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Conditional Sigma Factor Expression, Using the Inducible Acetamidase Promoter, Reveals that the *Mycobacterium tuberculosis sigF* Gene Modulates Expression of the 16-Kilodalton Alpha-Crystallin Homologue

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A chemically inducible acetamidase promoter-*sigF* fusion gene was integrated into the chromosome of *Mycobacterium bovis* BCG. Two-dimensional protein gel analysis permitted the identification of a number of protein spots whose expression was SigF related. One spot upregulated by inappropriate induction of *sigF* expression corresponded to the 16-kDa antigen alpha-crystallin.

Despite effective antimicrobial therapy, tuberculosis remains a leading cause of death worldwide. Identifying important regulatory genes which enable *Mycobacterium tuberculosis* to survive environmental stress could have important therapeutic implications for a disease for which treatment is prolonged and multidrug resistance a daunting problem worldwide. Alternate sigma factors are activated under specific stress conditions and control the expression of specific regulons (6). Several *M. tuberculosis* sigma factors, including SigB, SigC, SigE, SigF, and SigH, appear to be responsive to stress conditions (11, 12, 20).

The *sigF* gene in *M. tuberculosis* was discovered by using degenerate PCR and appears to be upregulated in stationary phase (4). It shows significant homology with stress response sigma factors in *Streptomyces coelicolor* and *Bacillus subtilis* (3). Since stress responses in vitro are likely to reflect the array of mycobacterial genes expressed upon exposure to the host's immune system, the identification of genes controlled by these sigma factors may provide insight into the pathogenesis of *M. tuberculosis*. Using a chromosomally integrated, chemically inducible *Mycobacterium smegmatis* acetamidase promoter fused to the *sigF* gene, we were able to express SigF inappropriately during exponential phase when it is absent in wild-type cultures of both *Mycobacterium bovis* BCG and *M. tuberculosis* (10, 13, 14, 16). By comparing two-dimensional gel protein patterns of SigF-expressing strains with noninduced cultures and wild-type *M. bovis* BCG, we sought to identify proteins that were SigF dependent. This conditionally expressing construct has been particularly useful as we have previously attempted to overproduce SigF by using other promoters and showed that high-level, constitutive production of SigF is lethal in *M. bovis* BCG (3).

Induction of *sigF* expression with the inducible acetamidase promoter. A 0.7-kb *sigF*-containing *Nde*I-*Spe*I segment from pYZ99 (Table 1) (4) and a 3-kb acetamidase promoter locus-containing *Bam*HI-*Nde*I fragment from pAMI1 (10) were ligated to one another and cloned into the *Bam*HI-*Spe*I-digested pNBV1 to produce pCK0218. This fusion contained the four open reading frames upstream of the *M. smegmatis* acetamidase gene up to the ATG of the acetamidase gene. The acetamidase ATG was followed by *sigF* codon 2 (ACG) in the correct translational reading frame. The *Bam*HI-*Spe*I acetamidase promoter-*sigF* fusion gene fragment was excised, ligated to *Pac*I linkers, and cloned into *Pac*I-digested pMH94, a mycobacterial integrative vector, to yield pCK0275 (9). Using *M. bovis* BCG (Pasteur strain) and *M. tuberculosis* CDC1551 (18) transformed with pCK0275, which results in the single-copy integration of the acetamidase promoter-*sigF* fusion gene (P_{ace}::*sigF*), we tested whether acetamide induction produced inappropriate SigF expression during logarithmic phase.

Whole-cell lysates of log-phase *M. bovis* BCG (optical density [OD], 0.5) containing an integrated copy of the fusion gene with and without acetamide induction were separated on a sodium dodecyl sulfate – 10% polyacrylamide gel electrophoresis minigel. Figure 1 shows a Western blot of lysates from these bacteria developed with anti-SigF antibodies. A 30-kDa band consistent with expression of SigF in exponential phase was seen in the acetamide-induced strain (Fig. 1, right lane) but not in the uninduced cells (middle lane). Purified SigF is shown in the left lane. A similar level of induction of SigF expression was seen with *M. tuberculosis* containing the fusion gene (data not shown). Purified SigF and polyclonal rabbit antibodies against SigF were prepared as described previously (3).

