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Open Reading Frame 3, Which Is Adjacent to the Mycocerosic Acid Synthase Gene, Is Expressed as an Acyl Coenzyme A Synthase in *Mycobacterium bovis* BCG

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The aim of this study was to test for expression of a 900-bp open reading frame (ORF), ORF3, located at the 5′ end of the mycocerosic acid synthase gene in *Mycobacterium bovis* BCG and to determine the nature of the ORF3 protein. ORF3 was expressed as a 61-kDa C-terminal fusion protein with glutathione *S*-transferase in *Escherichia coli*. Polyclonal rabbit antiserum, prepared against this fusion protein, cross-reacted with a 65-kDa protein in *M. bovis* BCG crude extracts. Since this protein was larger than that predicted from the nucleotide sequence (32 kDa), ORF3 was resequenced, revealing an ORF of 1,749 bp that encodes a 64.8-kDa protein containing 583 amino acids. Reverse transcription-PCR revealed that ORF3 is expressed in *M. bovis* BCG. The ORF3 product has a high degree of similarity to the acyladenylate family of enzymes. Immunoaffinity absorption chromatography was used to isolate the 65-kDa cross-reacting protein from *M. bovis* BCG. This purified protein catalyzed coenzyme A (CoA) ester synthesis of *n*-C_{10} to *n*-C_{18} fatty acids but not mycocerosic acids. ORF3 antibodies severely inhibited acyl-CoA synthase activities of the purified protein and extracts of *M. bovis* BCG, *Mycobacterium smegmatis*, and *E. coli*. They also showed immunological cross-reactivity with proteins in these extracts. Both the ORF3 protein and the acyl-CoA synthase activity were located in the cell cytosol or were loosely associated with the cell membrane. These results indicate that ORF3 encodes an acyl-CoA synthase-like protein.

Tuberculosis is the world's foremost cause of death from a single infectious agent. More than 30 million tuberculosis-related deaths and 90 million new infections are expected to occur in the last decade of this century (11). The major difficulty in treating mycobacterial infections is that the cell walls of these organisms have a high lipid content (50 to 60%) and consequently are highly resistant to attack by both the host's own immune system and antimicrobial agents (7). With the advent of multidrug-resistant mycobacterial strains, identification of potential new drug targets has become a critical need. Components unique to pathogenic mycobacterial cell wall synthesis (such as the mycolic acids, mycocerosic acids, and phthiocerols) (6) may be such selective targets, and inhibitors of such processes may be used in conjunction with conventional antimicrobial therapies to treat mycobacterial diseases (9).

In spite of accumulating evidence which highlights the importance of the nature and quantity of lipids in the cell wall, much basic information remains to be elucidated about the metabolism of such lipids. Mycocerosic acids are produced by a multifunctional synthase, mycocerosic acid synthase (MAS), which elongates *n*-C_{20} acids by successive reactions involving the incorporation of four C_3 units from methylmalonyl-coenzyme A (CoA) (34). When the *mas* gene was cloned and sequenced, an adjacent open reading frame (ORF), ORF3, was identified that showed homology to the acyladenylate family of enzymes, suggesting a role for the product of this gene in fatty acid metabolism. However, nothing was known about this ORF, which was also found adjacent to the *mas* gene in *Mycobacterium tuberculosis* (cosmid Y338) and *Mycobacterium leprae* (cosmid B1170). In this paper, we report the newly identified complete ORF3 and demonstrate that it is expressed in *Mycobacterium bovis* BCG. We also present immunological and enzymological evidence that the ORF3 translation product can catalyze acyl-CoA synthesis and report isolation of this enzyme produced by *M. bovis* BCG.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. Strain descriptions and cultivation conditions of all the mycobacterial and *Escherichia coli* strains used in this study have been presented previously (1, 37, 41).

Nucleic acid isolation and analysis. High-molecular-weight chromosomal DNA was isolated according to the method of Jacobs et al. (22). Total RNA was isolated from mid-log-phase *M. bovis* BCG cells as previously described (27). Plasmid DNA was extracted from *E. coli* DH5α cells by the alkaline lysis method (2). Construction of recombinant plasmids and Southern hybridization experiments were performed as outlined by Sambrook et al. (37). Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs, Life Technologies, and Amersham International and were used according to the manufacturers' recommendations.

Expression of ORF3 as a GST fusion protein. The DNA segment previously designated ORF3 (33) was amplified under standard conditions by PCR (Perkin-Elmer) to introduce a *Bam*HI and an *Eco*RI site at the 5′ and 3′ ends, respectively, by using the following primers: sense primer, 5′-GGGGGATCCCCATG GATTACGAAC-3′; antisense primer, 5′-GGGGAATTCTCACCCTCTCCTG CA-3′. The amplified DNA was cloned in-frame into *Bam*HI-*Eco*RI-digested pGEX-5X-3 to generate an ORF3-glutathione *S*-transferase (GST) fusion construct, pGEXRF3. *E. coli* BL21 was transformed with pGEXRF3, and induction of the recombinant protein with isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma) was performed according to standard protocols. Cells were lysed by sonication, and aliquots of the pellet and supernatant fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (28).

