Mycobacterium smegmatis mc² 155 fbiC and MSMEG_2392 are involved in triphenylmethane dye decolorization and coenzyme F₄₂₀ biosynthesis

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Mycobacterium can tolerate relatively high concentrations of triphenylmethane dyes such as malachite green and methyl violet. To identify mycobacterial genes involved in the decolorization of malachite green, a transposon mutant library of Mycobacterium smegmatis mc² 155 was screened for mutants unable to decolorize this dye. One of the genes identified was MSMEG_5126, an orthologue of Mycobacterium bovis fbiC encoding a 7,8-didemethyl-8-hydroxy-5-deazariboflavin (FO) synthase, which is essential for the biosynthesis of the electron carrier coenzyme F₄₂₀. The other gene identified was MSMEG_2392, encoding an alanine-rich protein with a DUF121 domain. The minimum inhibitory concentrations (MICs) for malachite green and methyl violet of the six fbiC mutants and two MSMEG_2392 mutants were one-third and one-fifth, respectively, of the MIC of the parent strain M. smegmatis mc² 155. Representative fbiC and MSMEG_2392 mutant strains were also sensitive to oxidative stress caused by the redox-cycling agents plumbagin and menadione, and the sensitivity was reversed in the complemented strains. HPLC analysis of representative fbiC and MSMEG_2392 strains revealed that, while the fbiC mutant lacked both coenzyme F₄₂₀ and FO, the MSMEG_2392 mutant contained FO but not coenzyme F₄₂₀. These results indicate that MSMEG_2392 is involved in the biosynthesis of coenzyme F₄₂₀.

INTRODUCTION

Dyes and dye products are major pollutants that are often mutagenic and toxic to a wide range of organisms. The human health impact of dyes and their degradation products has recently led the US Environmental Protection Agency to declare three classes of dyes, anthraquinone, azo and triphenylmethane dyes, and their degradation products, as hazardous materials (US Environmental Protection Agency, 2005). This stringent legislation makes it imperative that new and better methods and processes be developed to treat dye-contaminated wastewater.

The triphenylmethane dye malachite green is used in the textile industry as a fabric dye and in the aquaculture industry as an antiparasitic agent (Alderman, 1985; Schnick, 1988). It is soluble and stable in water at high concentrations. While it is toxic to mammalian cells (Bose et al., 2004, 2005; Fessard et al., 1999; Gupta et al., 2003; Littlefield et al., 1985; Stammati et al., 2005), several species of fungi and bacteria are known to decolorize and degrade malachite green through a variety of pathways.

In the fungi Coriolus versicolor f. antarcticus and Fomes sclerodermeus, a laccase is involved in the degradation of this dye (Levin et al., 2004; Papinutti & Forchiassin, 2004), while in the fungus Cunninghamella elegans, a cytochrome P450 monooxygenase has been implicated in its degradation (Cha et al., 2001). In intestinal bacteria, HPLC analysis of Clostridium perfringens and Lactobacillus acidophilus cultures incubated with malachite green showed that malachite green was converted to colourless leucomalachite green (Henderson et al., 1997). In addition, Jang et al. (2004) isolated Citrobacter strain KCTC 18061P, which is also very efficient in decolorization of malachite green. The enzyme triphenylmethane reductase, which catalyses the conversion of malachite green to colourless leucomalachite green, was purified and the gene cloned for heterologous expression in Escherichia coli. The Citrobacter triphenylmethane reductase is an NADH/NADPH-dependent enzyme of the isoflavone reductase family (Jang et al., 2005).

Mycobacteria are also able to tolerate and decolorize triphenylmethane dyes in concentrations higher than those...
that would inhibit the growth of most other microorganisms (Alderman, 1985; Culp & Beland, 1996). In fact, Middlebrook 7H10 medium, used for the cultivation of pathogenic mycobacteria such as *Mycobacterium tuberculosis*, contains malachite green to reduce fungal contamination during the long incubation times required for growth. Jones & Falkingham (2003) reported that the decolorization of malachite green by pathogenic mycobacteria such as *Mycobacterium chelonae* and *Mycobacterium aviun* is inhibited by the addition of metyrapone, suggesting the involvement of a cytochrome P450 in decolorization of malachite green in these organisms. In a related species, the actinomycete *Nocardia corallina*, the steps following decolorization of the triphenylmethane dye crystal violet result in Michler’s ketone and dimethylaminophenol (Yatome et al., 1991, 1993).

To identify mycobacterial genes involved in triphenylmethane dye decolorization, we screened a transposon mutant library of *Mycobacterium smegmatis*, a non-pathogenic saprophyte, for mutants unable to decolorize malachite green. We report the identification and characterization of transposon mutants disrupted in *fbiC* and the predicted gene MSMEG_2392 that are unable to decolorize malachite green and methyl violet.

