Targeted replacement of the mycocerosic acid synthase gene in *Mycobacterium bovis* BCG produces a mutant that lacks mycosides

(homologous recombination/gene replacement/tuberculosis)

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ABSTRACT A single gene (*mas*) encodes the multifunctional enzyme that catalyzes the synthesis of very long chain multiple methyl branched fatty acids called mycocerosic acids that are present only in slow-growing pathogenic mycobacteria and are thought to be important for pathogenesis. To achieve a targeted disruption of *mas*, an internal 2-kb segment of this gene was replaced with approximately the same size hygromycin-resistance gene (*hyg*), such that *hyg* was flanked by 4.7- and 1.4-kb segments of *mas*. Transformation of *Mycobacterium bovis* BCG with this construct in a plasmid that cannot replicate in mycobacteria yielded hygromycin-resistant transformants. Screening of 38 such transformants by PCR revealed several transformants representing homologous recombination with single crossover and one with double crossover. With primers representing the *hyg* termini and those representing the mycobacterial genome segments outside that used to make the transformation construct, the double-crossover mutant yielded PCR products expected from either side of *hyg*. Gene replacement was further confirmed by the absence of the vector and the 2-kb segment of *mas* replaced by *hyg* from the genome of the mutant. Thin-layer and radio-gas chromatographic analyses of the lipids derived from [1^{14}C]propionate showed that the mutant was incapable of synthesizing mycocerosic acids and mycosides. Thus, homologous recombination with double crossover was achieved in a slow-growing mycobacterium with an intron-containing *RecA*. The resulting *mas*-disrupted mutant should allow testing of the postulated roles of mycosides in pathogenesis.

Tuberculosis, caused by *Mycobacterium tuberculosis*, afflicts over 30 million people throughout the world, resulting in 3 million deaths per year (1). The increasing incidence of tuberculosis in patients with AIDS and the emergence of multidrug-resistant strains have further emphasized the need for more efficient control of this infectious disease (2–4). The unique cell-wall components of mycobacteria are thought to play a major role in their ability to resist the host defense mechanisms and multiply in the host (5–8). Inhibition of synthesis of one of the unique wall lipids (mycolic acids) has been a major part of a long-standing strategy for antimycobacterial therapy (9, 10). With the development of resistance against such drugs, other targets need to be identified (11). Biosynthesis of phenolic glycolipids (mycosides) that are found uniquely in pathogenic mycobacteria (12–14) could offer such targets. The major phenolic glycolipids are composed of glycosylated phenol-phthiocerol diesters of multiple methyl branched very long chain fatty acids called mycocerosic acids (15–17). A multifunctional enzyme that catalyzes the synthesis of such fatty acids has been purified (18), and the gene that encodes this mycocerosic acid synthase has been cloned (19).

Knock out of this gene could open up an avenue to directly test whether this group of unique lipids plays an essential role in pathogenesis and, thus, lead to novel therapeutic strategies against mycobacterial infections.

