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The 16-kDa α-crystallin (Acr) protein of *Mycobacterium tuberculosis* is required for growth in macrophages

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ABSTRACT **Although the 16-kDa α-crystallin homologue of *Mycobacterium tuberculosis* (MTB) is the dominant protein produced by stationary phase cultures *in vitro*, it is undetectable in logarithmically growing cultures. By growing bacilli at defined oxygen concentrations, *acr* transcription was shown to be strongly induced by mildly hypoxic conditions. Acr expression also was found to be induced during the course of *in vitro* infection of macrophages. The *acr* gene was replaced with a hygromycin resistance cassette by allelic exchange in MTB H37Rv. The resulting Δ*acr*::*hpt* strain was shown to be equivalent to wild-type H37Rv in *in vitro* growth rate and infectivity but was significantly impaired for growth in both mouse bone marrow derived macrophages and THP-1 cells. In addition to its proposed role in maintenance of long-term viability during latent, asymptomatic infections, these results establish a role for the Acr protein in replication during initial MTB infection.**

A large proportion of active tuberculosis cases arise not from initial infection with actively growing bacilli but from reactivation of previously implanted organisms that have been dormant or growing very slowly within the mammalian host (1, 2). This asymptomatic stage of the disease can last for decades during which time the bacilli are resistant to available chemotherapies (3–6). Examination of surgically removed human lung tissue from tuberculous lesions has demonstrated that bacilli are present in blocked airways of tuberculosis patients for years after conversion to sputum-negative status, but such bacteria do not appear to be metabolically active (7). Maintenance of nonreplicating stasis is thought to be mediated by reduced oxygen tension and nutrient limitation within a caseous granuloma (1, 8).

Cessation of *Mycobacterium tuberculosis* (MTB) metabolic activity can be induced *in vitro* by using a variety of stress conditions including simple aging of logarithmically growing cultures or growth of unagitated submerged cultures with concomitant oxygen limitation (9, 10). We have previously shown that the 16-kDa α-crystallin (Acr) (Hsp16.3) or Acr protein (encoded by the *acr* gene), which is undetectable during logarithmic growth of the tubercle bacilli, is strongly induced and becomes the dominant protein component of old, stationary phase viable cultures (11). Another recent report confirms the expression of Acr in stationary phase and correlates expression with a pronounced thickening of the cell wall of *Mycobacterium bovis* strain BCG (12). The Acr protein is found only in mycobacteria of the closely related tuberculosis complex, although a related protein has been found in *M. leprae* (13, 14). Acr is known to be expressed during human infection with virulent strains of MTB because 85% of sera from patients with pulmonary tuberculosis recognized this protein (15, 16).

The Acr family of small heat shock proteins act as ATP-independent chaperones and play an important role in maintaining the transparency of the vertebrate eye (17, 18). Bacterial homologs of this protein have been shown to be involved in spore formation in *Bacillus subtilis* (19) and are induced in response to various acute stresses in other microorganisms (20). Initially, the MTB Acr was characterized as a major membrane protein (15), but subsequent work has revealed that it is a potent, ATP-independent, chaperone whose complex oligomeric active structure probably consists of a trimer of trimers (11, 21). Recent immunogold-labeling experiments support an association of Acr with the mycobacterial cell wall as well as with other macromolecular structures (12). The previously identified differentially expressed antigen unique to resting bacilli characterized in the low-oxygen model of mycobacterial persistence and dormancy likely represents the same protein (22). Overexpression of Acr during log-phase growth of MTB slows both the growth rate of the organism and the post-stationary phase autolysis that often accompanies aging cultures. As a result, Acr has been invoked as a potentially important component facilitating survival of MTB during latent human infection (11, 12). These studies were initiated to elucidate the environmental signals to which Acr expression responds and to understand the role of the Acr protein in the intracellular growth of tuberculosis.

