by appropriate washes, and the proteins were precipitated with acetone. The protein pellet was solubilized in 2% sodium dodecyl sulfate (SDS) in 0.05 M Tris-HCl buffer (pH 6.8). The protein concentration was determined by using the BCA kit (Sigma).

SDS-polyacrylamide gel electrophoresis (PAGE) separation of the solubilized proteins was done according to a standard protocol (12). Preparative gel electrophoresis was done to purify Irep-28 protein, and the eluted protein was evaluated again by SDS-PAGE to confirm the purity of the band. The gel slice was cut out and sequencing was done in the laboratory of F. Altmann.

Sequence analysis of Irep-28. The sample was digested in-gel and S alkylated, and the tryptic peptides were separated by liquid chromatography and analyzed by tandem mass spectrometry (MS/MS) on a Waters Q-TOF Ultima global as described recently (11).

Patients. Patients attending the Tuberculosis Clinic at Nizam's Institute of Medical Sciences (Hyderabad, India) with signs and symptoms of pulmonary or extrapulmonary tuberculosis were examined. The patients included both males and females, irrespective of age. Based on the provisional clinical diagnosis of tuberculosis, the relevant investigations, including chest X-ray, Ziehl-Neelsen staining, and culture by BACTEC and LJ of pulmonary and/or extrapulmonary samples, were performed. In the present study, a total number of 44 definite cases of tuberculosis (confirmed clinically and by lab testing) were chosen. Serum samples were collected from all of these patients and were tested for antibodies to Irep-28. We also tested the serum by a commercial lateral flow device (Mycotest; GVK BioSciences, India) as described below.

Reactivity of tuberculosis serum with Irep-28. Initially, Western blotting analysis of the solubilized wall and membrane proteins using serum from selected tuberculosis patients at a dilution of 1:250 was done by standard protocols. Later, all serum samples (at 1:250 dilution) were subjected to dot blot analysis against the purified Irep-28 antigen (2.5 μg). In both blots, rabbit anti-human immunoglobulin G alkaline phosphatase conjugate (Bangalore Genei, India) was used, and they were developed by using nitrilotriacetic acid-BCIP (5-bromo-4-chloro-3-indolylphosphate) as substrate (Bangalore Genei, India) according to the manufacturer's instructions.

Reactivity of tuberculosis serum as tested by Mycotest. All serum samples were tested by using the commercial Mycotest (marketed by GVK Biosciences, India), which is a lateral flow device coated with recombinant antigens for secretory proteins specific for *M. tuberculosis* (with a sensitivity and specificity of 87 and 90%, respectively, according to the manufacturer's information). A total of 80 to 100 μl of serum was added, and the detection of a pink band in addition to the control band within 15 min after addition of serum was considered positive for tuberculosis.

RESULTS

Coordinated expression of Irep-28, mycobactin and carboxymycobactin. 14-day-old cultures of *M. tuberculosis* showed maximal expression of both mycobactin (Fig. 1A) and carboxymycobactin (Fig. 1B) at 0.02 μg of Fe/ml. With increasing iron in the growth medium (iron levels ranged from 0.02 to 12 μg of Fe/ml), there was a progressive lowering of the levels of both these siderophores, the decrease being appreciable from 4 to 12 μg of Fe/ml.

An analysis of the detergent and aqueous phases of Triton X-114-solubilized wall and membrane proteins (Fig. 2) showed the iron-regulated expression of a 28-kDa cell wall-associated protein (henceforth called Irep-28) in the detergent fraction. The coordinate regulation of Irep-28 with mycobactin and carboxmycobactin was evident, with a high level of expression of all of the three components when iron was at <1 μg of Fe/ml and almost complete repression at more than 8 μg of Fe/ml.