Gene replacement by homologous recombination provides a powerful means to create a null mutation in a specific gene and to study gene function(s). Although gene replacement was demonstrated in the fast-growing nonpathogenic mycobacterial species, *Mycobacterium smegmatis* (20, 21), such replacement has not been achieved in slow-growing mycobacteria such as *M. tuberculosis* and *Mycobacterium bovis* BCG. Attempts with these species have resulted either in apparent random integration of the transformed DNA by nonhomologous recombination (22) or integration of the DNA by homologous recombination only by single crossover, without any detectable allelic replacement by double crossover (23). Recently, a gene replacement in *M. bovis* BCG has been demonstrated (24), but the use of a replicating vector for delivery of the altered gene resulted in reciprocal and reversible recombination yielding a mixture of the recombinant and wild-type cells from the same clone. Here we report a mycocerosic acid synthase gene knock out in *M. bovis* BCG, achieved by homologous recombination with double crossover, resulting in a mutant that is demonstrated to be incapable of synthesizing mycosides because of its inability to synthesize mycocerosic acids.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. *Escherichia coli* DH5 α (Life Technologies, Gaithersburg, MD) was used for cloning and propagation of plasmids. For selection of transformants, *E. coli* was grown in Luria–Bertani broth or on Luria–Bertani agar containing 100 μg of ampicillin (Sigma) and/or 200 μg of hygromycin B (Calbiochem) per ml. *M. bovis* BCG was a Tice Bacillus Calmette–Guérin vaccine strain of *M. tuberculosis* var. *bovis* (Organon Teknika–Cappel). BCG cultures were grown in Middlebrook 7H9 medium (Difco) supplemented with albumin–dextrose complex (ADC; Difco) and 0.05% Tween 80 (M-ADC-TW broth) in 850-cm plastic roller bottles at 3 rpm at 37°C in a Roll-in rolling incubator (Bellco Glass). Mycobacteria 7H11 (Difco), supplemented with oleic acid–ADC (OADC; Difco), was used as an agar medium. Hygromycin B was used at a concentration of 50 $\mu\text{g}/\text{ml}$ for BCG transformants. BCG cultures were grown for 2–3 weeks in broth and for 3–4 weeks on agar medium.

Construction of a Plasmid for *mas* Gene Disruption. A cosmid containing the entire *mas* gene was identified by screening a genomic library of *M. bovis* BCG (provided by William Jacobs, Albert Einstein College of Medicine) with the *mas* gene segments, representing the catalytic domains of

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Abbreviation: ADC, albumin–dextrose complex.

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mycocerosic acid synthase (19), as probes. An 8.1-kb *Bst*BI fragment, containing the *mas* gene and its flanking regions, was isolated from the cosmid, made blunt-ended by filling-in reaction with Klenow polymerase, and cloned into the unique *Sma*I site of pUC19 (Life Technologies) to produce pATP9. A 2-kb internal fragment of the *mas*-coding region was deleted by digestion of pATP9 with *Stu*I and replaced with a 1.8-kb *hyg* gene from *Streptomyces hygroscopicus* (25), which has been demonstrated to be a better selectable marker than kanamycin for mycobacterial species (26). Plasmid pIJ963 (a gift from David Hopwood, John Innes Centre, Norwich, U.K.) was used as a source of the *hyg* cassette that was excised from the plasmid as a 1.8-kb *Bgl*II–*Bam*HI fragment, blunt-ended with Klenow enzyme, and ligated into the *Stu*I sites of the pUC19–*mas* gene backbone of pATP9 to generate the disruption construct pATP10. Following subcloning at each step, the structure of the construct was examined by restriction enzyme analysis, Southern hybridization, and sequencing of the ligation junctions. All DNA-manipulating enzymes used were purchased either from Life Technologies, New England Biolabs, or Amersham.

Electroporation of *M. bovis* BCG. BCG cultures were grown in M-ADC-TW broth in roller bottles to an OD₆₀₀ of 0.8–1.0 (7–9 days), and competent cells were made as described (27), except that the cells were exposed to 1.5% glycine for 24 h before harvest. A 0.1 ml aliquot of BCG cell suspension from the frozen stock was mixed with 2–3 μ g of plasmid DNA, the mixture was incubated on ice for 10 min and subjected to electroporation in a 0.2-cm cuvette with a BTX Electro Cell Manipulator (BTX, San Diego) at a setting of 2.5 kV, capacitance to 25 μ F, and pulse controller to 720 Ω . The cells were then placed on ice for 10 min, resuspended in 0.5 ml M-ADC-TW broth, transferred to a 15-ml conical polypropylene tube, and incubated overnight with shaking at 37°C. The transformed cultures were spread on three 7H11 agar plates containing hygromycin B (50 μ g/ml) and cycloheximide (Sigma; 10 μ g/ml) to prevent fungal contamination. Seeded plates were sealed with parafilm to prevent drying and incubated at 37°C for 3–4 weeks.