EXPERIMENTAL PROCEDURES

Growth of MTB Under Different Oxygen Tensions. MTB strain CSU93 was obtained from John Belisle, Colorado State University, Ft. Collins, CO, under the Tuberculosis Research Materials contract, National Institute of Allergy and Infectious Diseases. This organism and strain H37Rv (ATCC 27294) were maintained in roller flasks in Middlebrook media as described (11). For growth under a defined oxygen atmosphere, liquid cultures were propagated in insect cell culture flasks (Kontes) customized by equipping the side arms with hose fittings and 0.2-μm filters that were connected to a constant flow of premixed oxygen in nitrogen gas and vented into a Class II biological safety cabinet (Norco Gas, Missoula, MT). Gas flow was measured and adjusted to ≈20 air changes/hour within each flask. Growth was monitored by removal of aliquots at the indicated time points and measuring the OD at 650 nm.

***acr* Gene Replacement in *M. tuberculosis*.** The gene replacement vector was constructed by first removing the *Eco*R V

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Abbreviations: Acr, 16-kDa α-crystallin homolog; MTB, *Mycobacterium tuberculosis*; GFP, green fluorescent protein from *Aequorea victoria*; BMDM, murine bone marrow-derived macrophages; β-gal, β-galactosidase.
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restriction enzyme site from pBluescript II KS+ (Stratagene) by digestion with *Hinc*II and *Eco*R V. Klenow-mediated fill-in and religation resulted in the modified pBluescript II KS+ derivative, which was then digested with *Bam*HI and ligated with the purified 4.4-kb *Bam*HI fragment from pMV206H:α-Cryst (11). This construct was digested with *Eco*R V to remove the internal *acr*-containing 1-kb fragment, which starts 70 nt upstream of *acr*-coding region. The vector fragment was purified and ligated with a hygromycin resistance cassette, which has been described (23). This construct was linearized with *Ssp*I, and 2 μg of this DNA was used to transform H37Rv, which was then plated on 7H11-oleic acid-dextrose-catalase-hygromycin (Remel, Lenexa, KS) (50 μg/ml). Hygromycin-resistant single colonies were picked and grown to saturation in the presence of hygromycin. The bacteria were harvested by centrifugation, washed with PBS–Tween-80 (0.05%), and lysed in a Bead-Beater 8 (BioSpec Products, Bartlesville, OK) for 3 min. Lysates were analyzed by SDS/PAGE and Western blotting as described (11). Genomic DNA from the Δ*acr*::*hpt* strain was digested with *Pst*I and ligated into pBluescript KS+, which was then used to transform *Escherichia coli* to hygromycin resistance. A plasmid carrying the predicted 6-kb *Pst*I fragment was isolated and sequenced in entirety.

Macrophage Infections. Mouse bone marrow-derived macrophages (BMDM) were prepared as described (26). Briefly, cells eluted from mouse femurs were incubated in DMEM-20, containing 20% FCS, 5×10^{-5} M 2-mercaptoethanol (GIBCO/BRL), 20% L-cell conditioned growth media, and 10 μg/ml gentamicin for 4 days. After removal of nonadherent cells with Dulbecco's PBS with Mg^{2+} and Ca^{2+}, BMDM were washed off and concentrated by centrifugation at $200 \times g$ for 5 min. The pelleted cells were resuspended and incubated in 24-well tissue culture plates at 6×10^5 cells/well in DMEM-10 (10% FCS/2-mercaptoethanol, gentamicin/200 units/ml interferon-γ) for 2 days. Human macrophage-like THP-1 cells (ATCC 202-TIB) at a concentration of 8×10^5 cells per well in 24-well plates were incubated in RPMI medium 1640 with PMA (50 ng/ml) for 48 hr prior to infection. MTB strains were grown in rolling culture in 7H9-ADC-Tween with appropriate antibiotics (kanamycin, 25 μg/ml or hygromycin, 50 μg/ml) to an OD at 650 nm of 0.1–0.5. Aliquots of this culture were stored at −80°C and then titered from the frozen vial by plating serial dilutions in triplicate, at this low culture density in the presence of detergent very little clumping was observed and no further dispersion was necessary. Freshly thawed bacilli were diluted in RPMI medium or DMEM-10 and added to the macrophage monolayer in 24-well plates at an multiplicity of infection of 1. After 1-hr incubation at 37°C the innocula were removed from the wells, which were then washed five times with media. The macrophage cells were fed with fresh media and incubated in 5% CO_2 at 37°C. Cells in three identical wells were lysed at each timepoint with 0.4 ml of 1% Triton X-100, diluted in 7H9/ADC and plated in triplicate on 7H11-agar plates. Colony-forming units were determined after incubation at 37°C for 3 weeks. The GFP reporter experiments were done on glass coverslips containing a monolayer of THP-1 cells. After infection with the appropriate strain as above and incubation for the specified time period, cells were fixed with 2.5% glutaraldehyde-PBS at 4°C for 24 hr. Fluorescence microphotography was done by using a Nikon FXA photomicroscope equipped with a 60X planaphochromat objective.

