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Identification of a *Mycobacterium tuberculosis* Gene That Enhances Mycobacterial Survival in Macrophages

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Intracellular survival plays a central role in the pathogenesis of *Mycobacterium tuberculosis*. To identify *M. tuberculosis* genes required for intracellular survival within macrophages, an *M. tuberculosis* H37Rv plasmid library was constructed by using the shuttle vector pOLYG. This plasmid library was electroporated into *Mycobacterium smegmatis* 1-2c, and the transformants were used to infect the human macrophage-like cell line U-937. Because *M. smegmatis* does not readily survive within macrophages, any increased intracellular survival is likely due to cloned *M. tuberculosis* H37Rv DNA. After six sequential passages of *M. smegmatis* transformants through U-937 cells, one clone (p69) was enriched more than 70% as determined by both restriction enzyme and PCR analyses. p69 demonstrated significantly enhanced survival compared to that of the vector control, ranging from 2.4- to 5.3-fold at both 24 and 48 h after infection. DNA sequence analysis revealed three open reading frames (ORFs) in the insert of p69. ORF2 (1.2 kb) was the only one which contained a putative promoter region and a ribosome-binding site. Deletion analysis of the p69 insert DNA showed that disruption of ORF2 resulted in complete loss of the enhanced intracellular survival phenotype. This gene was named the enhanced intracellular survival (*eis*) gene. By using an internal region of *eis* as a probe for Southern analysis, *eis* was found in the genomic DNA of various *M. tuberculosis* strains and of *Mycobacterium bovis* BCG but not in that of *M. smegmatis* or 10 other nonpathogenic mycobacterial species. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis showed that all *M. smegmatis eis*-containing constructs expressed a unique protein of 42 kDa, the predicted size of Eis. The expression of this 42-kDa protein directly correlated to the enhanced survival of *M. smegmatis* p69 in U-937 cells. These results suggest a possible role for *eis* and its protein product in the intracellular survival of *M. tuberculosis*.

Mycobacterium tuberculosis is an important human pathogen responsible for 3.1 million deaths worldwide per year (9). Although both virulent and avirulent mycobacteria are internalized by monocytes and macrophages (35, 43), only pathogenic mycobacteria survive and replicate intracellularly (28). *M. tuberculosis* is resistant to macrophage killing, and its survival during phagocytosis and its subsequent multiplication within these professional phagocytes are critical to its pathogenesis. A variety of mechanisms have been suggested to contribute to the survival of *M. tuberculosis* within macrophages (15, 39), including inhibition of phagosome-lysosome fusion (2), inhibition of the acidification of phagosomes (41), resistance to killing by reactive oxygen intermediates (26) and reactive nitrogen intermediates (12, 27), and modification of the lipid composition of the mycobacterial cell membrane, thereby altering its capacity to interact with immune or inflammatory cells (19). However, little progress has been made in identifying the genes and their corresponding products responsible for these properties. Because they represent potentially interesting targets for novel drugs and vaccines, the identification of the mycobacterial products that promote intracellular survival remains a priority.

Recent development of genetic techniques applicable to studying mycobacteria is advancing our understanding of how mycobacteria survive in phagocytic cells (3, 7, 29, 31, 33). Several groups have recently used *Escherichia coli* to express *M. tuberculosis* and *Mycobacterium leprae* genes which may be involved in entry and survival within mammalian cells (3, 22, 35, 36). However, because of the diverse genetic and structural differences between mycobacteria and enterobacteria, *E. coli* systems may be limited in the number of tuberculosis virulence gene products which can be successfully expressed, processed, and exported or transported to appropriate functional sites.

New vectors and methodologies for the transformation of mycobacteria have been developed that allow for the study of virulent mycobacterial genes in their homologous hosts. *Mycobacterium smegmatis* was selected for use in these studies because it grows rapidly in the laboratory, readily expresses genes from other mycobacteria (20, 40, 44), and can be genetically manipulated by various techniques (21). Numerous genes from virulent mycobacteria have been expressed in *M. smegmatis*, including the superoxide dismutase gene from *M. tuberculosis* (46), genes for the production of glycopeptidolipid antigens and the Mig protein from *Mycobacterium avium* (8, 34), a gene expressing a 19-kDa glycosylated antigen from *M. tuberculosis* (16), the *noxR1* gene from *M. tuberculosis* (14), and the *tr-trx* gene from *M. leprae* (44).

In the present study, the vector pOLYG was used to construct a genomic plasmid library from the DNA of the virulent *M. tuberculosis* strain H37Rv. The library was then introduced into *M. smegmatis*. A human histocytic macrophage-like cell line, U-937 (18, 42), was used for selecting transformants with enhanced intracellular survival. U-937 cells may be converted from a nonadherent, weakly phagocytic form to an adherent, actively phagocytic state with phorbol esters and other agents. This cell line has been widely used and accepted as a model system for the study of macrophage interactions with a variety of intracellular pathogens (10, 11, 22, 30, 32, 35, 45) (C. Ja-

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gannath, E. Sepulveda, L. Srinivasan, R. M. Emanuele, and R. L. Hunter, Abstr. 97th Gen. Meet. Am. Soc. Microbiol., abstr. U-97, p. 560, 1997). *M. tuberculosis* H37Rv replicates and is not killed in U-937 cells (Jagannath et al., Abstr. 97th Gen. Meet. Am. Soc. Microbiol.), while *M. smegmatis* does not multiply and is readily killed. By serial passage through U-937 cells, an *M. smegmatis* transformant clone that showed enhanced intracellular survival over *M. smegmatis* containing the vector alone was isolated. Evidence that this enhanced intracellular survival phenotype is due to an *M. tuberculosis* H37Rv gene which we have named *eis* is presented.

MATERIALS AND METHODS

Strains and growth media. *M. smegmatis* 1-2c, a derivative of strain mc^26 selected for improved transformation efficiency (46), was grown in Middlebrook 7H9 broth (Difco) supplemented with 0.2% glycerol or plated on 7H10 agar (Difco) with 0.2% glucose at 37°C. *M. tuberculosis* H37Rv was grown in Middlebrook 7H9 broth as previously described (47). Hygromycin B at 50 μg/ml (Boehringer Mannheim) was used in mycobacterial media to maintain the presence of pOLYG. Luria-Bertani (LB) broth or agar with 200 μg of hygromycin B/ml or 100 μg of ampicillin/ml was used for growing *E. coli* transformants. Human macrophage-like U-937 cells (ATCC 1593.2 CRL) were grown in RPMI 1640 medium (Sigma) containing 10% fetal calf serum (FCS) (Atlanta Biologicals) under 5% CO_2 at 37°C.