Genomic DNA Isolation and Southern Blot Analysis. Genomic DNA from BCG cultures was extracted as described (27) by disrupting the cells with 425- to 600- μ m glass beads (Sigma) in a Mini-Beadbeater Cell Disruptor (Biospec Products, Bartlesville, OK). DNA samples were digested with restriction enzymes and fragments were separated on 0.8% agarose gels and transferred to nylon membrane (Nytran Plus; Schleicher & Schuell) followed by baking at 80°C for 2 h. Prehybridization, hybridization, and washing of the blot under high stringency conditions were carried out according to standard procedures (28). The DNA used as a probe was labeled with [α -³²P]dCTP by using Rediprime Random Primer Labeling Kit (Amersham) according to the manufacturer's instructions.

PCR Experiments. For screening of BCG transformants, PCR experiments were performed directly on the lysate obtained by boiling the BCG cells. The Expand Long Template PCR System (Boehringer Mannheim) was employed to amplify a 4.8-kb fragment (4.74-kb segment of *mas* plus primers), using the supplier's buffer 1 and 2% dimethyl sulfoxide with the following primers: 5'-GGAATCCCGTCGGTATCCG-CAAAGTAGCCAG-3' (*B1*) and 5'-GGACCTCGACGACCTGCAGGCATGCAAGC-3' (*H1*). PCR conditions were as follows: 2 min at 94°C, then 10 cycles of 30 sec at 94°C, 30 sec at 65°C, 4 min at 68°C, followed by 20 cycles of 30 sec at 94°C, 30 sec at 65°C, 4 min plus a linear increase of 20 sec for each successive cycle at 68°C, ending with 7 min at 68°C. Standard PCR was done to amplify 1.4-kb fragment by using *Taq* polymerase (Boehringer Mannheim) with 10% dimethyl sulfoxide and the following primers: 5'-CGGCCGGGCTTC-CATCCAGCTCGG-3' (*H2*) and 5'-GATTCAGCAGTC-

GGGGCTCCACTTC-3' (*B2*). The PCR conditions were as follows: 5 min at 94°C, 2 min at 60°C, 2 min at 72°C, followed by 29 cycles of 1 min at 94°C, 1 min at 60°C, 1 min at 72°C, ending with 10 min at 72°C. Both types of PCR were performed in a PTC-100 Programmable Thermal Controller (MJ Research, Watertown, MA).

Biochemical Analysis of Cell-Wall Lipids in Wild-Type and Mutant BCG Strains. To 90 ml of the 15-day-old culture of *M. bovis* BCG and its *mas*-disrupted mutant (BM-18) in 850 cm roller bottles, 49 μ Ci of Na[1-¹⁴C]propionate (specific activity, 55 Ci/mol; 1 Ci = 37 GBq; American Radiolabeled Chemicals, St. Louis) was added and incubation at 37°C continued at 3 rpm for 16 h. The cells collected by centrifugation at 17,000 g for 20 min were extracted with an excess of a 2:1 mixture of chloroform and methanol at room temperature. The total lipids were recovered by the Folch method (29), and aliquots were assayed for ¹⁴C. The total lipid was subjected to thin-layer chromatography on 20 $\times$ 20 cm 0.5-mm layers of silica gel G with 5% (wt/vol) methanol in chloroform as the solvent. For detection of lipids the chromatograms were sprayed with 5% K₂Cr₂O₇ in 50% sulfuric acid and heated for 10 min at 110°C. For analysis of the labeled lipids, the chromatograms were scanned for ¹⁴C by Berthold Tracemaster 20 TLC-linear analyzer, the silica gel from the areas of the labeled components, corresponding to phthiocerol and phenol-phthiocerol esters (30, 31), was scraped off and lipids were recovered by elution with ethyl ether. These ester fractions were refluxed with 5% KOH in 2-methoxyethanol containing 12% water for 3 h (32). The mixture was diluted with water acidified with HCl and extracted with chloroform; the solvent was then evaporated under reduced pressure. The residue was refluxed with 14% BF₃ in methanol for 1 h. The products, recovered by extraction with chloroform after dilution with water, were subjected to thin-layer chromatography on silica gel G plates with hexane/ethyl ether/formic acid (40:10:1, wt/vol) as the solvent. After scanning the plates for ¹⁴C, the labeled components were recovered and the methyl ester fractions that contained most of the ¹⁴C were subjected to radio gas-liquid chromatography on 3.2 mm $\times$ 2 m 3% OV-101 column with Varian model 3300 gas chromatograph attached to a Lablogic GC-RAM radioactivity monitor (INUS Systems, Tampa, FL) with a column temperature of 180–300°C, programmed at 20°C per min followed by a 6-min isothermal period with a carrier gas (He) flow of 30 ml/min. The labeled mycocerosic acids were identified as before (18). ¹⁴C in aliquots were assayed with a Beckman LS3801 liquid scintillation spectrometer with Scintiverse BD as the counting fluid at 95% efficiency for ¹⁴C.

