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Stationary Phase-Associated Protein Expression in *Mycobacterium tuberculosis*: Function of the Mycobacterial α-Crystallin Homolog

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The majority of active tuberculosis cases arise as a result of reactivation of latent organisms which are quiescent within the host. The ability of mycobacteria to survive extended periods without active replication is a complex process whose details await elucidation. We used two-dimensional gel electrophoresis to examine both steady-state protein composition and time-dependent protein synthetic profiles in aging cultures of virulent *Mycobacterium tuberculosis*. At least seven proteins were maximally synthesized 1 to 2 weeks following the end of log-phase growth. One of these proteins accumulated to become a predominant stationary-phase protein. N-terminal amino acid sequencing and immunoreactivity identified this protein as the 16-kDa α-crystallin-like small heat shock protein. The gene for this protein was shown to be limited to the slowly growing *M. tuberculosis* complex of organisms as assessed by Southern blotting. Overexpression of this protein in wild-type *M. tuberculosis* resulted in a slower decline in viability following the end of log-phase growth. Accumulation of this protein was observed in log-phase cultures following a shift to oxygen-limiting conditions but not by other external stimuli. The protein was purified to homogeneity from overexpressing *M. smegmatis* in two steps and shown to have a significant ability to suppress the thermal denaturation of alcohol dehydrogenase. Collectively, these results suggest that the mycobacterial α-crystallin protein may play a role in enhancing long-term protein stability and therefore long-term survival of *M. tuberculosis*.

More than 40 years after the introduction of effective chemotherapy, tuberculosis remains the single largest infectious cause of human mortality, resulting in about five deaths every minute (13). The failure to eradicate this disease is intimately linked to the pathogenesis of the organism. Initial infection with *Mycobacterium tuberculosis* only rarely leads to disease. Instead, infection is typically controlled by the host's immune system, and most viable bacilli are cleared. Not all bacteria are removed, however, and the remainder are capable of becoming inactive for decades before reactivating to cause clinical disease (37). The AIDS epidemic has exacerbated this problem. Patients latently infected with *M. tuberculosis* stand a 0.2% annual risk of reactivation, compared with the 5 to 10% annual risk borne by the human immunodeficiency virus-*M. tuberculosis* coinfected (13). The majority of the tuberculosis cases reported in the United States are the result of reactivation, not initial infection, and as many as 10 million to 15 million Americans have such latent infections (3). Worldwide the problem is even more serious: fully one-third of the entire human population may be latently infected with dormant bacilli (31). Clearly this delay in appearance of the active disease only complicates an already difficult problem, and new strategies which will allow the eradication of latent organisms are required. Such strategies will arise only through an improved understanding of the mechanisms by which the tubercle bacilli remains viable for such extended periods.

In the 1950s, prior to the advent of effective chemotherapy, it was possible to examine latent tuberculous lesions in human lung tissue following surgical removal (5, 35, 39). From these studies, it became apparent that viable bacilli were present in blocked airways for years following conversion to a sputum-negative status and that these bacteria can be cultured but are not actively growing. More recent work in the rabbit and mouse tuberculosis models suggests that the initial event in mycobacterial infection involves entry and multiplication within unactivated macrophages (9, 25). Following this rapid growth phase, infected macrophages and their bacillary cargo are surrounded and walled off by newly recruited activated macrophages to form the characteristic caseous granuloma (8). Granuloma formation appears to create an environment uniquely capable of restricting the growth of mycobacteria through a process involving apoptosis of infected monocytes (23). It is within these primary granulomas and following exponential growth that bacterial dormancy probably occurs. Although the environment inside the granuloma is unknown, many factors, including oxygen deprivation, nutrient depletion, low pH, toxic oxygen species, and other adverse conditions of this environment, could potentially contribute to the induction and maintenance of mycobacterial dormancy. As an initial effort to characterize the metabolic changes which allow *M. tuberculosis* to remain viable for such extended periods of time, we sought to characterize proteins which may be specifically synthesized during late exponential and stationary phase growth in vitro.

MATERIALS AND METHODS

Growth of organisms and two-dimensional (2D) gel sample preparation and conditions. *M. tuberculosis* H37Rv (ATCC 27294) and *M. smegmatis* mc^{2}155 were grown in Middlebrook 7H9 medium supplemented with albumin-dextrose-catalase (ADC; Difco, Detroit, Mich.) and 0.05% Tween 80 containing, when appropriate, kanamycin (25 μg/ml; Sigma, St. Louis, Mo.) or hygromycin (50 μg/ml; Calbiochem, La Jolla, Calif.). H37Rv was maintained at low optical density (OD) for several passages prior to evaluation of growth phase-specific protein expression by serial 1:10 subculture. For metabolic labeling, 10 OD units (e.g., 10 of organisms at the indicated time was removed from the main culture, 5 μl of [^{35}S]methionine (250 μCi, 50 mCi/ml; Dupont NEN, Boston, Mass.) was added,

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and the culture was incubated for 2 h. After 2 h, the bacteria were collected by centrifugation at 2,500 × *g* for 10 min, and the medium was removed. The cell pellet was resuspended in 0.5 ml of cold distilled water containing 1 mM phenylmethylsulfonyl fluoride and transferred to a 1.5-ml tube containing 250 mg of 0.1-mm-diameter glass beads. The sample was homogenized in a Bead-Beater 8 (BioSpec Products, Bartlesville, Okla.) for 3 min. The beads were allowed to settle as the sample cooled on ice, and 200 μl of lysate was removed to 200 μl of 2× first-dimension sample buffer (9.5 M urea, 2% Triton X-100, 5% β-mercaptoethanol, 1.6% 5/7 BioLyte ampholyte, and 0.4% 3/10 BioLyte ampholyte) and incubated for 1 h at room temperature before loading of 100-μl samples onto isoelectric focusing tube gels as specified by the manufacturer (Bio-Rad Laboratories, Richmond, Calif.). Second-dimension electrophoresis was carried out by using standard techniques on sodium dodecyl sulfate (SDS)–12% denaturing polyacrylamide gels (28). Gels were stained with 0.1% Coomassie brilliant blue R-250, photographed, dried, and examined following phosphor imaging.

Cloning and expression of the 16-kDa antigen and DNA manipulations. The open reading frame of the 16-kDa antigen was PCR amplified from *M. tuberculosis* H37Rv chromosomal DNA with a thermo-stable DNA polymerase (Pwo Polymerase; Boehringer Mannheim, Indianapolis, Ind.), using the following two primers: 5′-CGCGGATCCATTAGGAGGCATCAAATG-3′ and 5′-AAAACT GCAGCGGTCATCAGCACGGAC-3′ (from GenBank accession number S79751). The resulting product was precipitated and digested with *Bam*HI and *Pst*I and ligated into similarly digested pMV261 (30). *Escherichia coli* transformants were selected for kanamycin resistance, and DNA containing the appropriate insert was used to transform both *M. smegmatis* and *M. tuberculosis.*

The PCR-generated open reading frame was [α-^{32}P]dCTP labeled by using the Prime-a-Gene labeling system (Promega, Madison, Wis.) and used as a probe to screen an H37Ra cosmid library in pYUB18, which has been previously described (41). Colony lifts of this library produced three positive clones from 500 colonies screened. Southern blots of *Bam*HI digests of these three cosmids revealed a common 4-kb fragment carrying the 16-kDa antigen sequence, the same fragment as observed in chromosomal *Bam*HI digests. Southern blotting was done with QuickHyb hybridization solutions, and the blots were washed under stringent conditions as described by the manufacturer (Stratagene, La Jolla, Calif.). This 4-kb *Bam*HI fragment was gel purified and ligated into *Bam*HI-digested pMV206H (15) to make pMV206H:αCryst. Transformation into *E. coli* was used to screen for the correct-size insert and to produce sufficient DNA to transform both *M. smegmatis* and *M. tuberculosis*. Expression of the 16-kDa antigen in all constructs was confirmed by both analysis of Coomassie blue-stained polyacrylamide gels and Western blot (immunoblot) analysis using monoclonal antibody F24-2-3 (IT-4) (provided by the UNDP/World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases).