Construction of an *M. tuberculosis* H37Rv genomic DNA library. Genomic DNA was isolated from *M. tuberculosis* H37Rv as previously described (47). Briefly, fresh liquid cultures of H37Rv were inoculated with a 3-week starter culture and then grown for 7 days before harvesting. *M. tuberculosis* H37Rv DNA was partially digested with the restriction enzyme *Sau*3AI, and 4- to 12-kb fragments were recovered after electrophoresis on a 0.7% agarose gel. DNA was extracted from the gel and cloned into the *Bam*HI site of the plasmid pOLYG (17). The library was transformed into Max Efficiency *E. coli* DH5α (Gibco BRL) competent cells, plated on LB agar containing 200 μg of hygromycin B/ml, and incubated overnight at 37°C. Approximately 7,000 independent *E. coli* transformants were generated by using this plasmid library. Restriction analysis of plasmids isolated from *E. coli* transformants demonstrated that 81% had insert DNA with an average size of 5.4 kb. The library pool was mixed in LB–30% glycerol, and aliquots were frozen at −70°C. *E. coli* DH5α containing the *M. tuberculosis* plasmid library was amplified by growth on LB plates, and plasmids were isolated by following the Qiagen Maxi-plasmid purification protocol. The isolated plasmid library DNA was then used to transform *M. smegmatis*.

Electroporation of *M. smegmatis* and *E. coli*. *M. smegmatis* 1-2c was transformed via electroporation by the method of Snapper et al. (40). Electroporation was performed with a Gene Pulser (Bio-Rad). Electrocompetent *M. smegmatis* (0.4 ml) was pipetted into a 0.4-cm-gap-size cuvette with 1- to 10-μg aliquots of plasmid DNA and electroporated at settings of 2.5 kV, 25 μF, and 1,000 Ω. To analyze the recombinant clones, plasmid DNA was electroporated directly from *M. smegmatis* transformants to *E. coli* cells. Electrocompetent *E. coli* DH5α cells were prepared as previously described (4). Sixty microliters of *E. coli* cells was pipetted into a 0.1-cm-gap-size cuvette containing 40 μl of transformed *M. smegmatis* cells. Electroporation into *E. coli* was performed at settings of 2.5 kV, 25 μF, and 200 Ω.

Intracellular survival assay. The intracellular survival assay was devised by modifying the procedure of Ramakrishnan and Falkow (35) developed for *Mycobacterium marinum* and the mouse macrophage-like cell line J-774. Suspension cultures of U-937 cells were grown in RPMI 1640–10% FCS medium in 75-cm^2 tissue culture flasks for 3 days before overnight treatment with 0.4 μg of phorbol 12-myristate 13-acetate (PMA) (Sigma)/ml to transform U-937 into an adherent state. PMA-containing supernatants were removed the next day, and the adherent cells were washed with Hanks' Ca^{2+}- and Mg^{2+}-free balanced salt solution (HBSS). Adherent U-937 cells were released by gentle rocking with 3-mm-diameter glass beads. Released cells were recovered by centrifugation, washed in HBSS, quantitated with a hemocytometer, and diluted in RPMI 1640–10% FCS medium to a density of 2×10^5 cells per ml. U-937 cells (1 ml/well) were plated into each well of 24-well tissue culture plates (Costar) and incubated overnight under 5% CO_2 at 37°C.

Cultures of *M. smegmatis* 1-2c containing the *M. tuberculosis* plasmid library were grown on 7H10 agar plates with 50 μg of hygromycin B/ml for 3 to 4 days. Inocula were prepared by swabbing the plate growth into HBSS, sedimenting the mycobacteria at 1,300 × *g* for 10 min, resuspending in HBSS, vortexing for 30 s, and centrifuging again at 250 × *g* for 5 min. This yielded a supernatant consisting almost entirely of single mycobacterial cells, as observed by phase-contrast microscopy. On the assumption that an optical density at 650 nm (OD_{650}) of 0.1 equals 10^8 CFU per ml, the inoculum was diluted to a density of 2×10^6 CFU per ml in RPMI 1640–10% FCS containing 2.5% fresh human serum (Omega Scientific). U-937 cells resemble human mononuclear phagocytes (38) in that phagocytosis of mycobacteria is greatly augmented in the presence of fresh human serum (complement component C3). Aliquots of the inocula were routinely plated on 7H10 plates containing hygromycin B for CFU determinations (see below) to confirm that equivalent bacterial inocula were used in all experiments. After 20 min of opsonization at room temperature, 1 ml of inoculum was added to each well containing 2×10^5 U-937 cells to give a multiplicity of infection (MOI) of 10. At this MOI, no cytotoxicity was observed. The 24-well plates were incubated for 2 h at 37°C in a 5% CO_2 incubator. Acid-fast staining showed that after 2 h of incubation approximately half of the U-937 cells contained at least one *M. smegmatis* organism. The infected monolayers were then washed once with warm HBSS and treated with RPMI 1640–10% FCS containing 200 μg of amikacin per ml for 1 h at 37°C to kill extracellular organisms. The monolayers were washed again with HBSS, and those monolayers in which survival for 24 and 48 h was to be measured were incubated in medium containing 20 μg of amikacin/ml to prevent extracellular growth of any bacteria that might be released by premature lysis of infected U-937 cells. Cells in duplicate wells were lysed at 3, 24, and 48 h postinfection by adding 1 ml of water, waiting for 30 min, and vigorously pipetting five times to ensure cell lysis and the release of surviving intracellular bacteria. The lysates were serially diluted in 7H9 broth and plated onto 7H10 agar plates containing hygromycin B. CFU were counted after incubation at 37°C for 3 days. *M. smegmatis*(pOLYG) was included in every assay as a negative control. Clones that were recovered on plates at 24 and 48 h in each passage were pooled and passed through the U-937 survival assay for the next passage. A total of 6 passages were carried out to enrich for recombinant *M. smegmatis* with increased capacity for intracellular survival. After each step in the passage, bacterial pools were frozen at −70°C for further analysis.

Transmission electron microscopy. U-937 cell monolayers were prepared in 24-well tissue culture plates as described for the intracellular survival assay and infected with *M. smegmatis*(p69) at an MOI of 50. This higher MOI was used to assist in the observation of *M. smegmatis*(p69) in the transmission electron microscopy preparations. At 3 and 24 h after infection, monolayers were washed and fixed for 1 h at room temperature in 0.1 M phosphate buffer (pH 7.2) containing 2% glutaraldehyde. Cells from fixed monolayers were then released into suspension by use of a rubber policeman. Specimens were postfixed in 2% osmium tetroxide, dehydrated via graded alcohol steps, and embedded in Spurr low-viscosity resin. Sections were cut, stained with uranyl acetate and lead citrate, and viewed on a Philips CM12 transmission electron microscope.