The time course of expression of all of these three components was studied in organisms grown under high (8 μg of Fe/ml)- and low (0.02 μg of Fe/ml)-iron conditions (as described in Fig. 3). Irep-28, seen as a faint band in both low- and high-iron cultures on day 8, was expressed in significant amounts in low-iron cultures harvested on days 12 and 16, with negligible levels seen in the corresponding high-iron cultures. However, on day 20, the reappearance of Irep-28 in high-iron cultures, with the associated increase in the expression of carboxymycobactin (Fig. 3B), indicated the onset of conditions of iron limitation in these cells. Mycobactin levels took longer to rise, as observed after 25 days of growth (Fig. 3A).

Other proteins influenced by iron levels included Irep-130, Irep-26, and Irep-24 (Fig. 2B and D); the first two proteins were seen in the aqueous phase of the cell wall, and Irep-24

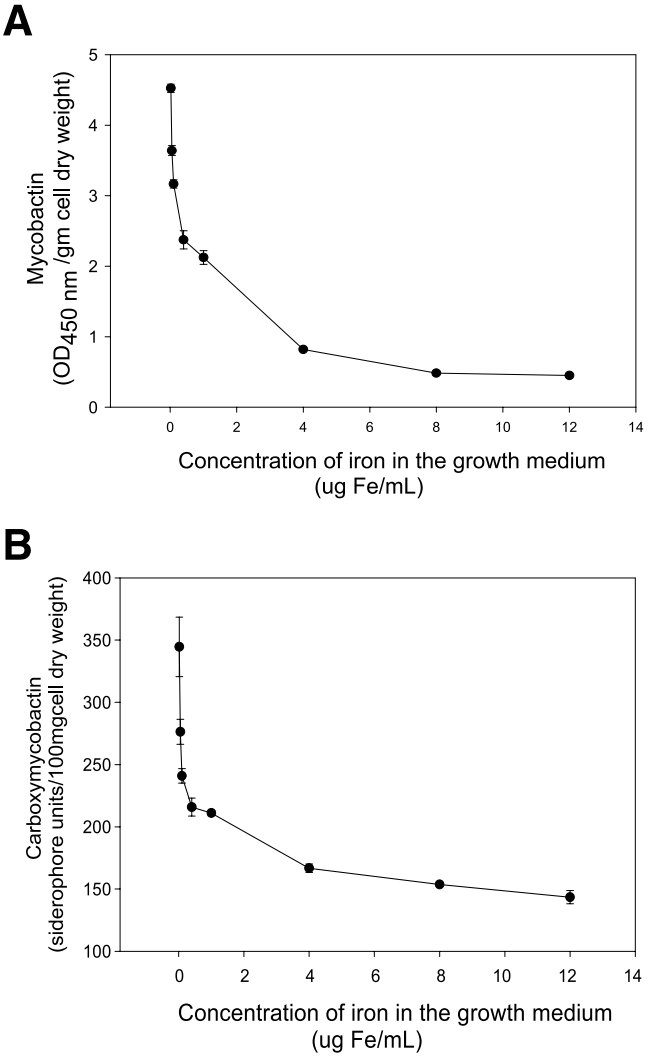


FIG. 1. (A) Iron-dependent expression of mycobactin in *M. tuberculosis*. The intracellular mycobactin was assayed in cells grown with 0.02, 0.05, 0.1, 0.2, 0.4, 1, 4, 8, and 12 μg of Fe/ml in the growth medium. As described in Materials and Methods, the values are represented as OD_{450} units/g of cell dry weight. The vertical bars represent the standard deviation of the mean from three independent experiments. (B) Iron-dependent expression of carboxymycobactin in *M. tuberculosis*. The extracellular carboxymycobactin was assayed by the Universal CAS assay in the culture filtrate of cells grown with 0.02, 0.05, 0.1, 0.2, 0.4, 1, 4, 8, and 12 μg of Fe/ml in the growth medium. As described in Materials and Methods, the values are represented as siderophore units/100 mg of cell dry weight. The vertical bars represent the standard deviation of the mean from three independent experiments.