Protein purification and in vitro assays. *M. smegmatis* mc^{2}155 carrying pMV206H:αCryst (1 liter) was grown to 24 h postsaturation in 7H9-ADC-Tween 80 containing hygromycin as described above. Bacterial cells were collected by centrifugation at 3,020 × *g* for 20 min, and the medium was removed. The pellet was resuspended in 10 ml of 20 mM Tris (pH 7.5)–0.1 M NaCl–1 mM phenylmethylsulfonyl fluoride (buffer 1), and the suspension was transferred to a precooled 15-ml Bead-Beater chamber which contained 10 g of 0.1-mm-diameter glass beads. The sample was lysed by bead beating for three 1-min pulses with a 1-min cooling period between pulses. The slurry was decanted from the beads to a centrifuge tube, which was then spun at 12,100 × *g* at 4°C for 15 min. The clarified supernatant (about 12 ml) was then centrifuged at 50,000 rpm (200,000 × *g*) in an SW60 rotor for 3 h. The pellet material from this centrifugation was resuspended in 2 ml of buffer 1 and applied to a Sephacryl S-200 column (2.5 by 40 cm) which had been preequilibrated in phosphate-buffered saline (50 mM sodium phosphate [pH 7.5], 100 mM NaCl) at 4°C. Fractions of 3 ml were collected and analyzed by measuring the OD at 260 nm (OD_{260}). Protein-containing fractions were further analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), and those fractions containing the purified 16-kDa antigen were pooled and concentrated in a Centricon-10 concentrator (Amicon, Inc., Beverly, Mass.). Protein concentration was determined by using the DC Protein Assay System (Bio-Rad). One liter of culture yielded approximately 6.3 mg of α-crystallin protein which was greater than 90% pure by SDS-PAGE. Thermal denaturation of horse liver alcohol dehydrogenase (ADH) was performed as previously described (17).

Expression during stress conditions. *M. tuberculosis* H37Rv (200 ml; grown in 7H9-ADC-Tween 80 as described above) was diluted to an initial OD_{650} of 0.01 and grown to an OD_{650} of 0.2. For short-term pulse-labeling, 10 ml of this culture was removed, hydrogen peroxide (10 and 100 mM) or ethyl alcohol (5%) was added simultaneously with [^{35}S]methionine (100 μCi), and the cultures were incubated for 2 h at 37°C. For low-pH conditions, pelleted *M. tuberculosis* cells were resuspended in 7H9-ADC-Tween 80 adjusted to pH 3.0 with acetic acid. For long-term labeling with low aeration, 10 ml was removed to a 15-ml screw-cap tube and left unagitated for 2 h before addition of [^{35}S]methionine as described above for 48 h. For 4°C, 10 ml of culture was placed on ice for 5 min, [^{35}S]methionine was added, and the culture was refrigerated for 48 h. For low-carbon medium ADC and glycerol were excluded from normal medium, and a centrifuged culture was resuspended in this medium along with [^{35}S]methionine for 48 h. For spent medium, an old culture (2 weeks postsaturation) was centrifuged and the supernatant was filtered through a 0.2-μm-pore-size filter; 10 ml of the pelleted culture was resuspended in an equal volume of this medium along with [^{35}S]methionine as described above. Following labeling, cells were collected by centrifugation, resuspended in 300 μl of water, added to 300 mg of 0.1-mm-diameter glass beads, and homogenized by bead beating for 3 min as described above. Protein was quantitated by using the DC Protein Assay System (Bio-Rad), and 10 μg of protein was used for SDS-PAGE and phosphor imaging analysis.

RESULTS

Discrete changes in protein profiles accompany the transition to stationary phase in vitro. To identify growth-phase-specific proteins, we examined both bulk protein composition (as evaluated by Coomassie blue staining) and novel protein synthesis (as evaluated by [^{35}S]methionine pulse-labeling) at various times during growth in rolling aerated cultures. Preliminary analysis revealed that both these patterns varied little during exponential growth of the organism provided that the culture was passed continuously at low OD in dispersed culture for several generations before the time course was begun. The doubling time of the organism changes significantly depending on the nature of the inoculum. For a culture inoculated with organisms passed several times at low OD, the doubling time was typically 15 to 22 h, while for organisms passed directly from older, high-OD cultures, initial doubling times as high as 60 h were not uncommon. This inoculum-dependent lag phase has been noted by other authors, particularly in regard to the transformability of *M. tuberculosis* complex organisms (2).

Figure 1 compares the steady-state protein levels (Fig. 1A to C) with newly synthesized protein profiles (Fig. 1D and F) of *M. tuberculosis* H37Rv at various points in the growth cycle. Near the end of log-phase growth, 2D gel profiles convey the complexity of the major protein constituents of *M. tuberculosis*. One week following the end of log-phase growth, the Coomassie blue-stained 2D profile had changed little but a novel synthetic response was apparent (Fig. 1B and E). The OD of cultures at this time point was stable, and the number of organisms had not yet decreased, as assessed by determination of CFU. At least seven proteins had accelerated synthetic rates, some of which are minor or barely detectable components by protein staining. One week following this time point (Fig. 1C and F), protein synthesis was still occurring (albeit at a significantly lower level; Fig. 1F represents only 1/10 of the counts per minute incorporated in Fig. 1D and E), but the overall synthetic profile appeared to resemble that at log phase. Synthesis at this point may reflect regrowth of some organisms following partial autolysis of the culture. Later time points (up to 16 weeks following saturation) revealed that protein synthesis continued at low rates even in very old cultures, in which the OD had declined and CFU count was less than 10% of saturation levels.

Identification of the growth phase-regulated 16-kDa protein as the *M. tuberculosis* α-crystallin homolog. One protein was particularly notable in that its abundance by Coomassie brilliant blue staining changed from barely visible (Fig. 2A, lane 1; see also Fig. 6B) to one of the dominant cellular proteins during the transition from log to stationary phase. Following the post-log-phase period, no significant level of synthesis of the 16-kDa protein was observed, even in cultures up to 16 weeks old, though the protein remained a dominant component of the total protein. This finding suggests that this protein may be exceptionally stable and have a low turnover rate. This protein was identified by N-terminal amino acid sequencing from 1D SDS-PAGE following large-scale protein preparation from 2-week postsaturation H37Rv. All of 17 N-terminal amino acid residues were found to match a previously identified protein variously referred to as the 14-, 16-, or 19-kDa