Recombinant DNA techniques. Standard techniques were used for plasmid isolation, endonuclease restrictions, DNA modifications, ligations, and plasmid transformations (37). Restriction endonucleases and other enzymes were used as recommended by the suppliers (Life Technologies and New England Biolabs).

Construction of *eis* deletion derivatives. The DNA insert of p69 was released from pOLYG by digestion with *Bam*HI and cut in the middle of open reading frame 2 (ORF2) by digestion with *Sph*I. The resulting 1.3- and 1.7-kb fragments were ligated into *Bam*HI-*Sph*I-digested pUC18 to yield the subclones p62-8 and p62-16, respectively. The 1.3-kb *Bam*HI-*Hin*dIII fragment from p62-8 was cloned into *Bam*HI-*Hin*dIII-digested pOLYG to obtain p69-8. The 1.7-kb *Bam*HI-*Hin*dIII fragment from p62-16 was then cloned into *Bam*HI-*Hin*dIII-digested pOLYG to obtain p69-16. p62-97 was created by digestion of p69 with *Apo*I and *Pvu*I. The *Pvu*I site was filled in with DNA polymerase. This 1.6-kb *Apo*I-*Pvu*I fragment carrying only ORF2 and its putative promoter region was isolated and subcloned into *Eco*RI-*Sma*I-digested pSP72 (Promega). p69-97 was constructed by inserting the 1.6-kb *Eco*RV-*Hin*dIII fragment from p62-97 into *Eco*RV-*Hin*dIII-digested pOLYG. p69-96 was made by inserting the 1.6-kb *Cla*I-*Hin*dIII fragment from p62-97 into *Cla*I-*Hin*dIII-digested pOLYG. The transcriptional orientation of p69-96 is opposite that of p69-97. ORF2 was disrupted by removing an internal 367-bp *Apa*I fragment from p69-96, creating p69-96A, which is an in-frame deletion. All p69 derivatives and p69 itself were then retransformed into *M. smegmatis* cells for use in the intracellular survival assay.

Nucleotide sequence data analysis. The nucleotide sequence of the DNA insert of p69 was determined with a DNA sequencer (Applied Biosystems model 373A) at the Laboratory of Molecular Systematics and Evolution, University of Arizona. Computation was performed by using the Genetics Computer Group sequence analysis software package (version 10, University of Wisconsin, Biotechnology Center) and the National Center for Biotechnology Information BLAST (1) network service.

PCR analysis for enrichment of p69 after serial passages through U-937 cells. Fifty isolates each from the third, fourth, fifth, and sixth rounds of *M. smegmatis* transformant passage in U-937 cells were analyzed for the presence of *eis* by using PCR amplification. After 5 days of growth on 7H10 agar containing 50 μg of hygromycin B/ml, individual colonies were picked and boiled in 50-μl aliquots of H_2O for 15 min, and then 5-μl aliquots of the lysed cells were added to separate 50-μl PCR mixtures containing 1× PCR buffer (Gibco BRL), 5 mM $MgCl_2$, 0.6 mM deoxynucleoside triphosphates, 4 μM each oligonucleotide primer (69.4F, 5′-GGATCCGTCAGACCCACCGAGCAT-3′, and 69.8R, 5′-CGGATCCCCATCCATGGCGTGT-3′; Gibco BRL), and 2.5 U of *Taq* DNA polymerase (Gibco BRL). Thermocycling reactions were performed in a Perkin-Elmer GeneAmp PCR System 2400 with the following parameters: an initial denaturing at 94°C for 2 min, a final additional extension at 72°C for 2 min, and 30 cycles of 94°C for 1.5 min, 56°C for 1.5 min, and 72°C for 1.5 min. For each set of reactions, pOLYG and p69 were included as negative and positive controls, respectively. PCR products were analyzed by electrophoresis on a 1% agarose gel with an *eis*-specific product expected to be 824 bp in length.

Southern hybridization. For isolation of genomic DNA of *M. smegmatis*, the procedure of Jacobs et al. (21) was followed. Genomic DNA of *M. tuberculosis* Erdman, *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra, and *M. bovis* BCG was kindly supplied by John T. Belisle of Colorado State University. These materials were provided through the National Institutes of Health (NIH, Rockville, Md.) contract entitled "Tuberculosis research materials and vaccine testing." Genomic DNA preparations isolated from *M. abscessus*, *M. aurum*, *M. avium*, *M. peregrinum*, *M. phlei*, *M. triviale*, *M. vaccae*, *M. chelonae*, *M. fortuitum*, and *M. gordonae* were received as a kind gift from Benjamin Schroeder and Clifton Barry III (NIH).

The DNA probe for Southern hybridization was prepared by using a PCR DIG Probe Synthesis Kit (Boehringer Mannheim). PCRs to generate a digoxigenin (DIG)-labeled probe included plasmid p69 as template DNA and primers 69.4F and 69.8R (described above). The reaction profile was identical to that used in PCR analysis of *M. smegmatis* transformant pools for the presence of *eis*. Samples of genomic DNA (5 μg) were digested with *Pst*I, and the resulting fragments were separated on a 1% agarose gel and transferred to a GeneScreen Plus membrane (NEN Research Products) by standard methods (37). Southern blots were prepared as previously described (6). DIG-labeled fragments were detected according to the manufacturer's directions by using the DIG DNA Labeling and Detection Kit (Boehringer Mannheim).

SDS-PAGE analysis of *M. smegmatis* transformants containing derivatives of *eis*. *M. smegmatis* transformants containing pOLYG, p69, p69-16, p69-8, p69-97, p69-96, and p69-96A were grown on Middlebrook 7H10 agar with 0.2% glucose and 50 μg of hygromycin B/ml for 3 to 4 days at 37°C before harvesting. Cells were swabbed into HBSS and pelleted by centrifugation at 12,000 × *g* for 10 min. Pelleted cells were resuspended in an equal volume of HBSS. One-milliliter aliquots of these cell suspensions were transferred to microcentrifuge tubes containing 0.25 g of 0.1- to 0.15-mm-diameter glass beads (Biospec Products, Bartlesville, Okla.). Cells were vortexed with glass beads for 30 min at room temperature by using a Vortex-Genie Turbomix device (Fisher Scientific). This procedure resulted in a 96% loss of cell viability, as determined by viable plate counts. After vortexing, the glass beads were allowed to settle before the supernatant was transferred. Protein concentrations of various lysates were determined by using the BCA Protein Assay Reagent (Pierce). Sodium dodecyl sulfate (SDS)-loading dye was mixed with 140-μg protein samples and boiled for 10 min before samples were loaded onto an SDS-polyacrylamide gel electrophoresis (PAGE) gel (12% polyacrylamide) with a 4% stacking gel (24). Samples were electrophoresed for 4 to 5 h at 200 V and 100 mA. Gels were stained with Coomassie brilliant blue and photographed by using an Alpha Imager 2000 Documentation and Analysis System (Alpha Innotech Corporation).