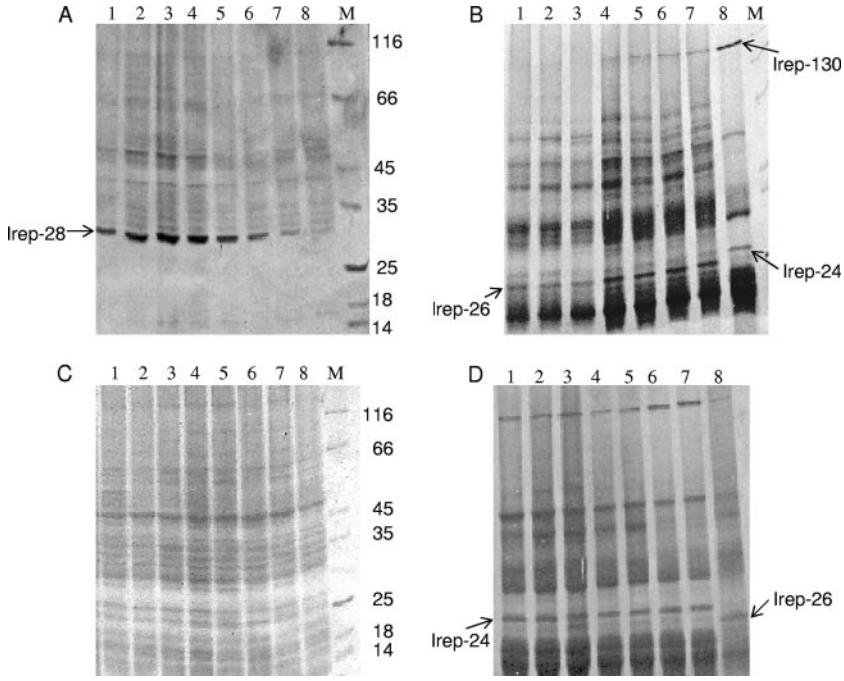


FIG. 2. Protein profile of cell wall and membrane fractions of *M. tuberculosis* grown under various iron concentrations. The detergent phase and aqueous phase of the cell wall (A and B, respectively) and the cell membrane (C and D, respectively) were separated on a 5 to 20% gradient gel with lanes 1 to 8 corresponding to cells grown with iron added at 0.02, 0.05, 0.1, 0.4, 1, 4, 8, and 12 μg of Fe/ml, respectively. The organisms were harvested after 2 weeks of growth. Lane M represents the molecular mass markers ranging from 116 to 14 kDa, as labeled in the figure. The different iron-regulated proteins, namely, Irep-28, Irep-24, Irep-26, and Irep-130, are indicated with arrows.

was extracted into the aqueous phase of the cell membrane. The cell wall-associated Irep-130 was seen at all concentrations of iron greater than 1 μg of Fe/ml. There appeared to be an association between Irep-26 and Irep-24, with a unique pattern of their expression between the wall and membrane fractions. At 12 μg of Fe/ml, Irep-26 was a predominant band in the cell wall, with Irep-24 expressed at all lower concentrations of iron; there was a reversal of this pattern of expression in the cell membrane.