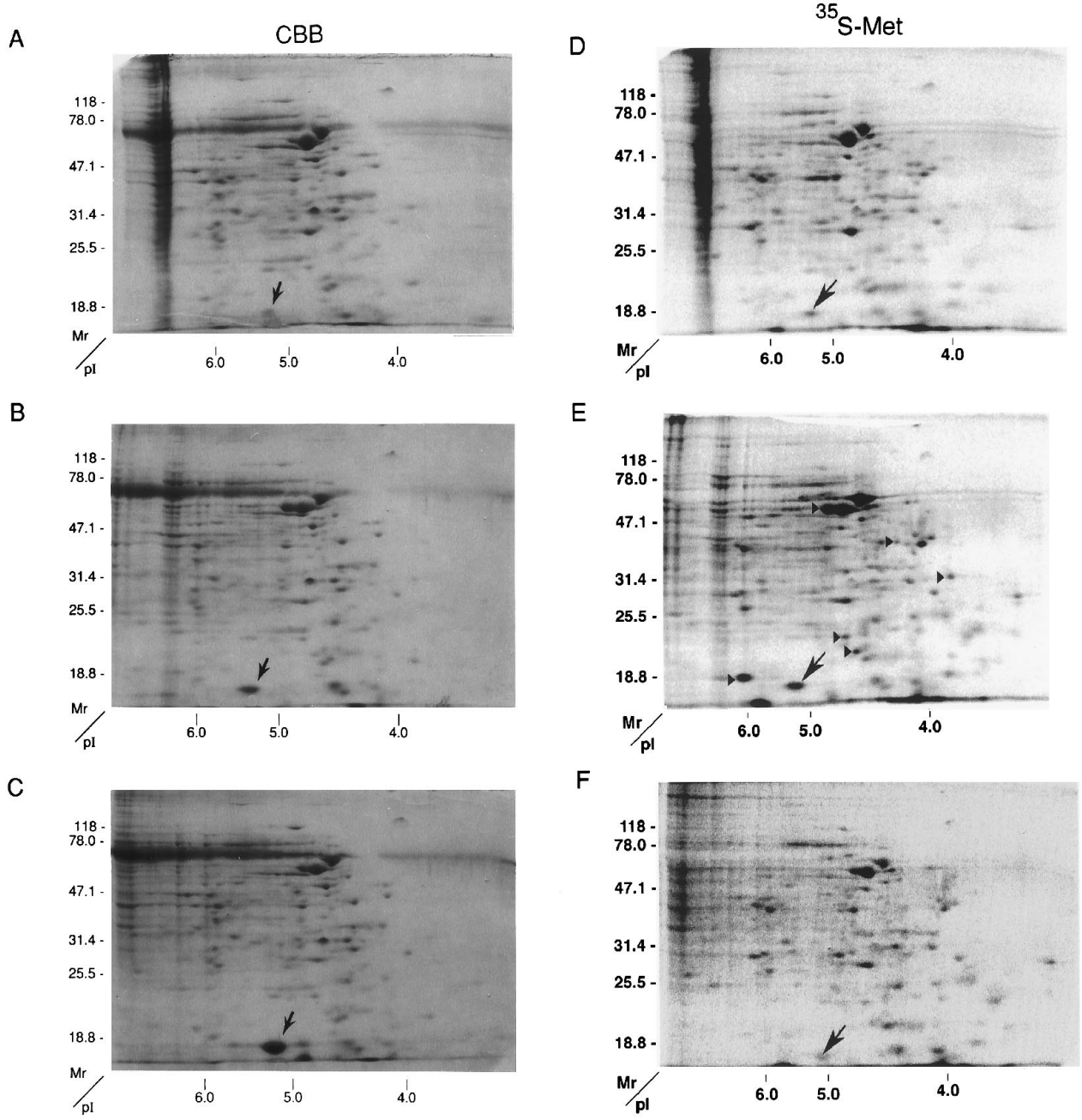

FIG. 1. Growth-phase-dependent protein composition and protein synthetic profiles of *M. tuberculosis* H37Rv. Coomassie brilliant blue (CBB)-stained patterns (A to C) and [^{35}S]methionine labeling patterns (D to F) for rolling aerated cultures at an OD_{650} of 1.0 (A and), 7 days later (B and E) (at this time point, the OD and CFU of the culture are neither increasing nor decreasing), and 14 days later (C and F) (at this time point the OD of the culture has declined to about 50% of the maximum value). In panels D and E, the phosphor imaging range is 0 to 100 cpm, while in panel F, the range is 0 to 25 cpm because of the lower level of protein synthesis occurring in this culture. One OD unit of labeled organism was subjected to isoelectric focusing after lysis (the left-to-right dimension) followed by SDS-PAGE (top-to-bottom dimension). Gels were stained with Coomassie brilliant blue and photographed before drying and phosphor imaging. The arrow indicates the position of the 16-kDa antigen; the arrowheads in panel E indicate proteins upregulated during the transition from log to stationary phase. Sizes are indicated in kilodaltons.

antigen or major membrane protein of *M. tuberculosis* (20, 33). The actual mass of this protein was measured as 16,100 Da by electrospray mass spectroscopy, confirming the predicted mass derived from the protein which was sequenced in its entirety by using overlapping proteolytic fragments (20). The nucleotide sequence encoding this protein has also been determined (GenBank accession number S79751), and monoclonal antibody reactivity has been extensively mapped (33).

To confirm the identity of this growth phase-associated protein as the previously identified *M. tuberculosis* antigen, we obtained a monoclonal antibody (IT-4) specific for the 16-kDa antigen from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Disease. As shown in Fig. 2B, lanes 1 to 4, the 16-kDa protein reactive with this antibody was, in fact, regulated by growth phase, since the protein was absent from blots with lysates from *M. tuberculosis*

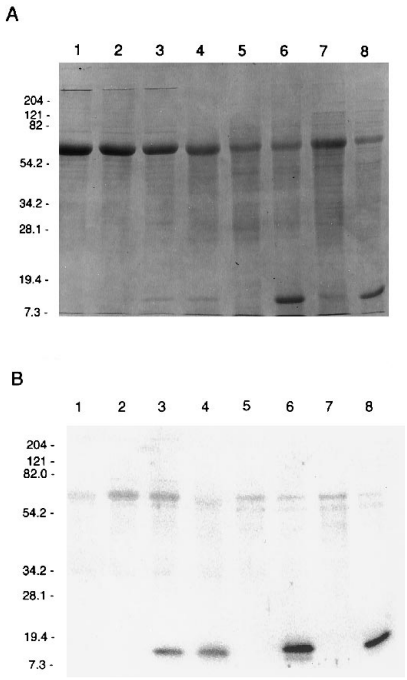

FIG. 2. Growth stage-specific expression and overexpression of the 16-kDa antigen. (A) Coomassie brilliant blue-stained SDS–12% polyacrylamide gel; (B) Western blot of the same gel blotted to Immobilon and probed with monoclonal antibody IT-4 specific for the 16 kDa antigen. Lanes 1 to 4, *M. tuberculosis* H37Rv at OD_{650} of 0.4 (lane 1), 0.7 (lane 2), and 1.8 (lane 3) and at stationary phase (2 weeks following growth cessation) (lane 4); lane 5, *M. smegmatis* mc^2155(pMV206H) (fresh overnight, OD of ca. 1.0); lane 6, *M. smegmatis* mc^2155(pMV206H:αCryst) (fresh overnight, OD of ca. 1.0); lane 7, *M. tuberculosis* H37Rv(pMV261) at an OD_{650} of 0.5; lane 8, *M. tuberculosis*(pMV261:αCryst) at an OD_{650} of 0.5. Each lane contains 10 μg of total protein from a lysed culture. Sizes are indicated in kilodaltons.

harvested at an OD of 0.4 or 0.7 and clearly present in lysates from cultures harvested following the end of log-phase growth. Figure 2A demonstrates that equivalent amounts of total protein were loaded on these blots. Using this antiserum, we also confirmed the observation of Lee et al. that this protein partitioned selectively into the cytoplasmic fraction when whole cells of stationary H37Rv were analyzed by Triton X-114 phase partitioning (data not shown) (20).