Reporter Gene Assays. Acr reporter vectors with luciferase and β-gal readouts were derived from the integrating pMH66 and pMH66Rev (27), which contain both the firefly luciferase (*luc*) and β-gal genes in opposite orientation and separated by a polylinker. The 254-nt region upstream of the initiator ATG of the *acr* gene was amplified by PCR and cloned as an *Xba*I/*Hin*dIII fragment into the *Spe*I/*Hin*dIII sites of pMH66 to create the *acr-luc* reporter pMH108. The same fragment cloned into pMH66Rev created the *acr-lac*Z reporter

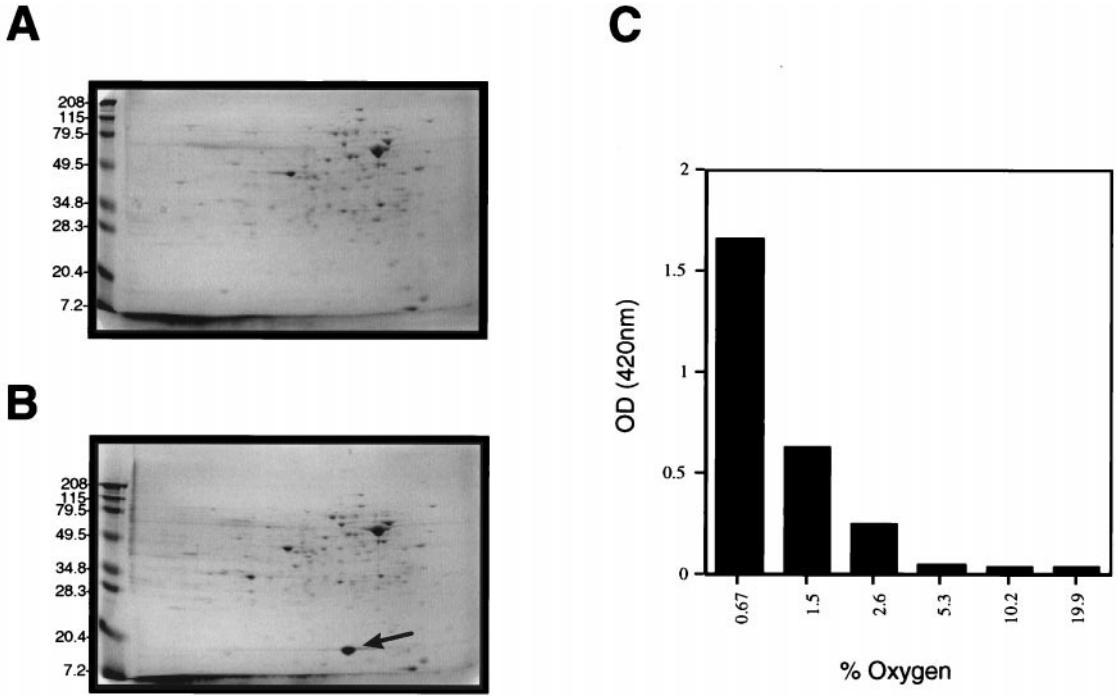

FIG. 1. Expression of Acr in CSU93 occurs only under reduced oxygen conditions. Two-dimensional gel-electrophoretic analysis of MTB strain CSU93 grown under 20% oxygen (*A*) and 1.3% oxygen (*B*). Molecular mass standards for the SDS/PAGE dimension are at the left in kDa and the isoelectric focusing is from basic (left) to acidic (right). Acr is the dominant protein spot in the lower part of the middle of the right gel (arrow). (*C*) Oxygen-dependent transcription of the *acr* promoter. A BCG strain transformed with an integrating vector carrying an *acr* promoter-driven β-gal reporter construct was grown to mid-log phase before splitting the culture and shifting the atmospheric oxygen content to the indicated level. After 6 hr, reporter activity was determined as described in *Materials and Methods*. In this short interval, no replication occurs and β-gal activity from equivalent numbers of organisms has been plotted.

pMH109. Luciferase activity from duplicate 150-ml aliquots of lysate was measured for 15 sec in a Wallac AutoLumat model 953B luminometer (Gaithersburg, MD) (24). For β-gal assays, *M. bovis* BCG strain Cannaught transformed with the *acr*-β-gal construct pMH109 was grown to early log phase (OD at 650 nm = 0.2) with shaking and aliquoted into flasks that were subsequently sealed with rubber septa. Gas mixtures of defined oxygen concentration in nitrogen were introduced and the flasks were incubated in a shaking water bath at 37°C. After 6 hr, 10-ml aliquots were removed and collected bacilli were lysed in a Bead-Beater 8 (BioSpec Products, Bartlesville, OK). *o*-Nitrophenyl β-D-galactoside assays with 200 μl of lysate were performed at 27°C for 30 min as described (27).

To construct the green fluorescent protein- (GFP) reporter vector, the 550-bp *Hin*dIII–*Xba*I fragment containing the terminator and putative *acr* promoter of pMH109 was ligated into the integrating vector pMV306. The 750-bp *Hin*dIII fragment containing the ORF of GFP was excised from pFPV27 (25) and inserted into this construct.

