

Use of Transposon Tn5367 Mutagenesis and a Nitroimidazopyran-Based Selection System To Demonstrate a Requirement for *fbiA* and *fbiB* in Coenzyme F₄₂₀ Biosynthesis by *Mycobacterium bovis* BCG

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Three transposon Tn5367 mutagenesis vectors (pAE94, pPR28, and pPR29) were used to create a collection of insertion mutants of *Mycobacterium bovis* strain BCG. A strategy to select for transposon-generated mutants that cannot make coenzyme F₄₂₀ was developed using the nitroimidazopyran-based antituberculosis drug PA-824. One-third of 134 PA-824-resistant mutants were defective in F₄₂₀ accumulation. Two mutants that could not make F₄₂₀-5,6 but which made the biosynthesis intermediate FO were examined more closely. These mutants contained transposons inserted in two adjacent homologues of *Mycobacterium tuberculosis* genes, which we have named *fbiA* and *fbiB* for F₄₂₀ biosynthesis. Homologues of *fbiA* were found in all seven microorganisms that have been fully sequenced and annotated and that are known to make F₄₂₀. *fbiB* homologues were found in all but one such organism. Complementation of the *fbiA* mutant with *fbiAB* and complementation of the *fbiB* mutant with *fbiB* both restored the F₄₂₀-5,6 phenotype. Complementation of the *fbiA* mutant with *fbiA* or *fbiB* alone did not restore the F₄₂₀-5,6 phenotype, but the *fbiA* mutant complemented with *fbiA* produced F₄₂₀-2,3,4 at levels similar to F₄₂₀-5,6 made by the wild-type strain, but produced much less F₄₂₀-5. These data demonstrate that both genes are essential for normal F₄₂₀-5,6 production and suggest that the *fbiA* mutation has a partial polar effect on *fbiB*. Reverse transcription-PCR data demonstrated that *fbiA* and *fbiB* constitute an operon. However, very low levels of *fbiB* mRNA are produced by the *fbiA* mutant, suggesting that a low-level alternative start site is located upstream of *fbiB*. The specific reactions catalyzed by FbiA and FbiB are unknown, but both function between FO and F₄₂₀-5,6, since FO is made by both mutants.

F₄₂₀ is a 7,8-didemethyl-8-hydroxy 5-deazaflavin electron transfer coenzyme that is present in few microorganisms. It was first described in detail in methanogens (11, 12). *Methanobacterium* F₄₂₀ contains two glutamates (12), while *Mycobacterium* contains primarily five- and six-glutamate forms (F₄₂₀-5 and F₄₂₀-6). Figure 1A shows the structure of F₄₂₀-5 (4). FO is a biosynthetic intermediate that contains the deazaflavin ring and ribityl, but lacks phosphate, lactyl, and glutamate moieties. In archaea, F₄₂₀ is essential for methylenetetrahydromethanopterin reductase, some hydrogenases, formate and methylenetetrahydromethanopterin dehydrogenases, some alcohol dehydrogenases, and quinone oxidoreductase activities (18, 21, 23, 26, 27, 47). F₄₂₀ is used by *Streptomyces* for tetracycline and lincomycin biosynthesis (8, 31, 39) and perhaps in mitomycin C biosynthesis (29). In *Mycobacterium* and *Nocardia*, F₄₂₀ is used by F₄₂₀-dependent glucose-6-phosphate dehydrogenase (35, 36) and is required for activation of the experimental antituberculosis drug PA-824 by *Mycobacterium tuberculosis* and *Mycobacterium bovis* strain BCG (45). The green alga *Scenedesmus*, the cyanobacterium *Synechocystis*, and the archaeon *Halobacterium* use F₄₂₀ in their photolyase (14, 15, 32).

The pathway by which F₄₂₀ is made has been explored with labeling approaches in methanogens (13, 22, 38). Also, recent enzymatic work with methanogens has shown that 2-phospho-

lactate serves as a precursor of the lactyl and phosphate groups of F₄₂₀ (17).

A simplified overview of the current hypothetical pathway is shown in Fig. 1B. The genes used for F₄₂₀ biosynthesis have never been described. Because of our interest in F₄₂₀ biosynthesis and in the metabolism of *M. tuberculosis*, we have undertaken a study to determine the genes involved in F₄₂₀ biosynthesis in *Mycobacterium* spp. Identification of these genes will enable us to create knockout mutants of *M. tuberculosis* to study in animal models in order to determine if F₄₂₀ is important for virulence. We believe that a role for F₄₂₀ in virulence is possible because preliminary studies have indicated that *M. bovis* mutants not producing F₄₂₀ are more sensitive than the wild-type strain to some oxidative stress agents (K.-P. Choi, unpublished observations).

We anticipated that mycobacteria would be more amenable than most other organisms for a study of F₄₂₀ biosynthesis for three reasons. First, since *Streptomyces* mutants lacking F₄₂₀ biosynthetic capabilities can grow without F₄₂₀ supplementation (8, 31, 39), we expected that F₄₂₀ would not be required for in vitro growth of the closely related mycobacteria, whereas in several archaea F₄₂₀ is essential for growth. Thus, with mycobacteria, no supplementation with F₄₂₀ should be required in the screening medium (F₄₂₀ is not commercially available and is expensive to produce, e.g., compared to the purchase of NADP.). Second, these organisms are considerably easier to grow than methanogens or *Archaeoglobus*. Third, several good systems exist for creating Tn5367 insertion mutants in *Mycobacterium* species (5, 30, 34).