Overexpression and species distribution of the 16-kDa α-crystallin protein. To further establish the identity of this protein and examine the distribution and phenotype of recombinant mycobacteria expressing it, we PCR amplified the open reading frame encoding this protein from *M. tuberculosis* genomic DNA and placed it in an *hsp60* promoter-driven mycobacterial expression plasmid, pMV261 (pMV261:αCryst) (30). This PCR product was also used as a probe to screen a cosmid library of H37Ra for the intact genomic locus. Positive cosmids were digested with *Bam*HI and subcloned into a promoterless pMV206 plasmid modified to carry resistance to hygromycin (pMV206H:αCryst) (15, 30). Clones containing the 16-kDa gene sequence on a 4- to 5-kb *Bam*HI fragment were selected and then further screened by SDS-PAGE and Western blotting. Both constructs were transformed into *M. tuberculosis* H37Rv and *M. smegmatis* mc^2155. Overexpression in *M. smegmatis* was higher with pMV206H:αCryst and resulted in protein production constitutively throughout the growth cycle of the organism (Fig. 2A and B, lanes 5 and 6). In *M. tuberculosis*, the expression level was higher with pMV261:αCryst and was independent of growth phase, suggesting that this expression is mediated by the *hsp60* promoter (Fig. 2A and B, lanes 7 and 8, harvested at low OD).

Although monoclonal antibody reactivity has been shown to be limited to members of the *M. tuberculosis* complex, the distribution of the gene sequence has not previously been described (33). We used the coding sequence of the 16-kDa antigen to screen a Southern blot containing *Bam*HI-digested genomic DNA from several mycobacterial species (Fig. 3). We observed positive hybridization signals from *M. tuberculosis* H37Ra, H37Rv, K10516 (an isoniazid-resistant clinical isolate), and BCG. We did not find hybridizing sequences in either the saprophytic *M. smegmatis* or the opportunistic pathogen *M. avium*. *M. leprae* has been previously reported to have a protein with homology to the α-crystallin family, although the relationship between this and the *M. tuberculosis* protein is somewhat uncertain, as the *M. leprae* homolog is only 29% identical at the amino acid level (1).

Phenotypic consequences of 16-kDa antigen overexpression. To assess possible functions for the 16-kDa antigen in stationary phase, we used two approaches. Our first approach involved examining overexpression of the 16-kDa protein in *M. smegmatis*. As shown in Fig. 2A, lane 6, we were able to express the protein to a high level in this organism, although the expression was unregulated. Nonetheless, an accumulation of the 16-kDa antigen occurred throughout the growth cycle, such that older cultures of *M. smegmatis* had significantly higher levels than did exponentially growing cultures, again suggesting a low turnover rate of the protein. Notably, this unregulated expression was from a plasmid-borne gene with no added promoter sequences, suggesting that expression was occurring either from the native 16-kDa antigen promoter or from an adventitious promoter sequence carried on pMV206. Nonetheless, high-level expression in *M. smegmatis* was attended by one striking phenotype. In growth curve analysis, 16-kDa antigen-expressing organisms formed uniformly much smaller colonies on plates than control organisms incubated for similar times. By scanning electron and conventional microscopy, no significant differences in colony morphology or bacterial aggregation could be observed (data not shown). In spite of this, when active log-phase cultures were evaluated for differences

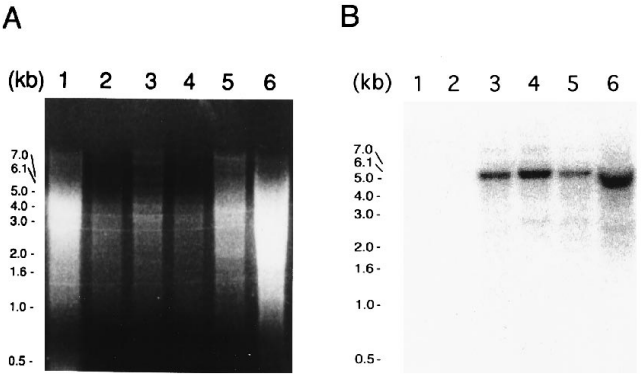

FIG. 3. Southern blot of various mycobacterial species with a 16-kDa antigen probe. (A) Ethidium bromide-stained 1.0% agarose gel; (B) Southern blot of the same gel, using the 16-kDa antigen coding sequence as a probe. The lanes represent *M. smegmatis* mc^2155 (lane 1), *M. avium* A5 (lane 2), *M. bovis* BCG Pasteur (lane 3), *M. tuberculosis* K10516 (an isoniazid-resistant clinical isolate) (lane 4), *M. tuberculosis* H37Ra (lane 5), and *M. tuberculosis* H37Rv (lane 6). Approximate sizes of the DNA fragments are indicated at the left. Hybridization was done under stringent conditions at 68°C followed by washing at 60°C as described in Materials and Methods.

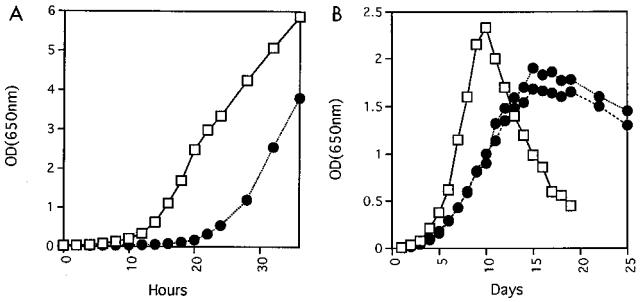

FIG. 4. Phenotypic consequences of recombinant 16-kDa antigen expression. (A) In *M. smegmatis* mc^{2}155, the expression of the 16-kDa antigen induces an extended lag phase. Log-phase organisms were subcultured to the same OD and allowed to grow to the end of log phase before being used to inoculate cultures to the same starting OD as shown. This experiment was performed several times (including serial passage following saturation from the same culture, with identical results), and the results shown are typical of the differences observed. Squares represent control mc^{2}155(pMV206H); circles represent mc^{2}155(pMV206H:αCryst). On plates, this phenotype was evident in the very small colony size associated with expression of the 16-kDa antigen upon plating of stationary-phase cultures. OD and CFU have been correlated (equivalently) in independent experiments with both strains by plating onto hygromycin-containing medium. (B) In *M. tuberculosis*, expression of the 16-kDa antigen results in a significant decrease in the post-log-phase drop in culture viability and slows the maximal log-phase growth rate [compare the control H37Rv(pMV261) (squares) with two clones of H37Rv(pMV261:αCryst) expressing high levels of the 16-kDa antigen in log phase (circles)]. Viability of these organisms was confirmed microscopically and by comparing rates of protein synthesis for wild-type cells and 16-kDa antigen expressors. For both graphs, samples were dispersed prior to measurement of the OD by low-power sonication to avoid clumping problems; the absence of clumping was verified by microscopic examination of samples.

in growth rate, none were observed. The presence of higher levels of protein was attended by even smaller colony size, and when identical log-phase cultures of expressing and nonexpressing organisms were allowed to grow to saturation and then diluted equivalently, a significant lag in growth was observed, although the ultimate log-phase rates were identical (Fig. 4A). The ultimate numbers of CFU formed upon saturation by these two strains were identical by plate counts of hygromycin resistant colonies; thus, this result is not due to outgrowth of wild-type colonies following antibiotic consumption. In addition, this lag phase was reproducible from serial cultures harvested at identical times postsaturation, which suggests that this lag is a consequence of 16-kDa antigen accumulation.