Production of antiserum against the ORF3 recombinant protein. Following separation by SDS-PAGE, the ORF3-GST fusion protein was excised by standard protocols and injected subcutaneously into New Zealand White rabbits with Freund's adjuvant. Three booster injections were administered at intervals of 2

weeks. The rabbits were bled by heart puncture 4 days after the final booster, and the serum was decanted after clot formation.

Immunoblotting. Electrophoresed proteins were transblotted onto an Immobilon polyvinylidene difluoride membrane (Millipore Corp., Bedford, Mass.) in 10 mM cyclohexylaminopropane sulfonic acid (CAPS) buffer (pH 11) and 10% methanol. ORF3 antibodies were diluted 1:1,000, and ^{125}I-labeled protein A was used as the secondary detection reagent.

DNA sequencing and analysis. Genomic DNA in the ORF3 region was sequenced by successive primer walking using pUC-based ORF3-containing plasmids as the template. Both strands were completely sequenced. Sequencing was performed by the chain termination method of Sanger et al. (38), using the Sequenase version 2.0 kit (U.S. Biochemical, Cleveland, Ohio). DNA Strider 1.2 and the Genetics Computer Group packages were used to analyze and align nucleotide and protein sequences.

Detection of ORF3 transcripts by reverse transcription-PCR (RT-PCR). *M. bovis* BCG total RNA was reverse transcribed into cDNA, using Superscript 11 reverse transcriptase (Gibco BRL) and random hexamer primers. The first strand of cDNA was subjected to PCR amplification using standard procedures (Perkin-Elmer). The amplified product was identified on a 0.9% agarose gel, and its identity was confirmed by hybridization with a ^{32}P-labeled ORF3 probe.

Cell extract preparation. Extracts of *M. bovis* BCG cells were prepared essentially as described by Kameda and Nunn (24).

Enzyme assays. Mycocerosic acids were synthesized in *M. bovis* BCG cells labeled with [1-^{14}C]propionic acid (specific activity, 59 Ci/mol). The mycoside fraction was isolated by thin-layer chromatography and hydrolyzed with 5% KOH in 2-methoxyethanol as previously described (35). Mycocerosic acids were purified by thin-layer chromatography on Silica Gel G with hexane-ethyl ether-formic acid (40:10:1 [vol/vol/vol]) as the solvent. The specific activity of each of the mycocerosic acids was 30 Ci/mol. [1-^{14}C]decanoic acid, [1-^{14}C]lauric acid, and [1-^{14}C]stearic acid, each with a specific activity of 50 to 60 Ci/mol, were obtained from American Radiolabeled Chemicals, St. Louis, Mo. Acyl-CoA synthase activity was determined by quantifying the amount of fatty acyl-CoA in the aqueous phase following extraction of the reaction mixtures with organic solvents (24). Acyl-CoA synthase activity is expressed in units of fatty acyl-CoA formed per milligram of protein. One unit is defined as the amount of enzyme which forms 1 nmol of fatty acyl-CoA per min. Lactate dehydrogenase activity was measured by standard protocols (39). Protein concentrations were determined by the Bradford method (5) with bovine serum albumin as standard.

Immunoaffinity purification of acyl-CoA synthase. Acyl-CoA synthase was purified from *M. bovis* BCG cells with a commercially available Immunopure protein A immunoglobulin G (IgG) orientation kit (Pierce, Rockford, Ill.). The following reagents were tested for their ability to elute native antigen from the immunosorbent column: (i) 100 mM triethylamine, pH 11.5, (ii) 100 mM glycine, pH 2.5, (iii) 5 M LiCl–10 mM phosphate, pH 7.2, (iv) 3.5 M $MgCl_2$–10 mM phosphate, pH 7.2, (v) 1% SDS, (vii) 2 and 8 M urea, (viii) 2 M guanidine HCl, (ix) 3 M thiocyanate, and (x to xviii) TE (50 mM Tris-HCl [pH 7.9], 0.1 mM EDTA) containing 30 to 50% ethylene glycol and 0.5 to 1.0 M NH_4SO_4 at pH 7 and pH 8. All eluted material was dialyzed against storage buffer (50 mM Tris-HCl [pH 7.9], 0.1 mM EDTA, 1 mM dithiothreitol, 150 mM ammonium sulfate, and 50% glycerol). The effectiveness of each reagent at eluting native protein from the immunoaffinity column was determined by assaying for acyl-CoA synthase activity.

Antibody inhibition of acyl-CoA synthase activity. IgG was incubated with both crude extract and purified acyl-CoA synthase for 1 h at 37°C, followed by 2 h at 4°C and centrifugation. The effect of anti-ORF3 IgG on acyl-CoA synthase activity was determined by the assays described above.

Nucleotide sequence accession number. The sequence reported in this publication is available in the GenBank database under accession number U75685.

RESULTS

Expression of ORF3 in *E. coli*. In a previous publication, we reported the existence of an ORF of approximately 900 bp, designated ORF3, at the 5′ end of *mas* (33). ORF3 was expressed as a C-terminal translational fusion with GST, using the construct pGEXRF3. The expressed protein was found as a major band at 61 kDa (Fig. 1A). The size of this protein is consistent with the predicted size of an ORF3-GST fusion protein. The recombinant protein was localized almost exclusively in the insoluble fraction. The ORF3 fusion protein, in an SDS-PAGE gel, was used to prepare polyclonal antiserum in rabbits. The 61-kDa band from the IPTG-induced cells showed significant immunological cross-reactivity with this antiserum, whereas no cross-reacting bands were visible in noninduced cell lysates (Fig. 1B). Therefore, the antiserum generated is specific for the ORF3 fusion protein.

