An open reading frame, ORF3, first identified adjacent to the mycocerosic acid synthase gene in *Mycobacterium bovis* BCG encodes a protein with acyl-CoA synthase (ACoAS) activity. Genes homologous to *acoas* are found adjacent to other multifunctional polyketide synthase genes in the mycobacterial genome. To test whether these gene products are necessary to esterify the fatty acids generated by the adjacent polyketide synthase gene products, the *acoas* gene was disrupted in *M. bovis* BCG using a suicide vector containing the *acoas* gene with an internal deletion and the hygromycin-resistant gene as selection marker. Allelic exchange at the *acoas* locus was confirmed by Southern hybridization and polymerase chain reaction amplification of both flanking regions expected from homologous recombination. Immunoblot analysis indicated that the 65-kDa ACoAS protein product was absent in the mutant. Chromatographic analysis of lipids derived from [1,14C]propionate showed that the mutant did not produce mycocerosyl lipids, although it produced normal levels of mycocerosic acid synthase. These results suggest that ACoAS is involved in the synthesis of mycocerosyl lipids of the mycobacterial cell wall.

Tuberculosis is the leading cause of death from a single infectious agent, accounting for approximately 26% of all preventable adult deaths in the world. It is estimated that approximately 0.6 billion people are infected with the causative agent, *Mycobacterium tuberculosis*, with 8–10 million new cases and 3 million deaths occurring annually (1). Mycobacterial cell walls have a very high lipid content (50–60%), which constitutes an effective permeability barrier to antimycobacterial therapies and contributes to the survival of this pathogen within the host (2). In addition, conventional antmycobacterial treatments largely directed against cell wall lipids unique to pathogenic mycobacteria have been rendered less effective because of the increasing incidence of multidrug resistance. The increased incidence of tuberculosis associated with the AIDS pandemic has provided an added impetus to identify alternative targets that would allow for the development of novel therapeutic drugs (3, 4).

Other classes of cell wall lipids found solely in pathogenic mycobacteria include the phenolphthiocerols (mycosides) and the phthiocerols (5, 6) both of which have been reported to play a key role in the host-pathogen interaction (7, 8). In each of these lipid classes, multimethyl-branched long chain fatty acids, known as mycocerosic acids, are esterified to two long chain diols, the phenolphthiocerols and the phthiocerols, respectively (5, 8). This laboratory has previously cloned and characterized the mycocerosic acid synthase gene (mas) (9) and has identified a gene cluster involved in phthiocerol and phenolphthiocerol synthesis (pps) in *Mycobacterium bovis* BCG (10). In addition, we recently reported that a small open reading frame, ORF3, is located at the 5′ end of the mas gene; amino acid sequence homology and enzyme assays using purified ORF3 protein indicated that this open reading frame encoded an ACoAS (11).

Genes homologous to *acoas* have been identified adjacent to the mas gene in *M. tuberculosis* (GenBank™ accession number Z83858) and *Mycobacterium leprae* (GenBank™ accession number U00010), as well as 5′ to a polyketide synthase gene and 3′ to a mas-like gene in *M. tuberculosis* (GenBank™ accession numbers Z74697, U00024, and Z77826). Given the high degree of homology among these genes (54–99% identity; 69–99% similarity), it seems likely that all these genes play a role in acyl transfer. The multiplicity and relative location of these genes adjacent to large polyketide synthase-like enzymes lead us to speculate that their gene products may be involved in the selective transfer of the acyl products of the neighboring synthase genes either directly or indirectly to the ultimate biological acceptors (11). Purified MAS fails to release mycocerosic acids, and mycocerosic acids are not found free in the cytosol but instead are esterified to the diols, which suggests the involvement of a separate transferase enzyme (12). A possible interaction of the mas and *acoas* gene products may be facilitated by their co-localization on the cell wall; immunogold labeling experiments indicate that MAS is associated with the cell membrane (13) and recent results also suggest that the ACoAS protein may be loosely bound to the membrane (11). However, in vitro studies indicated that purified ACoAS protein catalyzed the activation of mycocerosic acids to their corresponding thiosteres only at extremely low rates. Furthermore, ACoAS failed to stimulate MAS activity and did not cause measurable release of mycocerosic acids either in the free form or as phthiocerol derivatives when phenolphthiocerol or glycosylated phenolphthiocerol was provided as acceptor (11). Although such in vitro reconstitution experiments failed to reveal the function of ACoAS, additional factors may be necessary for this protein to function in the transfer of mycocerosic acids to the diol acceptors.