RESULTS

Construct for *mas* Gene Disruption. The *mas* gene from *M. bovis* BCG, composed of a 6.4-kb coding region, a 1.2-kb 5'-flanking region, and a 0.5-kb 3'-flanking region, was used for making the construct for gene disruption. An internal 2-kb segment from the open reading frame was replaced with a 1.8-kb *hyg* gene of approximately the same size as the selectable marker. This pUC-based construct (Fig. 1a) contains, in addition to the *hyg* gene that is known to be functional in mycobacterial species (26), an *E. coli* origin of replication but not a mycobacterial origin of replication. Therefore, hygromycin-resistant transformants can result only from integration of the plasmid into the mycobacterial genome.

Transformation of *M. bovis* BCG with the *mas* Gene-Disruption Construct. Because the circular plasmid gave low frequency of transformation with no homologous recombination, the plasmid linearized by cutting with *Xba*I in the multiple cloning site of the vector or *Xba*I–*Kpn*I-excised insert was electroporated. The linearized construct and the excised insert gave few (two to four) transformants per μ g DNA. Controls without DNA showed that *M. bovis* BCG did not yield spon-

FIG. 1. Schematic diagram of the strategy used for gene replacement at the *mas* locus in the *M. bovis* BCG genome. (a) The *mas* gene-disruption construct, pATP10, used for transformation; (b) BCG genome representing the *mas* gene and flanking regions; (c) gene organization expected from a homologous recombination with single crossover at the left arm between the disrupted copy of the gene and the wild-type gene in the BCG genome; and (d) genome organization expected from double crossover. Hatched boxes represent the *mas*-coding sequence plus the 5'- and 3'-flanking regions, and stippled boxes represent the sequences of pUC19, used as a nonreplicative vector. Black boxes denote the *hyg* gene, which is used as a disruption element as well as a selectable marker. Vertical double lines indicate the break-points of continuous sequence. Only relevant restriction enzyme sites are shown. B, *Bam*HI; Bs, *Bst*BI; K, *Kpn*I; S, *Sst*I; St, *Stu*I; X, *Xba*I. *bla*, ampicillin-resistance gene; *oriE*, *E. coli* origin of replication. B1/H1 and H2/B2 represent the primer recognition sites for 4.8- and 1.4-kb PCR products, respectively, from the disrupted BCG genome. P indicates the *Stu*I fragment of the *mas* gene used as the probe for the Southern blot in Fig. 3.

taneous hygromycin-resistant mutants at detectable frequency.

