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Interruption of the Phosphoglucose Isomerase Gene Results in Glucose Auxotrophy in *Mycobacterium smegmatis*

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Two glycerol utilization mutants of *Mycobacterium smegmatis* that were unable to utilize most carbon sources except glucose were isolated. Supplementation of these media with small amounts of glucose restored growth in the mutants; these strains are therefore glucose auxotrophs. The mutant phenotype is complemented by the gene encoding phosphoglucose isomerase (*pgi*), and direct measurement of enzyme activities in the mutants suggests that this gene product is absent in the auxotrophic strains. Mapping of the mutant allele by Southern analysis demonstrates the presence of a 1-kb deletion extending into the coding sequence of *pgi*. The possible roles of phosphoglucose isomerase in mycobacterial cell wall synthesis and metabolic regulation are discussed.

The resurgence of diseases caused by mycobacteria has spurred a new interest in these acid-fast organisms. Although current research is largely directed towards virulence mechanisms in pathogenic strains of mycobacteria, basic biochemical information about these organisms is essential for understanding their interactions with their hosts and for developing new therapeutics. Knowledge of intermediary metabolism of the mycobacteria has been fragmentary, in large part due to the low growth rates, tendency to clump in culture, and lack of genetics systems of members of this genus. Mycobacteria exhibit unusual and complex patterns of carbohydrate utilization. In *Mycobacterium tuberculosis*, *Mycobacterium phlei*, and *Mycobacterium smegmatis*, glycerol utilization is blocked until all other carbon sources, including glutamate and asparagine, have been consumed (3, 23, 38). Nevertheless, glycerol is considered the preferred carbon source under laboratory conditions. Growth of most species on this substrate results in greater mass per unit volume of growth medium (32).

Glycerol and its metabolic derivatives are at the interface of catabolic (glycolytic and oxidative pathways) and anabolic (lipid biosynthesis and gluconeogenesis) processes and therefore play important growth regulatory roles in cells grown on a variety of carbon sources. In addition, glycerol is an important precursor of key cell wall constituents. The presence of glycolipids such as the acyl glycerols and acylglucoses varies widely depending on the carbon source in the medium (4). A number of the enzymes of glycolysis, the hexose monophosphate shunt, and the Entner-Doudoroff pathways have been studied (reviewed in reference 26). However, carbon metabolism in mycobacteria has not been characterized at the genetic level and few genes encoding glycolytic enzymes have been cloned.

We have initiated a genetic approach to the study of carbohydrate metabolism in *M. smegmatis* by isolating and characterizing mutants unable to utilize certain substrates as sole carbon sources. In this study, we describe two strains obtained in a screen for glycerol utilization mutants. Unexpectedly, these mutants are unable to use any carbohydrate except glucose as a sole carbon source. Genetic complementation of the growth defect on glycerol was accomplished by expression of the gene encoding phosphoglucose isomerase (*pgi*), which we have cloned and sequenced. The mutants lack detectable levels of Pgi, a key enzyme in glycolysis that functions at the juncture of gluconeogenesis and catabolism. Finally, we demonstrate that *pgi* mutants in *M. smegmatis* are defective in the utilization of many carbohydrates as sole carbon sources because they are actually auxotrophic for glucose.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used are shown in Table 1. *M. smegmatis* mc^{2}155 (27) was used for all mycobacterial experiments. *Escherichia coli* DH5α was used to propagate DNA for cloning, sequencing, and transforming *M. smegmatis*. mc^{2}743 and mc^{2}744 were derived in this study. The plasmids used were pYUB18 and a cosmid library contained therein (13), pMVI203 (28), and pDT252, created in this study, which is pMVI203 bearing a 1.9-kb fragment of mc^{2}155 DNA encoding the *pgi* gene.

Media. *M. smegmatis* was grown as described previously (6). The complete medium used was Middlebrook 7H9 (liquid) or 7H10 (agar; Difco). Minimal media were composed of basal salts medium (0.1% KH_2PO_4, 0.25% $NaHPO_4$, 0.5% NH_4Cl, 0.2% K_2SO_4) supplemented with either glucose (1.0%) or glycerol (0.5%). For auxotrophy studies, glucose was added at a concentration of 0.01%, a level insufficient to support growth in the absence of another carbon source. All liquid cultures were supplemented with 0.05% Tween 80 (Sigma Chemical Co.). Kanamycin sulfate (Sigma) was used at 50 μg/ml.

Mutant isolation. *M. smegmatis* (mc^{2}155) was mutagenized by treatment with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG; Sigma) at 100 μg/ml in basal salts medium. After treatment at 30°C for 2 h, the cells were pelleted and washed three times in basal salts medium with 0.05% Tween 80. The cells were resuspended in Middlebrook 7H9 medium with glycerol at 5 g/liter and glucose at 10 g/liter and incubated at 37°C for 3 h to allow expression of mutant gene products and then diluted onto minimal plates containing glucose and glycerol with 0.05% Tween 80 to facilitate replica plating. Colonies were replica plated onto minimal glycerol plates, and those that did not grow on glycerol were collected from the master plates.