Other Procedures. Two-dimensional gel electrophoresis was performed as described (11). Genomic DNA was prepared from MTB by using Bose's protocol (28). Southern blotting was done with QuickHyb hybridization solutions, and the blots were washed under stringent conditions as described by the manufacturer (Stratagene). MTB was transformed by electroporation with various constructs at 37°C (29). Restriction endonucleases, DNA-modifying enzymes, and T4 DNA ligase were purchased from New England Biolabs. Plasmids or DNA fragments were purified and isolated with Qiagen plasmid kit, QIAquick Nucleotide Removal Kit or Gel Extraction Kit (Qiagen, Chatsworth, CA).

RESULTS

Acr Expression Is Regulated by Atmospheric Oxygen Concentration. Initial experiments to determine the nature of the environmental signal controlling Acr production demonstrated that transferring a culture from rolling to standing was sufficient to induce expression (11). To characterize further the signal responsible for induction, we then grew a recent clinical isolate of MTB (CSU93) under a constant flow of an atmosphere of defined oxygen concentration (Fig. 1). In rolling cultures during early logarithmic growth and under normal atmospheric oxygen pressure (20%), no Acr was visible by two-dimensional gel electrophoresis. However, reducing the oxygen concentration to 1.3% resulted in abundant production of the Acr protein (arrow in Fig. 1*B*). The simultaneous addition of [^{35}S]methionine and a low-oxygen atmosphere revealed that the synthesis of this protein was rapidly induced within 2 hr and that synthesis continued to increase through ≈48 hr (data not shown).

To investigate this regulation in more detail, a transcriptional fusion of the 254 nt upstream of the *acr* gene initiation codon to a promoterless *lacZ* gene was assembled in an integrating vector based on mycobacteriophage L5 (27, 30). Sealed early log-phase cultures of *M. bovis* BCG that had been transformed with this reporter were repeatedly purged with gas containing a defined concentration of oxygen in nitrogen, and these were grown for an additional 6 hr before assaying β-gal activity. In this time frame, significant promoter activity was observed at oxygen concentrations below 5.3% (Fig. 1*C*). This activity continued to increase as oxygen concentration was lowered, reaching a maximum of ≈35-fold induction in 0.7% oxygen. Activity of a constitutive, synthetic promoter (27) was unaffected under the same conditions (data not shown). The level of induction reported here may in fact be an underestimate because this assay is not linear at very high levels of activity.