We describe here our development of a strategy to select

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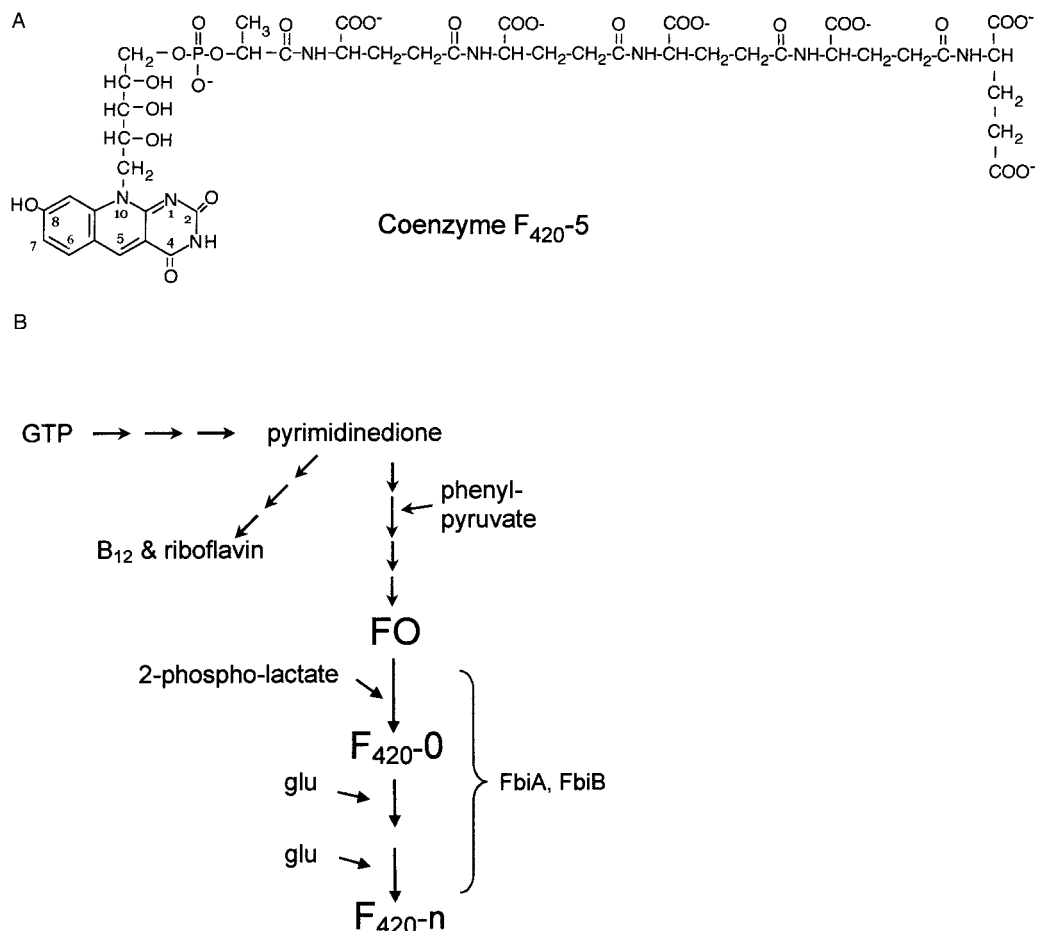


FIG. 1. (A) Structure of coenzyme F₄₂₀₋₅ from *Mycobacterium* spp.(4). (B) Overview of the hypothesized pathway for F₄₂₀ biosynthesis, which is based on our modifications of the reactions proposed by Bacher and Thauer and their colleagues (13, 22, 38), and including the role of 2-phospholactate proposed by Graupner and White (17).

transposon insertion mutants of *M. bovis* which cannot make F₄₂₀. We then demonstrate the utility of this method to identify the *M. bovis* homologues of the Rv3261 and Rv3262 genes from *M. tuberculosis* as essential for a step or steps in the pathway between FO and F_{420-5,6}, after the deazaflavin ring is formed.

MATERIALS AND METHODS

Bacteria, plasmids, and culture conditions. The bacteria and plasmids used in this study are given in Table 1. *M. bovis* was typically grown in Middlebrook 7H9 liquid medium or on 7H10 agar plates supplemented with 0.05% Tween 80 (liquid medium only), 0.2% glycerol, and Bacto Middlebrook ADC enrichment. *Escherichia coli* JM109 and pGEM were used as recombinant host and vector, respectively. *E. coli* was grown at 37°C on Luria-Bertani medium; ampicillin (100 µg/ml), isopropyl-β-D-thiogalactopyranoside (IPTG; 0.5 mM), and 3-indolyl-β-D-galactopyranoside (X-Gal; 80 µg/ml) were incorporated to select for and identify recombinants.

HPLC. F₄₂₀ and FO were separated by high-pressure liquid chromatography (HPLC) using a Beckman System Gold Nouveau 126 HPLC with a Shimadzu RF-10AxI fluorescence detector (excitation at 400 nm, emission at 470 nm). HPLC was performed using a method similar to that of Gorris (16), with modifications. An α-Bondpack C-18 (Supelco) column (3.9 mm by 300 mm) was eluted at 1 ml/min. Buffer A was 27.5 mM sodium acetate (pH 4.7) containing 2% acetonitrile; buffer B was 100% acetonitrile. The portion of buffer B in the elution buffer was varied as follows: 0 to 2 min, 0%; 2 to 6 min, 0 to 2% (linear gradient); 6 to 15 min, 2 to 10%; 15 to 22 min, 28%; 22 to 27 min, 28 to 0%.

Extracts of cultures to be examined were prepared by heating approximately equal volumes of a wet cell pellet and buffer A at >90°C for 15 min, followed by centrifugation to remove the pellet.

Molecular biology techniques. Chromosomal DNA was purified according to Husson et al. (19). Access reverse transcription (RT)-PCR System and Wizard minicolumns for plasmid purification were from Promega (Madison, Wis.). RNA was purified according to Mangan et al. (28) and processed with an RNeasy Kit (Qiagen). Standard *E. coli* cloning protocols were used (40). Primers are described in Table 1. Sequencing was done at the University of Iowa DNA Facility using an Applied Biosystems 373A sequencer. PA-824 (a nitroimidazopyran used to select F₄₂₀-minus mutants) was a gift of the PathoGenesis Corporation.

Insertion mutants of *M. bovis* were created with the phage phAE94 using techniques described by Bardarov et al. (5), but with the incorporation of PA-824 selection. Phage prepared from *Mycobacterium smegmatis* grown at 30°C was incubated with the target *M. bovis*. The infected culture was then grown at 37°C on 7H9-ADC agar medium containing 20 µg of kanamycin/ml, which did not allow growth of the phage due to a temperature-sensitive mutation. Colonies from these plates were combined and spread on plates of 7H9-ADC agar with kanamycin (20 µg/ml) and PA-824 (10 µg/ml). Colonies that grew on these plates were examined by PCR for the presence of uninterrupted *fgd* (*fgd* codes for F₄₂₀-dependent glucose-6-phosphate dehydrogenase [35, 36]). Insertion mutants with an intact *fgd* that survived this selection were further propagated and analyzed by HPLC for the presence of 5-deazaflavins.