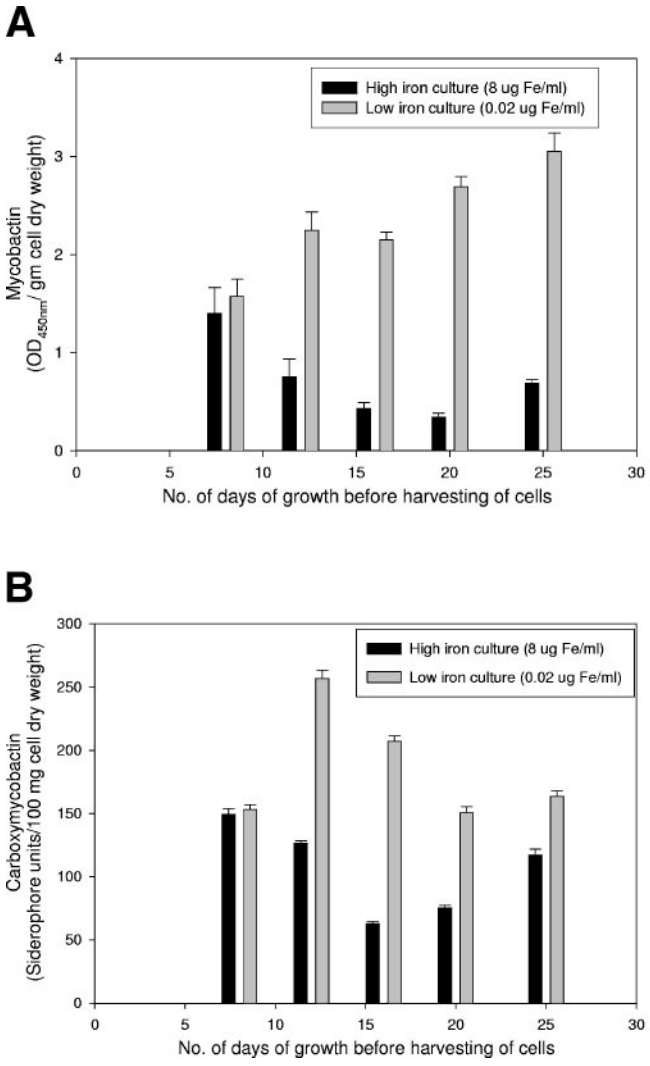
Characterization of Irep-28 as HupB protein. Irep-28 was purified by preparative gel electrophoresis and sequenced by MS/MS. Three distinctive peptides were found which comprised residues 20 to 34, 87 to 94, and 95 to 103. The suggested amino acid sequence in all three cases pointed at the partially histone-like protein P95109 (UniProt database). The C-terminal part of this protein is unusually rich in basic amino acids and hence cannot give rise to sequenceable ions by mass spectrometry. Figure 4 shows the amino acid sequence deduced from the gene of HupB; the boxed regions show the sequence of the three tryptic peptides identified by sequencing. The protein has (almost) identical homologs in other mycobacterial species such as *M. bovis* (Q9XB18) or *Mycobacterium paratuberculosis* (Q73VI9).

Immunoreactivity of Irep-28 with serum from tuberculosis patients. We detected antibodies to Irep-28 in the sera from tuberculosis patients. Figure 5 shows Irep-28 in the cell wall detergent fraction (corresponding to Fig. 2A) that reacted with serum from a confirmed tuberculosis patient, the intensity of the bands in the immunoblot corresponding to the iron-dependent expression of this protein. The detection of anti-Irep-28 antibodies in patients' serum was compared to other tests confirmatory for the disease. Of the 44 patients in the present study, culture by BACTEC was done in 40 cases, chest X-ray examination was performed for 41 cases, and 42 of the serum samples were tested by using the Mycotest device. A total of 28 of the 33 culture-positive cases and 33 of 39 chest X-ray-positive cases showed antibodies to Irep-28 (Table 1). The commercial Mycotest (with a sensitivity of 87% and a specificity of 90%) identified only 22 cases versus the 36 cases identified by immunoreactivity with Irep-28 (Table 1).

DISCUSSION

In this study, we show the coordinate regulation by iron of mycobactin, carboxymycobactin, and a 28-kDa cell wall-associated iron-regulated protein (Irep-28), identified by sequence analysis as the HupB protein. The antigenicity of this protein in human patients with tuberculosis as demonstrated by its immunoreactivity with serum samples from these patients is discussed here.