Acr Is Expressed During the Course of Macrophage Infections *in Vitro*. The induction of Acr expression in mildly hypoxic conditions suggested that the protein might be expressed in the course of active infections. The 254-nt sequence containing the *acr* promoter was fused to a promoterless *gfp* gene derived from *Aequorea victoria* inserted into the integrative mycobacterial vector pMV306. This construct was transformed into H37Rv, and expression was monitored by fluorescence microscopy. As expected, log-phase organisms grown aerobically *in vitro* were nonfluorescent whereas organisms grown under low-oxygen conditions were brightly fluorescent (data not shown). When these organisms were used to infect human macrophage-like THP-1 cells and observed under a fluorescence microscope, bacteria were easily observed within these cells 12–48 hr later (Fig. 2*B*). With the *acr* promoter region in the opposite orientation, no fluorescence was observed (Fig. 2*A*). To assess the macrophage-dependent induction of *acr* gene expression quantitatively, the H37Rv strain carrying the *acr* promoter-*lux* fusion was incubated with THP-1 cells and assays of luciferase activity were performed. Induction of the *acr* promoter was evident within 1 hr, while entry into macrophages was still underway. Within 4 hr, *acr*-promoter driven luciferase expression was induced >100-fold compared with organisms incubated identically without macrophages. Again, activity of a constitutive, synthetic promoter (27) was unaffected under the same conditions (data not shown). Bacilli suspended in cell culture media in tissue culture flasks did not induce *acr* expression even at 24 hr. (Fig. 2*C*).

Construction of a Δ*acr*::*hpt* Strain of *M. tuberculosis*. A strain of MTB lacking Acr was created by replacing the 1 kb *acr* gene embedded within a 4.4-kb genomic fragment with a hygromycin resistance cassette, yielding a construct with *acr* homologous upstream and downstream sequence surrounding

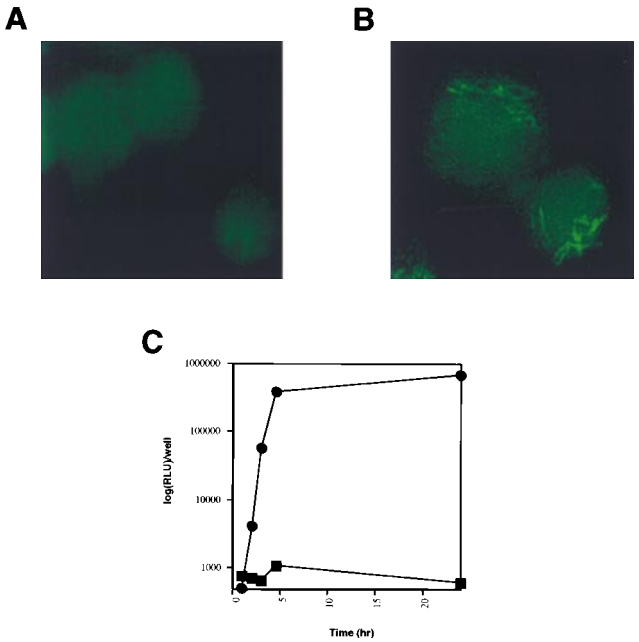

FIG. 2. Expression of Acr inside of macrophages. (*A* and *B*) A reporter fusion containing putative *acr* promoter was fused with the GFP. Transformants of H37Rv containing this reporter were used to infect THP-1 cells (a human macrophage-like cell line), which were analyzed by fluorescence microscopy. (*A*) Control fusion with the *acr* upstream region fused in the opposite orientation. (*B*) *acr*-driven expression of GFP. Fluorescent bacteria were visible as early as 12 hr after infection, and the intensity continued to increase up to 48 hr after infection. The photograph shown in *B* was taken 24 hr after infection and is typical of several infections at various time points. (*C*) *acr*-driven luciferase reporter fusion activity during the first 24 hr after infection of THP-1 cells. Circles show activity from H37Rv transformed with this construct during infection of THP-1 cells, and squares show a time-course with the same bacteria in a mock-infection without THP-1 cells.