Analysis of Hygromycin-Resistant Transformants. The transformants were screened by PCR with one set of primers (H1 and B1) representing the 5' terminus of the *hyg* gene and a unique sequence in the 5'-flanking region of the *mas* gene of the mycobacterial genome beyond that used for making the construct (Fig. 1 c and d). This set of primers would generate a 4.8-kb PCR product in the case of homologous recombination. PCR was also done with another set of primers representing the 3' terminus of the *hyg* gene (H2) and a unique sequence from the 3'-flanking region of *mas* in the *M. bovis* BCG genome adjacent to the expected integration site but absent from the vector construct (B2) (Fig. 1d). This set of primers would generate a 1.4-kb product if the transforming DNA integrates by homologous recombination into the mycobacterial genome. Integration by a double crossover would yield both 1.4- and 4.8-kb products with the two sets of primers. Analysis of 38 hygromycin-resistant transformants by such a PCR analysis showed three mutants with single crossover events, two yielding a 1.4-kb product and one (BM-19) yielding a 4.8-kb product. One mutant, designated BM-18, yielded both 1.4- and 4.8-kb products (Fig. 2), as expected from homologous recombination by double crossover causing replacement of the native *mas* gene by a disrupted copy of the gene (Fig. 1d). Because the transformant BM-19 (Fig. 1c) was generated by the single-crossover event at the 5' side of the *mas* locus, only the larger PCR product (4.8 kb) was obtained (Fig. 2A).

The gene disruption by double crossover was further analyzed by Southern hybridization. When the 2-kb internal segment of the *mas* gene that was replaced by the *hyg* gene was used as the probe, only the *mas* gene-disrupted mutant (BM-18) failed to show hybridization; the wild-type and the hygromycin-resistant transformants, including the single crossover integrant (BM-19), showed the expected hybridization bands (Fig. 3A). The *mas* gene-disrupted mutant and all of the other hygromycin-

resistant transformants hybridized with the *hyg* gene probe (Fig. 3B). As expected from double crossover event, DNA from the *mas* gene-disrupted mutant showed no hybridization with the vector probe, whereas all of the other transformants hybridized with the same probe (transformants generated with the excised insert did not contain vector) (Fig. 3C). To confirm that the PCR products generated from either side of the *hyg* gene represent the true flanking regions, *mas* gene segment encoding the acyl transferase domain of the synthase representing the 5' side and the gene segment encoding the acyl carrier protein domain of the synthase representing the 3' side

FIG. 2. Agarose gel electrophoresis of PCR products with the genomic DNA from wild-type BCG and its transformants, BM-18 and BM-19, as the templates. (A) Long-range PCR with primers B1/H1. (B) Standard PCR with H2/B2 primers. PCR products, obtained with each set of primers and the following templates, were subjected to electrophoresis. Lanes: 1, no template; 2, pATP10 construct; 3, genomic DNA from wild-type BCG; 4, genomic DNA from mutant BM-18; 5, genomic DNA from mutant BM-19; M, 1-kb DNA ladder as size marker.

FIG. 3. Autoradiograms of Southern blots of genomic DNA from wild-type BCG, double-crossover mutant BM-18, and a representative single-crossover integrant BM-19. (A) Genomic DNA (3–4 μ g) was digested with *Bam*HI, transferred to nylon membrane, and probed with 2-kb internal *mas* fragment (P in Fig. 1) that was replaced with the *hyg* gene. (B) DNA was digested with *Sst*I, blotted to membrane under the same conditions, and probed with 1.8-kb *hyg* gene. (C) *Bam*HI-digested DNA was processed under the same conditions and probed with 2.7-kb pUC19 vector DNA. Lanes: 1, wild-type BCG; 2, BM-18; 3, BM-19. Size markers are from the 1-kb DNA ladder.

were used as probes for Southern hybridization of the PCR products. As expected, only the 4.8-kb product hybridized with the acyl transferase probe and the 1.4-kb product hybridized with the acyl carrier protein probe (Fig. 4). The mutant was stable after repeated (eight times) subculturing with or without hygromycin over a period of 4 months.

Mycocerosic Acid Biosynthesis by *M. bovis* BCG and its *mas* Disrupted Mutant. To test whether *mas* gene disruption eliminated the ability of the organism to synthesize mycocerosic acid, [^{14} C]propionate incorporation into lipids by the wild-type BCG and its *mas*-disrupted mutant was examined. Overnight incubation with [^{14} C]propionate under sterile conditions resulted in the incorporation of 13.4% and 5.6% of the administered label into total lipids by the wild type and mutant, respectively. Thin-layer chromatographic analysis showed two major labeled components corresponding to phthiocerol ester fraction and phenol-phthiocerol ester fraction in the wild type, whereas no labeling was detected in the phenol-phthiocerol ester (mycoside) region in the *mas*-disrupted mutant (Fig. 5A). Charring of the chromatograms clearly showed two major components corresponding to phthiocerol