Comparison of protein expression in BCG P_{ace}::*sigF* with and without acetamide induction using 2-D gel electrophoresis. Recently, 2-D gels have been used extensively to examine culture filtrates as well as cell-associated proteins expressed by *M. tuberculosis* (15, 17, 19). In addition, 2-D gels have been used to compare bacterial proteins expressed during macrophage infection and under certain stress conditions (1, 8). We sought to identify SigF-dependent proteins by using 2-D gel electrophoresis. We compared protein patterns in whole-cell lysates of exponential-phase *M. bovis* BCG harboring the P_{ace}::*sigF* fusion gene with or without acetamide induction. Only small differences in spot intensity were noted on Coomassie blue staining of gels. Hence, we opted for a pulse-labeling approach to visualize recently synthesized proteins. For metabolic labeling, 250 μCi of [^{35}S]methionine and cysteine was added per 10 OD units of culture. Immediately

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TABLE 1. Plasmids and bacterial strains used

Plasmid	Description	Reference
pAMI1	4.2-kb *Bam*HI fragment of *M. smegmatis* genomic DNA in pUC18 (acetamidase promoter)	10
PYZ99	2.8-kb *Bam*HI fragment of *M. tuberculosis* genomic DNA (*sigF*)	4
pCK0218	3.7-kb acetamidase promoter::*sigF* fusion gene in pNBV1	This paper
pCK0230	3.7-kb fusion gene in pNEB193	This paper
pMH94	2.0-kb insert with *attP* and *int* into pUC119	9
pCK0275	3.7-kb fusion gene in pMH94	This paper
M. tuberculosis 102509	Δ*sigF* Hy^R *M. tuberculosis* CDC1551	Chen et al.[a]

[a] Chen et al., Abstr. 99th Gen. Meet. Am. Soc. Microbiol.

thereafter, half of each labeled culture was exposed to 0.2% acetamide. After a 4-h incubation, bacteria were pelleted by centrifugation, washed twice with sterile phosphate-buffered saline (PBS), and then resuspended with lysis solution (3 M urea, 0.5% Triton X-100, 3.25 μM dithiothreitol, 2% Pharmalyte, phenylmethylsulfonyl fluoride [100 μg/ml], leupeptin [2 μg/ml]). By using 0.1-mm-diameter glass beads, the samples were homogenized twice. The samples were centrifuged, and 250 μg of soluble protein was rehydrated overnight into freeze-dried isoelectric focusing strips (Pharmacia Biotech). First-dimension electrophoresis and second-dimension electrophoresis with 8 to 18% gradient gels were carried out in accordance with the manufacturer's instructions.

Proteins initially absent in exponential phase but inducible with the addition of acetamide were readily apparent by this approach. Figure 2 shows 2-D gels of exponential-phase (OD, 0.6) P_{ace}::*sigF M. bovis* BCG lysates induced with acetamide (Fig. 2A) and uninduced lysates (Fig. 2B). Several proteins appeared to be upregulated in the induced culture. The appearance of these spots was dependent upon the P_{ace}::*sigF* fusion as they were not present in acetamide-treated wild-type cell lysates (data not shown). In addition, proteins which were absent in the induced culture were noted in the uninduced cell lysates.

In another experiment, stationary-phase cultures were diluted only 1:50 and grown to ODs at 600 nm of 0.2 and >1.0. Figure 3 shows 2-D electrophoresis of these whole-cell lysates. A 16-kDa protein with an estimated isoelectric point of 5 was visible during early exponential phase with the addition of acetamide (Fig. 3A) although absent under the same conditions in uninduced lysates (Fig. 3B) and in wild-type lysates (Fig. 3C). In stationary-phase cultures where the native *sigF* gene is known to be expressed, the 16-kDa protein spot is uniformly present in all cell lysates. It appears to be relatively upregulated in acetamide-induced cell lysates of *M. bovis* BCG containing the fusion gene (Fig. 3D). This inducible spot correlates with the predicted molecular weight and isoelectric point of the alpha-crystallin homologue, Acr (21).

Analysis of 16-kDa protein. We then sought to determine if SigF was necessary for the elaboration of the 16-kDa protein in

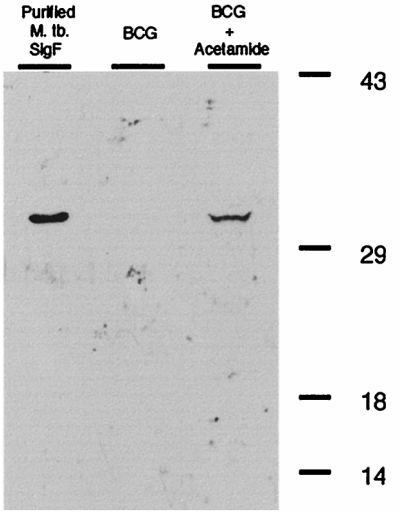

FIG. 1. Western blot of *M. bovis* BCG culture lysates incubated with anti-SigF antibody showing expression of SigF by use of the inducible acetamidase promoter from *M. smegmatis*. Lanes: left, purified *M. tuberculosis* SigF; middle, exponential-phase (OD, 0.5) uninduced BCG containing the P_{ace}::*sigF* fusion gene construct; right, exponential-phase BCG containing the P_{ace}::*sigF* fusion gene construct induced with acetamide.