Earlier studies in several mycobacterial species showed that concentrations of 0.02 and 4 μg of Fe/ml, respectively, indicated low-iron and high-iron conditions (25, 9). However, we did not find appreciable differences in the protein pattern in *M. tuberculosis* under similar growth conditions, although differences in the siderophore levels were seen (not shown). Here, in a detailed analysis, we found detectable levels of mycobactin, carboxymycobactin, and Irep-28 even at 4 μg of Fe/ml; at 8 μg of Fe/ml none of them were expressed. It is possible that a relatively higher level of the intracellular iron triggers the iron



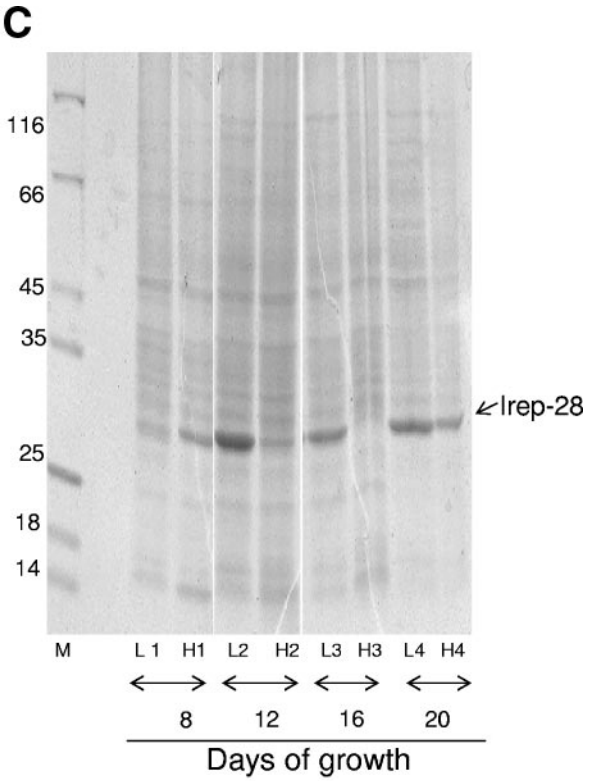


FIG. 3. Time course of expression of mycobactin, carboxymycobactin, and Irep-28 in *M. tuberculosis*. A 1-ml portion of a 14-day-old culture, resuspended to McFarland 4 in low-iron medium, was used to inoculate fresh medium with iron added at 8 μg of Fe/ml (high iron) and 0.02 μg of Fe/ml (low iron), respectively. Both mycobactin (A) and carboxymycobactin (B) were assayed as described in Materials and Methods in both high- and low-iron cultures harvested on days 8, 12, 16, 20, and 25, respectively. Mycobactin levels are expressed as OD_{450} units/g of cell dry weight. Carboxymycobactin values are expressed as siderophore units/100 mg of cell dry weight. The vertical bars represent the standard deviation of the mean from three independent experiments in both sets of experiments. (C) SDS-PAGE analysis of the detergent layer of Triton X-114-extracted cell envelope of *M. tuberculosis* harvested on days 8, 12, 16, and 20 grown as low-iron (lanes L1 to L4) and high-iron (lanes H1 to H4) cultures.

acquisition machinery in *M. tuberculosis* compared to other mycobacteria. Since a reflection of the iron status of the organisms is better understood by assaying all of the components of the iron acquisition machinery, we confirm here that Irep-28 is indeed regulated by iron. We observed that Irep-28 and carboxymycobactin were the first indicators of iron limitation due to nutrient depletion in the time course study. Mycobactin was slow to increase (seen in a 25-day-old culture), an observation that is in accordance with the view of Ratledge (19) that this siderophore is an intra-envelope iron storage molecule, holding the excess iron temporarily as ferric-mycobactin and releasing it slowly into the cytoplasm for immediate utilization and/or storage in the iron storage molecule bacterioferritin. Whether Irep-28 is a receptor for ferricarboxymycobactin is a hypothesis that requires further experimental proof.