Enzyme assays. Cell extracts were prepared by growing cells in the designated minimal medium to an A_{600} of 0.8 to 1.0. Cells were harvested by centrifugation at 3,000 × *g* for 10 min at 4°C and washed in 10 mM potassium phosphate buffer (1 mM EDTA and 1 mM dithiothreitol). The cell pellet was resuspended in an equal volume of buffer, and the cells were disrupted by passage twice through a French press at 18,000 lb/in^2. The extracts obtained were dialyzed overnight at 4°C in the same phosphate buffer and stored at −70°C.

Glucose phosphate isomerase activity was determined spectrophotometrically by measuring the decrease in A_{340} caused by reduction of β-NADP. Reactions of a total volume of 3 ml contained 42 mM glycylglycine, 3.3 mM D-fructose-6-

TABLE 1. Strains and plasmids used

Strain or plasmid	Description	Reference
Strains		
E. coli K-12 DH5α	*endA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1 recA1 gyrA*(Nal^r) *relA1* Δ(*lacIZYA-argF*)*U169* *deoR* [ϕ80d*lac* Δ(*lacZ*)M15]	12
M. smegmatis		
mc^2155	*ept-1*	27
mc^2743	*ept-1* Δ1(*pgi*)	This work
mc^2744	*ept-1 pgi2*	This work
Plasmids		
pYUB18		24
pMVI203		28
pDT252		This work

phosphate, 0.67 mM β-NADP, 3.3.mM $MgCl_2$, and 5.0 U of glucose-6-phosphate (G6P) dehydrogenase (8).

Triose phosphate isomerase activity was determined spectrophotometrically by measuring the decrease in A_{340} caused by reduction of β-NADP. Reactions of a total volume of 3 ml contained 280 mM triethanolamine, 245 mM glyceraldehyde-3-phosphate, 0.132 mM β-NADP, and 4 U of α-glycerolphosphate dehydrogenase (19).

α-Glycerol phosphate dehydrogenase activity was determined spectrophotometrically by measuring the decrease in A_{340} caused by reduction of β-NADP. Reactions of a total volume of 3 ml contained 290 mM triethanolamine, 2.5 mM dihydroxyacetone phosphate, and 0.13 mM β-NADP (14).

Complementation of mutants with wild-type genomic libraries. The carbohydrate utilization mutant strains mc^2743 and mc^2744 were transformed with a cosmid library prepared from mc^2155 (wild-type) DNA. This library was constructed by ligating 32- to 40-kb fragments of genomic DNA into pYUB18 (24). Transformants were plated on glucose minimal medium to estimate the transformation efficiency and on glycerol minimal medium to select complementing cosmids. mc^2743 was complemented for growth on glycerol by 12 cosmids (of 3×10^4 screened), and mc^2744 was complemented by 7 cosmids (of 6×10^3 screened). Cosmid DNA was isolated from two representative Glp^+ colonies of strains mc^2743 and mc^2744 and amplified in *E. coli* DH5α. These four cosmid DNAs were then used to transform mc^2743 and mc^2744. Each was fully capable of complementing mc^2743 and mc^2744 for growth on glycerol minimal plates. Two of the cosmids were then partially digested with *Sau*3A, and fragments in the range of 1,500 to 5,000 bp were isolated from an agarose gel. The *Sau*3A fragments were ligated into the *Bam*HI site of pMVI203. The resulting recombinant plasmids were pooled and used to transform mc^2743 and mc^2744. Nine plasmids were recovered. All nine complemented the glycerol-negative phenotype of mc^2743; mc^2744 was complemented fully by four plasmids. The four plasmids which complemented the glycerol-negative phenotype of both mc^2743 and mc^2744 showed identical restriction maps; the smallest of these, pDT252, bearing a 1.5-kb insert, was chosen for sequencing. That five plasmids did not complement both mutant strains suggests that mc^2744 may have an alternate or additional lesion, and this will be investigated further.

DNA sequencing and deletion mapping. The Erase-a-Base system (Promega) was used to generate a series of deletions from one end of the 1.5-kb insert in pDT252. The plasmid was digested with *Not*I and *Kpn*I and treated with exonuclease III for 0.5 to 10 min. Samples of the reaction were removed at 30-s intervals and separated on an agarose gel. DNA fragments differing by 200 bp in length were collected, treated with S1 nuclease and Klenow large fragment to introduce blunt ends, and self-ligated to produce a series of plasmids for sequencing. Sequencing was performed with an automated sequencer (Applied Biosystems).

Southern hybridization was performed to determine whether sequences found in the wild-type *pgi* region were missing in the mutant chromosome of mc^2743. Two micrograms of genomic DNA prepared from mc^2155 and mc^2743 was digested with various restriction enzymes and analyzed on agarose gels. The Southern transfer procedure used was described previously (15). The probe used for Southern analysis was the plasmid pDT252 containing the entire *pgi* gene.

Nucleotide sequence accession number. The 1,928-bp sequence bearing the phosphoglucose isomerase gene of *M. smegmatis* was deposited in GenBank under accession number U88433.