Statistical analysis of data. Results of assays for intracellular survival are expressed as means ± standard deviations from three independent experiments performed on different days. Differences between various groups were assessed by use of Student's *t* test. The level of significance was set at 0.001.

Nucleotide sequence accession number. The nucleotide sequence of the *eis* gene shown in Fig. 4 has been deposited in the GenBank database under accession no. AF144099.

RESULTS

Selection of *M. smegmatis* transformed with *M. tuberculosis* H37Rv DNA for enhanced survival in U-937 cells. To enrich for recombinants with increased ability for intracellular survival, *M. smegmatis* transformants containing the *M. tuberculosis* H37Rv DNA plasmid library were used to infect monolayers of U-937 as described in Materials and Methods. One hundred twenty independent clones randomly selected from the 3rd passage were screened individually in the intracellular survival assay to look for individual *M. smegmatis* recombinants with enhanced survival compared to *M. smegmatis* containing only the vector pOLYG. Twenty-one of the recombinants showed ≥2-fold-increased survival 48 h after infection (data not shown). One clone (p69) demonstrated significantly enhanced survival, ranging from 2.4- to 5.3-fold at 24 and 48 h after infection, respectively (Fig. 1), and was further characterized.

Electron microscopy was performed to confirm that *M. smegmatis*(p69) had indeed been internalized by U-937 cells and was present intracellularly. Figure 2 shows typical observations of intracellular *M. smegmatis*(p69) in the cytoplasm of U-937 cells. At 3 h postinfection, numerous *M. smegmatis* bacilli were present in the cytoplasm of U-937 cells either in tightly fitting phagosomes (Fig. 2A) or in more spacious phagosomes (Fig. 2B). The mycobacteria were also seen within

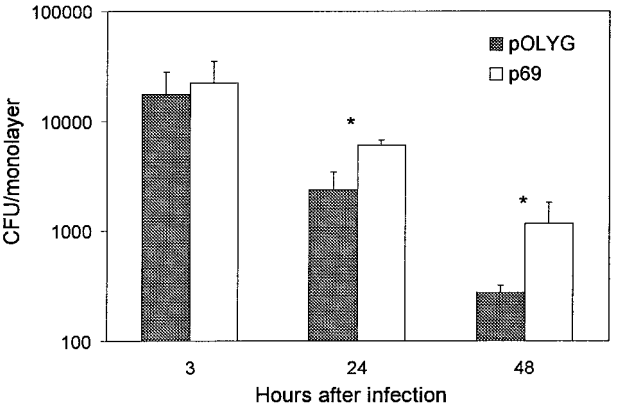

FIG. 1. Survival of *M. smegmatis* containing pOLYG or p69 in U-937 cells. As described in Materials and Methods, surviving intracellular bacteria were counted at 3, 24, and 48 h after infection. Data are means ± standard deviations from three independent experiments performed on different days. *, $P < 0.001$ compared to pOLYG at each time point.

phagosomes and not free in the cytoplasm. Similar electron micrographs were obtained at 24 h after infection (data not shown). These electron micrographs of *M. smegmatis* in U-937 cells are comparable to those of *M. smegmatis* in human mononuclear phagocytes (5).

The shuttle vector pOLYG is a multicopy, hygromycin B-resistant plasmid (17). Since hygromycin B was not present during serial passages and assays for survival in U-937 cells, it was necessary to verify that antibiotic selection pressure is not required to maintain the plasmid library in *M. smegmatis*. When infected monolayer lysates from different passages were plated on 7H10 agar plates with or without hygromycin B, equivalent numbers of CFU were recovered (data not shown). This demonstrates that hygromycin B is not required for maintenance of the genomic plasmid library in *M. smegmatis* during passage in the U-937 cells.

In the survival assay, amikacin (200 μg/ml) is used initially to kill uningested extracellular *M. smegmatis*. Following this 1-h treatment, 20 μg of amikacin/ml is present to prevent bacterial replication in the tissue culture medium. Theoretically, it might be possible to select for amikacin-resistant mutants in the survival assay. To clarify this, the MICs of amikacin were determined for *M. smegmatis*(pOLYG) and *M. smegmatis*(p69) recovered after passage in the survival assay and for unpassed *M. smegmatis* transformed with each vector. In both passaged and freshly transformed *M. smegmatis*(pOLYG) and *M. smegmatis*(p69), the MIC was 0.3 μg/ml. Thus, *M. smegmatis*(p69) has no increased resistance to amikacin.

We also sought evidence that amikacin kills extracellular microbes only and does not inhibit the intracellular survival of *M. smegmatis*. If the levels of amikacin used in the survival assay do indeed kill ingested *M. smegmatis*, then doubling the exposure of U-937 cells to the antibiotic should reduce intracellular survival. U-937 cells were pretreated with amikacin at 20 μg/ml for 48 h and then at 200 μg/ml for 1 h prior to infection with *M. smegmatis*(pOLYG) and *M. smegmatis*(p69) in the survival assay. A comparable population of U-937 cells not pretreated with the antibiotic was also infected with the same inocula, and the survival of the four sets of *M. smegmatis*-infected U-937 cells was then compared in the customary assay. Equivalent numbers of *M. smegmatis* transformants were recovered in both the amikacin-pretreated (two rounds of amikacin exposure) and non-pretreated (one round of amikacin