We also examined the effect of 16-kDa antigen overexpression in the wild-type *M. tuberculosis* background by transforming H37Rv with the *hsp60* promoter-driven construct lacking the native 16 kDa antigen promoter. As shown in Fig. 2B, lane 8, log-phase cultures of *M. tuberculosis* containing this plasmid expressed higher levels of the α-crystallin homolog than did stationary-phase cultures of the wild-type organism. When long-term cultures of these recombinant organisms and controls were evaluated, expression of the 16-kDa antigen was found to have an effect on the growth rate of these organisms (Fig. 4B). More importantly, overexpression led to higher total amounts of the protein by the beginning of stationary phase, and these amounts seemed to protect the organisms from autolysis, as signaled by the drop in OD seen with the control organisms. In fact, although the expressing organisms failed to achieve as high an OD as the controls, by 5 days after reaching their OD maxima, they maintained 100% of the saturation OD value, whereas the control organisms had only 30% of their maximal OD 5 days after the peak value was reached. The OD data have been correlated by reference to the number of CFU observed in control cultures at all of these points, and the OD reflects the actual number of CFU present. It was impossible to evaluate CFU data for 16-kDa antigen overexpressors because of their extremely slow growth rate when transferred to plates. Although early-log-phase cultures could be evaluated, the colonies compared with those of controls were very small. Late-log-phase and stationary-phase cultures, even after incubation of plates for 8 to 10 weeks, produced only extremely small, barely visible colonies. The colonies that did become visible appeared morphologically and by acid-fast staining to be *M. tuberculosis*, suggesting that these cultures contained viable bacilli, even at 16 weeks postsaturation. A higher number of viable organisms in old cultures of the 16-kDa antigen-expressing strain was also supported by the higher levels of [^{35}S]methionine incorporation in these very old cultures. In these experiments, the 16-kDa antigen-expressing organisms synthesized protein at higher levels than did vector controls (data not shown).

Purification of 16-kDa antigen and in vitro chaperone activity. Given the divergence between the *M. tuberculosis* α-crystallin homolog and other proteins in this family, we attempted to demonstrate that this protein possessed some common properties which might explain its activity in enhancing long-term viability of *M. tuberculosis*. To accomplish this, we purified this protein to near homogeneity as shown in Fig. 5A. This purification took advantage of the known tendency of proteins belonging to this family to aggregate and form oligomeric structures composed of as many as 32 monomers with an estimated molecular mass of about 800 kDa (4, 6). On the basis of this predicted size and the results of Lee et al. (20), we subjected extracts clarified at 10,000 × *g* to high-speed centrifugation at 200,000 × *g* and effected a substantial purification of the *M. tuberculosis* α-crystallin homolog, as shown in Fig. 5A, lane 2. Further purification was achieved by Sephacryl S-200 chromatography, during which the oligomerized protein elutes in the void volume. From overexpressing recombinant *M. smegmatis*, we were able in this way to isolate 6.3 mg of purified 16-kDa antigen from 1 liter of culture. This purification establishes that the *M. tuberculosis* homolog shares at least one

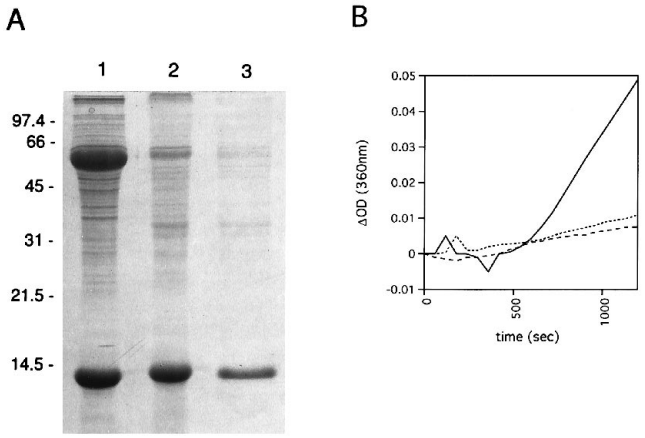

FIG. 5. Purified 16-kDa antigen has in vitro chaperone activity. (A) Coomassie brilliant blue-stained gel showing the level of protein expressed by *M. smegmatis* mc^{2}155(pMV206H:αCryst) (lane 1), the level of purity of 16-kDa antigen obtained by ultracentrifugation at 200,000 × *g* of clarified cell extract (lane 2), and the purity of combined fractions from Sephacryl S-200 chromatography (lane 3). Sizes are indicated in kilodaltons. (B) Time-dependent thermal denaturation curves of horse liver ADH in the absence of added 16-kDa antigen (solid line), in the presence of a 1:50 molar ratio of 16-kDa antigen to ADH (stippled line), and in the presence of a 1:10 molar ratio of 16-kDa antigen to ADH (dashed line). Molarity was calculated on the basis of an assumed molecular mass of 360,000 Da for the aggregated form of the 16 kDa antigen.