RESULTS

Isolation and complementation of glycerol utilization mutants. Glycerol-nonutilizing mutants of *M. smegmatis* were isolated as described in Materials and Methods. Of 40,000 colonies screened, two isolates, mc^2743 and mc^2744, were unable to use glycerol as a sole carbon source. mc^2743 and mc^2744 had colony morphologies and growth rates on glucose minimal plates similar to those of the wild-type parental strain, mc^2155 (data not shown).

Strains mc^2743 and mc^2744 were transformed with a cosmid library of mc^2155 (wild-type) DNA (see Materials and Methods). Several cosmids which complemented the glycerol deficiency in both strains were isolated. Analysis of restriction enzyme digests of four of the cosmids revealed overlapping inserts of similar sizes from the cosmids. An insert of 1.9 kb which complemented the deficiencies in strains mc^2743 and mc^2744 was subcloned from one of the complementing cosmids. The insert was cloned into pMVI203 to create pDT252. Initial restriction mapping of the clone and construction of internal deletions indicated that the entire 1.9 kb was required for complementation of growth on glycerol for both mutants (data not shown).

Characterization of the mycobacterial gene encoding phosphoglucose isomerase. The sequence of the region of wild-type *M. smegmatis* DNA complementing the two glycerol utilization mutants is shown in Fig. 1. The fragment contains a large single open reading frame (ORF) on one strand and two smaller ORFs on the opposite strand (data not shown). The single long ORF encodes a putative protein of 442 amino acids, beginning with GTG (Val) at position 420, with a predicted molecular mass of 47,649 Da. An alternative start codon is found at position 471, with a methionine codon (ATG), predicting a polypeptide product of 425 amino acids or 45,818 Da. The codon usage pattern is consistent with that of other mycobacterial genes, namely, a G+C content of >65%, due largely to G or C in the third position (36). To determine the identity of the ORF contained in the complementing fragment, we translated the 442-codon ORF and subjected the derived amino acid sequence to a homology search in GenBank by using FASTA. The predicted *M. smegmatis* sequence shows striking homology to that of the products of genes encoding the enzyme phosphoglucose isomerase (Pgi). Multiple sequence alignment demonstrated extensive amino acid homology between the *M. smegmatis* protein and the amino acid sequences of Pgi from *Homo sapiens* (37), *Saccharomyces cerevisiae* (31), *Leishmania mexicana* (22), *Bacillus stearothermophilus* (30), and *E. coli* (11) (data not shown). For example, human Pgi has 49.4% identity over a 447-amino-acid overlap, *S. cerevisiae* has 50.7% identity over a 436-amino-acid overlap, and *E. coli* has 52.9% identity over a 448-amino-acid overlap.

The mutant allele of mc^2743 was characterized by designing six pairs of primers to amplify the mutant gene in six overlapping fragments. It was observed that the 3′ end of the mc^2743 allele was resistant to amplification by PCR. Southern analysis identified a deletion of approximately 1 kb in the 3′ end of the *pgi* gene. Chromosomal DNA from wild-type and mutant cells was restricted with three enzymes (*Nco*I, *Pst*I, and *Sph*I) recognizing internal sites suggested by the sequence of the cloned fragment carrying the wild-type gene (data not shown). In all cases, there is an approximately 1-kb reduction in the size of one band of the mutant allele; a restriction map of the region is shown in Fig. 2. This result is consistent with the reversion frequency measured for this mutant, which is $<10^{-8}$ (data not shown). Precise determination of the deletion endpoints in the

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      10        20        30        40        50        60        70        80        90       100
1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
GGATCCACAGTCCGGTGGGG ACGGATGAAATCAGGTCTTC GATGCGCATACATCCATCAT GTACACCCTCGACACGTTCA CCGCACTAAGGTCGACACAA   100