Transposon insertions in F₄₂₀-minus mutants created with phAE94 were identified using a semirandom two-step PCR procedure modified from the original method (7). The primers that corresponded to the known transposon sequence, HOPS1 and HOPS2, were taken from Bardarov et al. (5). The random primer Semi-rand 2-1 was independently developed, but Semi-rand 4 was the same as

TABLE 1. Bacterial strains, transposon, plasmids, and DNA primers used in this study

Strain, transposon, plasmid, or primer	Description or DNA sequence	Source or reference
Strains		
<i>M. bovis</i> BCG	Montreal <i>M. bovis</i> strain BCG	C. Kendall Stover, PathoGenesis Corp.
<i>M. smegmatis</i> mc ² 155	Efficient plasmid transformant mutant (<i>ept-1</i>)	William R. Jacobs Jr., Albert Einstein College of Medicine (44)
<i>E. coli</i> JM109	RecA ⁻ , pGEM host strain	Promega, Madison Wis.
<i>M. bovis</i> <i>fbiA</i> mutant	<i>M. bovis</i> strain with <i>fbiA</i> ::Tn5367	This study
<i>M. bovis</i> <i>fbiB</i> mutant	<i>M. bovis</i> strain with <i>fbiB</i> ::Tn5367	This study
Transposon Tn5367	IS 1096 with kanamycin resistance cassette	Brigitte Gicquel, Institut Pasteur (5, 30, 34)
Plasmids and phasmid		
pGEM	T/A PCR cloning vector	Promega, Madison, Wis.
pFbiAB	pGEM containing <i>fbiA</i> and <i>fbiB</i>	This study
pPR28	<i>sacB</i> (Ts) delivery plasmid pPR27 with Tn5367 insert	Brigitte Gicquel, Institut Pasteur (34)
pPR29	<i>sacB</i> (Ts) delivery plasmid pPR27 with Tn5367 insert	Brigitte Gicquel, Institut Pasteur (34)
phAE94	Temperature-sensitive shuttle plasmid with a Tn5367 insert; contains <i>oriM</i> (-Ts); no replication at 37°C	William R. Jacobs Jr., Albert Einstein College of Medicine (5)
pSMT3	<i>Mycobacterium</i> complementation vector	Larry Schlesinger (33)
p3261	pSMT3 containing <i>fbiA</i> as 1,150-bp insert	This study
p3262	pSMT3 containing <i>fbiB</i> as 1,454-bp insert	This study
p61-62	pSMT3 containing <i>fbiA</i> and <i>fbiB</i> as 2,425-bp insert	This study
Sequencing primers (5' → 3')		
T7	TAATACGACTCACTATAGGG	Sigma-Genosys
SP6	CATACGATTTAGGTGACACTATAG	Sigma-Genosys
Primers for inverse and semirandom PCR and RT-PCR (5' → 3')		
Tnp2	GTGGACGTTGTTGCCATGGA	This study
Tnp3	CGCGTTGAGGATTCTTCACC	This study
Tnp4	CCATTAGCTTCTGCAGCAAC	This study
HOPS1	GGCGTAGGAACCTCCATCATC	Bardarov et al. (5)
HOPS2	CTTGCTCTCCGCTTCTTCTCC	Bardarov et al. (5)
YMB1	GTACGTCACCGTCATCATCG	This study
YMB2	GAGTCACGCTGTCACGTACA	This study
Semi-rand 2-1	GGCCACGCGTCGACTAGTAC-(N) ₁₀ -GCAGC	This study
Semi-rand 4	(CUA)-GGCCACGCGTCGACTAGTAC	Chun et al. (7)
F1	GAAACTTGGCACGCCATG	This study
R2	CTCGCATTTGGACACCACCTTG	This study
F3	CGATACGTGCCTTTCGGTTATC	This study
R4	CATGGTGTGGTGGATGAC	This study
F5	GGCTGCTAGATCGGATGA	This study
R6	TGTGCAGCCAGGTA AAAAAGG	This study
3260-2	GAATTGATCCGTCGGGAGGTTCT	This study
3260-3	CTCCGCCTCCTCACTCGATAGA	This study
3260-9	GTGGACGGATCCAACGTC	This study
3261-1	GCAATGAGTTGGTGGTCACC	This study
3262-2	CAAAGATCGTCGAGCCGATC	This study
3260C-1	CTGGATATCCGATGTGAAGGTCACCGTTCTG	This study
3260C-2	GTGAAGCTTGAATTGATCCGTCGGGAGGTTG	This study
3261C-2	CACAAGCTTACCTTGCTGGTAACCACCAC	This study
3262C-1	CATGATATCGGTGCGACCTTGGGGAGTGTA	This study
Primers for checking if <i>fgd</i> contained an insert		
FGD-left	TTCTCGCTG(G/T) C(C/G) TGGATGA	This study
FGD-right	CGGTTCCGCGACCTAGCA	This study

primer 4 of Chun et al. (7). Semirandom PCR was carried out as described previously (7), but with the #3 buffer from the Boehringer Mannheim Expand Long Template PCR kit, and Platinum *Taq* polymerase (Gibco).

Insertion mutants were created in *M. bovis* with plasmid pPR28 or pPR29 using techniques similar to those described by Pelicic et al. (34), but with incorporation of PA-824 selection. pPR28 (1 µg) produced in *E. coli* JM109 was electroporated into 0.1 ml of electrocompetent *M. bovis* cells. Electroporated cells were supplemented with 5 ml of 7H9-ADC liquid medium and incubated at 32°C for 1 day. Transformants were selected by growth on 7H9-ADC agar medium containing gentamicin (5 µg/ml) and kanamycin (20 µg/ml) for 4 to 6 weeks at 32°C. One colony was used to inoculate 100 ml of 7H9-ADC-kanamycin medium. After 3 weeks of incubation at 32°C with shaking at 170 rpm, 100 µl of this culture was spread on 7H9-ADC plates containing kanamycin (20 µg/ml),

PA-824 (5 µg/ml), and sucrose (2%, wt/vol) and then incubated at 39°C for 3 weeks. Colonies were picked and grown in 7H9-ADC liquid medium.

pPR29 (1 µg) produced in *E. coli* JM109 was electroporated into 0.4 ml of electrocompetent *M. bovis* cells. Electroporated cells were supplemented with 4 ml of 7H9-ADC and incubated at 32°C for 1 day. Transformants were selected by growth in 50 ml of 7H9-ADC liquid medium containing gentamicin (5 µg/ml) and kanamycin (20 µg/ml) for 4 to 6 weeks at 32°C. Cultures were centrifuged, the pellet (≈0.3 ml of packed cells) was diluted with 0.1 ml of 10% glycerol in water, and 0.2-ml fractions were spread-plated onto 7H10-ADC agar plates containing kanamycin (20 µg/ml), PA-824 (5 µg/ml), and sucrose (2%, wt/vol). These plates were grown at 39°C for 4 to 6 weeks, and the resultant colonies were used to inoculate 7H10-ADC agar medium or 7H9-ADC liquid medium supplemented with sucrose, kanamycin, and PA-824. Strains mutated by pPR28 or

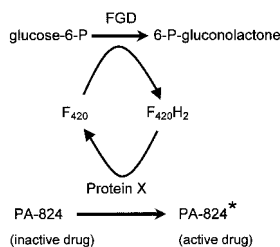


FIG. 2. Hypothesis for the role of F₄₂₀ in PA-824 activation. Protein X is a hypothetical enzyme that transfers electrons from F₄₂₀H₂ to PA-824.

pPR29 that survived this selection were further propagated and analyzed by HPLC for 5-deazaflavin. Dry weights were estimated by measuring the postboiling pellet volume (milliliters) and dividing by 8 ml/g (a conversion factor determined experimentally with *M. bovis*).