1 The abbreviations used are: ACoAS, acyl-CoA synthase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; kb, kilobase(s); MAS, mycocerosic acid synthase.
The recent development and application of targeted gene disruption technology to slow growing mycobacterial species (10, 14–16) allowed us to directly test the in vivo function of ACoAS in M. bovis BCG. In this paper, we report the targeted disruption of the acoas gene in M. bovis BCG and show that this mutant is incapable of producing mycocerosyl lipids, although it has normal levels of mycocerosic acid synthase activity. These results suggest that acoas-like genes function in the transfer of acyl groups, generated by the products of adjacent polyketide synthase genes, to their ultimate biological acceptors.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—Strain descriptions and cultivation conditions for all the mycobacterial and Escherichia coli strains used in this study have been presented previously (11). Generation of an acoas Gene Disruption Construct—A pUC19-based plasmid, D2, which had previously been subcloned from a MAS-containing M. tuberculosis BCG cosmid (10), contained the entire 1.749-kb coding region of the acoas gene, 0.8-kb 5′-flanking region and 0.4-kb 3′-flanking region. A 181-base pair BglII fragment internal to the acoas gene was deleted and replaced with the hygromycin gene (hyg) from S. hygroscopicus. The hyg gene was digested and the MAS-containing fragments were identified by slot-blot analysis using the MAS specific antibody and the Supersignal ULTRA chemiluminescence detection system according to the manufacturer’s recommendations (Pierce). MAS-containing fractions were pooled and concentrated by ultrafiltration using a PM30 membrane (Amicon, MA). MAS activity was quantified using labeled methylmalonyl-CoA as described previously (12), and activity is expressed as picomoles of methylmalonyl-CoA incorporated into hexane-extractable material per minute per milligram of protein. ACoAS activity was determined by quantifying the amount of fatty acyl-CoA in the aqueous phase following extraction of the reaction mixtures with organic solvents as described (21). ACoAS 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 minute. Protein concentrations were determined by the Bradford method (22) with bovine serum albumin as standard.

**RESULTS**

Disruption of the acoas Gene by Allelic Exchange—To examine the role of ACoAS in cell wall lipid metabolism, disruption of the acoas gene in M. bovis BCG was undertaken by allelic exchange. A pUC-based suicide plasmid, pORF3C35, was constructed containing the entire 1.749-kb acoas-coding region, 0.8-kb 5′-flanking region and 0.4-kb 3′-flanking region. An internal 181-base pair BglII fragment of acoas was replaced with a 1.8-kb hygromycin gene (hyg) as the selectable marker. Hygromycin-resistant transformants, generated with the linearized plasmid, were screened by PCR using a set of primers flanking the deleted internal segment of acoas (Fig. 1, primers A and B). This primer pair generates a single 0.7-kb PCR product from the wild type strain, 0.7-kb and 2.4-kb products in a mutant generated from a single crossover homologous recombination event, and a single 2.4-kb product in a mutant generated from double crossover homologous recombination. Analysis of approximately 500 hygromycin-resistant transformant colonies using this PCR screening strategy identified four mutants with single crossover events and two mutants with double crossover events. PCR amplification of representative single and double crossover mutants (RF3147 and RF320, respectively) using primers A and B is depicted in Fig. 2.

Disruption of acoas by allelic exchange was confirmed by further PCR analysis using two other sets of primers, each containing a hyg primer and a primer in the mycobacterial genome directly outside the acoas-flanking segments used to make the disruption construct (Fig. 1, primer pairs C and H1, and D and H2). In mutant RF320, these primer pairs generated 2.3- and 1.2-kb PCR products, respectively, sizes consistent with homologous recombination by double crossover, whereas in mutant RF3147 only the larger PCR product was obtained.
Fig. 1. Schematic organization of the construct used to disrupt the M. bovis BCG acoas gene by allelic exchange. Hatched, stippled, checkered, and unshaded boxes represent acoas coding sequences, acoas-flanking sequences, acoas internal deleted region, and regions on the mycobacterial genome outside those used to make the disruption construct, respectively. Black boxes represent the hyg gene, which was used as both a disruption element and selection marker. Relevant sites are indicated as follows: Bg-Bgl II, B-Bam HI, and S-Sph I. Primer pairs A/B, C/H1, D/H2 and E/F were used for PCR analysis of homologous recombination as described in the text. P1-P5 denote acoas segments used as probes in hybridization experiments with PCR products (Figs. 2–4) and genomic DNA (Fig. 5).

indicating that integration of the disruption construct occurred at the 5’ end of the acoas gene (Fig. 3A). To confirm that the PCR products generated from either side of the hyg gene represent genuine acoas-flanking segments, regions representing the 5’ and 3’ ends of acoas were used to probe these PCR products. As anticipated, the 5’ probe hybridized only with the 2.3-kb PCR product and the 3’ probe hybridized only with the 1.2-kb product (Fig. 3, B and C). Furthermore, primers specific for the internal deleted segment of acoas (Fig. 1, primers E and F) failed to amplify this region in mutant RF320, whereas a 167-base pair fragment was present in both the wild type and mutant RF3147 (Fig. 4A). The identities of these PCR products were confirmed by hybridization with the internal Bg/HI fragment deleted from acoas (Fig. 4B).