We show by sequencing the tryptic peptides of the purified protein that Irep-28 is the DNA-binding HU homologue HupB protein (*hupB* [*Rv2986c*]). This protein probably does not conform to the predicted size of 21.8 kDa for HupB protein due to the exceptionally high content of lysine and arginine residues that may result in an altered mobility in SDS-PAGE. Modification of the Triton X-114 extraction protocol (22, 23) allowed complete solubilization of the lipid-rich wall and membrane samples and the relatively easier detection and purification of Irep-28. Sequencing of Irep-28 was done after separation by single-dimension gel electrophoresis. With mycobacterial envelope proteins, Gu et al. (10) and Sinha et al. (23) showed that

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1          10         20         30         40         50         60
MNKAELIDVL TQKLGSDRRQ ATAAVENVVD TIVRAVHKGD SVTITGFGVF EQRRRAARVA
61         70         80         90         100        110        120
RNPRTGETVK VKPTSVPAFR PGAQFKAVVS GAQRLPAEGP AVKRGVGASA AKKVAKKAPA
121    130        140        150        160        170        180
KKATKAAKKA ATKAPARKAA TKAPAKKAAT KAPAKKAVKA TKSPAKKVTK AVKKTAVKAS
181    190        200        210   214
VRKAATKAPA KKAAAKRPAT KAPAKKATAR RGRK
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FIG. 4. Sequence of Irep-28 identified as HupB protein. MS/MS identified Irep-28 as P95109 (Swiss-Prot). The three tryptic peptides sequenced by MS/MS are represented in boxes.

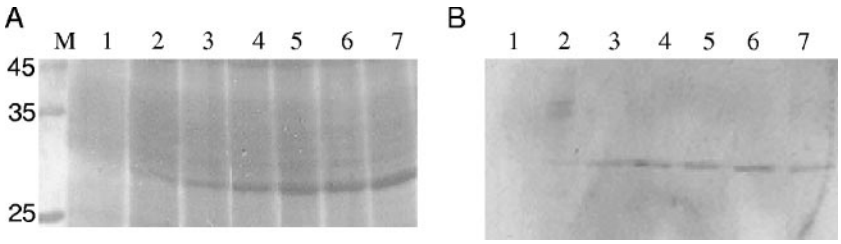

FIG. 5. Immunoreactivity of Irep-28 with patient serum. A Coomassie blue-stained gel pattern (A) and a blot developed with serum (B) from a patient who tested positive for *M. tuberculosis* are shown. Lanes 1 to 7 in both panels A and B represent cells grown with iron added at 12, 8, 4, 1, 0.1, 0.05, and 0.02 μg of Fe/ml, respectively.

one-dimensional gel electrophoresis followed by subjecting gel sections to peptide mass mapping through high-performance liquid chromatography–mass spectrometry was superior to two-dimensional gel electrophoresis followed by matrix-assisted laser desorption ionization–time of flight analysis. This protein is perhaps similar to the Irp28 reported by Calder and Horwitz in their initial study (3), but these authors did not emphasize it as an iron-regulated protein. In addition, they did not report it as an iron-regulated protein in their subsequent analysis by two-dimensional gel electrophoresis (27).

There are several reports on the clinical significance of HupB protein (4, 16) and the diagnostic potential of the *hupB* gene (17). The *M. tuberculosis hupB* gene product, first reported as a histone-like protein HLP_{Mt} (16), was shown to bind DNA and induce lymphoproliferation and antibodies in tuberculosis patients. Subsequently, the same group (17) used the *hupB* gene as a target for the differentiation of *M. tuberculosis* from *M. bovis* species from among other members of the *M. tuberculosis* complex and other mycobacterial species. In addition, antibodies to mycobacterial HupB were detected in patients with inflammatory bowel disease (4). In the present study, we found that most of the tuberculosis patients showed antibodies to HupB. Although other factors within the environment of the host could influence the expression of HupB, our observations on the coordinated expression of this protein with mycobactin and carboxymycobactin indicate that its expression in vivo may be influenced by iron limitation. Restricting the availability of iron is an important strategy for defense against bacterial infection (19). This includes the chelation of free iron by transferrin and lactoferrin and increased synthesis of transferrin and ferritin. Under in vivo conditions, *M. tuberculosis* grows within the phagocytic vacuoles of the macrophages, where the pH is between 6.1 and 6.5, values at which the maximum concentration of Fe^{3+} is between 1 and 10 ng/ml. However, the lactoferrin present within the macrophages may lower this further, thus making it necessary for the pathogen to adapt to this condition of iron restriction. As pointed out by Ratledge (19), treatment of anemia in a chronic infection such as tuberculosis needs to be done with caution, since iron supplementation would counter the body's defense mechanism of withholding iron.

