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Cell Wall Core Galactofuran Synthesis Is Essential for Growth of Mycobacteria

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The mycobacterial cell wall core consists of an outer lipid (mycolic acid) layer attached to peptidoglycan via a galactofuranosyl-containing polysaccharide, arabinogalactan. This structural arrangement strongly suggests that galactofuranosyl residues are essential for the growth and viability of mycobacteria. Galactofuranosyl residues are formed in nature by a ring contraction of UDP-galactopyranose to UDP-galactofuranose catalyzed by the enzyme UDP-galactopyranose mutase (Glf). In *Mycobacterium tuberculosis* the *glf* gene overlaps, by 1 nucleotide, a gene, Rv3808c, that has been shown to encode a galactofuranosyl transferase. We demonstrate here that *glf* can be knocked out in *Mycobacterium smegmatis* by allelic replacement only in the presence of two rescue plasmids carrying functional copies of *glf* and Rv3808c. The *glf* rescue plasmid was designed with a temperature-sensitive origin of replication and the *M. smegmatis glf* knockout mutant is unable to grow at the higher temperature at which the *glf*-containing rescue plasmid is lost. In a separate experiment, the Rv3808c rescue plasmid was designed with a temperature-sensitive origin of replication and the *glf*-bearing plasmid was designed with a normal original of replication; this strain was also unable to grow at the nonpermissive temperature. Thus, both *glf* and Rv3808c are essential for growth. These findings and the fact that galactofuranosyl residues are not found in humans supports the development of UDP-galactopyranose mutase and galactofuranosyl transferase as important targets for the development of new antituberculosis drugs.

The mycobacterial cell wall core consists of two layers. The highly impermeable outer layer is composed of mycolic acids, 70 to 90 carbon-containing lipids. The inner layer consists of peptidoglycan. These two layers are covalently tethered via the connecting polysaccharide arabinogalactan (2, 4, 12–14). Arabinogalactan itself (Fig. 1) contains three regions: the disaccharide linker, α-L-rhamnosyl-(1→3)-α-D-GlcNAc-(1→phosphate), which is attached to the peptidoglycan; a galactofuran $[\rightarrow 6)$-β-D-Gal*f*-(1→5)-β-D-Gal*f*-$(1]_{\sim 15}$ (where Gal*f* is galactofuranose), which is attached to the linker (4); and finally a complex mycolic acid-bearing arabinan, which is attached to the galactofuran (4).

Galactofuranosyl residues are formed in nature by the enzyme UDP-galactopyranose mutase (16, 23), which converts UDP-galactopyranose to UDP-Gal*f*. Although this activity was shown some time ago in penicillin fungus (22), only recently has the enzyme been isolated and its activity directly demonstrated to occur in *Escherichia coli* (16), *Klebsiella* sp. (9), and mycobacteria (23). Methods to assay the activity of the enzyme have been developed (10), and crystallographic structural study of the enzyme has commenced (11).

The location of galactofuran between the peptidoglycan and the mycolic acids strongly suggests that galactofuran is essential for mycobacterial growth (Fig. 1). Direct evidence of such a role exists for the similarly located arabinan (Fig. 1), because ethambutol, an effective antituberculosis drug, inhibits its formation (21). However, there are not yet any drugs known to directly inhibit the formation of galactofuran and other direct evidence supporting an essential role for galactofuran is lacking.

The gene encoding UDP-galactopyranose mutase in *Mycobacterium tuberculosis* has been identified as Rv3809c (23). Directly downstream from Rv3809c and overlapping it by a single nucleotide is Rv3808c, which was recently identified as a galactofuranosyl transferase (15), although the specific substrate and product were not identified. Downstream from Rv3808c are three more open reading frames (ORFs), separated from their upstream neighbors by 7, 29, and 89 bp. Thus, *glf* (Rv3809c) is very likely the first gene in an operon containing at least two and possibly up to five ORFs. The functions of Rv3807c, Rv3806c, and Rv3805c are unknown. In designing and analyzing knockout mutations of *glf*, this genetic organization must be borne in mind. Herein we present experiments that demonstrate that *glf* and Rv3808c are essential in *Mycobacterium smegmatis*. Our basic strategy was to show that *M. smegmatis* chromosomal *glf* could be knocked out only in the presence of appropriate rescue plasmids and then that loss of either the *glf* or the Rv3808c rescue plasmid correlated with the loss of the ability of the bacterium to grow.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* Top 10 electrocompetent cells (Invitrogen, Carlsbad, Calif.) were used for propagating all plasmids except for pCG76 where chemically competent DH5α (Life Technologies, Inc., Grand Island, N.Y.) cells were used. The bacteria were grown in Luria-Bertani (LB) broth and LB agar with appropriate antibiotics and incubated at 37°C routinely. A fast-growing mycobacterium (referred to in this paper as "mycobacterial lab strain") was used to isolate the DNA in the *glf* region, the sequence of which was shown to be nearly identical to that of *M. smegmatis* mc^2155 (see below for further details). *M. smegmatis* mc^2155 was used for allelic-exchange experiments and was grown in LB broth with 0.05% Tween 80 or on LB agar plates. Appropriate antibiotics were included, and incubations were at 30, 40, and 42°C, depending on the experiment. The growth curves of various *M. smegmatis*