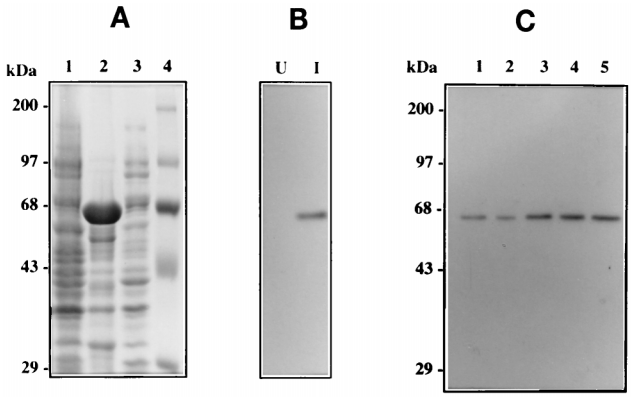

FIG. 1. (A) SDS-PAGE analysis of ORF3 recombinant protein expression in *E. coli*. Lane 1, uninduced pGEXRF3; lane 2, 10,000 × *g* pellet from IPTG-induced pGEXRF3; lane 3, 10,000 × *g* supernatant from IPTG-induced pGEXRF3; lane 4, molecular size marker. (B) Immunoblot analysis of ORF3 recombinant protein expression in *E. coli*. U, uninduced; I, induced. All induction experiments were performed with IPTG for 4 h at 37°C. (C) Immunoblot analysis of ORF3 expression in *M. bovis* BCG. Crude cell extracts prepared from cells harvested at optical densities at 600 nm of 0.2, 0.4, 0.8, 1.4, and 2.0 were used in lanes 1 to 5, respectively. Rabbit anti-ORF3 IgG was used as the primary antibody, and ^{125}I was used for detection.

Analysis of ORF3 expression in *M. bovis* BCG. Crude extracts of *M. bovis* BCG cells showed a major immunologically cross-reacting band at 65 kDa in Western blots; this band was detectable over the entire growth cycle of *M. bovis* BCG (Fig. 1C). The size of the primary translation product predicted from the ORF3 sequence is approximately 32 kDa; however, a band of this size was not detectable under any of the conditions examined. The size of the immunologically cross-reacting protein suggested that the ORF3 in *M. bovis* BCG might be larger than the one we originally identified.

Identification of the complete ORF3 and evidence for its transcription. ORF3 was sequenced by using a *mas*-containing cosmid (cosmid D) which had previously been isolated by screening an *M. tuberculosis* library with probes containing the ketoacyl synthase and acyl transferase domains of MAS (1a). The complete ORF3 comprises an ORF of 1,749 bp which encodes a 583-amino-acid polypeptide having a molecular weight of 64,800 (GenBank accession no. U75685). That this ORF is transcribed in *M. bovis* BCG was confirmed by the observation that RT-PCR with primers specific for the terminal regions of ORF3 yielded a single PCR product of 1.7 kb (Fig. 2A), corresponding to the expected size of an ORF3 transcript. The identity of this product was confirmed by hybridization with a ^{32}P-labeled ORF3 probe (Fig. 2B). The size of the immunologically cross-reacting protein found in *M. bovis* BCG cells is consistent with both the ORF and transcript sizes.

Southern blot analysis of *M. bovis* BCG genomic DNA using the entire ORF3 as the probe indicated that there is probably a single copy of ORF3 in the genome and that this gene has been cloned without rearrangements. With ORF3 as the probe, a single major hybridization band was detected in each of the digests restricted with enzymes which have no cleavage sites within ORF3 (*Not*I, *Kpn*I, *Eco*RI, and *Hin*dIII). *Xca*I, *Pvu*I, and *Bsp*EI each have a single restriction site within ORF3 and accordingly yielded two separate hybridization bands of the expected size in each case (Fig. 3). Since the hybridization was done under high-stringency conditions, homologous genes might not have been detected by this method.

Homology of the ORF3 product to acyladenylate enzymes. By using the Blast program from the National Center for

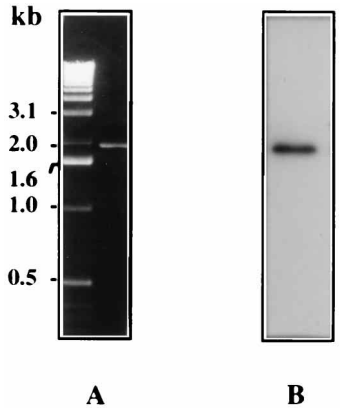

FIG. 2. RT-PCR detection of ORF3 transcripts in *M. bovis* BCG. (A) Ethidium bromide-stained RT-PCR product. (B) Southern blot of the RT-PCR product with ^{32}P-labeled ORF3 as probe. The expected size of the ORF3 RT-PCR product was 1,749 bp.

Biotechnology Information, the deduced amino acid sequence of ORF3 was compared to other sequences in the databases for homology. As expected, the highest degree of similarity observed was to other mycobacterial sequences, including a number of *M. tuberculosis* cosmids (cosmids Y338, tbc2, and SCY19G5) and an *M. leprae* cosmid (cosmid B1170). Overall, ORF3 showed 49 to 67% identity to these sequences and 63 to 81% homology when conserved amino acids are considered (Fig. 4).