Southern hybridization analysis confirmed that replacement of the acoas gene had occurred by allelic exchange in mutant RF320. When genomic DNA from the wild type and mutant strains was digested with BamHI and SphI, which results in excision of the hygromycin element from RF320, the wild type produced a single hybridization band at approximately 3 kb; RF3147 yielded three hybridization bands at 3, 2.2, and 0.8 kb; and RF320 produced two bands at 2.2 and 0.8 kb. This result is consistent with integration of the hyg-disrupted acoas gene and replacement of the wild type allele in mutant RF320 (Fig. 5).

Immunological and Biochemical Characterization of the acoas Gene-disrupted Mutant—When total proteins from the wild type and mutant RF320 were separated by SDS-PAGE and subjected to immunoblot analysis using an ACoAS specific antibody, the wild type showed a specific cross-reacting band of approximately 65 kDa, whereas this hybridizing band was not visible in the acoas-disrupted mutant (Fig. 6). This mutant expressed ACoAS activity at approximately 20% of wild type levels (0.17 units/mg protein versus 0.85 units/mg protein in the wild type) when 14C-labeled palmitic acid was used as the substrate. Therefore disruption of the acoas gene in mutant RF320 results in a failure to express a functional 65-kDa protein that encodes acyl-CoA synthase activity.

To examine the effect of acoas gene disruption on the synthesis of mycoseryl lipids in M. bovis BCG, incorporation of [1-14C]propionate into total lipids was examined in both the wild type and acoas-disrupted mutant. After 18 h of incubation, approximately 18 and 6% of the total administered 14C was incorporated into total lipids by the wild type and acoas-disrupted mutant, respectively. Thin-layer chromatography identified two major labeled lipid fractions in the wild type that corresponded to phenolphthiocerol esters (mycosides) and phthiocerol esters. However, in the acoas-disrupted mutant only the phthiocerol ester fraction was present (Fig. 7, left). Charring of chromatograms showed that only phthiocerol esters but not mycosides were present in the acoas-disrupted mutant, whereas the wild type contained both (Fig. 7, right). Approximately equal amounts of label were present in the mycosides and phthiocerol esters in the wild type (approximately 46 and 48%, respectively), whereas in the mutant the bulk of the label (approximately 75%) was in the phthiocerol esters. Following base hydrolysis, the majority of the label in
both strains (in excess of 80%) was found in the acyl portion of the lipids. Radio-gas chromatographic analysis of the methyl esters from the mycosides in the wild type indicated that 14C-labeled propionate was predominantly incorporated into C29 and C32 mycocerosic acids, whereas this fraction was missing from the acoas-disrupted mutant. The phthiocerol ester fraction of the wild type also contained significant quantities of the C29 and C32 mycocerosic acids, whereas only shorter chain fatty acids were present in the corresponding fraction from the acoas-disrupted mutant (Fig. 8). Thus, mycocerosic acids generated by MAS were not found in the lipids of the acoas-disrupted mutant.

To investigate whether the inability of the ACoAS disruptant to produce mycosides is a consequence of some type of interference in the expression of the mas gene, production of the MAS protein was monitored in both the wild type and the ACoAS-disrupted mutant strains. SDS-PAGE and immunoblot analysis...
sis indicated that a similar level of MAS protein was produced in both the wild type and the ACoAS-disrupted mutant (Fig. 9). In addition, MAS protein, partially purified by DEAE-Sepharose fractionation from the ACoAS disruptant, incorporated radiolabeled methylmalonyl-CoA into mycocerosic acids at a level similar to that observed with the wild type strain (approximately 34 pmol of methylmalonyl-CoA incorporated per min per mg protein) and radio-gas chromatographic analysis of the fatty acid methyl esters generated by both the wild type and mutant strains were identical (Fig. 10).