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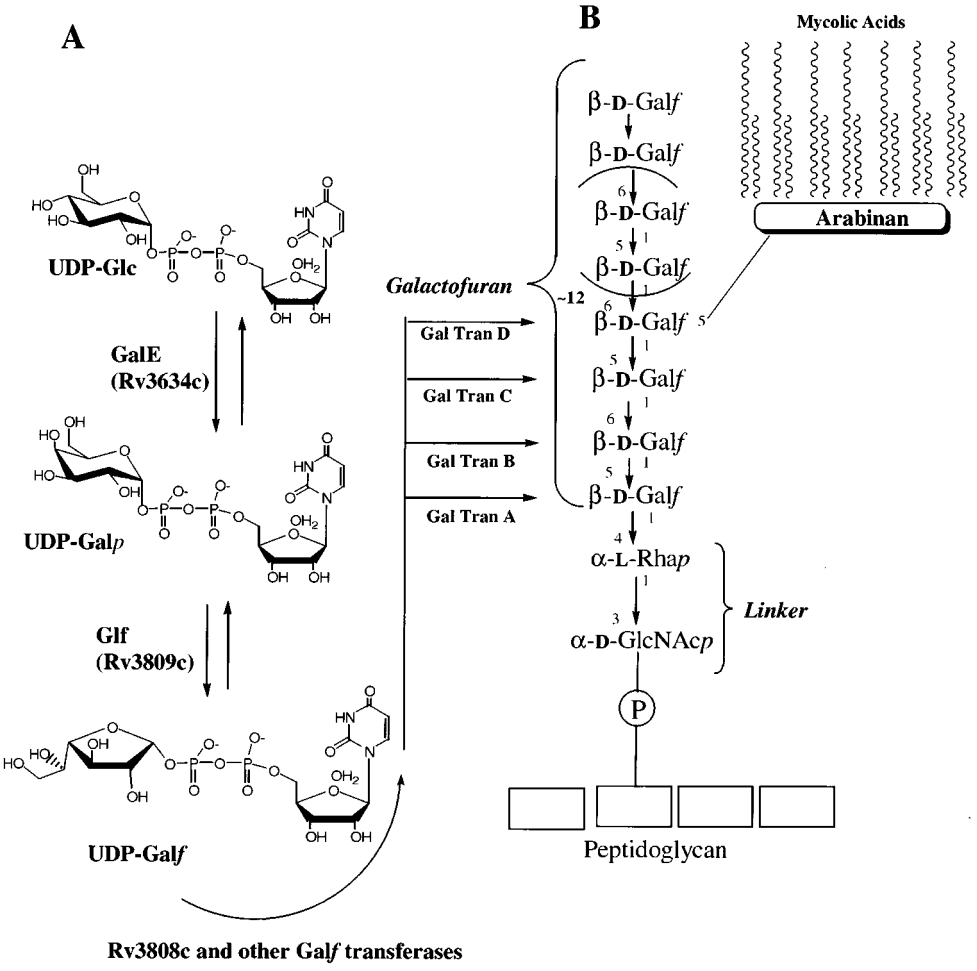

FIG. 1. Formation of UDP-Gal*f* (A) and galactofuran (B). The role of galactofuranosyl residues of linking peptidoglycan to mycolic acids via arabinan is also shown. From the galactofuran structure, it is estimated that four galactofuranosyl transferase activities (Gal Tran A to D) are needed to form the galactofuran (with the remaining residues being assembled by the activities Gal Tran C and D); it is possible that some of these activities are combined into one polypeptide. Rv3808c presumably encodes one or more of these galactofuranosyl transferase activities (15). Gal*p*, galactopyranose; Rha*p*, rhamnosylpyranose.

mc^2155 strains (see Fig. 6) were obtained by culturing the bacteria in 5 ml of LB broth containing 0.05% Tween 80 and monitoring the optical density at 600 nm. Antibiotics were as follows: for *M. smegmatis* mc^2155 containing plasmids pMVHG1:Rv3808c and pCG76:Tb*glf*, hygromycin was included in the medium at both temperatures, with streptomycin also being used at 30°C only; for *M. smegmatis* FP102 and *M. smegmatis* FP103 (see Table 1 for the plasmids in these strains), kanamycin (KAN) and hygromycin were included in the medium at both temperatures, with streptomycin also being used at 30°C only. The concentrations of antibiotics when used were as follows: 100 μg/ml for ampicillin; 5 μg/ml for gentamicin; 50 μg/ml (*E. coli*) and 25 μg/ml (*M. smegmatis*) for KAN; 100 μg/ml (*E. coli*) and 50 μg/ml (*M. smegmatis*) for hygromycin; and 20 μg/ml (*E. coli*) and 10 μg/ml (*M. smegmatis*) for streptomycin. Ten percent sucrose was added to the solid medium when required.

Transformation. Transformation of *E. coli* Top 10 and DH5α cells was done by following the protocol provided by the vendor. Electrocompetent *M. smegmatis* was made as described previously (18). Electroporation was done by setting the voltage and capacity of a Gene Pulser (Bio-Rad, Richmond, Calif.) to 2,500 V and 25 μF and the resistance of the pulse controller to 1,000 Ω. To prepare *M. smegmatis* strains containing both pMVHG1:Rv3808c and pCG76:TB*glf* (and for strains containing both pMVHG1:Tb*glf* and pCG76:Rv3808c), both plasmids were electroporated into bacteria at the same time and selection was done using both streptomycin and hygromycin.

DNA extraction, Southern blot analysis, and DNA sequencing. Mycobacterial genomic DNA was extracted as described previously (1). Genomic DNA was digested overnight by appropriate enzymes and then loaded onto a 0.8% agarose gel. The gel was run at 30 V overnight (20 to 24 h). Then the DNA was transferred to a Nytran Plus membrane (Schleicher & Schuell, Keene, N.H.). The DNA was fixed to the membrane by using a Stratalinker 2400 (Stratagene, La Jolla, Calif.). For Southern blots, the DNA probe was generated by using DIG High Prime Labeling and Detection Starter Kit I (Boehringer Mannheim, Indianapolis, Ind.). The 1,595-bp *Sma*I fragment containing ~90% of *glf* and 535 bp upstream of *glf* of the mycobacterial lab strain was used as the probe template (see below for isolation of this DNA fragment). DNA hybridization and detection were performed as recommended by the vendor. Sequences of double-stranded plasmids were obtained by Macromolecular Resources (Colorado State University) using an ABI Prism 377 automated DNA sequencer.