The most interesting result from this analysis is the homology of ORF3 to a number of enzymes which function via a common biochemical mechanism in which they activate their substrates as acyladenylates (Fig. 5). Acyladenylate-forming enzymes include acyl-CoA synthases (3, 12, 16–19, 43, 45), acyl-glycerol-3-phosphoethanolamine acyltransferase/acyl-acyl carrier protein synthase (21), acetyl-CoA synthase (13), coumaroyl-CoA ligase (29), luciferase (32), amino acid-activating

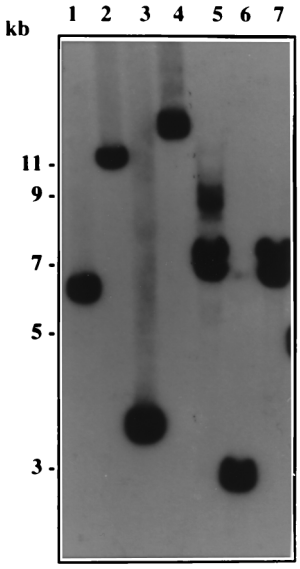

FIG. 3. Southern hybridization of *M. bovis* BCG genomic DNA digested with *Not*I (lane 1), *Kpn*I (lane 2), *Eco*RI (lane 3), *Hin*dIII (lane 4), *Xca*I (lane 5), *Pvu*I (lane 6), and *Bsp*EI (lane 7). The entire ORF3 was labeled with ^{32}P and used as the probe.

enzymes, and complexes involved in peptide and antibiotic biosynthesis (15, 31, 42, 44, 46). These enzymes display three characteristic domains which are highly conserved among the various members. Domain I, the most conserved of the three domains, contains the consensus sequence SGXXGXPKG, which is related to the stretch of glycine-rich sequences that form the phosphate binding loop (P-loop) found in ATP and GTP binding proteins. This region is believed to be involved in binding the AMP moiety of the acyl-AMP intermediate (40). ORF3 amino acid residues 168 to 187 display 40 to 60% sequence identity and 65 to 85% sequence similarity to domain I of the acyladenylate enzymes. Residues 327 to 347 display 14 to 24% sequence identity and 48 to 62% sequence similarity to domain II of the acyladenylate enzymes, whereas residues 441 to 464 display 25 to 50% sequence identity and 38 to 79% sequence similarity to domain III of the acyladenylate enzymes. The functional significance of the latter two domains is not known, but some reports have postulated their involvement in the formation of a hydrophobic pocket which accommodates the fatty acid moiety of the acyl-AMP intermediate (21).

Immunological evidence that ORF3 encodes an acyl-CoA synthase-like enzyme. Given the homology of the ORF3 protein to the acyladenylate enzymes, it appeared possible that ORF3 can encode an acyl-CoA synthase-like enzyme. Crude extracts of *M. bovis* BCG grown to mid-log phase express acyl-CoA synthase activities of approximately 0.85 U per mg of protein, when [^{14}C]palmitic acid is used as substrate. Addition of increasing amounts of anti-ORF3 IgG to *M. bovis* BCG cell extracts resulted in a progressively higher inhibition of acyl-CoA synthase activity, leading to a maximum inhibition of 70%, whereas preimmune serum did not affect acyl-CoA synthase activity (Fig. 6). This inhibition of acyl-CoA synthase activity by the anti-ORF3 IgG indicates either that this ORF directly encodes acyl-CoA synthase or that it encodes a protein that is immunologically similar to acyl-CoA synthase.

Purification of the ORF3 protein from *M. bovis* BCG. Since the antiserum prepared against the fusion protein cross-reacted with only a single protein in the *M. bovis* BCG extract, this protein must be encoded by ORF3. An immunoaffinity absorption procedure was used to purify the ORF3 protein from extracts of *M. bovis* BCG; ORF3 antibody conjugated to a protein A-immobilized matrix was used as an affinity ligand to isolate the ORF3 protein from a crude extract of mid-log-phase cells. In order to remove the bound protein from the affinity matrix, a variety of traditional elution conditions were attempted, including the use of low pH and the use of chaotropic salts, e.g., magnesium chloride. However, protein eluted in this manner retained little or no activity. Additional experiments involved the use of polyols and kosmotropic salts, at or near neutral pH, conditions which have previously been reported to be effective in breaking the antibody-antigen interaction while retaining high enzyme activity (30). A variety of polyol-salt concentrations were examined; the most effective one in obtaining enzymatically active ORF3 protein was 10 mM Tris (pH 7.5) containing 30% ethylene glycol and 0.5 M NH_4SO_4.