the resistance marker (Fig. 3*A*). This plasmid construct, which was incapable of replicating in *M. tuberculosis*, was linearized and used to transform MTB H37Rv. Cultures from hygromycin-resistant colonies (we obtained 10–20 colonies/μg of DNA) were analyzed by SDS/PAGE and Western blot by using a mAb specific to the Acr protein (11). Out of 100 such colonies, one, MTB H37Rv Δ*acr*::*hpt*, was shown to lack immunoreactivity and was characterized further. Chromosomal DNA from this strain was analyzed by Southern blot and had acquired the hygromycin resistance cassette used for gene replacement, although lacking the *acr* gene sequence (Fig. 3*B*). In addition, this strain still possessed the *cma*1 gene, a marker that uniquely identifies the pathogenic mycobacteria (31). The two-dimensional gel-electrophoretic profile of this strain grown under 1% oxygen conditions confirmed the lack of the Acr protein (Fig. 3*C*). Reisolation of the chromosomal fragment carrying the hygromycin resistance cassette and sequence analysis of the junction regions outside the homologous regions carried by the initial knockout construct confirmed the resistance gene was inserted by homologous recombination into the *acr* locus. Interestingly, no compensatory changes in protein expression were observed in H37Rv Δ*acr*::*hpt* compared with the wild-type strain.

H37Rv Δ*acr*::*hpt* Is Impaired for Growth Within Macrophages. When we examined growth of the H37Rv Δ*acr*::*hpt* mutant *in vitro* no significant differences were observed compared with the parental wild-type strain, even under low oxygen conditions (Fig. 4*A*). These results do, however, show a decline in growth rate as oxygen tension was reduced. There was no significant difference in sensitivity to *in vitro* killing by hydrogen peroxide between H37Rv and H37Rv Δ*acr*::*hpt* (data not shown). We then examined growth of organisms within the human macrophage-like cell line THP-1 (Fig. 4*B*). Infectivity was unaffected but growth of H37Rv Δ*acr*::*hpt* was substantially reduced compared with wild type over the course of a 10-day infection. Growth of the knockout organism in primary mouse bone marrow-derived macrophages also was notably reduced over the course of a 6-day infection (Fig. 4*C*). In both cases, equivalent numbers of organisms were present 2 hr after infection, indicating that the mutant was not impaired for macrophage attachment or entry, only growth. Log-phase organisms were used in both of these infections, so that neither strain was expressing Acr initially. Infections with wild-type organisms grown to stationary phase with high-levels of Acr were identical to organisms grown to log-phase in long-term macrophage infections (data not shown). These infections did not reveal a significant difference in survival in macrophages during early infection, regardless of Acr status.

DISCUSSION

The ability of MTB strains to grow under reduced oxygen tension *in vitro* is directly correlated with the ability of such strains to cause disease (32, 33). In addition to our earlier work associating Acr with MTB dormancy (11), we have now shown that expression of *acr* is rapidly and powerfully induced *in vivo* on entry into macrophages or *in vitro* under oxygen-limitation.