Construction of vectors. pFP101 was constructed as follows. A partial mycobacterial lab strain genomic DNA library was constructed by isolation of *Sma*I-digested fragments of DNA from the strain of approximately 1,600 bp, ligation into the pCR-Blunt vector (Invitrogen), and maintenance in *E. coli* Top 10 cells. The *glf* gene was found to be contained in a 1,595-bp fragment by colony hybridization (6) using the entire *M. tuberculosis glf* gene (23) as a probe. Sequencing revealed that the fragment contained 535 bp upstream of the ATG start site and 1,060 bp of the *glf* sequence (approximately 90% of the *glf* gene). Plasmid pBluescript II SK(+) (Stratagene) was treated sequentially with *Bam*HI, mung bean nuclease, and T4 DNA polymerase to remove the *Bam*HI site to facilitate later cloning operations. The 1,595-bp *glf*-containing fragment was cut out from the pCR-Blunt vector with *Eco*RI and inserted into the *Eco*RI site of pBluescript II SK(+) (which lacks a *Bam*HI site) to yield plasmid pFP5. A 1.2-kb KAN resistance cassette was cut out with *Bam*HI from plasmid pUC4K, filled in

TABLE 1. Key bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Reference or source
Strains		
M. smegmatis mc^{2}155	Strain harboring all plasmids used herein	19
Mycobacterial lab strain	A fast-growing mycobacterium. Source of *glf* DNA used to prepare pFP101. The identity of this strain is uncertain (it was originally thought to be *M. smegmatis* mc^{2}155). Its sole use in this study was as a source of *glf* DNA, and its sequence is essentially the same as that of *M. smegmatis* mc^{2}155 *glf* DNA	This study
M. smegmatis FP101	*M. smegmatis* mc^{2}155 with pFP101 integrated into the *glf* locus (orientation 1 in Fig. 3)	This study
M. smegmatis FP102	*M. smegmatis* FP101 which has undergone a second crossover event in the presence of pMVHG1:Rv3808c and pCG76:TB*glf*	This study
M. smegmatis FP103	*M. smegmatis* FP101 which has undergone a second crossover event in the presence of pMVHG1:TB*glf* and pCG76:Rv3808c. The genome of FP103 should be identical to the genome of FP102, with the strains differing only in which rescue plasmids they carry	This study
Plasmids		
pPR27	Temperature-sensitive mycobacterial origin of replication. Carries the *sacB* gene, *gen*, and the *E. coli* origin of replication	17
pFP101	pPR27 derivative carrying *glf*::*kan* and the *xylE* gene (Fig. 2)	This study
pVV16	pMV261 (20) with the hygromycin resistance cassette (*hyg*), a KAN resistance cassette (*kan*), and P*hsp60*	8
pMVHG1	pVV16 derivative with *kan* deleted, *hyg*	This study
pMVHG1:Rv3808c	Rescue plasmid for Rv3808c; P*hsp60 hyg* (Fig. 2)	This study
pMVHG1:TB*glf*	Rescue plasmid for *glf*; P*hsp60 hyg*	This study
pCG76	*E. coli* and *Mycobacterium* shuttle vector carrying a temperature-sensitive mycobacterial origin of replication and the streptomycin resistance cassette (*str*)	8
pCG76:TB*glf*	Temperature-sensitive plasmid carrying the *M. tuberculosis glf* gene (under the control of P*hsp60*) and *str* (Fig. 2)	This study
pCG76:Rv3808c	Temperature-sensitive rescue plasmid carrying the *M. tuberculosis* Rv3808c gene (under the control of P*hsp60*) and *str*	This study

with the Klenow fragment of DNA polymerase I, and inserted into the Klenow fragment-filled *Bam*HI site of the *glf* gene carried by pFP5, yielding plasmid pFP6. A 2.8-kb *glf*::*kan* fragment was cut by *Eco*RI from pFP6 and then moved to the *Eco*RI site of plasmid pXYL4, a plasmid carrying the *xylE* gene from *Pseudomonas putida* (3), which yielded plasmid pFP7. Finally, the 3.8-kb (*glf*::*kan*)::*xylE* fragment was cut by *Bam*HI from pFP7 and put into the *Bam*HI site of pPR27 (Table 1) to yield plasmid pFP101 (Fig. 2), the vector used to achieve allelic replacement at the *glf* locus of *M. smegmatis*. As shown in Fig. 2, pFP101 has the mycobacterial temperature-sensitive origin of replication from the parent plasmid pPR27. Thus, it can replicate at 30°C but is efficiently lost at 39°C and above (17). Plasmid pFP101 also harbors the counterselectable marker *sacB* from *Bacillus subtilis* (17) for use in selection of the double-crossover event in the presence of sucrose. To check the orientation of *xylE*, *kan*, and *glf*, pFP101 plasmid was digested with *Bam*HI and a 3.8-kb fragment was purified by using a QIAEX II kit. The 3.8-kb fragment was digested with *Xba*I and *Sma*I, and analysis of the restriction fragments by gel electrophoresis showed that the orientations of *kan, glf,* and *xylE* were as shown in Fig. 2 and 3. The genes *xylE*, *kan*, and *sacB* are all transcribed from their own promoters.