SDS-PAGE analysis of the immunopurified protein preparation revealed the presence of a single band of approximately 65 kDa (Fig. 7), which corresponds to the size predicted from the ORF3 sequence and the size of the cross-reacting band revealed by Western analysis. The purified protein showed high acyl-CoA synthase activity (55.25 U/mg of protein). The overall purification was 65-fold, with a yield of approximately 25%. Antibody prepared against the ORF3 protein inhibited the acyl-CoA synthase activity of the purified protein, even

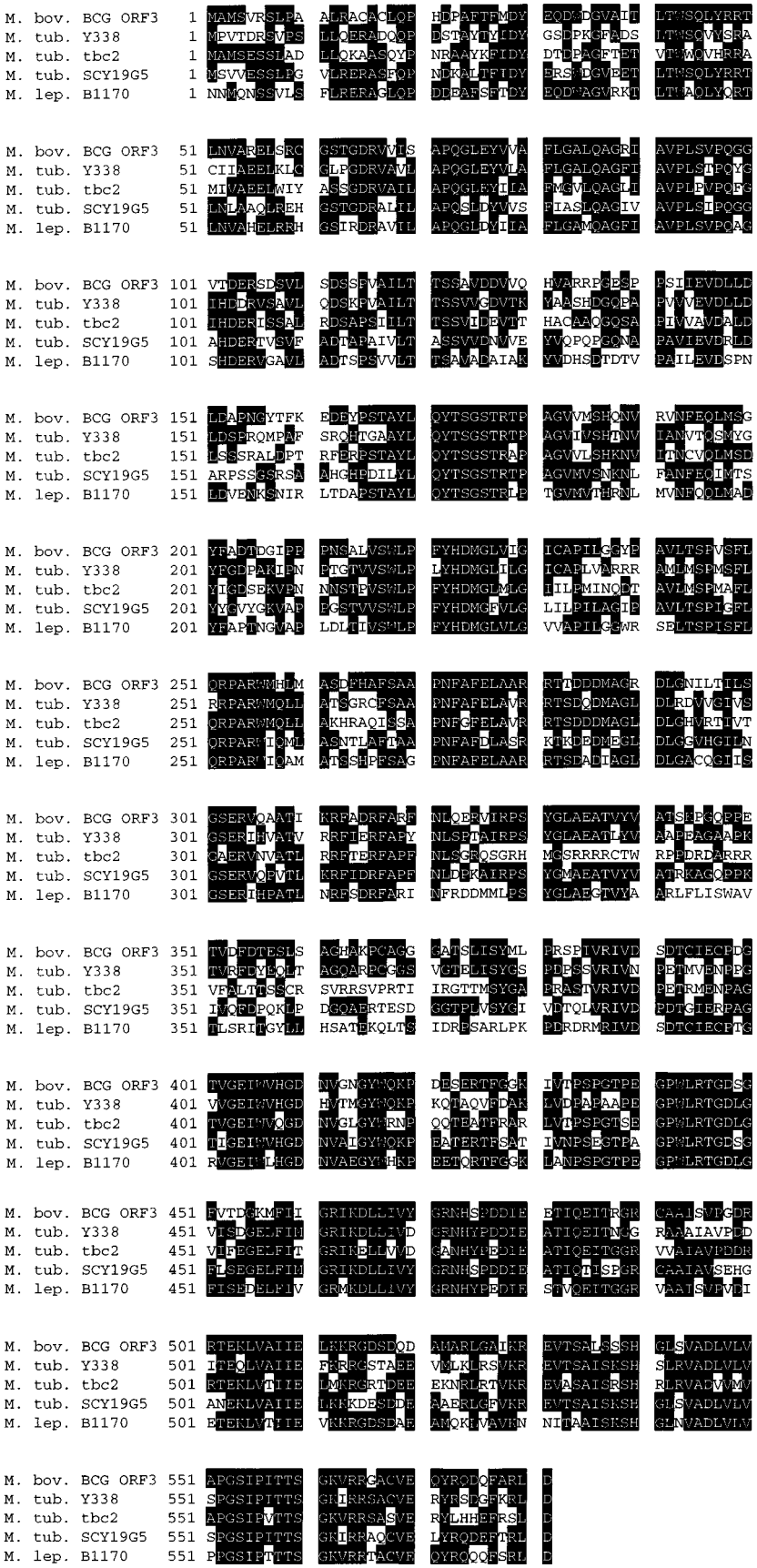

FIG. 4. Homology of the predicted amino acid sequence of the ORF3 described in this study (M. bov. BCG ORF3) to those of other ORF3-like sequences found in the *mas*-containing and polyketide synthase-containing *M. tuberculosis* cosmid Y338 (M. tub. Y338), the polyketide synthase-containing *M. tuberculosis* cosmid tbc2 (M. tub. tbc2), the *mas*-like gene-containing *M. tuberculosis* cosmid SCY19G5 (M. tub. SCY19G5), and the *mas*-containing *M. leprae* cosmid B1170 (M. lep. B1170). Domains I, II, and III are represented by amino acids 168 to 187, 327 to 347, and 441 to 464 of the ORF3 sequence, respectively.