Transposon insertion sites created with pPR28 and pPR29 were found by an inverse PCR technique similar to that described by David Bowtell (<http://www.pmc.unimelb.edu.au/manual/molbiol>). With inverse PCR, chromosomal DNA from mutants was digested with *EagI*. Two *EagI* sites in the transposon of pPR28 and pPR29 allowed a short internal section of the transposon to be excised, and at some distance upstream and downstream of the insertion other *EagI* sites resulted in chromosomal DNA cleavage. This resulted in two independent segments with *EagI*-cut ends, with DNA adjacent to the insertion site and either the left or right portion of the transposon. These segments were ligated to create circular DNA. To identify the pPR28 sites, inverse PCR was conducted with primers YMB1 and YMB2. To identify the pPR29 sites, inverse PCR was conducted using TNP3 and TNP4 (for molecules containing the left transposon component) or HOPS1 and TNP2 (for molecules containing the right transposon component). Fragments of approximately 500 to 1,500 bp were usually obtained and were then gel purified and subcloned into pGEM, and one round of single-strand sequencing was conducted.

M. bovis homologues of both Rv3261 and Rv3262 were amplified by *Pfu* DNA polymerase PCR using primers 3260-2 and 3260-3. A terminal A overhang was introduced onto both ends of this product by *Taq* polymerase, and the final product was cloned into pGEM to make pFbiAB. Using this clone as a template, PCR products were constructed with an *EcoRV* site on the 5' end and a *HindIII* site on the 3' end. The insert containing both *fbiA* and *fbiB* that was used to create p61-62 was constructed using primers 3260C-1 and 3260C-2. The insert containing *fbiA* used to create p3261 was constructed using primers 3260C-1 and 3261C-2. The insert containing *fbiB* used to create p3262 was constructed using primers 3262C-1 and 3260C-2. These inserts were subcloned in frame into pSMT3 after digesting this plasmid with both restriction enzymes. pSMT3 was used as an expression vector to complement insertion mutants (33), using hygromycin-supplemented liquid or agar medium (50 µg/ml for *M. bovis*).

Electrodeposition/electroporation to confirm that pSMT3 containing an insert was present in complemented cells was performed by a modification of the method described by O Gaora (33). One *M. bovis* colony was transferred to 100 µl of 10% glycerol in water along with ≈0.1 ml of 1-mm glass beads in a 1.5-ml screw-cap plastic tube, and the tube was shaken for 30 s in a Mini Beadbeater (Biospec Products, Bartlesville, Okla.) at 5,000 rpm to disrupt clumps but not break all cells. After the beads settled, 50 µl of the supernatant was used for electroporation of *E. coli*, which were then plated on LB-hygromycin (250 µg/ml) to select transformants. Plasmids prepared from the *E. coli* were then examined by agarose gel electrophoresis. The remaining supernatant from the Beadbeater was spread on 7H10-ADC medium supplemented with sucrose and kanamycin, and after growth, cells were examined to confirm F₄₂₀ content.

RT-PCR was used to determine if genes were coexpressed in an operon. The reverse transcriptase reaction was performed with Access RTase at 48°C (45 min) according to instructions provided by the manufacturer. PCR was performed with primer pairs F1 and R2, F3 and R4, and F5 and R6 (Table 1), and the products were analyzed by agarose gel electrophoresis. RT-PCR to determine if the *fbiB* transcript was made by *fbiA*::Tn5367 was conducted with primer pairs 3260-9 and 3262-2, and 3261-1 and 3262-2.

Computer analysis of sequences. Comparison of derived amino acid sequences to the NCBI protein database was performed by the NCBI BlastP (1) and ψ -Blast (2) programs (<http://www.ncbi.nlm.nih.gov>). Comparison of *M. tuberculosis* sequences to *M. bovis* sequences not in the NCBI database was made by the TBLASTN program at the Sanger Centre (<http://www.sanger.ac.uk>). Genes in

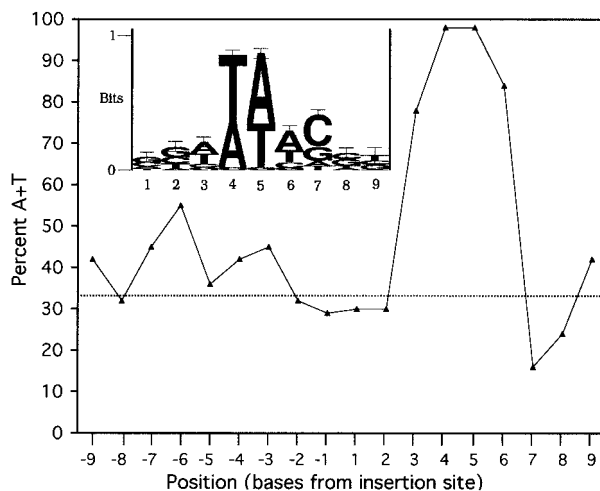


FIG. 3. Sequence characteristics of the Tn5367 transposon insertion sites. In the large graph, percent A+T at each position to the right (positive values) and to the left (negative values) of the insertion site is given for nine bases on both sides of the 31 gene insertion sites observed in our experiments and the 8 duplicated bases (positive values 1 to 8) for an additional 19 insertion sites reported by others (5, 30, 34). The horizontal dotted line indicates the A+T content of the *M. tuberculosis* genome (34.4%). The inset graph provides a sequence logo plot to graphically emphasize the incidence of individual base types, if they predominate at positions 1 to 9.

the *Mycobacterium leprae* genome were examined at the Institut Pasteur Leprosoma site (<http://genolist.pasteur.fr>). Sequences were aligned using ClustalW (46) or PIMA (43) multiple alignment programs with default settings available at the Baylor College of Medicine Search Launcher (<http://dot.imgen.bcm.tmc.edu>). Alignments were prepared for examination by shading with the Boxshade program at the Swiss Institute for Experimental Cancer Research (<http://www.isrec.isb-sib.ch>). Relationships of protein sequences were examined by the NCBI COGs program.