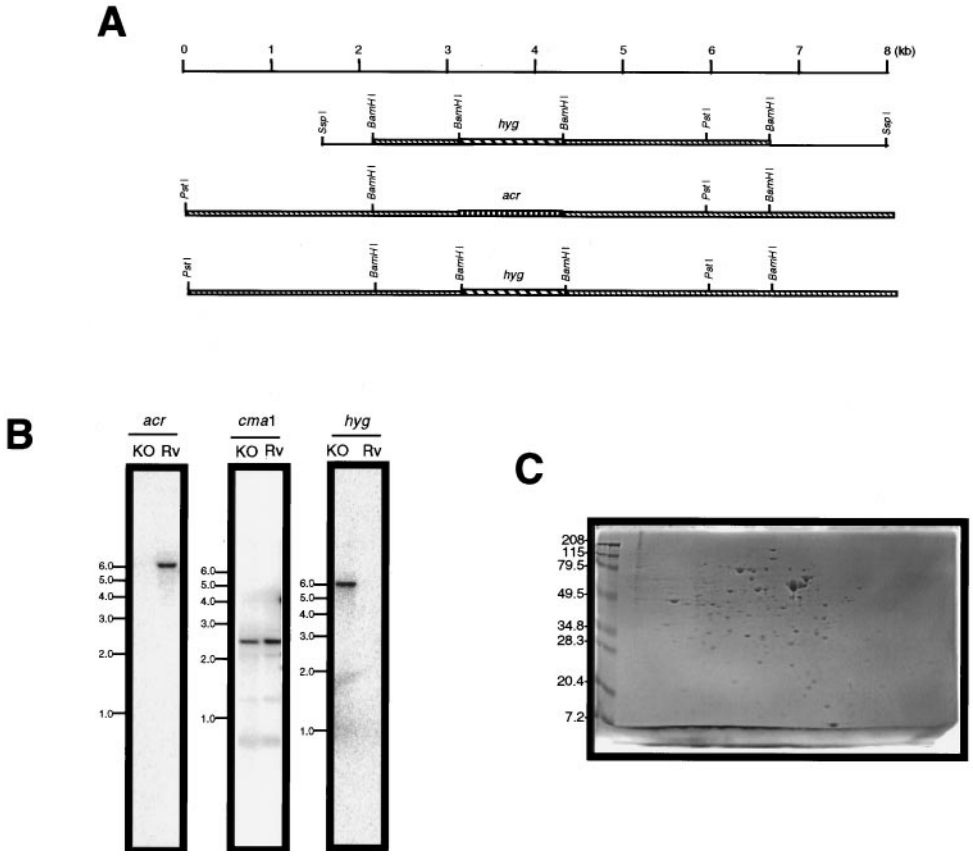

FIG. 3. Construction of MTB H37Rv Δ*acr*::*hpt*. (*A Top*) Map of linearized pBluescript construct used for gene replacement; solid dark line on each side represents vector sequence. (*Middle*) Map of genomic locus containing the *acr* gene and showing relevant restriction sites. (*Bottom*) Map of genomic locus isolated from the knockout construct. The region between the *Pst*I sites was rescued from genomic DNA of H37Rv Δ*acr*::*hpt* by selecting for hygromycin resistance in *E. coli* and was sequenced in entirety verifying the shown genomic order and the junctions of the hygromycin resistance marker and the MTB chromosome. (*B*) Southern blot analysis of *Pst*I-digested chromosomal DNA isolated from the parent H37Rv (*Right* lanes) and H37Rv Δ*acr*::*hpt* (*Left* lanes, labeled KO) using the *acr* gene (*Left*), the *cma*1 gene (*Center*), and the hygromycin resistance cassette (*Right*) as probes. DNA size standards are shown on the left of each blot in kDa. (*C*) Two-dimensional gel electrophoresis of a low-oxygen grown sample of H37Rv Δ*acr*::*hpt*. The parental strain grown under the same conditions was indistinguishable from the profile shown in Fig. 1*B*.