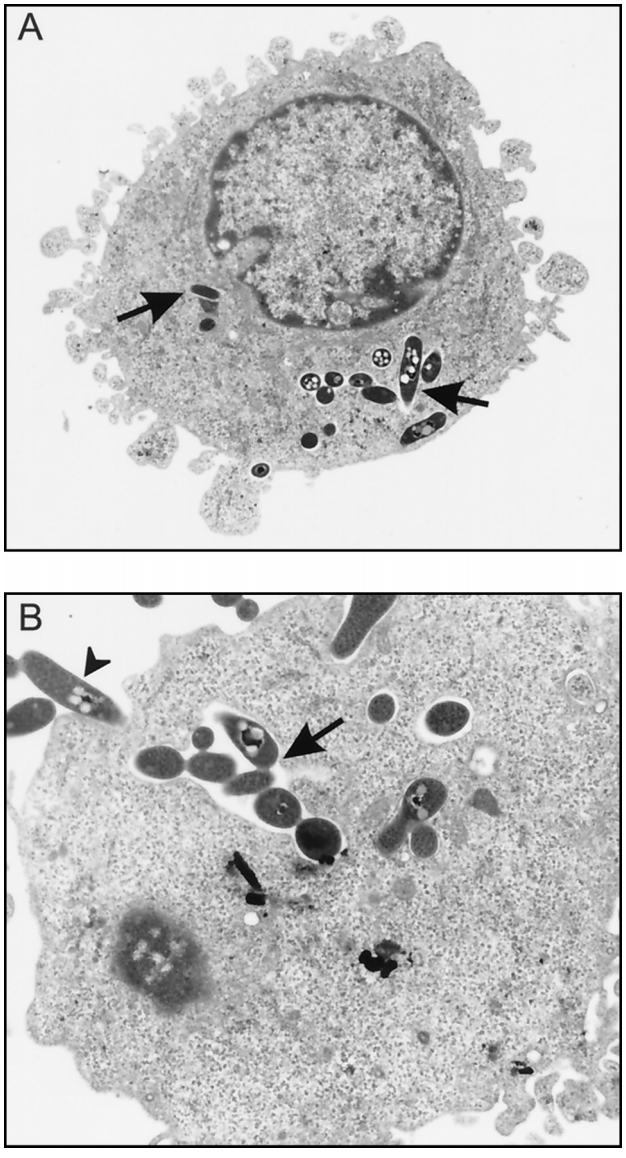

FIG. 2. Electron micrographs demonstrating intracellular *M. smegmatis*(p69) within U-937 cells at 3 h after infection. (A) Several *M. smegmatis*(p69) bacilli (arrows) are present in tight-fitting phagosomes in the cytoplasm of the macrophage-like cell. Magnification, ×8,500. (B) Higher magnification of an area of another U-937 cell which contains *M. smegmatis*(p69) in a more spacious phagosome (arrow). The arrow-head denotes bacilli probably in the early stages of internalization. Magnification, ×13,600.

exposure) groups at 3, 24, and 48 h after infection. Therefore, amikacin is not significantly internalized into U-937 cells to levels that interfere with the intracellular survival of *M. smegmatis*.

Restriction digestion and sequence analysis of p69. To facilitate analysis of p69, the plasmid was directly transformed by electroporation from *M. smegmatis* into *E. coli* DH5α cells. Restriction mapping indicated that p69 contained a 2.99-kb DNA insert. The p69 insert was sequenced and analyzed. The insert DNA was GC rich (68% G+C) and was identical to a nucleotide sequence found in the *M. tuberculosis* genome database (13). This is a 9-kb region in which none of the potential ORFs has a known function. Nucleotide sequencing revealed the presence of three potential ORFs designated ORF1, ORF2, and ORF3s in the insert (Fig. 3). These ORFs correspond to the hypothetical genes *Rv2417c*, *Rv2416c*, and *Rv2415c*, respectively, in the *M. tuberculosis* genome (13). The deduced amino acid sequences of the three ORFs were used in searches of the GenBank/EMBL and SWISSPROT databases with the BLAST and FASTA programs. Some homology (34% identity) was found between ORF2 and a hypothetical 45-kDa protein (*orf5*), of unknown function, downstream from the *amfC* gene of *Streptomyces griseus*, which is involved in aerial mycelium formation in this microbe (23). Only one of the three ORFs encoded on the p69 insert, ORF2, contains both a putative promoter region and a ribosome binding site (Fig. 3 and 4). These results suggest that ORF2 of p69 is likely to be the gene which confers the enhanced intracellular survival phenotype on *M. smegmatis*. ORF2 can potentially express a 42.2-kDa protein (387 amino acids).

Subcloning and deletion analysis of ORF2. Deletion analysis of the p69 insert DNA was carried out to verify that intact ORF2 is indeed essential for the enhanced intracellular survival phenotype observed in *M. smegmatis*(p69) (Fig. 3). Plasmid p69-8 contains a complete ORF1 and the 5′ half of ORF2, while plasmid p69-16 contains the 3′ half of ORF2 and an intact ORF3. Both plasmids p69-96 and p69-97 contain a 1.6-kb *Apo*I-*Pvu*I fragment with the intact ORF2 and its putative promoter region, but in opposite orientations. Plasmid p69-96A is an in-frame deletion of p69-96, in which ORF2 has been disrupted by removal of the 367-bp *Apa*I fragment. These deletion constructs were electrotransformed into *M. smegmatis* and compared in the intracellular survival assay with p69 and pOLYG as controls (Fig. 5). *M. smegmatis* transformants with p69-8, p69-16, and p69-96A all contain a disrupted ORF2 and survived no better than pOLYG transformants. *M. smegmatis* containing p69-96 and p69-97 had levels of intracellular survival comparable to that of p69 and significantly higher than that of pOLYG ($P < 0.001$). The activity of ORF2 in either orientation provides evidence that ORF2 can be expressed from its own promoter. These results confirm that the *M. tuberculosis* gene ORF2 is directly responsible for the enhanced intracellular phenotype associated with p69 in *M. smegmatis*. Therefore, ORF2 was named the enhanced intracellular survival (*eis*) gene.

Efficiency of selection of *eis*-bearing transformants by U-937 cells. With the identification of *eis*, it became possible to measure its rate of selection during serial passages in U-937 cells. Ten independent clones were selected at random from the 3rd to 6th passages of the *M. smegmatis* transformant pools. Plasmids from these clones were isolated and characterized by digestion with *Bam*HI and *Sma*I to determine the percentage with the same restriction digest patterns as p69. In addition, 50 independent clones were randomly selected from each of these same passages and two *eis*-specific oligonucleotide primers (Fig. 3) were used in PCR analysis for the presence of the *eis* gene in each clone. Results showed that the *eis*-bearing transformants, undetectable in the 3rd passage, were enriched by the 6th passage to 70 or 88% of the transformant pool, as determined by restriction digestion or PCR analysis, respectively (Fig. 6). Similar results were obtained in a second independent series of passages of the recombinant library in the U-937 survival assay.