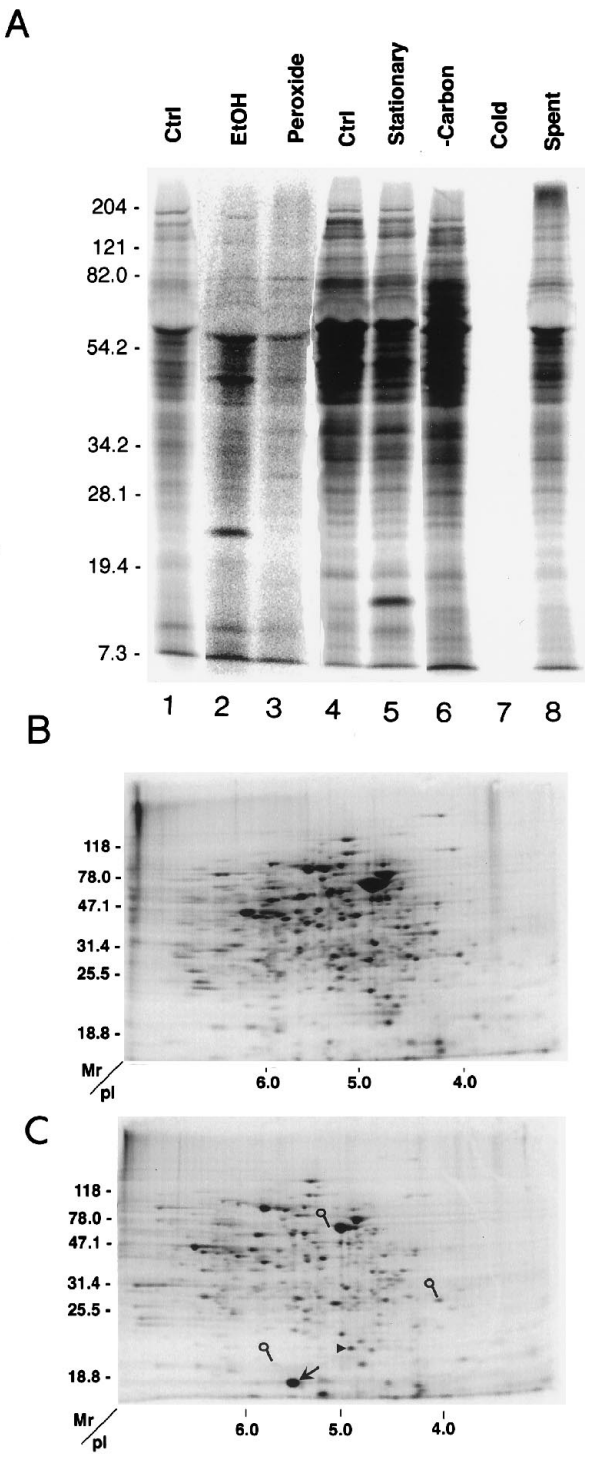

FIG. 6. Induction of 16-kDa antigen expression by oxygen tension. (A) Log-phase rolling cultures of *M. tuberculosis* H37Rv at an OD_{650} of 0.2 were pulse-labeled with [^{35}S]methionine for 2 h (lane 1) or 48 h (lane 4) (controls [Ctrl]) in the presence of various stress conditions as described in Materials and Methods. Neither 5% ethanol (EtOH) nor 10 mM hydrogen peroxide (lane 2 or 3, respectively) induced expression of the 16-kDa antigen in a 2-h labeling. The PhosphorImager scale for lanes 4 to 8 is higher because of the much longer labeling time. Lane 5 shows the upregulation of a band comigrating with the 16-kDa antigen upon shifting of the culture from a rolling, aerated flask to a stationary, unagitated flask for 48 h. This band comigrates with the 16-kDa antigen by 2D gel electrophoresis as well (data not shown). Resuspension of the log-phase culture in medium without a carbon source (lane 6) does not upregulate this protein, nor does cold shock (lane 7) or resuspension in medium collected from a stationary culture of *M. tuberculosis* (lane 8). (B and C) Two-dimensional gel electrophoretic analysis of samples from lane 4 (B) and lane 5 (C) confirming that the upregulated protein is, in fact, the 16-kDa antigen (arrow). In addition, one other protein (arrowhead) common to the synthetic response observed in stationary phase (Fig. 1) is seen. Several other proteins which are also synthesized during the transition to stationary phase are not synthesized in response to the transfer to a stationary, unagitated culture (circles). Sizes are indicated in kilodaltons.

important property with other α-crystallin small heat shock proteins (sHSPs) in that it forms high-molecular-weight aggregates in vivo.

To establish that this protein possessed the chaperone function ascribed to other sHSPs (17), we assayed the ability of the protein to suppress the in vitro thermal denaturation of ADH (Fig. 5B). ADH is known to undergo thermal denaturation at temperatures above 39°C. This denaturation is accompanied by exposure of hydrophobic surfaces which subsequently aggregate and scatter light, a phenomenon which can be monitored by observing the intensity of light transmission at 360 nm (17). sHSPs are thought to act as molecular surfactants, stabilizing proteins from unfolding by nonspecific weak interactions with properly folded structures (7). Even at a molar ratio of 50:1 (ADH/purified 16-kDa antigen aggregates) or 0.013 mg of sHSP plus 0.130 mg of ADH, the *M. tuberculosis* homolog completely ablates the thermally induced precipitation which signals denaturation of ADH at 48°C, an activity comparable to that observed with α-crystallin isolated from bovine eye tissue (17). This activity is specific to α-crystallins (7); similar assays using bovine serum albumin have no stabilizing effect at comparable or higher weight ratios (data not shown). Another function which has been suggested for sHSPs is the nonspecific inhibition of protease activity (34). Unlike other members of this family, the purified *M. tuberculosis* 16-kDa antigen was not active in inhibiting proteolysis of a chromophoric elastase substrate by porcine pancreatic elastase (data not shown).

Regulation of 16-kDa antigen expression by environmental stress. Although the 16-kDa antigen is clearly a member of the family of sHSPs, it does not appear to be induced during heat shock (33). Although most other members of this family appear to be responsive to thermal induction, at least one other example of an sHSP which is developmentally regulated has been described (26). To clarify the relationship of this protein to various stress conditions, we examined induction of this protein under the stress conditions shown in Fig. 6. Exposure to some conditions such as hydrogen peroxide treatment, low pH, and alcohol shock elicits a relatively fast novel synthetic response (28). Nonetheless, none of these conditions resulted in the induction of the 16-kDa antigen (Fig. 6, lanes 1 to 3). Less toxic stresses were evaluated for a longer labeling period, since overall protein synthesis rates were much lower (data not shown). Neither carbon starvation, cold shock, nor resuspension of log-phase cultures in spent media collected from old cultures induced detectable levels of this protein. Lowering the aeration of mid-log-phase cultures by stopping agitation did, however, result in a dramatic upregulation of the 16-kDa antigen (Fig. 6, lane 5), suggesting that one environmental cue to which this protein is responsive is oxygen tension.

DISCUSSION

Stationary phase in the tuberculosis complex bacteria has only begun to be explored. In this study, we defined stationary phase as commencing at the end of log-phase growth and showed that it was associated with the differential expression of at least seven proteins whose functions may be related to

maintaining cell viability. The physical and metabolic changes which accompany stationary phase are largely unknown, although such cells have been reported to be more resistant to antimycobacterial agents, have altered pathways for glyoxylate metabolism, and are able to resume growth synchronously (37). We have demonstrated that the 16-kDa antigen of *M. tuberculosis* is specifically expressed during the transition to stationary phase. Further, expression of this protein appears to render the recombinant organisms less susceptible to autolysis following saturation of the culture in vitro. The slow initial growth rate observed in both recombinant organisms suggests that the lag phase associated with growth of organisms of the *M. tuberculosis* complex may be related to the necessity to dilute out or degrade the 16-kDa antigen in order to achieve maximal log-phase growth rates. The small colony size evident in both *M. tuberculosis* and *M. smegmatis* suggests some 16-kDa antigen-related inhibition of growth on solid media.

Through the pioneering work of Wayne, an in vitro system for evaluating anaerobic or microaerophilic cultures of *M. tuberculosis* in which a population of bacilli is induced to grow in unagitated suspension culture has been developed (37). In this model, organisms growing in the layer nearer the surface are thought to grow aerobically and then to slowly adapt to anaerobic conditions as they settle through an oxygen gradient. Although these organisms are capable of shifting back to growth aerobically, apparently synchronously (36), the down-shift to this condition is difficult to evaluate since the initial culture is extremely asynchronous. The relationship between organisms grown in submerged culture and organisms characterized as stationary phase in the present study is important to consider. Wayne and Diaz reported that a sudden shift of log-phase cultures from aerobic growth to anaerobic submerged culture resulted in death of the culture (38). Thus, under these conditions, production of this protein is presumably not protective, possibly because a high enough protein concentration is not achieved before cell death. On the other hand, our experiments with aging cultures and overexpressing organisms suggest that production of this protein at some concentration is protective. These conditions (shifting from aerobic to anaerobic submerged cultures) are similar to those found to induce expression of the 16-kDa antigen as shown in Fig. 6B and C. These induction conditions are obviously not a precise mimic for the transition to stationary phase observed in aging cultures. In fact, a comparison of the 2D gel profiles of induced proteins in Fig. 6B and C with those in Fig. 1E reveals that although 16-kDa antigen induction is common to the two conditions, many of the other proteins identified as induced in Fig. 1E are not induced in Fig. 6B and C. Thus, although anoxia may represent part of the environmental signal resulting in stationary phase, additional environmental changes may be involved. The cultures themselves should not be oxygen deprived, the surface area-to-volume ratio is very high, and the culture vessels were opened frequently over the course of the month-long growth curve for sampling. Thus, the shift-down to lower oxygen conditions may occur through the production of cell wall material which renders the organism less permeable or as a result of clumping of multiple organisms, with the interior of these clumps becoming increasingly microaerophilic or anaerobic. We routinely disperse organisms by low-power sonication for sampling and OD measurements, but in older cultures especially, clumping clearly occurs. Alternatively, the 16-kDa protein may be induced in response to a gradual shift to anaerobic conditions or an unknown condition which occurs during the transition to stationary phase in aerated culture.