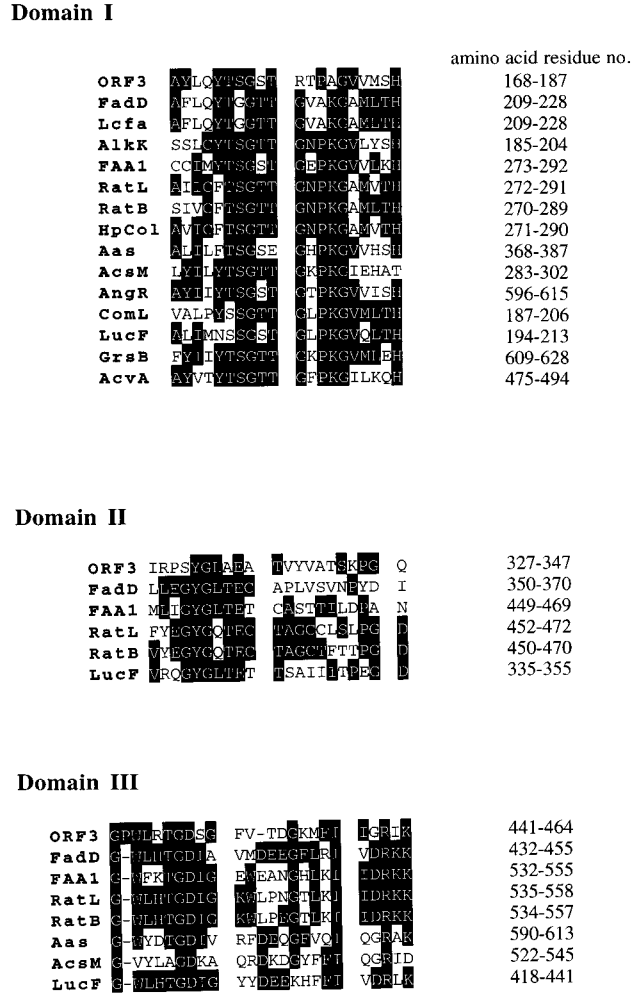

FIG. 5. Homology of the predicted amino acid sequence of ORF3 to other synthases. Acyl-CoA synthases from *E. coli* (FadD [3]), *Haemophilus influenzae* (Lcfa [16]), *P. oleovorans* (AlkK [45]), *R. palustris* (HbaA [18]), *S. cerevisiae* (FAA1 [12]), rat liver (RatL [43]), rat brain (RatB [17]), *Homo sapiens* (HpCol [19]), 2-acyl-glycerol-3-phosphoethanolamine acyltransferase/acyl-acyl carrier protein synthetase from *E. coli* (Aas [21]), acetyl-CoA synthase from *Methanothrix soehngenii* (AcsM [13]), an iron uptake regulatory gene from *Vibrio anguillarum* (AngR [15]), 4-coumarate CoA ligase from *P. crispum* (ComL [29]), luciferin monooxygenase from *Luciola cruciata* (LucF [32]), gramicidin S synthase 2 from *Bacillus brevis* (GrsB [44]), and δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthase from *Aspergillus nidulans* (AvcA [46]) are shown.

more severely than that observed with the crude extract (Fig. 6). The rates of CoA ester formation observed for n-C_{10}, n-C_{12}, n-C_{16}, and n-C_{18} fatty acids were 39, 201, 55, and 35 U/mg of protein, respectively. These relative activities are similar to those observed with the fatty acyl-CoA synthase from *E. coli*. The purified enzyme from *M. bovis* BCG showed extremely low activity (<0.02 U/mg of protein) with the multiple *methyl*-branched mycocerosic acids.

Distribution of acyl-CoA synthase activity and the ORF3 cross-reacting protein in *M. bovis* BCG cell extracts. When *M. bovis* BCG extracts were assayed for acyl-CoA synthase, 58% of activity was located in the high-speed supernatant fraction and 29% of activity was located in the membrane wash fraction. The remaining 13% of activity was localized in the membrane fraction. Lactate dehydrogenase, a membrane-bound marker enzyme, was found exclusively in the cell pellet. Immunoblot analysis reveals that the distribution of the ORF3 cross-reacting protein paralleled that of acyl-CoA synthase activity. About 53 and 32% of the 65-kDa protein that cross-reacted with the anti-ORF3 antibodies were found in the supernatant and membrane wash fractions, respectively, whereas only 15% was found in the cell pellet. In addition, hydropathy analysis did not identify any obvious helical hydrophobic domains indicative of ORF3 as a transmembrane protein (data not shown). These results show that both the acyl-CoA synthase activity and the ORF3 cross-reacting protein are loosely associated with the membrane, in a manner similar to that of acyl-CoA synthase in *E. coli* (24).

Immunological cross-reactivity of ORF3 with proteins from *Mycobacterium smegmatis* and *E. coli*. Anti-ORF3 protein antibodies cross-reacted with a single band of approximately 70 kDa in crude extracts of *E. coli* and with two proteins of approximately 65 and 85 kDa in *M. smegmatis* crude extracts (Fig. 8). In both cases, the cross-reacting proteins were located in the soluble fractions of the extracts.

Addition of anti-ORF3 antibodies to cell extracts inhibited acyl-CoA synthase activities in both *E. coli* and *M. smegmatis*. In *E. coli*, the pattern of antibody inhibition of acyl-CoA synthase activity was similar to that observed with *M. bovis* BCG. However, in *M. smegmatis*, inhibition of acyl-CoA synthase was less severe than that observed with *M. bovis* BCG; only a 40% inhibition of acyl-CoA synthase activity was observed with *M. smegmatis* (Fig. 6). Inhibition of *E. coli* and *M. smegmatis* acyl-CoA synthase activities by ORF3 antibody provides further evidence in favor of our conclusion that ORF3 encodes an acyl-CoA synthase enzyme.