TABLE 1. Immunoreactivity of Irep-28 versus culture (BACTEC), chest radiography, and Mycotest (commercial lateral flow device)[a]

Technique and result	No. of isolates		
	+	−	Total
Culture (BACTEC)			
+	28	5	33
−	5	2	7
Total	33	7	40
Chest X-ray			
+	33	6	39
−	2	0	2
Total	35	6	41
Mycotest			
+	20	16	36
−	2	4	6
Total	22	20	42

[a] The results of analysis of 44 serum samples from confirmed tuberculosis patients are presented. The detection of antibodies to Irep-28 is compared to conventional diagnostic tests, including culture, chest radiography, and the commercial antibody-detection lateral flow device Mycotest. The results show the high degree of correlation of the detection of anti-Irep-28 with culture and chest X-ray compared to the Mycotest.

The association of HupB with mycobactin and/or carboxymycobactin remains to be understood. The failure of mycobactin-defective mutants to grow within macrophages and the recent evidence for the new role for mycobactin as an iron-scavenging molecule (13) indicate that it probably plays an important role in the adaptation of *M. tuberculosis* within the environment of the host. As rightly pointed out by Barry and Boshoff (2), despite considerable knowledge of the chemistry and biosynthetic machinery of these two siderophores, there are several gaps in our understanding of their role in iron acquisition under conditions prevailing within the host. Even less is known of the association of these molecules with cell envelope proteins. Ongoing studies are focusing on the association of HupB with either or both siderophore molecules and understanding its role in iron acquisition. The antigenicity of this protein in tuberculosis patients and the specificity of the gene for *M. tuberculosis* complex (17) make it a candidate diagnostic marker and a potential drug target.