TGAGCGCTGACATCACCGAA ACTCCCGCATGGCAGGCTTT GTCGGACCACCACGCCGAGA TCGGCGACCGGCATCTGACA GAACTGTTTGCTGACGATCC   200

CGCGCGGGGAACCGAGTTGG CGTTGACCGTCGGGGATCTC TACATCGACTACAGCAAGCA CCGCGTCACGCGGCGCACCC TGGACCTGCTCGTCGACCTG   300

GCGCGCGCGGCCGGGTTGGA GGAGCGCCGGGATGCGATGT TCGCCGGCGAGCACATCAAC ACCTCCGAGGACCGCGCGGT GCTGCACACCGCGCTGCGCT   400

CCCGCGGGACGCCAAGCTGG TGGTGGACGGTCAGGACGTG GTCGCCGATGTGCACGACGT GCTGGACCGCATGGGCGATT TCACCGATCGGTTGCGCAGC   500
                     V   V  D  G  Q  D  V   V  A  D  V  H  D  V   L  D  R  M  G  D  F   T  D  R  L  R  S
GGAGAGTGGACCGGCGCCAC CGGCGAACGCATCACCACGG TGGTCAACATCGGCATCGGC GGCTCGGATCTGGGTCCGGT GATGGTGTACGACGCGCTGC   600
G  E  W  T  G  A  T   G  E  R  I  T  T  V   V  N  I  G  I  G   G  S  D  L  G  P  V   M  V  Y  D  A  L  R
GCCATTACGCCGACGCCGGC ATCTCGGCGCGCTTCGTGTC CAACGTCGACCCCGCCGATC TGGTCGCCAAACTCGACGGC CTGGAGCCGGCGAAGACGTT   700
  H  Y  A  D  A  G   I  S  A  R  F  V  S   N  V  D  P  A  D  L   V  A  K  L  D  G   L  E  P  A  K  T  L
GTTCATCGTCGCCTCCAAGA CGTTCTCGACGCTGGAGACG CTGACCAATGCGACCGCGGC GCGGCGCTGGCTCACCGACG CCCTGGGTGACGCCGCGGTG   800
 F  I  V  A  S  K  T   F  S  T  L  E  T   L  T  N  A  T  A  A   R  R  W  L  T  D  A   L  G  D  A  A  V
GCCAAGCATTCGTCGCGGTG TCCACCAACAAGAAGCTGGT CGACGAAGTTCGGCATCAAC ACCGACAACATGTTCGGGTT CTGGGACTGGGTGGGCGGGC   900
A  K  H  S  S  R  C   P  P  T  R  S  W  S   T  K  F  G  I  N   T  D  N  M  F  G  F   W  D  W  V  G  G  R
GCTACTCGGTGGACAGTGCG ATCGGGTTGAGCGTGATGGC CGTGATCGGCAAGGAACGCT TCGCCGAGTTCCTGGCCGGG TTCCACATCGTCGACGAGCA  1000
  Y  S  V  D  S  A   I  G  L  S  V  M  A   V  I  G  K  E  R  F   A  E  F  L  A  G   F  H  I  V  D  E  H
CTTCCGCACCGCCCCGCTGC ACCAGAACGCTCCGGCGCTG CTGGGGCTGATCGGGCTGTG GTACTCGAATTTCTTTGGCG CGCAATCCCGTGCGGTGCTG  1100
 F  R  T  A  P  L  H   Q  N  A  P  A  L   L  G  L  I  G  L  W   Y  S  N  F  F  G  A   Q  S  R  A  V  L
CCCTACTCCAACGACCTGTC ACGGTTCGCGGCGTACCTGC AGCAGTTGACCATGGAATCC AACGGCAAGTCGGTACGCGC CGACGGCACACCGGTGTCCA  1200
P  Y  S  N  D  L  S   R  F  A  A  Y  L  Q   Q  L  T  M  E  S   N  G  K  S  V  R  A   D  G  T  P  V  S  T
CCGACACCGGTGAGATCTTC TGGGGCGAGCCGGGCACCAA CGGCCAGCACGCCTTCTACC AGTTGCTGCACCAGGGCACC CGACTGGTGCCCGCGGACTT  1300
  D  T  G  E  I  F   W  G  E  P  G  T  N   G  Q  H  A  F  Y  Q   L  L  H  Q  G  T   R  L  V  P  A  D  F
CATCGGGTTCTCCCAGCCCA CCGACGACCTGCCCACGGCC GATGGCACCGGCAGCATGCA CGACCTGCTGATGAGCAACT TCTTCGCCCAGACCCAGGTG  1400
 I  G  F  S  Q  P  T   D  D  L  P  T  A   D  G  T  G  S  M  H   D  L  L  M  S  N  F   F  A  Q  T  Q  V
CTGGCATTCGGAAAGACCGC CGACGCAATAGCTTCCGAGG GCACCCCCGCAGATGTGGTG CCGCACAAGGTGATGCCCGG CAACCGGCCCACCACCAGCA  1500
L  A  F  G  K  T  A   D  A  I  A  S  E  G   T  P  A  D  V  V   P  H  K  V  M  P  G   N  R  P  T  T  S  I
TCCTCGCCACCAAGCTCACC CCGTCGGTCGTCGGGCAGTT GATCGCCCTCTACGAACATC AGGTGTTCACCGAAGGCGTG ATCTGGGGCATCGACTCGTT  1600
  L  A  T  K  L  T   P  S  V  V  G  Q  L   I  A  L  Y  E  H  Q   V  F  T  E  G  V   I  W  G  I  D  S  F
CGACCAGTGGGGCGTGGAAC TCGGCAAGACGCAGGCCAAG GCCCTGCTCCCGGTTCTGAC CGGCGACAAATCTCCTGCCG CGCAGTCGGATACGTCCACC  1700
 D  Q  W  G  V  E  L   G  K  T  Q  A  K   A  L  L  P  V  L  T   G  D  K  S  P  A  A   Q  S  D  T  S  T
GACGCCCTGGTGCGGCGCTA CCGGACCGAGCGGGGCCGGC CCGCTTAGGCTTCGGCGTCG GCTTCTGCGTCCGAACGCCT GGTCCAGCCCAGGATCATCG  1800
D  A  L  V  R  R  Y   R  T  E  R  G  R  P   A  .
CGGGCAGGCAGCTGCCGATC ATCAAGGCCGCCAAGGCCAT CAGCGCACCGGCATAGGCCA GGTCGCGGATGGTCTTGGCG TCGGCGCCGGTTCAGCTGCG  1900

TCGACACGATGATCCAGCTG CAGAATTC                                                                            1928
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FIG. 1. Nucleotide and deduced amino acid sequences of the *Eco*RI-*Bam*HI fragment containing the phosphoglucose isomerase gene of *M. smegmatis*. Two putative start codons are underlined.

pgi gene of mc^{2}743 and characterization of the mutant allele of mc^{2}744 are under way.