DISCUSSION

An ORF called ORF3 has been found adjacent to *mas* in several pathogenic mycobacteria (cosmids Y338 and B1170) (33). The evidence in favor of ORF3 encoding an acyl-CoA synthase-type enzyme includes the following results. (i) Analysis of the ORF3 amino acid sequence indicates that it has a high degree of similarity to each of the three domains typical of the acyladenylate enzymes. In particular, the presence of a highly conserved ATP binding site is consistent with ORF3 encoding acyl-CoA synthase, since the initial reaction of this enzyme would involve interaction of the ATP binding region with the substrate to form an enzyme-bound adenylated intermediate. (ii) ORF3-specific antibodies were used to isolate a purified protein of 65 kDa from *M. bovis* BCG. The size of this cross-reacting protein is in good agreement with the size of the acyl-CoA synthase proteins present in *E. coli* (3) and in *Pseudomonas oleovorans* (45). The purified protein displayed high acyl-CoA synthase activity, with straight-chain fatty acids containing 10 to 18 carbon atoms. Although overall activities are lower, this pattern of broad substrate specificity with medium- and long-chain fatty acids is identical to that of the *E. coli* acyl-CoA synthase enzyme (24). Addition of ORF3-specific antibodies severely inhibited enzyme activity. (iii) Both the ORF3 cross-reacting protein and the acyl-CoA synthase activity are located in the soluble fraction or are loosely associated with the membrane in *M. bovis* BCG extracts. Previous studies have reported that the *E. coli* (3) and the *P. oleovorans* (45) acyl-CoA synthases are also located in the cell cytosol or are loosely associated with the membrane. (iv) Analysis of the 5′ region upstream of ORF3 indicates that it has promoter elements identical to those present in the *E. coli* acyl-CoA synthase gene (*fadD* [3]) (unpublished data).

The ORF3 gene isolated in this study is expressed in *M. bovis* BCG; this has been demonstrated through the detection of

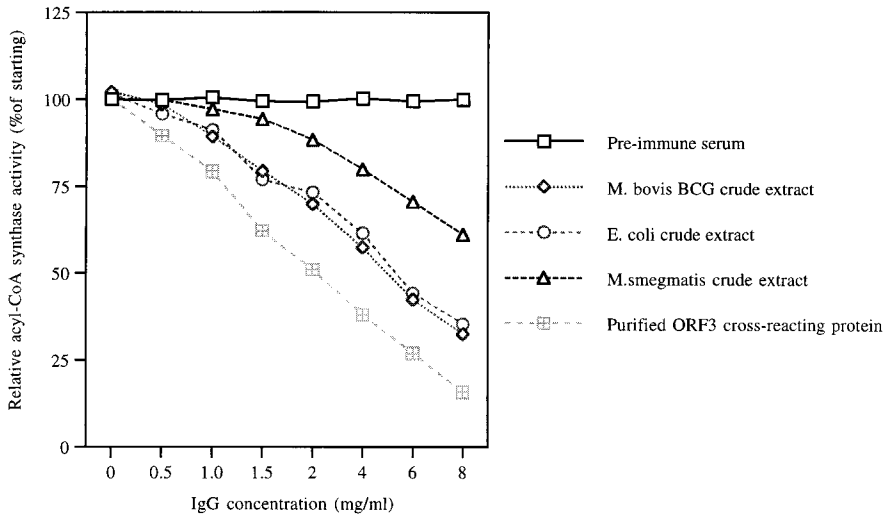

FIG. 6. Inhibition of *M. bovis* BCG acyl-CoA synthase activities with antibodies raised against ORF3. The synthase activity is expressed as a percentage of the value for the control without antibody.

ORF3 transcripts, the isolation of an ORF3 cross-reacting protein which displays acyl-CoA synthase activity (this study), and promoter expression studies which show that the acyl-CoA synthase gene is strongly expressed in *M. bovis* BCG (unpublished data). Expression of this acyl-CoA synthase gene would permit fatty acyl-CoA formation in mycobacteria and is in agreement with a number of studies which indicate that fatty acyl-CoA plays a key role in intermediary fatty acid metabolism and in the regulation of enzyme activity in mycobacteria. Key enzymes of the acyl-CoA-dependent de novo fatty acid synthesis and chain elongation systems and the β-oxidation pathway have been detected in mycobacterial species (26, 48, 49). In addition, acyl groups are incorporated from acyl-CoA into membrane lipids (34, 35). Long-chain fatty acyl-CoAs also feedback inhibit de novo fatty acid synthesis (4). In *E. coli*, long-chain fatty acyl-CoA compounds induce expression of the β-oxidation genes (*fad* regulon), by directly interacting with the negative transcriptional regulator FadR (10). Whether such regulation is operational in mycobacteria is not known.

The biological significance of ORF3 expression in *M. bovis* BCG has not been determined. *E. coli* FadD$^-$ strains display reduced acyl-CoA synthase activity and reduced fatty acid transport rates and consequently are unable to incorporate exogenous fatty acids into membrane phospholipids (36). Additional mutants partially defective in acyl-CoA synthase activity also display altered acyltransferase activities and altered relative rates of acylation of the glycerolphosphate acyltransferase pathway, the system governing the fatty acid composition of *E. coli* membrane phospholipids (20). In order to determine if ORF3 plays a similar role in mycobacterial lipid metabolism, targeted disruption of the ORF3 gene is currently under way.