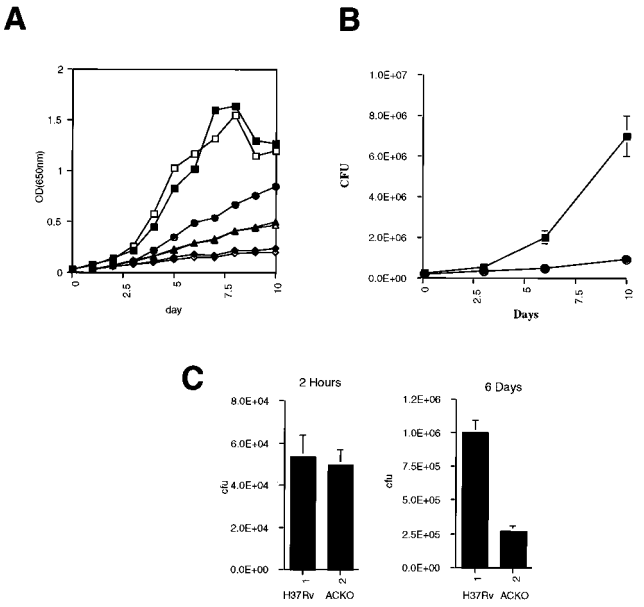

FIG. 4. Growth of H37Rv Δ*acr*::*hpt in vitro* and *in vivo*. (*A*) Growth of the parent (open symbols) and H37Rv Δ*acr*::*hpt* (closed symbols) under defined oxygen conditions. Each pair of lines represents growth under a continuous flow of a premixed oxygen in nitrogen atmosphere at 20% (squares), 5% (circles), 2.5% (triangles), and 1.25% (diamonds). Growth was monitored daily by optical density at 650 nm. (*B*) Growth of wild-type H37Rv (squares) and H37Rv Δ*acr*::*hpt* (circles) in THP-1 cells. Each point is a mean of total bacilli per well in triplicate wells, and the result shown is typical of several repetitions of this experiment. (*C*) Growth of the wild-type H37Rv (bars 1) and H37Rv Δ*acr*::*hpt* (bars 2) in primary mouse bone-marrow derived macrophages. (*Left*) Colony-forming units are shown immediately following infection (2 hr after infection) and on the *Right* 6 days later. The results shown are the average of triplicate wells plated at multiple dilutions.

These data invite reevaluation of both of the nature of the intracellular milieu experienced by MTB and the physiological role of the Acr protein during infection. Human alveolar macrophages are presumably exposed to higher oxygen concentrations than other leukocytes (34), but the actual oxygen tension within these cells is unknown. Although additional means of regulation cannot be excluded, the robust (>100-fold) induction of *acr*-luciferase fusions on infection of cultured macrophages, similar in magnitude to the maximum achievable under hypoxic conditions *in vitro*, suggests that the oxygen concentration within these macrophages is low (35). Thus, contrary to the long-standing idea that low-oxygen conditions are unique to the caseous granuloma, growth under oxygen limitation may be the norm for tubercle bacilli and *in vitro* propagation techniques at atmospheric oxygen may be atypical.

Because of its earlier association with MTB *in vitro* dormancy models, the role of Acr in intracellular growth within macrophages described here was unexpected. In other systems and in *M. tuberculosis*, Acr has been shown to function as a chaperonin, protecting other cellular proteins from degradation (17, 18). However, the hypoxic conditions that induce Acr expression seem unlikely to result in protein instability. Oxygen limitation may instead serve as a signal of the hostile intracellular environment, with Acr expression necessary to protect against other stresses inherent to that environment. In this regard, it is curious that the knockout strain is not more sensitive to oxidative stress such as hydrogen peroxide treatment and that stresses such as low pH, nutrient deprivation or oxidative stress fail to induce Acr expression (11). Although the possibility remains that the suppressed intracellular growth phenotype of H37Rv Δ*acr*::*hpt* has been caused by a secondary mutation or polar effect on downstream gene expression, a simpler interpretation is that the Acr protein directly protects against the effects of toxic metabolites through its chaperonin activity. The nature of the intracellular bacteriocidal effector against which Acr protects MTB during growth in macrophages remains unknown.

Production of Acr exacts a toll in terms of growth rate of expressing organisms *in vitro* therefore production is most likely essential to growth under intracellular conditions. The repression of expression in atmospheric oxygen levels suggests the possibility that this regulatory mechanism may have been maintained to promote rapid growth in a highly oxygenated environment. Such an environment might occur uniquely in the context of the *in situ* alveolar macrophage or it may occur later in the infectious cycle of tuberculosis when caseous granulomas undergo liquefaction and the lung wall disintegrates (8). In this scenario, down-regulation of Acr protein expression may contribute to transmission of disease by facilitating rapid extracellular growth of the organism to high titer prior to discharge in aerosolized sputum. The availability of the H37Rv Δ*acr*::*hpt* strain will allow for testing of such hypotheses in animal models of both persistence and acute infection as well as providing a potential vehicle for the production of a new live vaccine for tuberculosis.

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