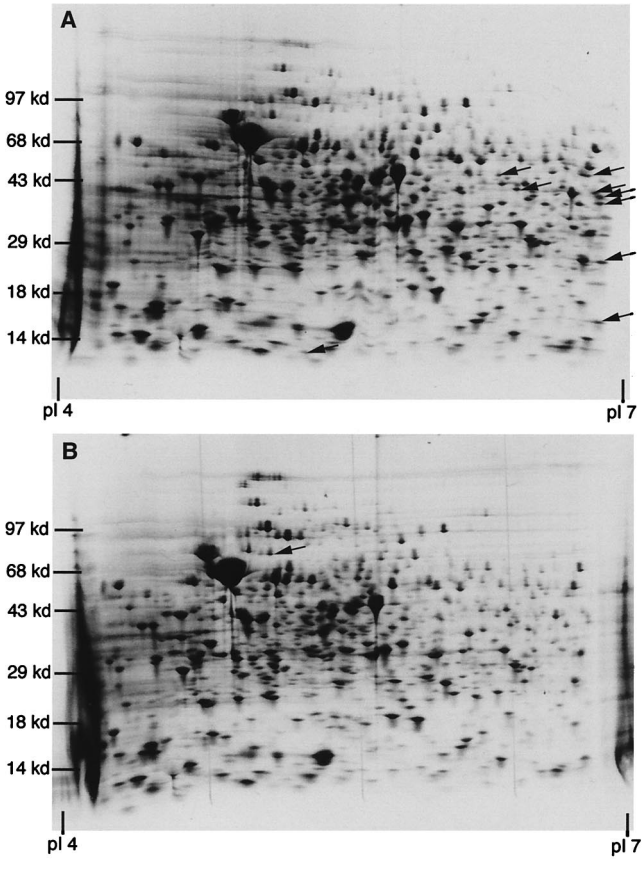

FIG. 2. 2-D polyacrylamide gel electrophoresis of P_{ace}::*sigF M. bovis* BCG whole-cell culture lysates without acetamide (B) and after 4 h of induction with acetamide (A). All cultures were treated for 4 h with [^{35}S]cysteine-methionine. Arrows denote protein pattern differences between gels.