There is a possible alternative explanation for the enrichment of *eis*-bearing transformants during library passage in U-937 cells. It may be that *eis*-bearing transformants grew faster in the medium used to prepare the inocula for each round of selection in U-937 cells. However, the growth curves of *M. smegmatis*, *M. smegmatis*(pOLYG), and *M. smegmatis*(p69) in 7H9 medium were found to be indistinguishable

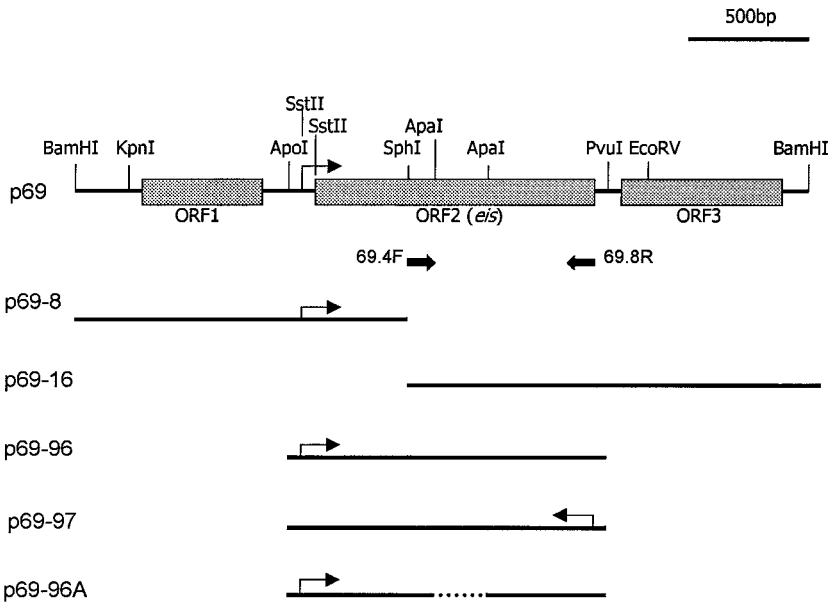

FIG. 3. Restriction map of p69 and its deletion derivatives. Shaded boxes represent the three ORFs revealed by sequencing. An arrow indicates the putative promoter. Solid arrows, primers 69.4F and 69.8R, used in the PCR; dotted line, in-frame deletion.

(data not shown). Therefore, these results show that the intracellular survival assay efficiently enriches for the transformants with enhanced survival phenotypes and that *eis* confers a real survival advantage on *M. smegmatis* containing this gene.

Demonstration of *eis* only in *M. tuberculosis* and *M. bovis* BCG. If *eis* contributes to the survival of *M. tuberculosis* in macrophages, it might be present in pathogenic species but absent in nonpathogenic species. The genomic DNAs of a number of mycobacterial species were examined by Southern analysis using a PCR-generated DIG-labeled probe to detect the presence of *eis* (Fig. 7). The *eis* gene was identified as a 12-kb band present only in *M. tuberculosis* H37Rv, H37Ra, and Erdman and in *M. bovis* BCG. None of the nonpathogenic mycobacterial species tested, including *M. smegmatis*, hybridized with the DIG-labeled *eis* probe. These results demonstrate that *eis* occurs only in pathogenic mycobacteria and their laboratory-produced derivatives.

Identification of the putative gene product of *eis*. To assay for the presence of an *eis* gene product, *M. smegmatis* transformants containing vector alone, p69, or deletion derivatives of p69 were lysed by vortexing with glass beads. Proteins from the various cell lysates were separated on a 12% SDS–PAGE gel. Figure 8 shows that in the presence of an intact *eis* gene (p69, p69-97, and p69-96), there is a unique Coomassie blue-stained band corresponding to a protein matching the predicted size of Eis (42 kDa). This protein band is not present in *M. smegmatis* transformants containing *eis* gene deletion constructs (p69-16, p69-8, and p69-96A). Therefore, the appearance of this 42-kDa protein directly correlates with enhanced intracellular survival of the various transformants containing an intact *eis* gene (compare Fig. 5 and 8).

DISCUSSION

Previous workers have used similar approaches to look for genes of *M. tuberculosis* needed for survival in macrophages. Arruda et al. cloned DNA from avirulent *M. tuberculosis* H37Ra and isolated the *mce* gene, which augmented the ability of *E. coli* to enter and survive in nonphagocytic HeLa cells (3). Mundayoor and Shinnick (29) passed recombinant DNA libraries of *M. leprae* in *E. coli* through a mouse macrophage-like cell line to enrich for clones with increased survival in those host cells. Using pOLYG as a cloning vector, Wieles et al. (44) cloned the thioredoxin-thioredoxin reductase gene of *M. leprae* into *M. smegmatis* and showed that the transformant was less rapidly killed by human mononuclear phagocytes than *M. smegmatis* with pOLYG alone.

In the present study, the human macrophage-like cell line U-937 was used to show that a clone of avirulent *M. smegmatis* transformed with DNA from the virulent *M. tuberculosis* strain H37Rv exhibited significantly enhanced intracellular survival for at least 48 h. Evidence indicates that the prolonged survival of this clone (p69) in U-937 cells resulted from the presence of a *M. tuberculosis* gene we have designated *eis* and that disruption of this gene was followed by loss of the enhanced intracellular survival phenotype.

Identification of *eis* using *M. smegmatis* and U-937 cells suggests the potential of this system for identifying additional genes contributing to the survival of virulent mycobacteria in macrophages. Of the 21 clones isolated from the 3rd passage of *M. smegmatis* transformants in U-937 cells, only p69 has been characterized in detail. The restriction patterns of many of the remaining 20 clones are not the same as that of p69, which suggests that further investigation may lead to identification of additional genes contributing to the intracellular survival of mycobacteria. A limitation of this system is the size of the H37Rv DNA insert used in this library, 5.4 kb on average. This effectively limits the probability of finding more than one gene of interest in a single DNA insert. It is possible that cosmid libraries of *M. tuberculosis* DNA, with insert sizes of up to 40 kb, may be used to identify virulence factors that require the concerted action of several separate genes or the presence of an entire operon.