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REFERENCES

1. Reference deleted.
2. **Barry, C. E., and H. Boshoff.** 2005. Getting the iron out. Nat. Chem. Biol. **3:**127–128.

3. **Calder, K. M., and M. A. Horwitz.** 1998. Identification of iron-regulated proteins of *Mycobacterium tuberculosis* and cloning of tandem genes encoding a low-induced protein and a metal transporting ATPase with similarities to two-component metal transport systems. Microb. Pathog. **24:**133–143.
4. **Cohavy, O., G. Harth, M. A. Horwitz, M. Eggena, C. Landers, C. Sutton, S. Targan, and J. Braun.** 1999. Identification of a novel mycobacterial histone H1 homologue (HupB) as an antigenic target of pANCA monoclonal antibody and serum immunoglobulin A from patients with Crohn's disease. Infect. Immun. **67:**6510–6517.
5. **Cole, S. T., et al.** 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete sequence. Nature **393:**537–544.
6. **De Voss, J. J., K. Rutter, B. G. Schroeder, and C. E. Barry III.** 1999. Iron acquisition and metabolism by mycobacteria. J. Bacteriol. **181:**4443–4451.
7. **De Voss, J. J., K. Rutter, B. G. Schroeder, H. Su, Y. Zhu, and C. E. Barry III.** 2000. The salicylate-derived mycobactin siderophores of *Mycobacterium tuberculosis* are essential for growth in macrophages. Proc. Natl. Acad. Sci. USA **97:**1252–1257.
8. **Dussurget, O., and I. Smith.** 1998. Interdependence of mycobacterial iron regulation, oxidative-stress response and isoniazid resistance. Trends Microbiol. **6:**354–358.
9. **Hall, R. M., M. Sritharan, A. J. M. Messenger, and C. Ratledge.** 1987. Iron transport in *Mycobacterium smegmatis*, occurrence of iron-regulated envelope proteins as potential receptors for iron uptake. J. Gen. Microbiol. **133:**2107–2114.
10. **Gu, S., J. Chen, K. M. Dobos, E. M. Bradbury, J. T. Belisle, and X. Chen.** 2003. Comprehensive proteomic profiling of the membrane constituents of a *Mycobacterium tuberculosis* strain. Mol. Cell. Proteomics **2:**1284–1296.
11. **Kolarich, D., R. Leonard, W. Hemmer, and F. Altmann.** 2005. The N-glycans of yellow jacket venom hyaluronidases and the protein sequence of its major isoform in *Vespula vulgaris*. FEBS J. **272:**5182–5190.
12. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
13. **Luo, M., E. A. Fadeev, and J. T. Groves.** 2005. Mycobactin-mediated iron acquisition within macrophages. Nat. Chem. Biol. **3:**149–153.
14. Reference deleted.
15. Reference deleted.
16. **Prabhakar, S., P. S. Annapurna, N. K. Jain, A. B. Dey, J. S. Tyagi, and H. K. Prasad.** 1998. Identification of an immunogenic histone-like protein of *Mycobacterium tuberculosis*. Tuberc. Lung Dis. **79:**41–53.
17. **Prabhakar, S., A. Mishra, A. Singhal, V. M. Katoch, S. S. Thakral, J. S. Tyagi, and H. K. Prasad.** 2004. Use of the *hupB* gene encoding a histone-like protein of *Mycobacterium tuberculosis* as a target for detection and differentiation of *M. tuberculosis* and *M. bovis*. J. Clin. Microbiol. **42:**2724–2732.
18. **Ratledge, C., and L. G. Dover.** 2000. Iron metabolism in pathogenic bacteria. Annu. Rev. Microbiol. **54:**881–941.
19. **Ratledge, C.** 2004. Iron mycobacteria and tuberculosis. Tuberculosis **84:**110–130.
20. **Rodriguez, G. M., and I. Smith.** 2003. Mechanisms of iron regulation in mycobacteria: role in physiology and virulence. Mol. Microbiol. **47:**1485–1494.
21. **Schwyn, B., and J. B. Neilands.** 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. **160:**47–55.
22. **Sinha, S., S. Arora, K. Kosalai, A. Namane, A. S. Pym, and S. T. Cole.** 2002. Proteome analysis of the plasma membrane of *Mycobacterium tuberculosis*. Comp. Funct. Genomics **3:**470–483.
23. **Sinha, S., K. Kosalai, S. Arora, A. Namane, P. Sharma, An. N. Gaikwad, P. Brodin, and S. T. Cole.** 2005. Immunogenic membrane-associated proteins of *Mycobacterium tuberculosis* revealed by proteomics. Microbiology **151:**2411–2419.
24. **Sritharan, M., and C. Ratledge.** 1989. Co-ordinated expression of the components of iron transport (mycobactin, exochelin, and envelope proteins) in *Mycobacterium neoaurum* FEMS Microbiol. Lett. **60:**183–186.
25. **Sritharan, M., and C. Ratledge.** 1990. Iron-regulated envelope proteins of mycobacteria grown in vitro and their occurrence in *Mycobacterium leprae* grown in vivo. Biol. Metals **2:**203–208.
26. **Sritharan, M.** 2000. Iron as a candidate in virulence and pathogenesis in mycobacteria and other microorganisms. World J. Microbiol. Biotechnol. **16:**769–780.
27. **Wong, D. K., J. Gobin, M. A. Horwitz, and B. W. Gibson.** 1999. Characterization of exochelins of *Mycobacterium avium*: evidence for saturated and unsaturated acid for acid and ester forms. J. Bacteriol. **178:**6394–6398.
28. Reference deleted.