The *glf* rescue plasmid (pCG76:TB*glf*) was prepared as follows. The entire *M. tuberculosis glf* gene was cut with *Hin*dIII and *Sal*I from plasmid pMMRS1 (23) and ligated into the *Hin*dIII and *Sal*I sites downstream of the heat shock promoter P_{hsp60} in plasmid pMV261 (20). The P_{hsp60}-*glf* fragment was then cut with *Xba*I and *Hpa*I, blunt ended, and inserted into *Xba*I-cut and blunt-ended pCG76 (Table 1), yielding plasmid pCG76:TB*glf* (Fig. 2). Plasmid pCG76 carries the same temperature-sensitive mycobacterial replication origin as pFP101 and thus can replicate at the permissive temperature of 30°C but is cured at 39°C and above (8). The *glf* rescue plasmid in pMVHG1 was made by inserting the P_{hsp60}-*glf* fragment described directly above into the *Xba*I and *Hpa*I sites of pMVHG1 (Table 1).

The Rv3808c rescue plasmid (pMVHG1:Rv3808c) was prepared by cloning an Rv3808c-containing fragment (cut by *Nde*I and *Hin*dIII from plasmid Rv3808c-pCR-Blunt [15]) into the *Nde*I and *Hin*dIII sites downstream of P_{hsp60} in plasmid pMVHG1 (Table 1). The temperature-sensitive rescue plasmid pCG76:Rv3808c was prepared by cutting out Rv3808c with P_{hsp60} from pMVHG1:Rv3808c with *Xba*I and *Hin*dIII, end blunting, and insertion into the *Xba*I site (end blunted) of pCG76.

RESULTS

DNA sequence of the mycobacterial lab strain *glf*. A DNA fragment hybridizing with *M. tuberculosis glf* from the mycobacterial lab strain was cloned as a 1,595-bp *Sma*I fragment as described under Materials and Methods. Sequencing of the fragment revealed that it contained 535 bp upstream and 1,060 bp downstream of the ATG start codon of *glf* (approximately 90% of the ORF). During this study, sequence data of *glf* and surrounding regions of *M. smegmatis* mc^{2}155 appeared on The Institute for Genomic Research web site (http://www.tigr.org/). There were only eight base pair differences in the 1,595-bp fragments of DNA from the two bacteria, and the deduced amino acid sequences for Glf were identical. Thus, the 1,595-bp DNA fragment could be readily used for the homologous-recombinant experiments that are discussed below.

Construction of the *glf* replacement plasmid (pFP101) and obtaining the first homologous-recombination event. Essentially the same strategy was used to replace *glf* as was used recently to replace *pgsA* (8). Hence, plasmid pFP101 was constructed. This plasmid carried the 1,595-bp fragment described above that had been modified so that a KAN cassette disrupted the *glf* gene (*glf*::*kan*) (Fig. 2). The plasmid has a temperature-sensitive mycobacterial origin of replication that facilitates obtaining recombinant strains that have undergone a single homologous-recombination event at the *glf* locus. It also harbors the *sacB* counterselectable marker (17) and the *xylE* colored marker (3). Plasmid pFP101 was electroporated into *M. smegmatis* mc^{2}155, and transformants were selected on LB broth-

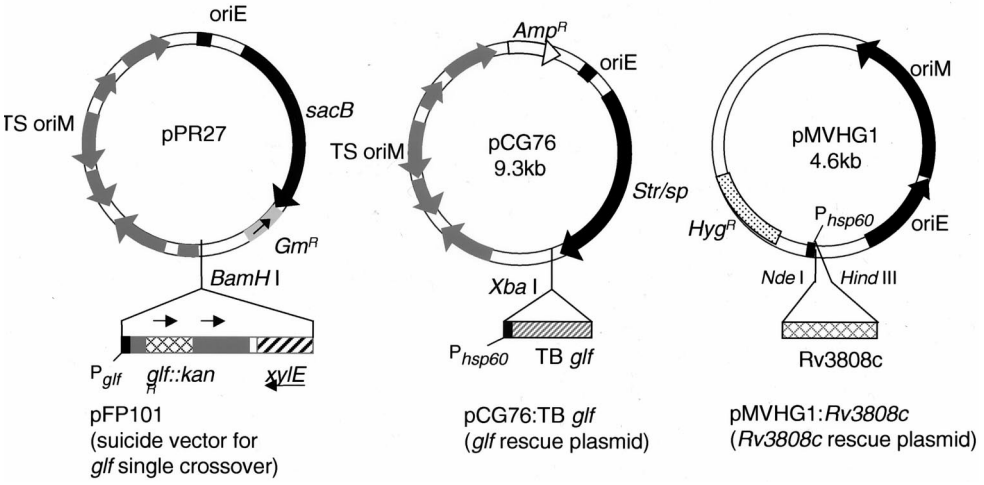

FIG. 2. Key plasmids (pFP101, pCG76:TB*glf*, and pMVHG1:Rv3808c) used in this study. The plasmids pCG76:Rv3808c and pMVHG1:TB*glf* are strictly analogous to CG76:TB*glf* and pMVHG1:Rv3808c. TS oriM, temperature-sensitive *oriM*; Amp^R, ampicillin resistance cassette; Gm^R; gentamicin resistance cassette; Str/sp, streptomycin/spectinomycin resistance cassette; Hyg^R, hygromycin resistance cassette.