Characterization of *pgi* mutants. The enzyme Pgi exerts considerable control at a pivotal point at the juncture of three metabolic pathways, i.e., the Embden-Meyerhoff-Parnass, the Entner-Doudoroff, and the phosphogluconate pathways (Fig. 3) (see Discussion). We examined the growth of the parental strain (mc^{2}155) and the two glycerol utilization mutants (mc^{2}743 and mc^{2}744) on nine carbon sources, which is summarized in Table 2. Each of the three strains carries the *E. coli*-mycobacterium shuttle vector, pMVI203, containing a kanamycin resistance gene and a multiple cloning site (28).

The noncomplemented mutant strains mc^{2}743 and mc^{2}744 grow only on glucose and poorly on galactose: no other carbohydrate tested (glycerol, fructose, succinate, Casamino Acids, gluconate, sorbitol, or ribose) was able to serve as a sole carbon source for growth. This suggests that the metabolic requirement is G6P or some derivative thereof, since of the nine carbon sources tested, only galactose typically enters the glycolytic pathway above G6P (Fig. 3). Pgi, which catalyzes the conversion of G6P to fructose-6-phosphate, is the only source of G6P during growth on substrates other than glucose or galactose.

We tested the growth of the wild-type and mutant strains carrying the complementing plasmid, pDT252, on these carbon sources. Table 2 shows that the wild-type and mutant strains grow equally well on glycerol minimal media in the presence of *pgi* carried on a multicopy plasmid (pDT252). This indicates

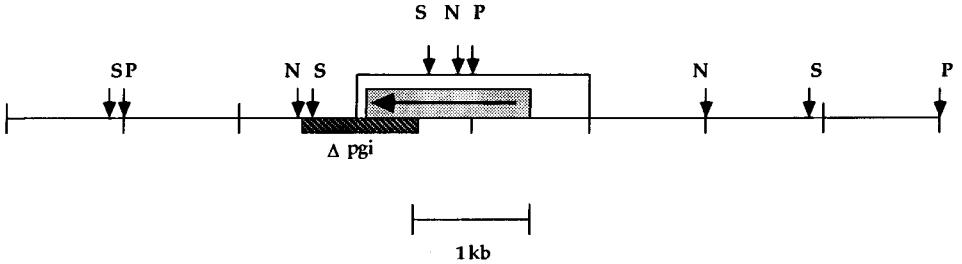

FIG. 2. Restriction map of the *pgi* region of *M. smegmatis*. *Pst*I (P), *Nco*I (N), and *Sph*I (S) were used to analyze chromosomal DNA of mc^{2}155 and mc^{2}743 by Southern analysis (see Materials and Methods). The sequenced region is indicated by an unshaded box. A horizontal arrow indicates the direction of transcription of the *pgi* ORF (shaded area). A hatched bar indicates the extent of deletion in mutant strain mc^{2}743.