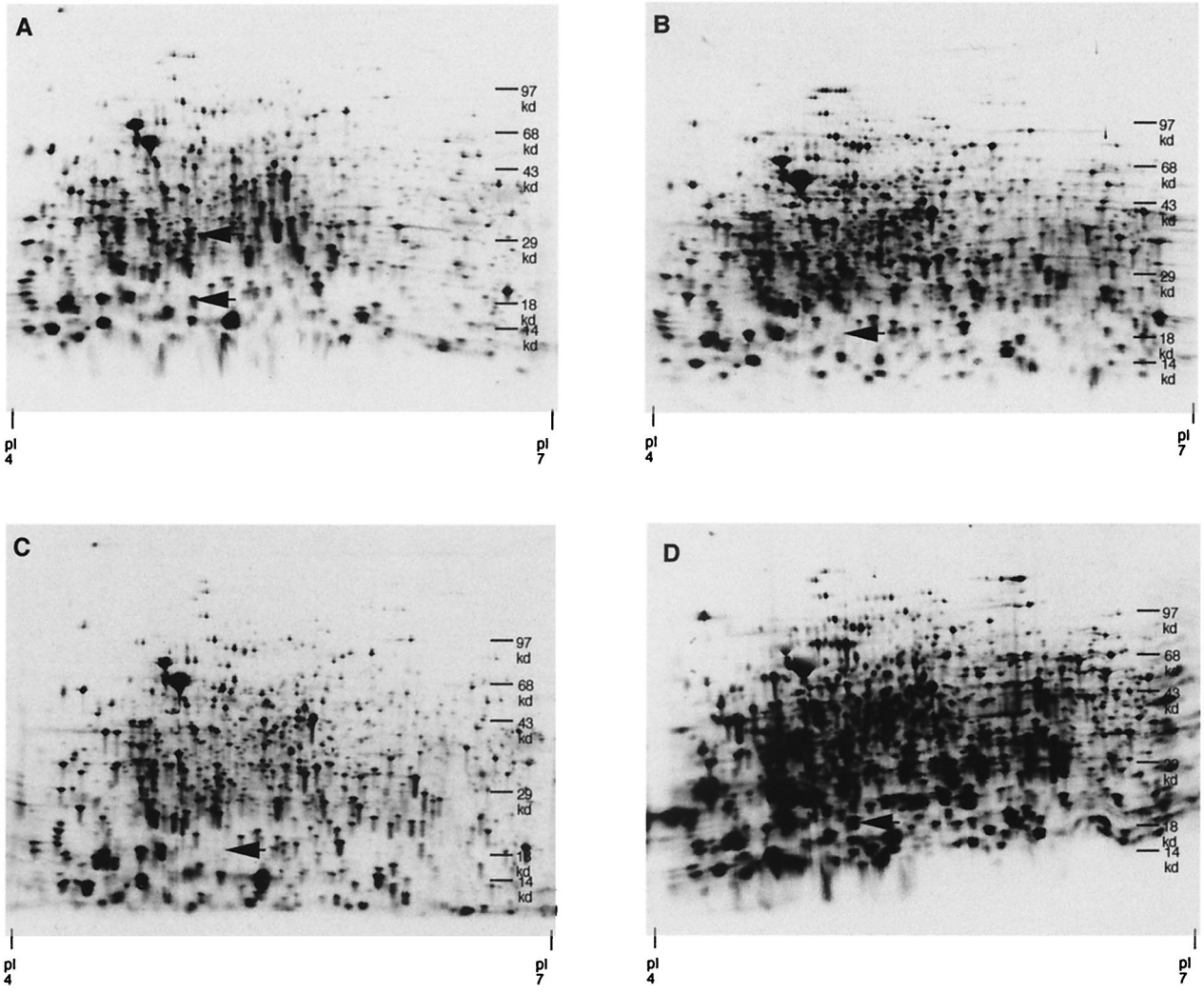

FIG. 3. A comparison of wild-type *M. bovis* BCG and the P_{ace}::*sigF* strain during early exponential phase (OD, 0.2) and stationary phase (OD, >1.0) with and without acetamide. Whole-cell lysates labeled with [^{35}S]methionine-cysteine of both strains were compared by 2-D gel electrophoresis. (A) Exponential-phase P_{ace}::*sigF* BCG with acetamide; (B) exponential-phase P_{ace}::*sigF* BCG without acetamide; (C) exponential-phase wild-type BCG with acetamide; (D) stationary-phase P_{ace}::*sigF* BCG with acetamide.

aerobically grown cultures. Using polyclonal rabbit antibodies directed against Acr, we compared 2-D Western blots of wild-type *M. tuberculosis* stationary-phase cultures (Fig. 4A) and stationary-phase cultures of an *M. tuberculosis sigF* knockout strain (*M. tuberculosis* Δ*sigF*) (Fig. 4B) (P. Chen, R. E. Ruiz, and W. R. Bishai, Abstr. 99th Gen. Meet. Am. Soc. Microbiol. 1999, abstr. U-101, p. 653, 1999). After gel electrophoresis, proteins were transferred to nitrocellulose membranes by gravity diffusion overnight at 4°C and then blocked with 5% nonfat milk in PBS with 0.1% Tween 20 (PBS-T) for 1 h. Membranes were then incubated overnight in PBS-T with rabbit-specific polyclonal antibody at the appropriate concentration at 4°C. After washing the membranes, they were incubated with anti-rabbit horseradish peroxidase-conjugated antibody for 2 h and then with a chemiluminescent substrate for autoradiograph exposure. Polyclonal rabbit antibodies against Acr were the gift of C. E. Barry (Bethesda, Md.).

While the anti-alpha-crystallin antibody nonspecifically binds to other heat shock protein complexes, it shows a discrete spot at the predicted molecular weight and isoelectric point of 5 for Acr. This 16-kDa protein uniformly appears only in wild-type cultures and is completely absent in *M. tuberculosis* Δ*sigF* culture lysates.

To further establish the identity of the 16-kDa protein, we excised the protein and performed sequence analysis on the gel piece through the Harvard Microchemistry Facility where samples are proteolytically digested, separated by microcapillary high-pressure liquid chromatography, and analyzed with tandem mass spectrometry. Correlating these results with known sequences by using the algorithm Sequest, three unique polypeptide protein fragments, including a hypothetical protein (Rv3686c), lumazine synthase (RibH, Rv1416) involved in the riboflavin synthesis pathway, and Acr (HspX, Rv2031c), were identified in the excised gel piece (5). The molecular weights and isoelectric points of all three of these proteins are similar. In summary, the 16-kDa SigF-dependent spot was both immunoreactive with anti-Acr antibodies and found by mass spectrometry to contain peptide sequences matching Acr.