A computer program was written in C++ to examine any genome for sites with high, medium, and low probability for Tn5367 insertion [4(A/T), 3(A/T), and 2(A/T), respectively] (3). This program was used to examine the *M. tuberculosis* nucleotide sequence (9) at the Sanger Centre web site to identify the frequency and locations of all such sequences in the *M. tuberculosis* genome and to calculate maximum distances and average distances between high-, medium-, and low-probability sites. To characterize Tn5367 insertion sites, the duplicated 8-bp sequences of all 50 available sequences were analyzed by the WebLogo program (41) (available at <http://www.bio.cam.ac.uk/cgi-bin/seqlogo/logo.cgi>). Chi square analysis was used to examine the significance of the AT percentages.

RESULTS AND DISCUSSION

Transposon mutagenesis and selection for PA-824-resistant F₄₂₀-minus mutants of *M. bovis*. Using recently reported transposon mutagenesis methods (5, 34), we were confident that we could create insertion mutants of *Mycobacterium* spp. that would not produce F₄₂₀. However, it was not obvious how to identify mutants which did not make F₄₂₀ except to screen thousands of colonies by HPLC. As a rapid alternative to HPLC, we tried direct spectrofluorometric analysis of cell extracts, but the low levels of F₄₂₀ present compared to the amounts produced by methanogens and the presence of other fluorescent material made this unsuitable for mutant screening (E. Schoenberger, unpublished).

We then encountered an approach to enrich the mutant population for F₄₂₀-minus mutants using an experimental antituberculosis drug (a nitroimidazopyran, PA-824) (45). F₄₂₀-

dependent glucose-6-phosphate dehydrogenase (FGD) (35, 36) is required to activate PA-824, which can then kill members of the *M. tuberculosis* complex; other mycobacteria (e.g., *M. smegmatis*) are not inhibited (45). We developed the hypothesis (Fig. 2) that PA-824 is activated by a reductive reaction that is dependent on reduced F_{420} ($F_{420}H_2$). Thus, some *M. tuberculosis* or *M. bovis* mutants that are resistant to this drug would be defective in FGD activity or unable to produce F_{420} . This allowed us to substantially reduce the number of mutants to be examined by HPLC.

The transposon vectors phAE94, pPR28, and pPR29 all successfully transformed *M. bovis*, giving rise to colonies on plates supplemented with kanamycin and PA-824. While the phAE94 method was initially easier and less susceptible to generating sibling mutants, the plasmid method proved superior due to its ability to routinely generate large numbers of mutants. We examined cell extracts from 134 cultures derived from these colonies by HPLC and compared F_{420} and FO levels to those in the wild-type strain. FO levels were considerably lower than F_{420} levels, and in some cases it was difficult to determine conclusively if FO was absent. About one-third ($n = 48$) of the PA-824-resistant mutants were defective in F_{420} accumulation.

Identification of genes interrupted in F_{420} -minus mutants. With either inverse PCR or semirandom two-step PCR, we located transposon insertion sites in PA-824-resistant mutants. Among 48 PA-824-resistant F_{420} -minus mutants, we identified 31 distinct genes with a transposon insertion. Several genes were found more than once due to independent insertions or siblings. Insertions in 19 genes were found in mutants which had an F_{420} -minus and FO-minus phenotype, and interruptions in 12 genes were found in mutants with an F_{420} -minus and FO-plus phenotype.

Characteristics of Tn5367 insertion sites. As shown in Fig. 3, examination of our transposon insertion sites and sites reported by others (5, 30, 34) showed that in the 8-bp duplicated region, positions 4 and 5 are nearly 100% A or T and positions 3 and 6 are very high in A+T; this is a significant deviation from the 34% A+T genome content ($\alpha < 0.001$). Position 7 is G+C rich (16% A+T; $\alpha < 0.005$). The inset sequence logo portrays the approximately equal amounts of A and T at positions 3 to 6 and the predominance of C at position 7. This agrees with the general observations (that the duplicated region was AT-rich) by others who had each examined a smaller number of insertions (5, 30, 34).

The sequence on the other side of the insertion site (the unduplicated region corresponding to positions -1 through -9), for which we have data for only our mutants, was not conserved; it was not AT-rich (Fig. 3), nor did any bases predominate (data not shown). We cannot confidently conclude if position -6 is GC rich ($\alpha < 0.025$).

We wondered if this transposon would insert into most mycobacterial genes, which have a G+C content of 65.6%. We examined the *M. tuberculosis* H37Rv genome with a computer program to determine how many genes had sites that Tn5367 would insert into with high probability (four adjacent A's or T's) or with a moderate probability (three adjacent A's or T's) (3). Of the 3,924 genes in the genome, 103 (2.6%) do not contain at least one high-probability site, but all have a moderate-probability site. All of our 31 transposon-inserted genes

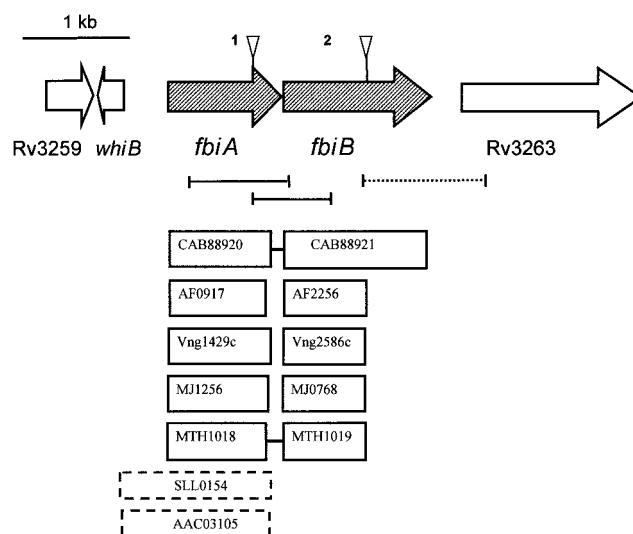


FIG. 4. Gene arrangement of the *fbiAB* cluster in *M. bovis* BCG, corresponding transposon insertion sites, and alignment of these genes with homologs from other microorganisms. Triangles indicate transposon insertion sites. CAB, *Streptomyces coelicolor*; MTH, *Methanobacterium thermoautotrophicum*; AF, *Archaeoglobus fulgidus*; Vng, *Halobacterium* sp.; SLL, *Synechocystis* sp.; AAC, *Nostoc* sp. Numbers refer to gene designations in the corresponding genome sequences. Rv3259 and Rv3263 are used to indicate the *M. bovis* homologs of these *M. tuberculosis* H37Rv genes, since the *M. bovis* sequence is not yet annotated, and thus *M. bovis* genes have not been assigned unique designations. The lines below the *M. bovis* genes indicate the sizes and locations of RT-PCR products expected; a solid line indicates that a product was seen, and a dotted line indicates that no product was observed. The primer pairs used were (from left to right) F1-R2, F3-R4, and F5-R6. Boxes with solid lines indicate genes identified with default NCBI Blast settings. Boxes with dashed lines indicate genes identified with ψ -Blast iterations. Boxes connected by bars indicate the genes are adjacent, and those not connected are not adjacent. Figure is approximately to scale, with boxes indicating the sizes of all putative homologous genes.