KAN plates at 30°C. One transformant was then propagated in LB broth-KAN at 30°C and then plated onto LB broth-KAN plates at 42°C. Since the temperature-sensitive plasmid is able to replicate at 30°C but not at 42°C, the KAN-resistant colonies that appear on plates have necessarily integrated part or all of the vector into their chromosome. Single homologous-recombination events upstream and downstream from the KAN resistance gene resulting in genotypes 1 and 2 are shown in Fig. 3. The important difference between the two genotypes is that genotype 1 (Fig. 3) results in an intact *glf*-containing operon and that genotype 2 (Fig. 3) results in both the lack of an intact *glf* gene (since the introduced 1,595-bp fragment does not encode the entire Glf protein) and the possibility that genes downstream of *glf* that are dependent on the natural promoter of *glf* are not expressed. Illegitimate recombination which would leave ORFs Rv3809c through Rv3805c fully intact may also occur. Finally, a double-crossover event would lead to the disruption of the *glf* gene and presumably would affect the expression of the downstream genes. Analysis of 17 colonies from the 42°C plate by Southern blotting after digestion with *Nru*I (Fig. 4) revealed that 10 colonies were able to grow on KAN due to illegitimate recombination and that seven colonies

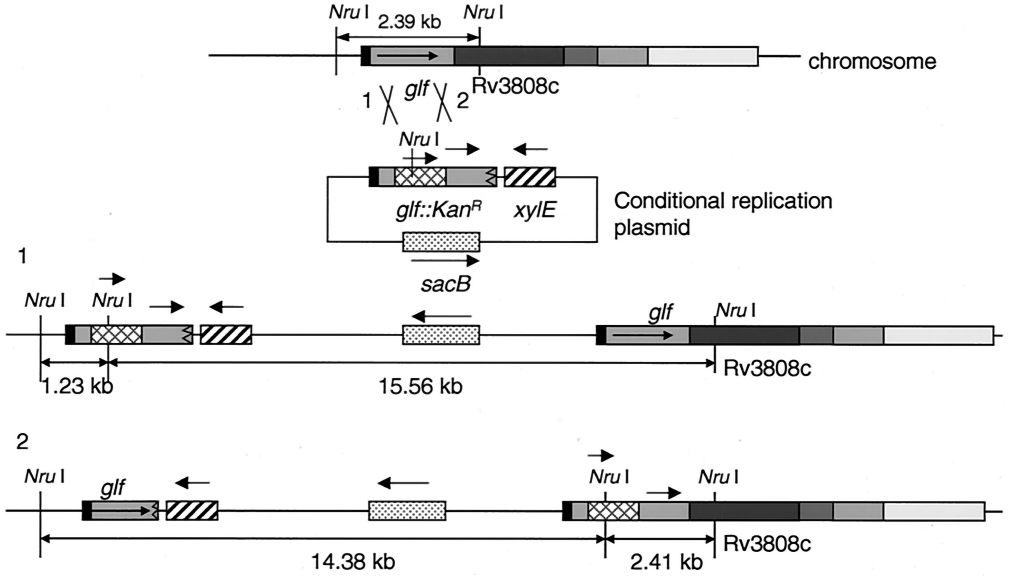

FIG. 3. Two possible pathways for homologous recombination between pFP101 and the *M. smegmatis* chromosome. Crossover upstream of *kan* yields a functional *glf* gene (with its promoter) along with functional Rv3808c and any transcriptionally linked genes further downstream. Crossover downstream from *kan* yields no functional *glf* gene, and the interrupted *glf* gene upstream from Rv3808c is likely to inhibit transcription of Rv3808c and any transcriptionally linked genes downstream from it. If *glf,* Rv3808c, or any other downstream ORF expressed from the *glf* promoter is essential, only single-crossover events of type 1 should occur. Also illustrated are the *Nru*I fragments used to distinguish which single-crossover event occurred.

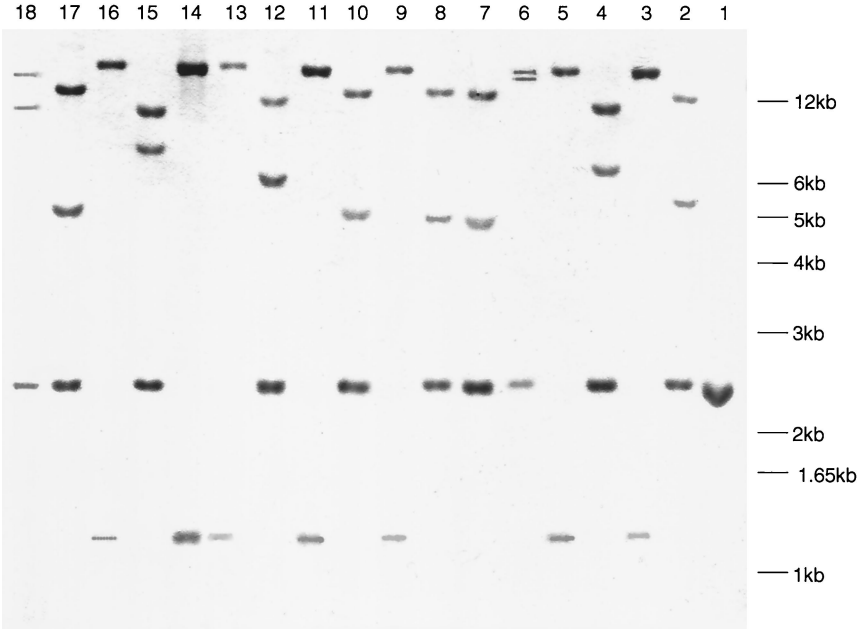

FIG. 4. Southern blot analysis of *M. smegmatis* of the single homologous-recombination event at the *glf* locus of *M. smegmatis*. Lane 1 is wild-type *M. smegmatis*; lanes 2 to 18 are 17 clones selected from the LB broth-KAN plates at 42°C. The DNA was cleaved with *Nru*I, and the 1,595-bp *glf*-containing fragment was used as the probe template. The DNAs in lanes 3, 5, 9, 11, 13, 14, and 16 resulted from type 1 homologous recombination (Fig. 3), as bands at 1.2 and 15.6 kb are evident, whereas the DNAs in lanes 2, 4, 6, 7, 8, 10, 12, 15, 17, and 18 come from clones with illegitimate recombination, as wild-type *glf* at 2.4 kb is evident, as are the expected two bands of various sizes.

came from homologous-recombination pathway 1 (Fig. 3 and 4 and the legend to Fig. 4). We detected no colonies that arose from homologous-recombination pathway 2 (Fig. 3) or from a double-crossover event. These results suggested that either *glf* and/or a gene(s) downstream from *glf* is essential. One of the seven colonies that came forth from homologous-recombination pathway 1 was propagated for further experiments and named *M. smegmatis* FP101 (Table 1).