FIG. 4. Southern blots of 4.8- and 1.4-kb PCR products from BM-18 and BM-19. (A) The long-range PCR products with B1/H1 primers from BM-18 and BM-19 were probed with the acyl transferase domain fragment of the *mas*, present on the 5' side of the gene. (B) The standard PCR products with B2/H2 primers from BM-18 and BM-19 were probed with the acyl carrier protein domain fragment of the *mas*, present on the 3' side of the gene. Lanes: 1, BM-18; 2, BM-19. The size markers are from the 1-kb DNA ladder.

FIG. 5. Radio thin-layer chromatographic analysis of total lipids derived from [^{14} C]propionate in *M. bovis* BCG and its *mas*-disrupted mutant. (A) Autoradiograms. (B) Chromatograms charred with the $K_2Cr_2O_7/H_2SO_4$. WT, wild type; Mu, mutant BM-18; My, mycoside standard.

ester waxes and mycosides in the wild-type but in the *mas*-disrupted mutant only phthiocerol esters could be found with no bands corresponding to mycosides (Fig. 5B). Nearly 40% and >50% of the ^{14} C contained in the total lipids was found in the phthiocerol and phenol-phthiocerol ester fractions, respectively, in the wild type, whereas most (98%) of the label in the lipids in the mutant was contained in phthiocerol esters. When the acyl and alcohol portions of the ester fractions were separated by base hydrolysis, most of the label ($\approx$ 90%) was found to be in the acyl portion both in the wild-type and the *mas*-disrupted mutant. In the wild type almost 10% of the label in the phenol-phthiocerol ester fraction was contained in a polar material tentatively identified as phenol-phthiocerol (data not shown). Radio-gas chromatographic analysis of the methyl esters of the fatty acids showed that the major part of the label was contained in C_{29} and C_{32} mycocerosic acids of the phenol-phthiocerol esters in the wild type (Fig. 6), whereas this ester fraction was absent in the mutant. The phthiocerol ester fraction of the wild type also contained significant amounts of C_{29} and C_{32} mycocerosic acids, whereas the corresponding fraction from the mutant contained only shorter fatty acids with no mycocerosic acids (Fig. 6). Obviously, *mas* gene-disrupted mutant could not synthesize mycocerosic acids.

DISCUSSION

Mycocerosic acid-containing lipids, being found only in pathogenic mycobacterial cell walls, have been suggested to be important for pathogenicity (12–14). However, direct evidence for such a hypothesis is lacking. Replacement of gene(s) involved in the biosynthesis of such lipids would be one approach to make a direct test. Earlier efforts to achieve targeted gene disruption in slow-growing mycobacteria were unsuccessful as only nonhomologous recombinations were observed (22). Attempts to achieve homologous recombination by increasing the efficiency of transformation yielded mutants with single crossover in *M. bovis* BCG, but without gene replacement (23). Efforts to increase probability of

FIG. 6. Radio gas-liquid chromatograms of the methyl esters from the phthiocerol esters and phenol-phthiocerol esters derived from [1-¹⁴C]propionate in *M. bovis* BCG and its *mas*-disrupted mutant. (A) Fatty acids from phenol-phthiocerol esters from wild type. (B) Fatty acids from phthiocerol esters from wild type. (C) Fatty acids from the phthiocerol esters from the *mas*-disrupted mutant. (There were no phenol-phthiocerol esters in the mutant.) The retention times of methyl esters of *n*-fatty acids with carbon atoms indicated are shown by arrows. M1 and M2 (shaded) are C₂₉ and C₃₂ mycocerosic acids that were identified as before (18).

double crossover by increasing the level of exposure to the transforming DNA with the use of a high-copy-number replicating vector resulted in double crossover, but the recombination was reciprocal and reversible and, thus, yielded mixed cultures without producing stable mutants (24). In these slow-growing mycobacteria with a *RecA* carrying a protein intron (33), gene replacement by double crossover has not been observed even though in *Mycobacterium intracellulare*, with an intronless *RecA*, replacement of an introduced gene was recently reported (34).