had a high-probability site. We conclude that, although this transposon is not truly random for insertion in mycobacteria, insertion is very likely in >97% of *M. tuberculosis* and *M. bovis* genes, and ultimately all genes will be interrupted if a large number of mutants are examined.

***M. bovis* mutants with an insertion in Rv3261 or Rv3262 homolog.** Of 18 genes interrupted by pPR29 which gave an F_{420} -minus phenotype, one each had an independent insertion in the adjacent *M. bovis* homolog of *M. tuberculosis* H37Rv sequences Rv3261 (790 bp downstream from the start codon) and Rv3262 (801 bp downstream from the start codon), as shown in Fig. 4. We have named these genes *fbiA* and *fbiB*, respectively, for F_{420} biosynthesis. These mutants made no F_{420} but did make FO. An example of the HPLC profile of the *fbiA* mutant is given in Fig. 5, in comparison with the wild-type strain. The *fbiB* mutant had about the same HPLC profile as the *fbiA* mutant at the scale shown in Fig. 5 (data not shown). Since FO is produced by both mutants, the products of these genes were expected to be involved in steps between FO and F_{420} -5,6 (Fig. 1B).

We cloned and sequenced a 2,718-bp region from *M. bovis* containing *fbiA* and *fbiB*, creating pFbiAB. Our sequences for

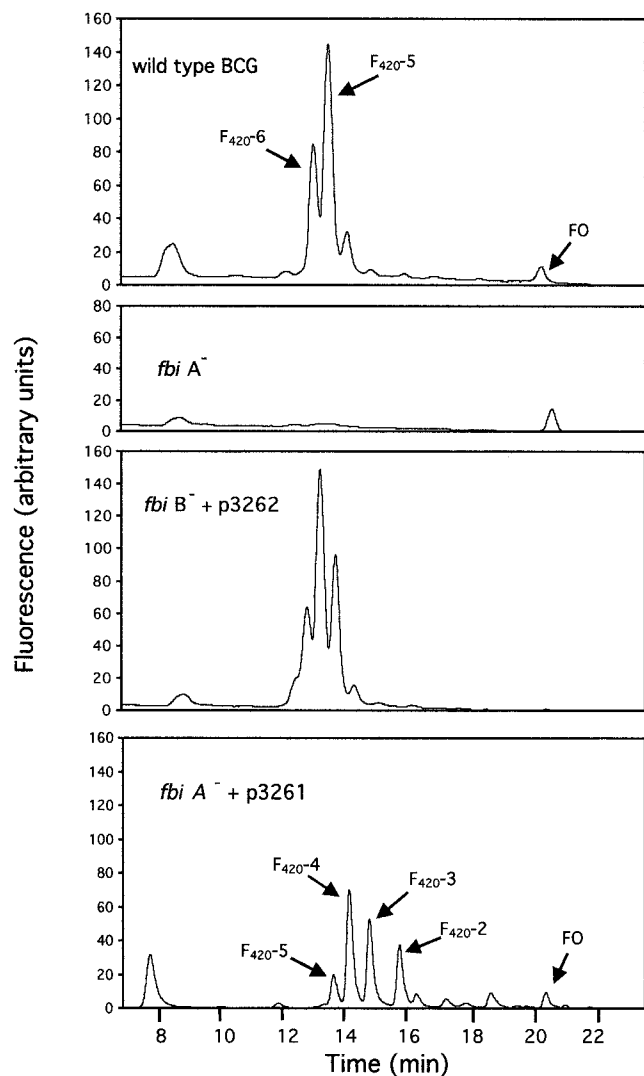


FIG. 5. HPLC elution profiles of extracts made from *M. bovis* BCG wild-type, the *fbiA* mutant (Rv3261 homolog interrupted), the *fbiB* mutant (Rv3262 homolog interrupted) that was complemented with p3262, and the *fbiA* mutant that was complemented with p3261.

both genes precisely matched the Rv3261 and Rv3262 sequences and the Sanger database *M. bovis* homologs of these genes. As shown in Table 2, Blast analysis of the deduced sequence of FbiA against the NCBI database revealed that this gene has homologs in all fully sequenced microorganisms which make F₄₂₀, including *M. tuberculosis* (9), *M. leprae* (10), *Archaeoglobus fulgidis* (25), *Halobacterium* sp. (32), *Methanococcus jannaschii* (6), *Methanobacterium thermoautotrophicum* (42), and *Synechocystis* sp. (24). However, the *Synechocystis* homolog was not identified by default BlastP settings, but was identified by ψ -Blast (2).

When MTH1018 was analyzed by BlastP against *Synechocystis* alone, a weak hit was obtained with SLL0154 (score = 44; $E = 10^{-5}$). The ψ -Blast analysis also revealed a homolog from a *Nostoc* sp. (also known as *Anabaena*), which may contain F₄₂₀ (since it is a cyanobacterium), although the genome of this organism has not been sequenced. We are cautiously optimistic that these weak hits in cyanobacteria have identified true functional homologs of FbiA, but will wait for further supportive data (e.g., complementation of our *fbiA* mutant with *Synechococcus fbiA* and the *M. bovis fbiB* on the same plasmid) before reaching a final decision.

Strong FbiA hits were also obtained with *Streptomyces coelicolor* (37), another F₄₂₀ producer for which the genome sequence is largely completed but not yet annotated. Figure 4 provides an overview of the arrangement of these homologs (the *M. leprae* homolog, ML0759, which was the same size as Rv3261, is listed in Table 2 but was omitted from Fig. 4 to conserve space). Homologs for FbiB were found in all sequenced F₄₂₀-producing organisms except *Synechocystis* (Table 2 and Fig. 4). The *Mycobacterium*, *Streptomyces*, and *Methanobacterium fbiA* and *fbiB* homologs were adjacent, while in the other organisms these two genes were distant from each other. *M. tuberculosis* and *M. bovis* have exactly the same gene arrangement in the region shown in Fig. 4. *M. leprae* has a very similar arrangement, except that another open reading frame (ORF) (ML0756) was identified between *fbiB* and the Rv3263 homolog.