Construction of rescue plasmids. Before attempting the second crossover event, two sets of rescue plasmids were constructed. It could not be predicted if the KAN resistance cassette would introduce a polar effect (i.e., lack of transcription of Rv3803c) or not. It is possible that transcription of mRNA could continue on past the KAN resistance cassette since no obvious termination sequence is present as part of the cassette. However, there are also many ways that the presence of the KAN resistance gene might result in the downstream Rv3808c gene not being transcribed or translated. Therefore, the first set of rescue plasmids consisted of pCG76:TB*glf* and pMVHG1:Rv3808c (Table 1 and Fig. 2). The pMVHG1:

TABLE 2. Percentages of *xylE*-negative and xylE$^+$ colonies found on second-crossover selection plates[a] of *M. smegmatis* FP101 with various rescue plasmids

M. smegmatis FP101 with the following plasmid(s)	Antibiotic(s) used (same on both broth and plates)[f]	% of yellow colonies	% of white colonies
None	KAN	99	1
pCG76 and pMVHG1[b]	KAN, STR, HYG	99	1
pCG76:TB*glf*	KAN, STR	90	10[c]
pMVHG1:Rv3808c	KAN, HYG	99	1
pMVHG1:Rv3808c and pCG76:TB*glf*	KAN, STR, HYG	0	100[d]
pMVHG1:TB*glf* and pCG76:Rv3808c	KAN, STR, HYG	0	100[e]

[a] The strains were grown in LB broth with the antibiotics indicated in the table and then plated on LB broth plates containing 10% sucrose and the antibiotics indicated here.

[b] This is a control for the effect of the empty pCG76 and pMVHG1 plasmids.

[c] Twelve of the white colonies were tested for the double-crossover event by Southern blot analysis after digestion of their DNAs with *Nru*I, using the 1,595-bp *glf*-containing fragment as a probe. None of the colonies (data not presented) yielded the 1.23- and 2.41-kb *glf*-containing bands expected after a genuine (Fig. 5) second crossover event.

[d] Twenty-four of the white colonies were tested for the double-crossover event by Southern blot analysis after digestion of their DNAs with *Nru*I followed by probing with *glf*. Thirteen yielded the 1.23- and 2.41-kb *glf*-containing bands expected for the genuine double-crossover strain. The Southern blot of three of these is presented in Fig. 5.

[e] All 17 colonies tested for the double-crossover event by Southern blot analysis after digestion of their DNAs with *Nru*I using the 1,595-bp *glf*-containing fragment as the probe were found to be genuine double-crossover strains. The reason for the slightly different result here from that with the other pair of rescue plasmids (see footnote *d*) is unclear.

[f] STR, streptomycin; HYG, hygromycin.

Rv3808c was merely *M. tuberculosis* Rv3808c under the control of P_{hsp60} (Table 1); pCG76:TB*glf* was *glf* under the control of P_{hsp60} but in a plasmid with the same temperature-sensitive origin of replication used in pFP101, so that later experiments to cure the cells of this plasmid could be performed. The second set of rescue plasmids was complementary to the first set and consisted of pCG76:Rv3808c and pMVHG1:TB*glf* (Table 1). In this case, Rv3808c was in the plasmid containing the temperature-sensitive promoter and could be cured in later experiments. *M. smegmatis* FP101 bacteria (Table 1) containing various combinations of these plasmids were then prepared.

Second crossover attempts and events. Single-colony isolates of *M. smegmatis* FP101 (Table 1) containing no rescue plasmid and various rescue plasmids were grown in LB medium (containing appropriate antibiotics as reported in Table 2) at 30°C and then plated onto LB broth-sucrose plates at 30°C. The resulting colonies (Table 2) were analyzed for their XylE phenotype (a yellow color develops in colonies expressing *xylE* when they are sprayed with catechol). Colonies that have undergone a second crossover should both be able to grow on sucrose and have lost the XylE marker; colonies that can grow on sucrose but still express *xylE* are likely to be *sacB* mutants rather than arising from the second crossover event. Thus, only the white colonies were candidates for the second crossover event occurring. Examination of Table 2 revealed that a small number (10%) of white colonies were formed when only pCG76:TB*glf* was present. Analysis of 12 of these colonies by Southern blot analysis showed that none of them resulted from the second crossover event (data not presented). In contrast, when one of the rescue plasmid pairs, pCG76:TB*glf* and pMVHG1:Rv3808c or pCG76:Rv3808c and pMVHG1:TB*glf*, was present, 100% of the colonies were white. Southern blot analysis (Table 2 and Fig. 5) revealed that, in these cases, genuine second crossover events occurred. This outcome strongly suggested that both *glf* and Rv3808c are essential and expressed from the same promoter in *M. smegmatis* and that the KAN resistance cassette for some reason introduces a polar mutation. No information on whether Rv3807c, Rv3806c, and Rv3805c are needed for growth of *M. smegmatis* was obtained in these experiments as these genes may be expressed from a different promoter than the *glf* promoter. One of the colonies showing the genuine second crossover event with the rescue plasmid pair pCG76:TB*glf* and pMVHG1:Rv3808c was named *M. smegmatis* FP102 and propagated for further experiments; one of the colonies showing the genuine second crossover event with the rescue plasmid pair pCG76:Rv3808c and pMVHG1:TB*glf* was named *M. smegmatis* FP103 and propagated for further experiments (Table 1).