Still unanswered is the question of how the *eis* product acts to prolong the survival of *M. smegmatis*, and perhaps ultimately that of *M. tuberculosis*, in macrophages. Numerous mycobacterial activities must be required for long-term survival of these

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CGAAATTCGTCGCTGATTCTCGCAGTGGCGTCACGCTGGCGGGGCTACCCGCATCGCGTG   60
      -35              -10
ATCCTTTGCCAGACACTGTCGTCGTAATATTCACGTGCACGTGGCCGCGGCATATGCCAC  120
                                RBS
AGTCGGATTCTGTGACTGTGACCCTGTGTAGCCCGACCGAGGACGACTGGCCGGGGATGT  180
                                                          M  F
TCCTACTGGCCGCGGCCAGTTTCACCGATTTCATCGGCCCTGAATCAGCGACCGCCTGGC  240
  L  L  A  A  A  S  F  T  D  F  I  G  P  E  S  A  T  A  W  R
GGACCCTGGTGCCCACCGACGGAGCGGTGGTGGTCCGCGATGGTGCCGGCCCGGGTTCTG  300
  T  L  V  P  T  D  G  A  V  V  V  R  D  G  A  G  P  G  S  E
AGGTGGTCGGGATGGCGCTGTACATGGATCTGCGGTTGACGGTGCCTGGTGAAGTGGTGC  360
  V  V  G  M  A  L  Y  M  D  L  R  L  T  V  P  G  E  V  V  L
TCCCGACCGCCGGTCTCAGTTTCGTCGCGGTGGCGCCGACGCATCGCCGGCGCGGCTTGC  420
  P  T  A  G  L  S  F  V  A  V  A  P  T  H  R  R  R  G  L  L
TGCGCGCGATGTGCGCCGAACTGCACCGCCGCATAGCCGATTCCGGCTATCCGGTCGCGG  480
  R  A  M  C  A  E  L  H  R  R  I  A  D  S  G  Y  P  V  A  A
CACTGCATGCTAGCGAGGGCGGCATCTACGGCCGGTTCGGCTACGGGCCCGCTACCACCT  540
  L  H  A  S  E  G  G  I  Y  G  R  F  G  Y  G  P  A  T  T  L
TGCATGAGCTGACGGTCGACCGACGCTTCGCGCGCTTTCACGCCGACGCACCGGGCGGCG  600
  H  E  L  T  V  D  R  R  F  A  R  F  H  A  D  A  P  G  G  G
GCCTAGGTGGCAGCAGCGTCCGGTTGGTCAGACCCACCGAGCATCGCGGCGAGTTTGAGG  660
  L  G  G  S  S  V  R  L  V  R  P  T  E  H  R  G  E  F  E  A
CGATCTACGAGCGATGGCGCCAGCAGGTGCCGGGCGGGCTGCTACGCCCGCAGGTGCTCT  720
  I  Y  E  R  W  R  Q  Q  V  P  G  G  L  L  R  P  Q  V  L  W
GGGACGAGCTGCTGGCAGAATGCAAAGCCGCGCCCGGTGGAGACCGTGAATCGTTCGCGT  780
  D  E  L  L  A  E  C  K  A  A  P  G  G  D  R  E  S  F  A  L
TACTGCATCCCGACGGGTACGCGCTGTACCGGGTGGATCGCACCGATCTCAAGCTAGCGC  840
  L  H  P  D  G  Y  A  L  Y  R  V  D  R  T  D  L  K  L  A  R
GCGTCAGCGAACTCAGGGCGGTAACCGCAGATGCGCATTGTGCGTTGTGGCGGGCCCTGA  900
  V  S  E  L  R  A  V  T  A  D  A  H  C  A  L  W  R  A  L  I
TTGGCCTCGACTCCATGGAGCGAATCAGCATCATCACCCATCCACAGGACCCGTTACCCC  960
  G  L  D  S  M  E  R  I  S  I  I  T  H  P  Q  D  P  L  P  H
ACCTGCTCACCGATACCCGACTGGCCCGCACTACCTGGCGCCAGGACGGCCTGTGGTTGC 1020
  L  L  T  D  T  R  L  A  R  T  T  W  R  Q  D  G  L  W  L  R
GCATCATGAACGTACCGGCCGCACTCGAGGCGCGTGGTTACGCTCACGAAGTTGGCGAGT 1080
  I  M  N  V  P  A  A  L  E  A  R  G  Y  A  H  E  V  G  E  F
TTTCCACGGTCCTCGAGGTATCCGATGGCGGCCGGTTCGCGCTCAAGATCGGTGACGGCC 1140
  S  T  V  L  E  V  S  D  G  G  R  F  A  L  K  I  G  D  G  R
GTGCGCGGTGTACCCCGACCGATGCGGCAGCCGAGATCGAAATGGATCGGGACGTACTGG 1200
  A  R  C  T  P  T  D  A  A  A  E  I  E  M  D  R  D  V  L  G
GCAGCCTTTACCTTGGAGCGCACCGCGCTTCGACGTTAGCCGCCGCTAACCGGTTGCGCA 1260
  S  L  Y  L  G  A  H  R  A  S  T  L  A  A  A  N  R  L  R  T
CCAAAGATTCCCAGCTGCTTCGTCGACTCGACGCGGCGTTTGCCAGTGATGTTCCCGTCC 1320
  K  D  S  Q  L  L  R  R  L  D  A  A  F  A  S  D  V  P  V  Q
AGACCGCGTTCGAGTTCTGAAGGCCGTGCTAGGCCGGCGCTAGGCTGACGGGCTTTTCGG 1380
  T  A  F  E  F  *
CGTGGTCAGCGACCCGCGTGCTGCGCGCCGGCTTCGGTCGCCACACGCCATGGATGGGGA 1440

TCGGCCGCGCGGCTCAGCACTCGCGGATCGCGTTGCCGAGTACACTCTCGATCGCGGTGA 1500
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FIG. 4. Nucleotide sequence of the 1.5-kb DNA fragment containing the *eis* gene and deduced amino acid sequence of Eis. Asterisk, stop codon. The putative promoter region and the ribosome binding site (RBS) are underlined.

organisms in human phagocytes. These include housekeeping activities necessary for intracellular growth and survival, activities needed to defend the mycobacteria against stressful conditions, and activities specifically evolved to promote entry, proliferation, and latency in host cells (25). The finding that *eis* is present only in virulent mycobacteria or their laboratory-generated derivatives and not in *M. smegmatis* and numerous

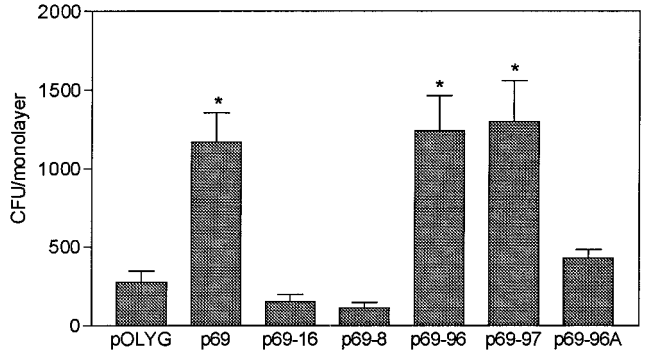

FIG. 5. Survival of *M. smegmatis* containing pOLYG, p69, and deletion derivatives of p69 in U-937 cells. Surviving intracellular bacteria were counted 3, 24, and 48 h after infection, but only the 48-h values are presented. Data are means ± standard deviations from three independent experiments performed on different days. *, $P < 0.001$ compared to pOLYG.