**DISCUSSION**

The process by which pathogenic mycobacteria transfer multimethyl-branched mycocerosic acids onto the diol acceptors on the cell wall is poorly understood. Previous results indicate that the *M. bovis* BCG mas gene lacks a chain-terminating thioesterase domain (9) and that mycocerosic acids remain attached to the synthase (12). Mycocerosic acids are found exclusively as esters of phenolphthiocerol and phthiocerol (7, 8), suggesting the involvement of a separate transferase system that mediates direct transfer of the newly synthesized mycocerosic acids from the synthase to the diol acceptors. Recently, we identified a small open reading frame at the 5' end of the mas gene, ORF3, which displays distinct homology to a number of ACoAS-like enzymes and purified ORF3 protein has ACoAS activity (11). However, we could not demonstrate the involvement of ACoAS in the *in vitro* activation of mycocerosic acids, mycocerosic acid release from MAS, or transfer of mycocerosyl groups from MAS to the diols (11). Therefore, we resorted to the *in vivo* disruption of acoas to determine the possible role played by this gene in mycocerosyl lipid synthesis.

The recent successful application of allelic exchange technology to slow-growing mycobacterial species made such an approach feasible. To date, reciprocal recombination has been achieved using the mas gene (16), the pps gene cluster (10), and the ureC gene (14) in *M. bovis* BCG, and the leuD gene in *M. tuberculosis* (15). In the present study, the following evidence clearly showed that the *M. bovis* BCG acoas gene was disrupted by homologous recombination. Mutant RF320 lacks the internal segment deleted from the acoas gene, contains the hyg gene, and PCR amplification using a combination of hyg and flanking
primers show that the disrupted allele has integrated into the correct region on the mycobacterial genome. Furthermore, the mutant fails to produce the 65-kDa protein encoded by \textit{acoas} and has a drastically decreased ACoAS activity.

Disruption of the \textit{acoas} gene in \textit{M. bovis} BCG results in the production of a mutant that lacks the ability to produce mycosides and mycocerosyl phthiocerol esters. Radiolabeling studies with [1-\textsuperscript{14}C]\textit{propionate}, used to specifically label mycocerosic acids, show that even though mycocerosylated phenolphthiocerol esters are abundant in the wild type, this fraction is absent in the \textit{acoas} disruptant. In addition, an examination of charred chromatograms confirms the total absence of mycoside production in the mutant. Radio-gas chromatography indicates that mycocerosic acids are significant components of the phthiocerol wax esters in the wild type strain. However, in the ACoAS disruptant mycocerosyl phthiocerols were not present; only shorter chain fatty acids were esterified to the phthiocerols.

Studies using the \textit{acoas} mutant clearly show that this strain produces mycocerosic acid synthase. Partial purification of MAS by anion-exchange chromatography from the wild type and mutant strains yielded similar elution profiles. The purified proteins from both strains were discernible at similar levels following SDS-PAGE and immunoblot analysis, displayed comparable specific activities, and generated similar relative proportions of radiolabeled products from methylmalonyl-CoA. Therefore, it appears that while mycocerosic acid synthase is produced at wild type levels in the mutant, mycocerosic acids are not esterified onto the diol acceptors on the cell wall. This finding directly implicates ACoAS in the process by which mycocerosic acids are incorporated onto the diol acceptors in the cell wall of \textit{M. bovis} BCG. With mandatory coupling of the synthesis and transfer, MAS would be unable to produce mycocerosic acids in the absence of a functional transfer mechanism. Alternatively, it is also possible that disruption of \textit{acoas} affected the supply of the acyl-CoA primers required for the elongation of methylmalonyl-CoA by MAS and thus prevented mycocerosyl lipid synthesis. We consider this an unlikely possibility because highly homologous \textit{acoas} genes are present near other polyketide synthase-like genes in the mycobacterial genome and these \textit{acoas} genes are likely to encode acyl-CoA synthases that would have substituted for the disrupted one unless there is strict substrate specificity. Considering the composition of the long chain methyl-branched fatty acids present in the mycobacteria, \textit{C\textsubscript{16}}, \textit{C\textsubscript{18}}, and \textit{C\textsubscript{20}} primers would be adequate for the synthesis of all of these acids. If the acyl-CoA synthases encoded by the multiple \textit{acoas} genes produce any of these primers, mycocerosic acids or their homologues would have been produced by the mutant as MAS is known to be able to use any of these primers (12). On the other hand it would appear more likely that the \textit{acoas} products are specific for the transfer of the elongated acids generated by the synthases encoded by the gene adjacent to each \textit{acoas} in the genome. Such a specificity may not only depend on the nature of the acyl chain but also on the interaction of this enzyme with a membrane bound partner that is probably involved in the acyl transfer to the final acceptor.