Neither *M. smegmatis* FP102 nor *M. smegmatis* FP103 grow at 40°C. As a final experiment to confirm that UDP-galactopyranose mutase and the galactofuranosyl transferase are essential for growth, *M. smegmatis* FP102 containing plasmids pCG76:TB*glf* and pMVHG1:Rv3808c and *M. smegmatis* FP103 containing plasmids pCG76:Rv3808c and pMVHG1:TB*glf* were shown to be unable to grow at 40°C (Fig. 6), a temperature at which pCG76 and its insert are lost. These experiments were conducted at 40°C rather than at 42°C because it was found that *M. smegmatis* mc^{2}155 containing pCG76:TB*glf* and pMVHG1:Rv3808c was unable to grow at 42°C, presumably due to the stress of harboring two plasmids.

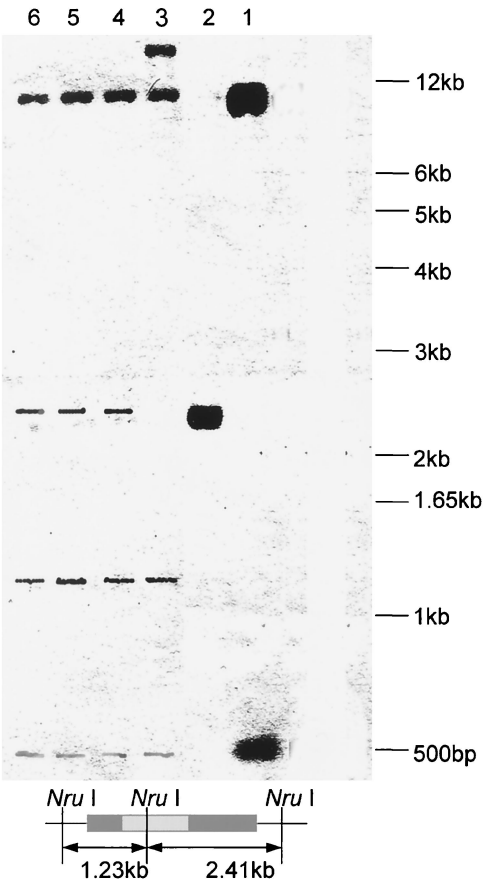

FIG. 5. Southern blot analysis of *M. smegmatis* FP102 (the *glf* knockout strain) containing plasmids pMVHG1:Rv3808c and pCG76:TB*glf*. The DNA was cleaved with *Nru*I, and the 1,595-bp *glf*-containing *M. smegmatis* fragment was used as the probe template. Lanes 1 to 3 are controls; lanes 4 to 6 are positive for the second crossover event. Lane 1, plasmid pCG76:TB*glf* only; lane 2, *M. smegmatis* mc^{2}155 wild type; lane 3, *M. smegmatis* FP101 (first single-crossover bacterium [see also Fig. 4]) with plasmids pMVHG1:Rv3808c and pCG76:TB*glf* before selection on sucrose for the second crossover event; lanes 4 to 6, three colonies of *M. smegmatis* FP102 (Table 1) containing plasmids pMVHG1:Rv3808c and pCG76:TB*glf*. The origins of the bands at 1.2 and 2.4 kb in *M. smegmatis* FP102 are illustrated. Both the wild type (lane 2) and M. *smegmatis* FP102 yield a band near 2.4 kb (2.39 kb for the wild type and 2.41 kb for *M. smegmatis* FP102). However, the band near 2.4 kb also hybridizes with a probe made from the KAN resistance cassette in the case of *M. smegmatis* FP102 (lanes 4 to 6) but does not hybridize in the case of wild-type *M. smegmatis* (lane 2) (data not presented). The bands in lanes 3 to 6 (*M. smegmatis* FP102) at ≈11 and 0.5 kb come from plasmid pCG76:TB*glf* (see lane 1) being present in *M. smegmatis* FP102.

DISCUSSION

The inability to form a second recombination event, i.e., a knockout of *glf* in the absence of both *glf* and Rv3808c rescue plasmids (Table 2), coupled with the inability of *M. smegmatis* FP102 and FP103 to grow without pCG76:TB*glf* and pCG76:Rv3808c, conclusively demonstrates that UDP-galactopyranose mutase and the galactofuranosyl transferase are neces-