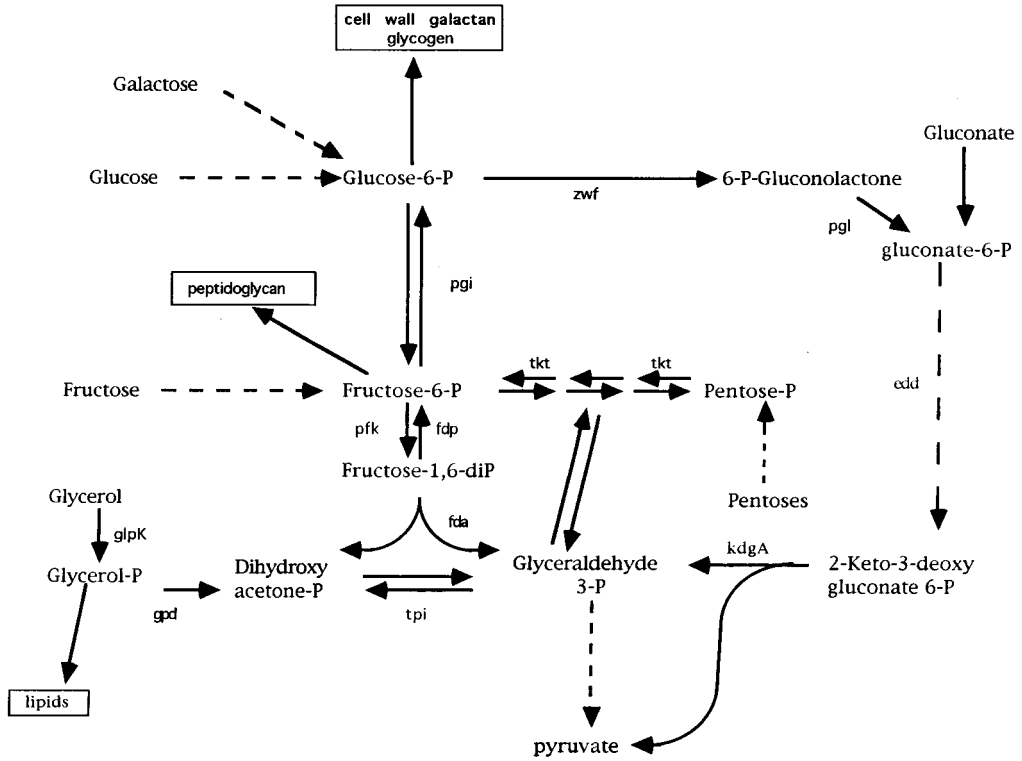

FIG. 3. Intermediary metabolic pathways in bacteria known to be present in mycobacteria (26). The routes to cell wall components are indicated.

that the *pgi* gene is sufficient to restore wild-type growth abilities to the two mutants.

To corroborate these phenotypic observations, levels of three glycerol and glucose metabolic enzymes were measured in extracts prepared from wild-type cells, the *pgi* mutants, and all three strains carrying the cloned copy of the *pgi* gene. We prepared extracts from wild-type and mutant cells carrying either the cloning vector pMVI203 or the *pgi* plasmid pDT252 growing in either glucose or glycerol. Table 3 shows the levels of Pgi in wild-type cells and in mc^{2}743 and mc^{2}744. The wild-type strain had high levels of Pgi in both glycerol and glucose minimal medium, indicating constitutive expression of the enzyme. However, the mutant strains mc^{2}743 and mc^{2}744 showed at least a 1,000-fold reduction in Pgi activity when grown on glucose. In contrast, the presence of the cloned copy of the complementing gene restored Pgi activity to the mutant strains.

As controls for the cell extracts and growth conditions, we measured the levels of α-glycerol phosphate dehydrogenase (GlpD) and triose phosphate isomerase (Tpi). The former should be regulated in response to a carbon source (i.e., repressed by glucose) (3), whereas the latter should be constitu-

TABLE 2. Growth patterns of the wild type and glycerol mutants on various substrates[a]

Carbon source (concn)	Growth pattern[b]					
	mc^{2}155(pMVI203)	mc^{2}743(pMVI203)	mc^{2}744(pMVI203)	mc^{2}155(pDT252)	mc^{2}743(pDT252)	mc^{2}744(pDT252)
Glucose	+	+	+	+	+	+
Glycerol	+	−	−	+	+	+
Glucose + glycerol	+	+	+	+	+	+
Fructose	+	−	−	+	+	+
Sorbitol	+	−	−	+	+	+
Ribose	+	−	−	+	+	+
Galactose	+	±	±	+	+	+
Gluconate	+	−	−	+	+	+
Succinate	+	−	−	+	+	+
Succinate + glycerol	+	−	−	+	+	+
Casamino Acids	+	−	−	+	+	+
Glucose (0.1%)	±	±	±	ND[c]	ND	ND
Glucose (0.01%)	−	−	−	ND	ND	ND
Glycerol + glucose (0.01%)	+	+	+	ND	ND	ND

[a] Cells were streaked for single colonies on minimal basal salts plates supplemented with the indicated carbon sources at 10 g/liter, except as indicated.
[b] Symbols: +, large single colonies; ±, small single colonies; −, no single colonies.
[c] ND, not done.

TABLE 3. Levels of phosphoglucose isomerase, triose phosphate isomerase, and α-glycerol phosphate dehydrogenase activities in wild-type and mutant strains with and without the *pgi* gene carried on plasmid pMVI203[a]

Strain	Activity[b]		
	Pgi	Tpi	GlpD
Glycerol grown			
mc^{2}155(pMVI203)	59.6 ± 0.5	16.3 ± 0.2	12.4 ± 0.4
mc^{2}743(pMVI203)	NG[c]	NG	NG
mc^{2}744(pMVI203)	NG	NG	NG
mc^{2}155(pDT252)	120.9 ± 0.1	9.8 ± 0.03	12.5 ± 0.6
mc^{2}743(pDT252)	81.0 ± 0.8	14.5 ± 0.1	12.4 ± 0.4
mc^{2}744(pDT252)	78.2 ± 0.2	7.9 ± 0.05	0.9 ± 0.1
Glucose grown			
mc^{2}155(pMVI203)	51.8 ± 0.8	10.8 ± 0.2	<0.6
mc^{2}743(pMVI203)	<0.6	8.2 ± 0.2	<0.6
mc^{2}744(pMVI203)	<0.6	8.5 ± 0.3	<0.6
mc^{2}155(pDT252)	141.7 ± 0.3	11.3 ± 0.3	<0.6
mc^{2}743(pDT252)	95.2 ± 0.5	11.0 ± 0.9	<0.6
mc^{2}744(pDT252)	82.4 ± 0.3	9.4 ± 0.1	<0.6

[a] Assays were performed three times on two extracts.
[b] Activity is expressed in units per milligram of cell extract.
[c] NG, no growth (mutants do not grow in glycerol minimal medium).