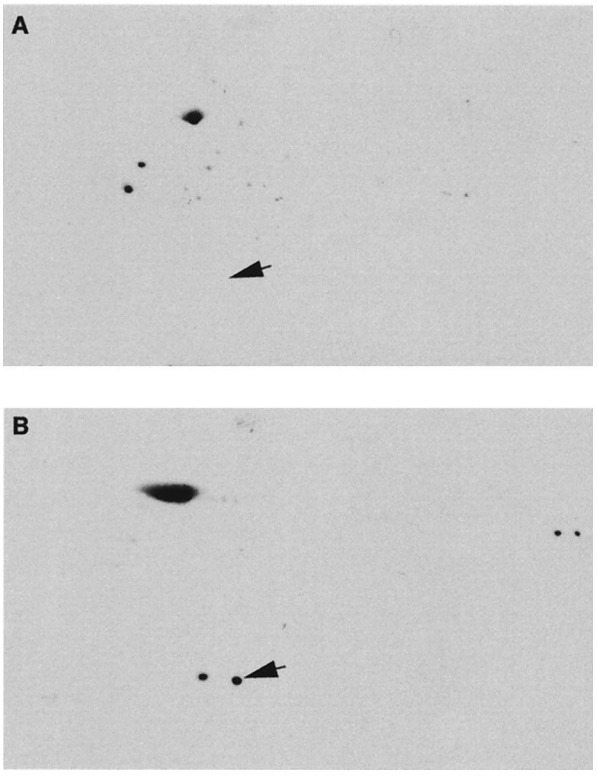

FIG. 4. 2-D gel Western blot of *M. tuberculosis* culture lysates incubated with anti-alpha-crystallin antibody. (A) Western blot of the stationary-phase *M. tuberculosis sigF* knockout strain lysate; (B) Western blot of stationary-phase wild-type *M. tuberculosis* culture lysate. The arrows show the location of the alpha-crystallin protein.

The 16-kDa alpha-crystallin homologue, Acr, is a member of the small heat shock protein superfamily. Proteins in this family act as molecular chaperones preventing the thermal aggregation of proteins and suppressing denaturation (2). The *M. tuberculosis* Acr protein has been shown to accumulate in the transition to stationary phase similar to SigF. However, as noted by Yuan et al., SigF may not be the sole regulator of alpha-crystallin expression as many of the conditions shown to upregulate SigF expression have no effect on the expression of the 16-kDa protein (12, 21). Moreover, the expression of the *acr* gene has been shown to be complex, with evidence for a strong promoter and a repressor binding site modulating expression (Y. Yuan, D. D. Crane, D. R. Sherman, M. Hickey, and C. E. Barry, Proceedings of the Thirty-Second U.S.-Japan Cooperative Medical Science Program, Tuberculosis-Leprosy Research Conference, Cleveland, Ohio). In other recent data from Hu and Coates, Acr appears to be posttranscriptionally modified to account for its accumulation in stationary phase rather than solely transcriptionally regulated (7).

In agreement with Yuan et al., our data show that in ordinary exponential-phase culture, very little alpha-crystallin can be detected even with SigF induction using the acetamidase promoter (21). In contrast, in cultures recently diluted from stationary phase, SigF induction leads to alpha-crystallin expression. Furthermore, in *M. tuberculosis sigF* knockout strains, alpha-crystallin is absent in stationary-phase cultures. Overall, alpha-crystallin expression appears to be controlled by transcriptional and translational mechanisms of expression and accumulation which may be modulated by different stress conditions such as anaerobiosis. Our data suggest that alpha-crystallin requires SigF in conjunction with other stationary-phase elements in order to be produced at detectable levels in stationary phase under aerobic culture conditions.

We have used the *M. smegmatis*-inducible acetamidase promoter fused to *sigF* to produce the transcription factor at a nonphysiologic time. 2-D gel electrophoresis comparisons reveal several candidate proteins that may be regulated by SigF either as a direct transcriptional effect or indirectly through other regulatory proteins controlled by SigF. In addition, Western blots of 2-D gels offer an alternative method for separating and distinguishing individual proteins. Using these techniques, we have shown a putative link between two genes which are upregulated in the transition to stationary phase: *sigF*, encoding an alternative sigma factor, and *acr*, encoding the alpha-crystallin homologue.

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