Transcription in the *fbiAB* cluster. As shown in Fig. 4, the *whiB* gene upstream of *fbiA* is transcribed in the opposite direction. Downstream of *fbiB* there is a 296- or 371-bp gap

TABLE 2. Blast analysis results when *M. bovis* BCG FbiA and FbiB were compared to homologs from other F₄₂₀ producers^a

FbiA				FbiB			
Homolog (organism)	Score	E value	% identity	Homolog (organism)	Score	E value	% identity
Rv3261 (<i>M. tuberculosis</i>)	575	1×10^{-163}	89	Rv3262 (<i>M. tuberculosis</i>)	655	0	81
ML0759 (<i>M. leprae</i>)	450	1×10^{-125}	70	ML0758 (<i>M. leprae</i>)	531	1×10^{-150}	65
CAB88920 (<i>Streptomyces</i>)	315	2×10^{-85}	52	CAB88921 (<i>Streptomyces</i>)	251	9×10^{-66}	39
AF0917 (<i>Archaeoglobus</i>)	126	3×10^{-28}	30	AF2256 (<i>Archaeoglobus</i>)	84	2×10^{-15}	30
MJ1256 (<i>Methanococcus</i>)	112	5×10^{-24}	25	MJ0768 (<i>Methanococcus</i>)	78	1×10^{-13}	26
Vng1429c (<i>Halobacterium</i>)	96	5×10^{-19}	27	Vng2586c (<i>Halobacterium</i>)	73	5×10^{-12}	31
MTH1018 (<i>Methanobacterium</i>)	83	4×10^{-15}	26	MTH1019 (<i>Methanobacterium</i>)	65	1×10^{-9}	27
SLL0154 (<i>Synechocystis</i>)	ψ 272	ψ 3 $\times 10^{-72}$	17				
AAC03105 (<i>Anabaena</i>)	ψ 268	ψ 6 $\times 10^{-71}$	17				

^a Default BlastP analysis of FbiA and FbiB sequences versus NCBI database, using a low-complexity filter, except that ψ -BlastP iteration was used for identification of *Synechocystis* and *Anabaena* genes. The low-complexity filter results in a conservative identity score, since sequences of low complexity are counted as not identical. Without the filtering, identity is usually higher; e.g., the *M. tuberculosis* and *M. bovis* sequences are 100% identical but are given as 89 and 81% identical in the BlastP results. Original papers describing genome or partial sequences: *M. tuberculosis* (9), *M. leprae* (10), *S. coelicolor* (37), *A. fulgidis* (25), *Halobacterium* sp. (32), *Methanococcus jannaschii* (6), *Methanobacterium thermoautotrophicum* (42), *Synechocystis* sp. (24), and *Nostoc* sp. (also known as *Anabaena*; NCBI database; data submitted by H.-S. Yoon and J. W. Golden).

TABLE 3. F_{420} production by wild-type *M. bovis* BCG, by *fbiA* and *fbiB* mutants, and by mutants complemented with these genes^a

Complementation status	$F_{420-5,6}$ level (pmol/culture)			$F_{420-2,3,4}$ level (pmol/culture)		
	Wild type	<i>fbiA</i> mutant	<i>fbiB</i> mutant	Wild type	<i>fbiA</i> mutant	<i>fbiB</i> mutant
Uncomplemented	448	6	5	83	4	15
Control with pSMT3	ND ^b	22	8	ND	15	7
Complemented (p3261)	ND	62	6	ND	438	6
Complemented (p3262)	ND	16	363	ND	12	201
Complemented (p61-62)	ND	464	ND	ND	136	ND

^a Cultures were of approximately the same packed cell volume (about 200 mg [wet weight] of cells) taken from a 100-ml liquid culture or the surface of one petri plate. *M. bovis* wild-type F_{420} production in this experiment was 0.02 nmol per mg of dry cell weight.

^b ND, not determined.

(depending on the start codon chosen) before the start of Rv3263. Downstream of Rv3263, *rmlA2* (not shown in Fig. 4) is transcribed in the opposite direction. This arrangement left open the possibility that *fbiA*, *fbiB*, and Rv3263 were transcribed together or that some or all were transcribed independently. Thus, we used RT-PCR to examine transcription from these three genes.

As shown in Fig. 4, mRNA species existed which coded for significant sections of both *fbiA* and *fbiB*. The sizes of the amplified sections within these two genes were as predicted. However, no product was obtained when primers were used to see if mRNA arising from both *fbiB* and Rv3263 was present (Fig. 4). This supports the concept that *fbiA* and *fbiB* are expressed as an operon which does not include Rv3263. We also used RT-PCR to examine mRNA production by the *fbiA* mutant, since it was possible that significant *fbiB* transcripts could result from a promoter upstream of *fbiB*. Using mRNA at the same concentration as above, we saw no *fbiB* transcript, but when the mRNA level was increased 10-fold, a weak but significant band of the correct size was observed. This suggests that although most *fbiB* transcription occurs from a promoter upstream of *fbiA*, a small but possibly significant amount of monocistronic *fbiB* transcription may occur from an alternative site.

Complementation of *fbiA* and *fbiB* mutants. Using complementation, we confirmed that interruption of these genes was responsible for the F_{420} -minus phenotype. PCR of the cloned *M. bovis fbi* genes yielded subclones containing each gene separately (p3261 and p3262) and one clone containing both genes (p61-62). The strong mycobacterial promoter in pSMT3 located upstream of the cloned gene(s) ensured adequate expression. Electrodeposition confirmed that pSMT3 in complemented cells contained an insert of the correct size.

Figure 5 shows examples of the HPLC profiles of extracts from the wild-type strain, the *fbiA* mutant, the *fbiB* mutant complemented with p3262, and the *fbiA* mutant complemented with p3261. Transformation of the *fbiA* mutant with p61-62 (HPLC data not shown) and of the *fbiB* mutant with p3262 (HPLC data shown in Fig. 5) resulted in full restoration of $F_{420-5,6}$ production, as shown quantitatively in Table 3 for both complemented mutants. The *fbiA* mutant complemented with p3262 did not make $F_{420-5,6}$ (Table 3); FO was the only significant 5-deazaflavin peak observed.