Acyl-CoA synthases from different species are immunologically cross-reactive. ORF3-specific antibodies cross-reacted with a single protein in both *M. bovis* BCG and *E. coli* extracts and with two proteins in *M. smegmatis* extracts. The presence of a single cross-reacting band in *E. coli* is in agreement with studies which indicate that this organism contains a single form of acyl-CoA synthase, although the molecular weight of the cross-reacting protein is slightly greater than that reported previously (3). Anti-ORF3 protein antibodies inhibit acyl-CoA

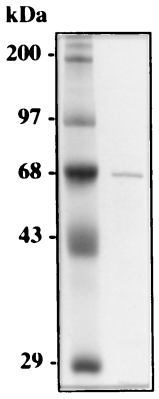

FIG. 7. Coomassie blue-stained SDS-polyacrylamide gel of the acyl-CoA synthase protein purified from *M. bovis* BCG.

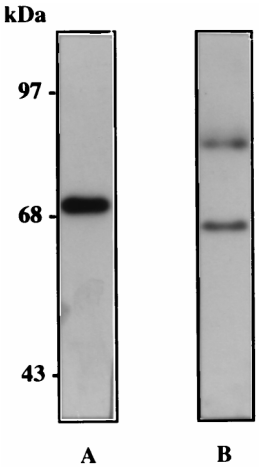

FIG. 8. Immunoblot analysis of ORF3 expression in *E. coli* (A) and *M. smegmatis* (B). Rabbit antiserum prepared against the expressed ORF3 of *M. bovis* BCG was used as the primary antibody and ^{125}I-labeled protein A was used for detection.

synthase activities in a similar manner in both *M. bovis* BCG and *E. coli* crude extracts. These observations suggest that the acyl-CoA synthase against which the antibodies were prepared is the major acyl-CoA synthase in both of these organisms. If other enzymes contribute to the observed acyl-CoA synthase activity, they are immunologically similar to the acyl-CoA synthase present in the extract. The smaller of the two cross-reacting proteins in *M. smegmatis* is identical in size to that found in *M. bovis* BCG, and may represent an authentic acyl-CoA synthase enzyme. The identity of the larger protein is not known; it is possible that *M. smegmatis* may contain more than one form of acyl-CoA synthase. Interestingly, antibodies against the ORF3 protein inhibit acyl-CoA synthase activity to a much lesser extent in *M. smegmatis* extracts than in extracts of *M. bovis* BCG and *E. coli*. This observation may indicate that a second enzyme with fatty acyl-CoA-forming capabilities is functional in *M. smegmatis*. In the hydrocarbon-utilizing yeast *Candida lipolytica*, two acyl-CoA synthase genes are present, acyl-CoA synthase I, which is responsible for the formation of fatty acyl-CoA destined to be used exclusively in chain elongation, and acyl-CoA synthase II, which provides acyl-CoA solely to the β-oxidation pathway (25). Multiple acyl-CoA synthase enzymes have also been detected in *Rhodopseudomonas palustris*, *Saccharomyces cerevisiae*, and *Petroselinum crispum* (14, 23, 29).

The genes encoding MAS and ORF3 in *M. bovis* BCG (33), *M. tuberculosis* (cosmid Y338), and *M. leprae* (cosmid B1170) are in close proximity. ORFs homologous to ORF3 are also present 5′ to the polyketide synthase gene and 3′ to a *mas*-like gene (cosmids Y338, tbc2, and SCY19G5) in *M. tuberculosis*. Given the high degree of similarity between these ORF3-like genes, it seems probable that their gene products would catalyze biosynthetic processes involving acyl transfer. Furthermore, the multiplicity and relative location of these genes on the mycobacterial genome lead to the speculation that their gene products may selectively transfer the acyl-containing products of the neighboring synthase genes either directly to CoA or to alternative biological acceptors. Purified MAS fails to release mycocerosic acids (35), and mycocerosic acids are not found free in the cell cytosol but instead are esterified selectively to the phthiocerols and phenolphthiocerols on the cell wall (8, 47), suggesting the involvement of a separate transfer enzyme. Immunogold labeling experiments indicate that MAS is associated with the cell membrane (1b), and results from the present study also suggest that the ORF3 protein may be loosely bound to the membrane. Because of the demonstrated acyl transfer capabilities of ORF3 and its cellular location in the vicinity of MAS, we tested for the involvement of ORF3 in both mycocerosic acid activation and mycocerosic acid release and transfer to the phthiocerols. However, direct activation of mycocerosic acids to their corresponding thioesters occurred only at extremely low levels. In addition, the ORF3 protein failed to stimulate MAS activity and did not cause measurable release of mycocerosic acids either in the free form or as phenolphthiocerol derivatives when phenolphthiocerol or glycosylated phenolphthiocerol was included in the reaction mixture (data not shown). Therefore, at least under the conditions employed in this study, the ORF3 protein does not appear to play a direct role in mycocerosic acid metabolism. Recent studies suggest that in *E. coli* the acyl-CoA synthase and the acyltransferase exist in transient membrane complexes which allow direct transfer of acyl groups from enzyme-bound fatty acyl-CoA to the transferase (20). The possibility exists that factors as yet unidentified may also be required for the involvement of ORF3 in the release of mycocerosic acids from MAS and their subsequent transfer to the phthiocerols.

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