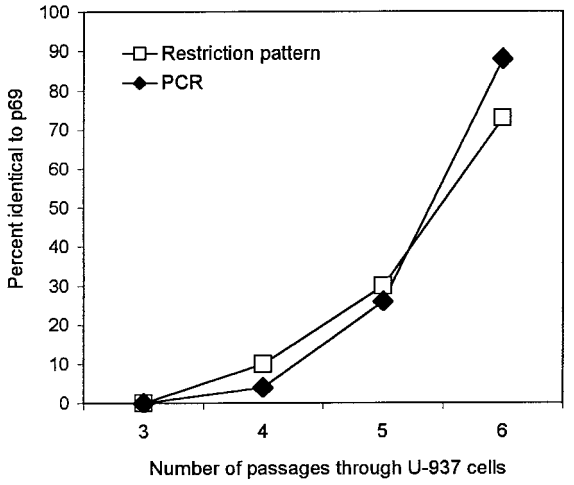

FIG. 6. Enrichment of p69 during passage through U-937 cells. Plasmid restriction patterns were analyzed for 10 clones randomly selected from each passage. PCR analysis was performed on 50 clones randomly selected from each passage. Details of the methodology are given in Materials and Methods.

other nonpathogenic mycobacteria argues against *eis* being a mere housekeeping gene.

A 42-kDa protein has been identified as the putative *eis* gene product, Eis. Two lines of evidence support this conclusion. First, the unique size of the protein matches the size predicted for an *eis* gene protein product. Second, the appearance of the 42-kDa protein in *M. smegmatis* transformants containing an intact *eis* gene directly correlates with the enhanced intracellular phenotype conferred by the gene in *M. smegmatis*. Exploratory studies suggest that Eis expressed in *M. smegmatis* is surface located. Such a location suggests participation of the Eis protein in the interactions between the surfaces of mycobacteria and the membranes of host cells, either in entry or during the subsequent sojourn of the mycobacteria in phagosomes. Studies are currently in progress to demonstrate that Eis is expressed in *M. tuberculosis* and to determine its cellular location in the pathogen.

The increased survival in U-937 cells conferred on *M. smegmatis* by the presence of the *eis* gene is modest but still significant, considering that it is the result of introducing a single

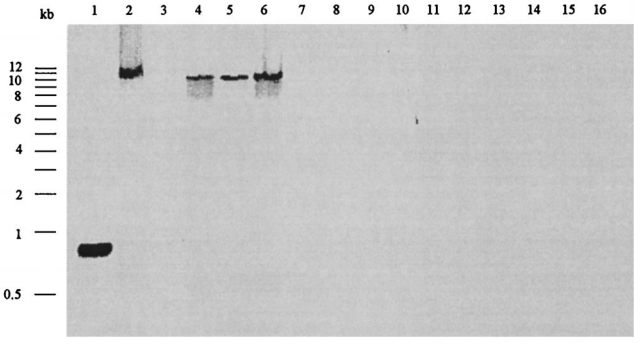

FIG. 7. Southern blot analysis for the presence of *eis* in *Mycobacterium* spp. The 824-bp DIG-labeled PCR product from *eis* was hybridized to *Pst*I-digested chromosomal DNA. Lanes: 1, 824-bp PCR product of *eis* obtained with primers 69.4F and 69.8R; 2, *M. bovis* BCG; 3, *M. smegmatis* 1-2c; 4, *M. tuberculosis* H37Rv; 5, *M. tuberculosis* Erdman; 6, *M. tuberculosis* H37Ra; 7, *M. abscessus*; 8, *M. aurum*; 9, *M. avium*; 10, *M. gordonae*; 11, *M. peregrinum*; 12, *M. phlei*; 13, *M. triviale*; 14, *M. vaccae*; 15, *M. chelonae*; 16, *M. fortuitum*.

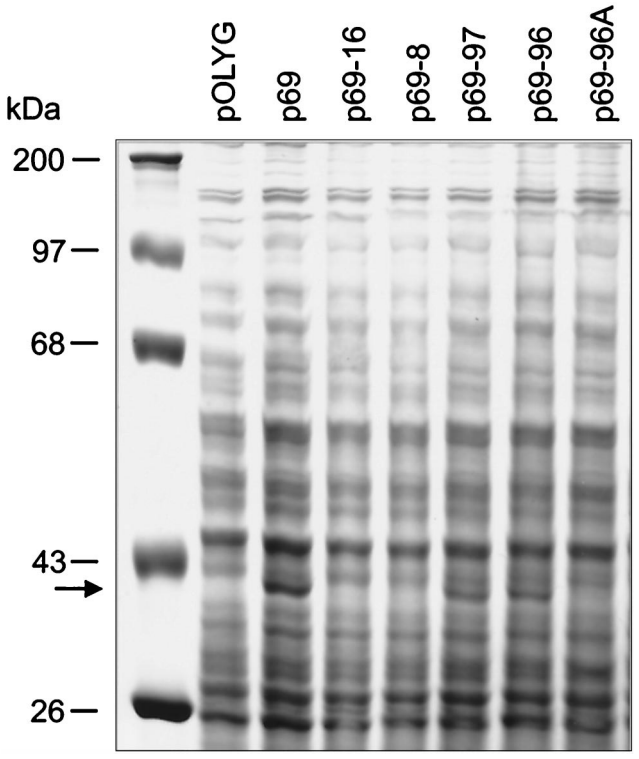

FIG. 8. SDS-PAGE analysis of *M. smegmatis* transformants. *M. smegmatis* transformants containing vector alone (pOLYG), p69, or deletion derivatives of p69 were lysed by vortexing with glass beads. Protein samples (140 μg of total protein per lane) were subjected to electrophoresis by using a 12% SDS–PAGE gel. A unique Coomassie blue-stained band corresponding to a 42-kDa protein (arrow) is present only in lanes of lysates from *M. smegmatis* transformants containing an intact *eis* gene (p69, p69-97, and p69-96) and not from transformants containing *eis* gene deletions (p69-16, p69-8, and p69-96A). Protein molecular size standards (in kilodaltons) are shown on the left.

gene from a highly pathogenic bacterium into a nonpathogenic relative. Since the survival-enhancing effect of *eis* may depend on its overexpression in the multicopy vector pOLYG, it will be necessary to see if *eis* placed in a single-copy chromosomal location still enhances *M. smegmatis* intracellular survival. To examine if *eis* also prolongs the survival of virulent *M. tuberculosis* H37Rv, *eis* deletion mutants of this organism must be studied in human macrophages and in animal models.

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