The 16-kDa antigen is related to the broad family of sHSPs, although this relationship is based on amino acid identities ranging from 27 to 31% in comparison with, for example, the sHSP from soybean. In fact, an examination of the α-crystallin domain of the *M. tuberculosis* homolog reveals that only 21 of 32 highly conserved residues are present or conservatively substituted (7). We have shown that although this sequence homology is not high, the mycobacterial sHSP possesses functional similarities with the proteins in this family. The defining characteristic of proteins in this family lies in their chaperone function, which was first demonstrated through their ability to suppress the thermally induced denaturation of other proteins, a property thought to contribute to their proposed physiological role in maintaining lens clarity in the vertebrate eye (17). This finding has been extended by recent studies which have demonstrated that this function also serves to preserve heterologous protein function as well as structure (16, 18). This chaperone activity has been shown to confer thermoresistance as well as increased resistance to lethal hydrogen peroxide treatments when cells express sHSPs (22, 32). We were unable to demonstrate either of these phenotypes in *M. smegmatis* recombinants expressing the *M. tuberculosis* 16-kDa antigen, although the effect on growth rate on plates was a complicating factor in this analysis. We were able to demonstrate that *M. tuberculosis* overexpressing this protein displayed an enhanced resistance to autolysis following the end of log-phase growth. In addition, the in vitro data generated by using purified protein suggest that this protein is functionally homologous to the sHSP family.

Previous reports have characterized this protein as cell wall associated primarily on the basis of the ability to pellet this protein at $100{,}000 \times g$ (20). These authors noted that the sHSP partitions into the cytoplasmic fractions when subjected to Triton X-114 phase partitioning, a result that we have confirmed. The present results suggest that the protein forms high-molecular-weight aggregates, as is characteristic of other proteins in the sHSP family, and that this aggregation may be responsible for the association with the cell wall. It should also be noted, however, that other α-crystallin proteins have been found to have a peripheral association with wall proteins and that this family of proteins is thought to function primarily through the interactions of exposed hydrophobic surfaces with other proteins in a denatured or partially denatured state (10, 24). These results make a precise cellular location for this protein difficult to assess. Other authors have suggested this protein is surface exposed, but such claims must be evaluated with caution since the protein is very abundant in older organisms and surface coating could be the result of autolysis of older organisms in mixed cultures, depending on culture conditions.

A survey of patients with active tuberculosis demonstrated that 85% showed a positive reaction to the purified *M. tuberculosis* sHSP, suggesting that this protein is important during infection and is expressed in vivo (20). Both the *M. tuberculosis* and the *M. leprae* homologs have recently been shown to be the focus of T-cell responses in patients infected with one of these organisms (1, 14). It also seems likely that the previously reported antigen differentially expressed between replicating and resting cells of *M. tuberculosis* (40) is the 16-kDa antigen described in this report. There is also some evidence that the *M. leprae* homolog of this protein is upregulated in response to intracellular growth from promoter fusions monitored during infection in in vitro-cultured murine macrophages (11). Although such studies fail to provide information directly related to persistence during latent infections, they do suggest that this protein may be induced in response to such stresses as are encountered during an infection.

The ability to persist following log-phase growth is a com-

mon theme in bacterial metabolism. In gram-negative organisms such as *E. coli*, the phenomenon whereby cells conduct an orderly shut-down of metabolism so as to preserve the ability to grow following changing environmental conditions has been studied in considerable detail (29). In addition to exhibiting changes in metabolic rate, starved or stationary-phase cells undergo numerous changes in cell wall structure, surface properties, and chromosome structure. Some bacteria, e.g., *Bacillus subtilis*, form fully differentiated spores which enable them to survive extended periods without nutrients (27). These diverse systems have common characteristics, among which is the use of developmentally linked programs of protein expression in the face of a shut-down of bulk protein synthesis. In *E. coli*, several regulons, including the regulon dependent on the alternate sigma factor KatF (19, 21), operate during starvation. Many of the proteins produced during stationary phase, including the proteins involved in carbon starvation, the catalase HPII, exonuclease III, and others (29), are dependent on KatF for their expression. Recently, a possible functional equivalent of KatF was identified in *M. tuberculosis* and named SigF. This sigma factor was shown to be strongly induced during the transition from log to stationary phase (12). The 16-kDa antigen is probably not solely regulated by the recently identified mycobacterial SigF, since several other stimuli shown to upregulate SigF do not appear to affect expression of this protein (Fig. 6) (12).

The coregulation of other proteins during this transition suggests that the 16-kDa antigen may still be a part of a larger regulon triggered upon entry into stationary phase in the pathogenic mycobacteria. Studying the proteins which constitute such a regulon will increase our understanding of the factors important to survival of dormant bacilli and may suggest targets for therapeutic intervention to eradicate such organisms. The specificity of the 16-kDa antigen to the *M. tuberculosis* complex organisms and the leprosy bacilli suggests that it may fulfill some function uniquely required by these pathogens. The diversity of this protein family among many eukaryotes and prokaryotes further suggests that the relevant function may be somewhat general (7). Thus, the protective effect of this protein may not be directly related to the immediate stress which triggers its synthesis. Instead, we propose that this protein may serve as a general cellular protectant through an enhanced stability of the proteins required for revivability. Oxygen tension may simply serve as the signal to produce proteins, such as the 16-kDa antigen, required for long-term survival and persistence. Obviously the construction of strains deleted for this gene or its regulator would allow a direct assessment of the contribution of this protein to stationary-phase survival and may offer significant potential as a component deletion in an attenuated vaccine strain.