The results presented here clearly show that the native *mas* gene in *M. bovis* BCG was disrupted by homologous recombination with double crossover. This conclusion is supported by the findings that the *mas* gene segment that was replaced with the *hyg* gene and the vector used for introducing the disrupted gene were not present in the genome of the mutant, and the *hyg* gene was present. Furthermore, the PCR products generated from both flanking sides of the introduced *hyg* gene clearly showed homologous recombination with double crossover. Our success in obtaining this gene replacement may be related to the size of homologous DNA segments introduced. It has been reported that the frequency of homologous recombination increased with increasing length of the homologous DNA segment introduced, and double crossover was enhanced by longer and shorter stretches of DNA homology on either side of the selectable marker in both yeast and mammalian systems (35, 36). In the murine embryonic stem cells, homologous recombination increased most dramatically when the length of the flanking region was increased up to 4.2 kb with some further increase when the length reached 6 kb and no further increases with longer inserts (36). The large size of the *mas* gene readily provided us a means to design a construct

that contains a 4.7-kb and a 1.4-kb homologous sequences flanking the *hyg* selection marker. The previous approaches with slow-growing mycobacteria did not use such a strategy. It is also possible that our use of the GC-rich *hyg* gene from *Streptomyces*, rather than the previously used transposon-derived kanamycin-resistance gene, made it more compatible for the GC-rich mycobacterial genome. Replacement of an internal segment of the native gene with a similar size *hyg* gene could have prevented loop formation or strand stress, that could have been detrimental for double recombination.

If there is only a single copy of *mas* gene in the *M. bovis* BCG genome, as suggested by Southern hybridization studies (19), replacement of this gene should be expected to make the mutant incapable of synthesizing mycocerosic acids. The use of [1-¹⁴C]propionate to specifically label mycocerosic acids clearly showed that the mutant was incapable of synthesizing mycocerosic acids. The wild-type *M. bovis* BCG incorporated significant amounts of the labeled propionate specifically into phthiocerol ester wax and glycosyl phenol-phthiocerol esters (mycoside). In the *mas*-disrupted mutant, labeled mycocerosic acids were absent in the acyl portion of both ester groups. Since [1-¹⁴C]propionate is also incorporated into phenol-phthiocerol, albeit much less than that incorporated into the mycocerosic acids, the observation that no labeled mycosides were found in the *mas*-disrupted mutant suggests that in the absence of mycocerosic acids acylated phenol-phthiocerols are not produced, presumably because the cells do not substitute other fatty acids for mycocerosic acids in the acylation steps. No mycoside was seen in the charred thin-layer chromatograms of the total lipids from the mutant, whereas the mycoside band in the wild type was obvious. If other fatty acids could be substituted, mycosides should be detectable in the mutant even

if they were not labeled. Thus, in the cell wall of the mutant the normal mycosides are not substituted by other mycosides with a fatty acid composition different from that of the wild type. This finding is consistent with our previous conclusion that the synthase probably transfers mycocerosyl groups directly to phenol-phthiocerol (18). The absence of a significant structural component of the cell wall, that has been postulated to be involved in the ability of the organism to multiply in the host, might have important biological consequences in the mycobacterial interactions with the host. The availability of the *mas* gene-disrupted mutant should allow exploration of these biological consequences.

Note Added in Proof. Two other gene replacements in *Mycobacteria* have recently been reported (37, 38).

We thank W. R. Jacobs for supplying the *M. bovis* BCG cosmid library and D. A. Hopwood for the plasmid containing the hygromycin-resistance cassette. We thank Norvin Fernandes for assistance with radio-gas chromatographic analysis, Debra Gamble for the reproduction of photographs, and Sharon Hollander for her secretarial assistance. This work was supported in part by Grant R01 AI35272 from the National Institutes of Health.

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