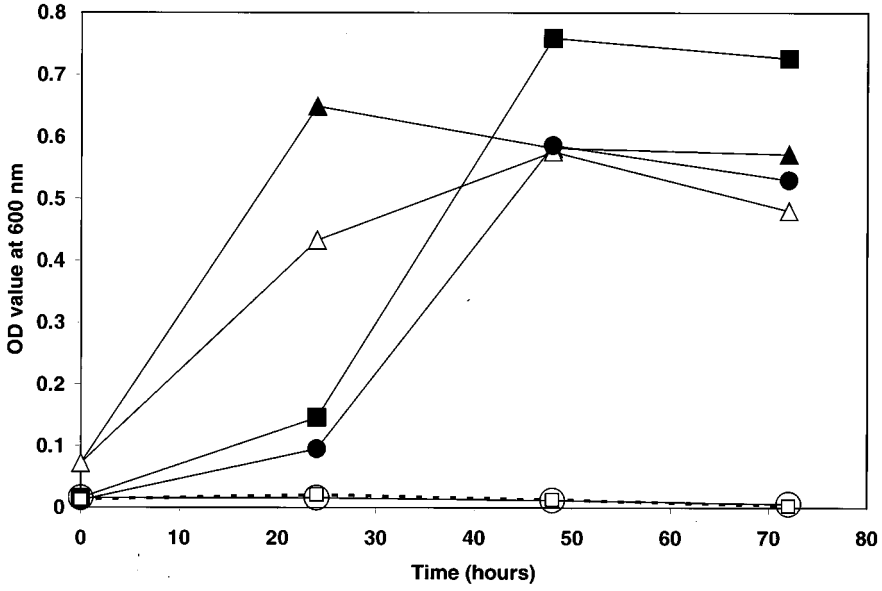

FIG. 6. Growth curves of *M. smegmatis* strains at 30 and 40°C. Shown are results with *M. smegmatis* mc^{2}155 containing plasmids pMVHG1:Rv3808c and pCG76:TB*glf* at 30°C (▲) or at 40°C (△), *M. smegmatis* FP102 (Table 1) at 30°C (●) or at 40°C (○), and *M. smegmatis* FP103 (Table 1) at 30°C (■) or at 40°C (□). The medium was LB broth in all cases, and antibiotics were present as detailed in Materials and Methods. *M. smegmatis* mc^{2}155 containing plasmids pCG76:Rv3808c and pMVHG1:TB*glf*, a second control construct, grew at both 30 and 40°C (data not presented). The slight lag in growth seen for the two knockout strains of *M. smegmatis* at 30°C is likely due to a weaker inoculum, as evidenced by the optical density (OD) at 600 nm at time zero.

sary for the viability of *M. smegmatis*. Even though *glf* was the only gene knocked out in strains FP102 and FP103, the fact that inserted DNA, *kan*, caused a polar effect on the gene Rv3808c allowed us to determine that Rv3808c is also essential. It is not surprising that our experiments demonstrate that *kan* causes a polar effect, as there are several possible ways that transcription or translation may be stopped and/or not initiated downstream of *kan*. However, the mechanism for the polar effect has not been determined. The experiments were done with *M. smegmatis* due to the fast-growth characteristics of this organism and the availability of a temperature-sensitive origin of replication for it. With respect to other mycobacteria, we have shown that the basic structure of the cell wall core of all mycobacteria is indistinguishable by ^{13}C nuclear magnetic resonance and oligosaccharide profiling (5). In addition, *glf* and Rv3808c are found in the genomes of *M. tuberculosis*, *Mycobacterium bovis, Mycobacterium avium*, *M. smegmatis*, and *Mycobacterium leprae.* The presence of these genes in *M. leprae* is of special note due to the fact that the *M. leprae* genome is smaller than that of *M. tuberculosis*, many potential ORFs are pseudogenes, and half of the DNA is noncoding (7). It therefore seems likely that only more necessary genes are present in *M. leprae.* Thus, the similarity of the cell wall core structure along with the identical genetic organizations around *glf* argue strongly that UDP-galactopyranose mutase and the galactofuranosyl transferase encoded by Rv3808c are essential in all mycobacteria, including *M. tuberculosis*.

Demonstration that UDP-galactopyranose mutase and one of the galactofuranosyl transferases are essential for mycobacterial growth is part of a logical sequence of a long-range *M. tuberculosis* drug development program. Initially, the cell wall core arabinogalactan was structurally characterized (2, 4, 13, 14). This led to the recognition that inhibiting the formation of any of three fundamental structural components of the arabinogalactan, L-rhamnosyl, D-arabinofuranosyl, and D-galactofuranosyl residues, was a logical approach to developing new tuberculosis drugs because of the key structural roles of these components (4) and the lack of these three glycosyl residues in humans. The next logical step required determining how these components were biosynthesized and led, in the case of galactofuranosyl residues, to the expression and characterization of UDP-galactopyranose mutase (23) and recognition that Rv3808c encodes a galactofuranosyl transferase (15). The next step was to prove that the formation of galactofuranosyl residues is essential for growth of the mycobacterium, and this has now been accomplished. Following this, inhibitors of the mutase and/or galactofuranosyl transferases are being sought. The enzyme inhibitors that gain entry into the mycobacterium and thus inhibit the growth of *M. tuberculosis* will then be candidates for further development. To identify the enzyme inhibitors, facile assays amenable to a microtiter plate format for UDP-galactopyranose mutase are currently being developed based on the release of radioactive formaldehyde from UDP-6-[^{3}H]Gal*f* by periodate. Assays for the transferase will require determination of the exact substrates of the enzyme; then a scintillation proximity assay where the acceptor is attached to scintillation proximity assay beads may be feasible.

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ADDENDUM IN PROOF

It has now been determined that the galactofuranosyl transferase encoded by Rv3808c is a bifunctional enzyme adding a Gal*f* residue to both 5-linked Gal*f* and 6-linked Gal*f* (i.e., activities C and D [Fig. 1]) by Kremer et al. (L. Kremer, L. G. Dover, C. Morehouse, P. Hitchin, M. Everett, H. R. Morris, A. Dell, P. J. Brennan, M. R. McNeil, C. Flaherty, K. Duncan, and G. S. Besra, 13 April 2001. J. Biol. Chem. 10.1074/jbc.M102022200).

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AUTHOR'S CORRECTION

Cell Wall Core Galactofuran Synthesis Is Essential for Growth of Mycobacteria

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Volume 183, no. 13, p. 3991–3998, 2001. Page 3994: The direction of the open reading frame of the kanamycin resistance cassette (*kan*) shown in Fig. 2 and 3 is incorrect. In fact, the open reading frame is in the opposite direction of what is shown in those figures, and thus, it is in the opposite direction of the open reading frame of *glf*. Recognition of the proper orientation of *kan* may explain why the insertion of *kan* into *glf* caused a polar effect on Rv3808c directly downstream of *glf* since the direction of transcription of *kan* is away from Rv3808c.