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REFERENCES

1. **Adams, E., A. Basten, R. Prestidge, and W. J. Britton.** 1995. T cell clones from a non-leprosy exposed subject recognize the *Mycobacterium leprae* 18-kD protein. Clin. Exp. Immunol. **102:**58–64.
2. **Aldovini, A., R. N. Husson, and R. A. Young.** 1993. The *uraA* locus and homologous recombination in *Mycobacterium bovis* BCG. J. Bacteriol. **175:** 7282–7289.
3. **American Thoracic Society.** 1992. Control of tuberculosis in the United States. Am. Rev. Respir. Dis. **146:**1623–1633.
4. **Arrigo, A.-P., J. P. Suhan, and W. J. Welch.** 1988. Dynamic changes in the structure and intracellular locale of the mammalian low-molecular-weight heat-shock protein. Mol. Cell. Biol. **8:**5059–5071.
5. **Auerbach, O., G. L. Hobby, M. J. Small, T. F. Lenert, and J. V. Comer.** 1955. The clinicopathologic significance of the demonstration of viable tubercle bacilli in resected lesions. J. Thorac. Surg. **29:**109–132.
6. **Behlke, J., G. Lutsch, M. Gaestel, and H. Bielka.** 1991. Supramolecular structure of the recombinant murine small heat shock protein hsp25. FEBS Lett. **288:**119–122.
7. **Caspers, G.-J., J. A. M. Leunissen, and W. W. de Jong.** 1995. The expanding small heat-shock protein family, and structure predictions of the conserved "α-crystallin domain." J. Mol. Evol. **40:**238–248.
8. **Dannenberg, A. M., Jr.** 1993. Immunopathogenesis of pulmonary tuberculosis. Hosp. Pract. **Jan. 15:**51–58.
9. **Dannenberg, A. M., Jr., and G. A. W. Rook.** 1994. Pathogenesis of pulmonary tuberculosis: an interplay of tissue-damaging and macrophage-activating immune responses—dual mechanisms that control bacillary multiplication, p. 459–483. *In* B. R. Bloom (ed.), Tuberculosis: pathogenesis, protection, and control. American Society for Microbiology, Washington, D.C.
10. **Das, K. P., and W. Surewicz.** 1995. Temperature-induced exposure of hydrophobic surfaces and its effect on the chaperone activity of α-crystalin. FEBS Lett. **369:**321–325.
11. **Dellagostin, O. A., G. Esposito, L.-J. Eales, J. W. Dale, and J. J. McFadden.** 1995. Activity of mycobacterial promoters during intracellular and extracellular growth. Microbiology **141:**1785–1792.
12. **DeMaio, J., Y. Zhang, C. Ko, D. B. Young, and W. R. Bishai.** A stationary phase, stress response sigma factor from *Mycobacterium tuberculosis*. Proc. Natl. Acad. Sci. USA **93:**2790–2794.
13. **Dolin, P. J., M. C. Raviglione, and A. Kochi.** 1994. Global tuberculosis incidence and mortality during 1990–2000. Bull. W. H. O. **72:**213–220.
14. **Frischia, G., H. M. Vordermeier, G. Pasvol, D. P. Harris, C. Moreno, and J. Ivanyi.** 1995. Human T cell responses to peptide epitopes of the 16-kD antigen in tuberculosis. Clin. Exp. Immunol. **102:**53–57.
15. **George, K. M., Y. Yuan, D. R. Sherman, C. E. Barry III.** 1995. The biosynthesis of cyclopropanated mycolic acids in *Mycobacterium tuberculosis*. J. Biol. Chem. **270:**27292–27298.
16. **Groenen, P. J. T. A., K. B. Merck, W. W. de Jong, and H. Bloemendal.** 1994. Structure and modification of the junior chaperone α-crystallin—from lens transparency to molecular pathology. Eur. J. Biochem. **225:**1–19.
17. **Horwitz, J.** 1992. α-Crystallin can function as a molecular chaperone. Proc. Natl. Acad. Sci. USA **89:**10449–10453.
18. **Jakob, U., M. Gaestel, K. Engel, and J. Buchner.** 1993. Small heat shock proteins are molecular chaperones. J. Biol. Chem. **268:**1517–1520.
19. **Lange, R., and R. Hengge-Aronis.** 1991. Identification of a central regulator of stationary phase gene expression in *E. coli*. Mol. Microbiol. **5:**49–59.
20. **Lee, B.-Y., S. A. Hefta, and P. J. Brennan.** 1992. Characterization of the major membrane protein of virulent *Mycobacterium tuberculosis*. Infect. Immun. **60:**2066–2074.
21. **McCann, M. P., J. P. Kidwell, and A. Matin.** 1991. The putative σ factor KatF has a central role in development of starvation-mediated general resistance in *Escherichia coli*. J. Bacteriol. **173:**4188–4194.
22. **Mehlen, P., J. Briolay, L. Smith, C. Diaz-Latoud, N. Fabre, D. Pauli, and A. P. Arrigo.** 1993. Analysis of the resistance to heat and hydrogen peroxide stresses in COS cells transiently expressing wild type or deletion mutants of the *Drosophila* 27-kDa heat-shock protein. Eur. J. Biochem. **215:**277–284.
23. **Molloy, A., P. Laochumroonvorapong, and G. Kaplan.** 1994. Apoptosis, but not necrosis, of infected monocytes is coupled with killing of intracellular Bacillus Calmette-Guerin. J. Exp. Med. **180:**1499–1509.
24. **Raman, B., T. Ramakrishna, and C. M. Rao.** 1995. Temperature dependent chaperone-like activity of alpha-crystallin. FEBS Lett. **365:**133–136.
25. **Rook, G. A. W., and B. R. Bloom.** 1994. Mechanisms of pathogenesis in tuberculosis, p. 485–501. *In* B. R. Bloom (ed.), Tuberculosis: pathogenesis, protection, and control. American Society for Microbiology, Washington, D.C.
26. **Sauer, U., and P. Durre.** 1993. Sequence and molecular characterization of a DNA region encoding a small heat shock protein of *Clostridium acetobutylicum*. J. Bacteriol. **175:**3394–3400.
27. **Setlow, P.** 1981. Biochemistry of bacterial forespore development and spore germination, p. 13–28. *In* H. S. Levinson, D. J. Tipper, and A. L. Sonenshein (ed.), Sporulation and germination. American Society for Microbiology, Washington, D.C.
28. **Sherman, D. R., P. J. Sabo, M. J. Hickey, T. M. Arain, G. G. Mahairas, Y. Yuan, C. E. Barry III, and C. K. Stover.** 1995. Disparate responses to oxidative stress in saprophytic and pathogenic mycobacteria. Proc. Natl. Acad. Sci. USA **92:**6625–6629.
29. **Siegele, D. A., and R. Kolter.** 1992. Life after log. J. Bacteriol. **174:**345–348.
30. **Stover, C. K., V. F. de la Cruz, T. R. Fuerst, J. E. Burlein, L. A. Benson, L. T. Bennett, G. P. Bansal, J. F. Young, M. H. Lee, G. F. Hatfull, et al.** 1991. New use of BCG for recombinant vaccines. Nature (London) **351:**456–460.
31. **Sudre, P., G. ten Dam, and A. Kochi.** 1992. Tuberculosis: a global overview of the situation today. Bull. W. H. O. **70:**149–159.
32. **van den Ijissel, P. R. L. A., P. Overkamp, U. Knauf, M. Gaestel, and W. W.**

de Jong. 1994. αA-crystallin confers cellular thermoresistance. FEBS Lett. **355:**54–56.
33. **Verbon, A., R. A. Hartskeerl, A. Schuitema, A. H. J. Kolk, D. B. Young, and R. Lathigra.** 1992. The 14,000-molecular-weight antigen of *Mycobacterium tuberculosis* is related to the alpha-crystallin family of low-molecular-weight heat shock proteins. J. Bacteriol. **174:**1352–1359.
34. **Voorter, C. E. M., W. de Haard-Hoekman, K. B. Merck, H. Bloemendal, and W. W. de Jong.** 1994. Elastase inhibition by the C-terminal domains of α-crystallin and small heat-shock protein. Biochim. Biophys. Acta **1204:**43–47.
35. **Wayne, L. G.** 1960. The bacteriology of resected tuberculous pulmonary lesions. II. Observations on bacilli which are sustainable but which cannot be cultured. Am. Rev. Respir. Dis. **82:**370–377.
36. **Wayne, L. G.** 1977. Synchronized replication of *Mycobacterium tuberculosis*. Infect. Immun. **17:**528–530.
37. **Wayne, L. G.** 1994. Dormancy of *Mycobacterium tuberculosis* and latency of disease. Eur. J. Clin. Microbiol. Infect. Dis. **13:**908–914.
38. **Wayne, L. G., and G. A. Diaz.** 1967. Autolysis and secondary growth of *Mycobacterium tuberculosis* in submerged culture. J. Bacteriol. **93:**1374–1381.
39. **Wayne, L. G., and D. Salkin.** 1956. The bacteriology of resected tuberculous pulmonary lesions. I. The effect of interval between reversal of infectiousness and subsequent surgery. Am. Rev. Tuberc. Pulm. Dis. **74:**376–387.
40. **Wayne, L. G., and H. A. Sramek.** 1979. Antigenic differences between extracts of actively replicating and synchronized resting cells of *Mycobacterium tuberculosis*. Infect. Immun. **24:**363–370.
41. **Yuan, Y., R. E. Lee, G. S. Besra, J. T. Belisle, C. E. Barry III.** 1995. Identification of a gene involved in the biosynthesis of cyclopropanated mycolic acids in *Mycobacterium tuberculosis*. Proc. Natl. Acad. Sci. USA **92:**6630–6634.