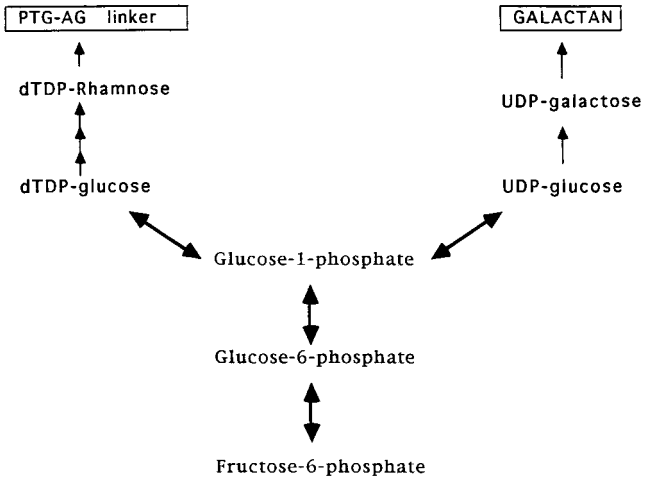

FIG. 4. Relevant cell wall synthetic pathways. Enzymes whose activity has been identified in mycobacteria are indicated (see Discussion).

tively expressed. Tpi is found at similar levels in extracts from all six strains (Table 3). As expected, GlpD levels were barely detectable in glucose-grown cells of all six strains, suggesting that the mutants bear no severe regulatory defects with respect to glycerol and glucose regulation of glycerol enzymes (3). However, mc^{2}744(pDT252), which exhibits wild-type levels of Pgi activity, does show slightly reduced levels of GlpD activity when grown on glycerol (Table 3). The basis of this sole difference between the two *pgi* mutants is under examination.

Pgi mutants of *M. smegmatis* are glucose auxotrophs. Since the *pgi* mutants of *M. smegmatis* grow only on glucose, we tested the possibility that they actually require glucose for growth. We supplied extremely low concentrations of glucose in media composed of other carbon sources. Glucose at a concentration of 0.01% is not sufficient to support the growth of wild-type *M. smegmatis* in the absence of another carbon source (Table 2). When this low concentration of glucose was added to glycerol (Table 2) or to the other carbon sources listed in Table 2, mc^{2}743 and mc^{2}744 grew at wild-type levels in every case (data not shown). This experiment demonstrates that low levels of glucose are required for growth of mycobacterial *pgi* mutants on substrates other than glucose, indicating an absolute requirement in these mutants for glucose or G6P.

DISCUSSION

We have isolated mutants of *M. smegmatis* lacking a functional gene encoding the enzyme phosphoglucose isomerase (*pgi*). These mutants can grow only on glucose or galactose as a sole carbon source. However, any carbon source will support wild-type levels of growth if glucose is provided in trace amounts. We used the mutants to clone the gene encoding *pgi* and showed that its sequence is highly homologous to that of the *pgi* genes of other species. We measured the levels of Pgi in the wild type and mutants and showed that there is no detectable activity of Pgi in the mutants. Complementation of the mutants with the cloned copy of *pgi* restored both intracellular levels of Pgi and the wild-type growth phenotype when tested on a large number of carbon sources. Finally, we demonstrated that the *pgi* mutants are glucose auxotrophs, since (i) the mutants grow only on glucose as the sole carbon source and (ii) supplementation of carbon sources other than glucose with trace amounts of glucose restores wild-type growth.

The absolute requirement for glucose by *pgi* mutants of *M. smegmatis* may be explained by examining the structure of the mycobacterial cell wall and its synthetic pathways. The major polysaccharide is arabinogalactan (AG), a large polymer positioned by covalent linkage between the peptidoglycan (PTG) and mycolic acid (MA) (18). MA, constituting close to 50% by weight of the cell wall mass, is likely responsible for the notorious impermeability of the mycobacterial cell. The ability to synthesize any of the components of the PTG-AG-MA network, or cell wall core, is expected to be essential, and the enzymes involved in its synthesis are targets of current or future antimycobacterial drugs (17).

A key feature in this covalent polymeric network is the linker disaccharide phosphate which links the PTG to the AG (16). The structure of the PTG-AG linker is L-rhamnose-(1-3)-D-*N*-acetylglucosamine-(1-P-6)-D-muramic acid (16). As illustrated in Fig. 4, G6P, via glucose-1-phosphate, is the precursor for the L-rhamnose component of the linker molecule. The donor of this component, dTDP-rhamnose, is synthesized from glucose-1-phosphate and TTP (18) in the same manner as it is in gram-negative bacteria, which use dTDP-rhamnose to insert rhamnose into lipopolysaccharide (LPS) O antigens (39). Thus, inability to synthesize G6P will inhibit cell wall core formation and lead to loss of viability of the cell.