However, as shown in Table 3, complementation of the *fbiA* mutant with p3261 led to a slight but significant production of $F_{420-5,6}$ (comprised only of F_{420-5}). As shown in Fig. 5, this complementation also led to the accumulation of compounds

that are almost certainly $F_{420-2,3,4}$ (F_{420-2} , F_{420-3} , and F_{420-4}), since coinjection of standard F_{420-2} and $F_{420-5,6}$ showed precisely coincident elution with putative F_{420-2} and F_{420-5} , and the fluorescent properties of these peaks are consistent with their being F_{420} species. Prior experience with F_{420-4} also agrees with this conclusion (4). It is possible that smaller peaks at 17 to 20 min include F_{420-0} and F_{420-1} . $F_{420-2,3,4}$ peaks of this magnitude were not observed in the uncomplemented mutants or in mutants complemented with p3262 or p61-62, but the wild-type strain and some of the mutants often showed small peaks in this region that were about one-tenth (or less) of the size seen in the *fbiA* mutant complemented with p3261. The appearance of several F_{420} species with fewer than five or six glutamates also suggests that glutamates are not added exclusively as an already assembled five- or six-glutamate piece, but are instead added individually, at least after F_{420-2} is formed.

The discovery of intermediates in the p3261-complemented *fbiA* mutant led us to more carefully examine the HPLC profiles of the *fbiA* and *fbiB* mutants. As shown in Fig. 6, aside

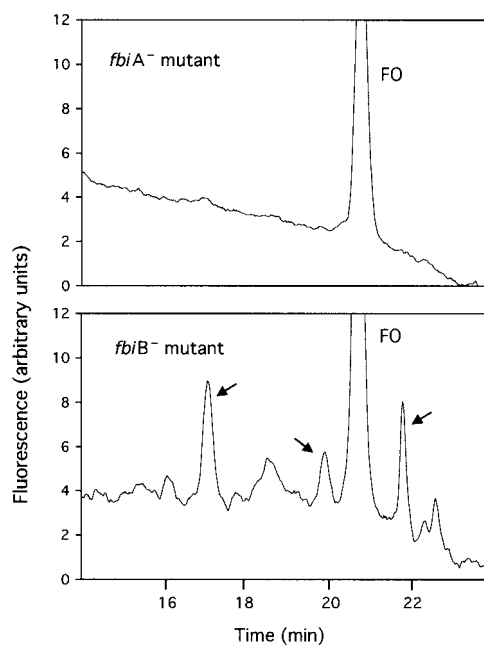


FIG. 6. HPLC elution profiles of extracts made from the *fbiA* mutant and the *fbiB* mutant. Compare the scale to the HPLC data in Fig. 5; the scale here is enhanced by 13.3-fold.

from FO, the uncomplemented *fbiB* mutant accumulated material showing up as three fluorescent HPLC peaks (indicated by arrows) that eluted between 16 and 22 min. The peaks at 18.5 and 22.5 min were not always seen in the *fbiB* mutant. Note that the scale in Fig. 6 is considerably magnified compared to Fig. 5, allowing these peaks to be seen. The three *fbiB* mutant peaks were not seen with the *fbiA* mutant. Peaks eluting in this region are expected to contain no glutamate, and thus these peaks could arise from F₄₂₀-0 or closely related structures. This would be consistent with a role for FbiA in making F₄₂₀-0 (F0-phosphate-lactyl), and with the inability of the *fbiB* mutant to add glutamates to F₄₂₀-0.

The complementation data clearly show that FbiB is essential, since replacing the inactive gene with an active one fully restores the original phenotype. The complication in interpreting these data is the behavior of the *fbiA* mutant which has been complemented with p3261. If the genes are monocistronic, we would expect to see full restoration of F₄₂₀-5,6 production when the *fbiA* mutant was complemented with p3261, but instead, although a little F₄₂₀-5 was made, mostly the smaller species were made. If the two genes were entirely transcribed as an operon from a promoter or promoters upstream of *fbiA*, then we should see no F₄₂₀-2,3,4,5 made when the *fbiA* mutant is complemented with p3261, since no FbiB could be produced. A reasonable explanation of these data, in light of the transcriptional data, is that although the *fbiA* mutant makes no FbiA, it can make a small amount of FbiB due to a leaky polar effect, i.e., a small amount of *fbiB* transcript is made from an alternative start site just upstream of *fbiB*.

Consideration of the roles of FbiA and FbiB. Since the mutated strains make FO, FbiA and FbiB must play roles in the later portion of the pathway shown in Fig. 1B, between FO and F₄₂₀-*n*. This portion of the pathway involves the construction, by mostly unknown mechanisms, of a side group that is composed of a phosphate, a lactyl, and five or six glutamyl groups. Although it would be logical to initially add a phosphate to FO by an FO kinase (in analogy to flavin mononucleotide biosynthesis), our repeated efforts to demonstrate such a reaction using ATP or GTP have been unsuccessful (20). Recent work with methanogens has demonstrated that 2-phospholactate is a precursor of the phosphate and lactyl groups of F₄₂₀ (17), suggesting that FO kinase may not be present in mycobacteria. It also makes sense that an enzyme transfers individual glutamyl groups to F₄₂₀-0 (since we see intermediates with two, three, four, five, and six glutamyl groups), but we have not sought such an enzyme yet.

We have examined protein sequence databases with several search methods (COGS, BlastP, and ψ -Blast) to deduce possible functions for FbiA and FbiB and have conducted multiple alignments of FbiA and FbiB with homologs from other F₄₂₀ producers. None of these methods provided firm support for assignment of specific functions to FbiA and FbiB, which is not surprising because these enzymes have never been studied. However, taken together, our data suggest three hypotheses. (i) FbiA is involved in making F₄₂₀-0, and FbiB participates in the addition of glutamates to F₄₂₀-0. (ii) FbiA and FbiB are subunits of one enzyme which adds glutamates to F₄₂₀-0; neither protein is active in glutamyl transfer by itself. (iii) FbiB is involved in making F₄₂₀-0, and FbiA adds glutamates to F₄₂₀-0. Our data cannot confidently distinguish between the three

hypotheses. Nonetheless, we are most attracted to hypothesis i, that FbiA participates in the conversion of FO to F₄₂₀-0, and that FbiB plays a role in the addition of glutamates. This hypothesis is consistent with intermediate accumulation in the *fbiB* mutant, a lack of intermediate accumulation in the *fbiA* mutant, and a leaky polar effect seen with the *fbiA*-complemented *fbiA* mutant. Development of assays in *Mycobacterium* species and with overexpressed FbiA and FbiB for the 2-phospholactate and glutamyl transfer steps will be very useful for examination of these hypotheses.

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