The observed lack of mycocerosyl lipids in the \textit{acoas} disruptant is consistent with the participation of ACoAS in mycocerosyl transfer to the diols. Previous studies have shown that only mycocerosic acids are esterified to phenolphthiocerol and

![Fig. 10. Radio gas-liquid chromatograms of methyl esters synthesized from [methyl-\textsuperscript{14}C] methylmalonyl-CoA by MAS protein partially purified by DEAE-Sepharose fractionation from \textit{M. bovis} BCG wild type (A) and ACoAS disruptant RF320 (B). Enzyme assays were performed as described previously (12) using \textit{n-C\textsubscript{20}}-CoA primer.](http://www.jbc.org/)

that shorter chain fatty acids cannot substitute in the acylation steps; a mas mutant that lacks the ability to synthesize mycocerosic acids also fails to generate any mycosides or mycocerosic acid-containing phthiocerols (16). Recent studies revealed that unlike MAS, a purified synthase that produces short chain branched acids releases its products directly as free fatty acids (23), which can then be esterified directly to the acceptors without requiring a separate ACoAS transferase system. Therefore, in the absence of a coupling of shorter chain branched fatty acid synthase and ACoAS transferase activities, disruption of ACoAS still permits synthesis of shorter chain fatty acids and their subsequent incorporation into the phthiocerol esters.

A possible explanation for the inability of ACoAS to cause mycocerosic acid transfer from MAS to the diols in vitro may be that this enzyme alone is not sufficient to effect transfer. In E. coli, ACoAS forms a transient membrane complex with a separate acyltransferase to promote the direct transfer of acyl moieties from enzyme bound fatty acyl-CoA to the transferase (24). At the C terminus of the mas gene, a short open reading frame has been identified which shares homology with polyketide synthase-type condensation enzymes that are involved in ester and amide bond synthesis (9, 25). This enzyme may function with ACoAS in transferring mycocerosic acids from MAS to the diols without involving release of free acids and activation of such released acids as discrete separate steps.

Genes displaying homology to the M. bovis BCG acoas gene have been identified adjacent to mas (GenBankTM accession number Z83858), mas-like genes (GenBankTM accession numbers Z77826 and Z97188) and polyketide synthase-like genes (GenBankTM accession numbers U00024, Z74697, and Z84725) in the M. tuberculosis genome. An acoas homologue is also present on the M. leprae genome adjacent to mas (GenBankTM accession number U00010). Overall, acoas-like genes located adjacent to polyketide synthases display 45–99% identity. Despite this high degree of conservation, acoas-like genes do not appear to be functionally interchangeable. We succeeded in creating a stable M. bovis BCG mutant with an altered pattern of lipid metabolism even though the other acoas-like genes were presumably intact in this mutant. The presence of additional acoas-like genes in the vicinity of large multifunctional polyketide synthases further supports our suggestion that the product of each acoas-like gene functions specifically with the products of the neighboring synthase gene to directly transfer their products to the appropriate acceptors on the mycobacterial cell wall.

Termination of a biosynthetic process, catalyzed by the product of a multifunctional synthase gene, by a small protein encoded by an adjacent open reading frame appears to be a commonly used mechanism not only in the synthesis and assembly of complex polyketides but also in the synthesis of other natural products. A coupling of multifunctional synthase and transferase activities is believed to occur during gramicidin biosynthesis; a thioesterase-like domain, located at the 5’ end of the gramicidin synthase gene and displaying homology to fatty acid thioesterases, has been suggested to function as a transacylase in the chain termination step (26, 27). Similar mechanisms also appear to be involved in the synthesis of nonribosomal peptide synthases such as δ-(1-α-aminoadipyl)-l-cysteinyl-d-valine synthase, which is involved in penicillin and cephalosporin biosynthesis (28, 29) and the bialaphos antibiotic synthesizing gene cluster (30). In mycobacteria each acoas-like gene, located adjacent to each of the many multifunctional polyketide synthases, probably functions at the terminal step to channel the product of the synthase to the appropriate acceptor in the cell wall as demonstrated in this paper for mycocerosyl lipid synthesis.

Acknowledgments—We thank Linda Rogers and Norvin Fernandes for assistance with radio-gas chromatographic analysis.

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An Acyl-CoA Synthase (acoas) Gene Adjacent to the Mycocerosic Acid Synthase (mas) Locus Is Necessary for Mycocerosyl Lipid Synthesis in Mycobacterium tuberculosis var. bovis BCG

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J. Biol. Chem. 1998, 273:8033-8039.
doi: 10.1074/jbc.273.14.8033

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