A second component of the mycobacterial cell wall requiring G6P for its synthesis is the galactan residue of AG. Again, by analogy with other polysaccharide synthetic systems (33, 34), G6P is converted to G1P and thence to UDP-glucose; an activity has been identified in mycobacteria which converts UDP-glucose to UDP-galactose for transfer into the AG complex (16). Inhibition of AG synthesis is lethal for the mycobacterial cell and is most likely the mechanism of action of ethambutol (29).

Pgi is a key enzyme in the pathway of cell wall biosynthesis in mycobacteria. Our observation that 0.01% glucose (10^{-5} g/ml) supplies sufficient precursor for cell wall core synthesis is supported by calculations of the amount of carbon directed into L-rhamnose and galactan from G6P, assuming that there is 1 rhamnose molecule for every 33 galactose molecules and that

the cell wall galactan is 1% of the dry weight of the cell (approximately 3×10^{-6} g/ml of saturated culture) (17a, 21).

Phosphoglucose isomerase mutants have been well characterized in a number of other genetic systems. The salient phenotypes of *Bacillus subtilis* (25) and *E. coli* (35) mutants lacking phosphoglucose isomerase are (i) very slow growth on glucose and galactose, (ii) normal growth on carbon sources entering glycolysis below the block, such as fructose and glycerol, and (iii) normal growth on gluconate, which is metabolized via either the Entner-Doudoroff pathway or the hexose monophosphate shunt. The slow growth on glucose is presumed to be the result of the inefficiency of growth on these latter pathways. *pgi* mutants of *E. coli* can grow on gluconate as the sole carbon source because they do not require G6P or any of its derivatives for growth (8, 18); gluconate is metabolized via the pentose phosphate or Entner-Doudoroff pathways (Fig. 3). The glucose auxotrophy of the *pgi* mutants of *M. smegmatis* described here predicts that these mutants cannot grow on gluconate, since the enzymes leading from G6P to gluconate-6-phosphate are unidirectional (Fig. 3). Table 2 shows that this is indeed the case.

In contrast, *pgi1* mutants of *S. cerevisiae* cannot grow on glucose as the sole carbon source, yet they are glucose auxotrophs (1). *pgi1* null mutants of *S. cerevisiae* have been well characterized. These strains cannot grow on glucose, presumably because flux through the hexose monophosphate shunt is inadequate to support growth (5). Yet, *pgi1* null cells also require glucose for growth and sporulation on other substrates (1). Indeed, the locus is also known as *CDC30*, since *cdc30* and *pgi1* cells arrest late in nuclear division at the nonpermissive temperature (7). A number of *CDC* genes encode intermediary metabolic genes. For example, *CDC19* encodes a temperature-sensitive pyruvate kinase (20). The sensitivity of *S. cerevisiae pgi1* null strains to high concentrations of glucose may explain the cell cycle phenotype as the result of accumulation of G6P in arrested cells, since many phosphorylated intermediates are toxic when allowed to accumulate (1).

In addition to its involvement in yeast cell cycle control, phosphoglucose isomerase has recently been implicated in growth phase gene regulation in *E. coli*. The *pgi* gene was identified in a screen for insertions which affect in *trans* the expression of a *lacZ* fusion to *osmY*, a gene regulated by the stationary-phase sigma factor RpoS (2). The authors of the *E. coli* study propose that UDP-glucose, a derivative of G6P, is an intracellular signal molecule in the control of expression of a number of *rpoS*-regulated genes. While the mycobacterial *rpoS* itself has not been cloned, a mycobacterial homolog of *sigF* (*B. subtilis* and *Streptomyces coelicolor*) and *sigB* (*B. subtilis*) has recently been cloned (13) and the regulatory targets of this and other putative stationary-phase and stress response regulatory molecules will soon be identified. It will be interesting to see whether *pgi* mutants of *M. smegmatis* are similarly altered in expression of *rpoS*-regulated genes, and these experiments are under way.

That *E. coli* and *S. typhimurium pgi* null mutants can grow, albeit slowly, on glucose suggests that there is no requirement for G6P and its derivatives in these species (35). Thus, *pgi* mutants of *S. typhimurium* grown on gluconate have incomplete LPS, since G6P cannot be formed from gluconate (9). In *pgi* mutants of *E. coli*, complete polymers of LPS or glycogen are produced only in the presence of glucose, and this feature can be exploited in the study of LPS or glycogen synthesis (10). Similarly, the mutants described here could serve as useful labeling strains. *M. smegmatis* appears to have an absolute requirement for glucose or a derivative thereof: growth of the cells on succinate or glycerol with trace labels of high-specific-activity [^{14}C]glucose would direct radiolabel only into those lipid moieties derived from glucose. We have initiated the construction of *pgi* deletion mutants to investigate the roles of *pgi* in carbohydrate metabolism and cell wall synthesis in the pathogenic slow-growing species such as *M. tuberculosis* and *Mycobacterium bovis.*

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