**METHODS**

**Bacterial strains and culture conditions.** *M. smegmatis mc² 155* (a generous gift of W. R. Jacobs, Albert Einstein College of Medicine, Bronx, New York, USA) was the parent strain from which transposon mutants were constructed. *E. coli* DH5α was used as the host strain for cloning experiments. *M. smegmatis mc² 155* was grown in Middlebrook 7H9 broth (Difco) with 0.05% Tween 80 and supplemented with either OADC (oleic acid, albumin, glucose, catalase supplement) or 1% glucose. *M. smegmatis mc² 155* was also grown on Middlebrook 7H10 solid medium (Difco) with 0.5% glycerol supplemented with OADC or 1% glucose. In addition, *M. smegmatis mc² 155* and *E. coli* cultures were grown on Lennox L (LB) broth (with 0.5% Tween 80) and supplemented with either OADC (oleic acid, albumin, glucose, catalase supplement) or 1% glucose. *M. smegmatis mc² 155* was grown in Middlebrook 7H9 broth (Difco) with 0.05% Tween 80 and supplemented with either OADC (oleic acid, albumin, glucose, catalase supplement) or 1% glucose. In addition, *M. smegmatis mc² 155* and *E. coli* cultures were grown on Lennox L (LB) broth (with 0.5% Tween 80) and supplemented with either OADC (oleic acid, albumin, glucose, catalase supplement) or 1% glucose. In addition, *M. smegmatis mc² 155* and *E. coli* cultures were grown on Lennox L (LB) broth (with 0.5% Tween 80) and supplemented with either OADC (oleic acid, albumin, glucose, catalase supplement) or 1% glucose.

**Molecular biology techniques.** Genomic DNA was isolated from *M. smegmatis mc² 155* and *M. smegmatis* mutant cultures as described by Larsen (2000). Standard recombinant DNA techniques such as restriction digestions, ligations and transformations were performed as described by Sambrook et al. (1989). The strains, plasmids and oligonucleotides used in this study are described in Table 1.

**Construction and screening of transposon library.** The transposon library (EZ : : TN <KAN-2>Tnp transposase and Tn5-based transposon with kanamycin-resistance marker) was constructed according to the manufacturer’s instructions (Epicerent Technologies). Briefly, 1 µl of the EZ : : TN transposase was electroporated into competent *M. smegmatis mc² 155* cells that had been prepared according to Snapper et al. (1988). The cells were then plated on LB solid medium with 25 µg kanamycin ml⁻¹ and allowed to grow for 2–3 days at 37 °C. Fourteen thousand colonies were picked and grown on LB solid medium in single-well ELISA plates for 2–3 days at 37 °C, then replica-plated into 96-well ELISA plates containing LB broth with 0.5% Tween and 25 µg kanamycin ml⁻¹. Malachite green was added to a final concentration of 0.1 mg ml⁻¹ to cultures in late-exponential phase or stationary phase. After the addition of malachite green, the cultures were incubated at 37 °C for 2–3 additional days and any mutant that failed to decolorize malachite green was rescreened in 1 ml liquid medium with 0.1 mg malachite green ml⁻¹ using cultures diluted to OD₆₀₀ 0.05. To further confirm the mutant phenotype, mutants were also streaked on LB agar medium supplemented with 0.001, 0.01, or 0.1 mg ml⁻¹ malachite green or methyl violet.

**Identification of the site of insertion of transposon.** A 1 µg amount of genomic DNA from each malachite green decolorizing mutant listed in Table 1 was digested with restriction enzymes *PstI*, *SalI*, or *SacI*, and 0.25 µg of the restriction-digested genomic DNA was self-ligated overnight at 16 °C. PCR amplification of the ligation mixture was performed using reverse primers Kan-2-Fp-1 and Kan-2-Rp-1 (Table 1), provided in the EZ : : TN <KAN-2>Tnp transposome kit. The PCR product was gel purified, then cloned into pCR2.1 for sequencing. The sequence obtained was compared with the TIGR *M. smegmatis* mc² 155 annotated genome sequence (http://www.tigr.org) using the BLASTN program (Washington University BLAST version 2.0). To determine the insertion site and orientation of transposon within *fbiC*, PCR amplifications were performed with primer sets *fbiC*-5′ and Kan-2-Fp-1, *fbiC*-3′ and Kan-2-Rp-1; or with primer sets *fbiC*-5′ and Kan-2-Rp-1, *fbiC*-3′ and Kan-2-Fp-1 (Table 1). In the same manner, to determine the insertion site and orientation of transposon within the predicted gene MSMEG_2392, PCR amplifications were performed with primer sets 2392-5′ and Kan-2-Fp-1, 2392-3′ and Kan-2-Rp-1; or with primer sets 2392-5′ and Kan-2-Rp-1, 2392-3′ and Kan-2-Fp-1 (Table 1).

**Complementation of fbiC and MSMEG_2392 mutants.** The *M. smegmatis* mc² 155 *fbiC* gene and 231 bp upstream of the start codon, presumably containing the promoter region, was amplified from genomic DNA using primers *fbiC*-5′ and *fbiC*-3′ (Table 1) and cloned into pCR2.1. The forward and reverse primers had XhoI and HindIII sites engineered into them for cloning into pHINT, a *M. smegmatis* shuttle vector that integrates into the attB site on the mycobacterial chromosome (Garbe et al., 1994) (Table 1). For cloning *fbiC* into pHINT, both pCR2.1 vector containing *fbiC* and empty pHINT vector were digested with XhoI and HindIII, the *fbiC* insert was gel purified, ligated to the digested pHINT vector, transformed into *E. coli* and plated onto LB agar containing 100 µg hygromycin ml⁻¹. After confirmation by restriction-digest analysis of the purified recombinant plasmid, the construct, pHINT*fbiC*, was electroproporated into competent cells from the mutant strains and plated onto LB agar supplemented with 25 µg kanamycin ml⁻¹ and 75 µg hygromycin ml⁻¹. In a similar manner, for complementation of mutants disrupted in MSMEG_2392, the MSMEG_2392 gene and 236 bp upstream of the start codon, presumably containing the promoter region, was amplified from genomic DNA using primers 2392-5′ and 2392-3′, also containing XhoI and HindIII sites, and cloned into pHINT (Table 1). The resulting construct, pHINT2392 (Table 1), was used to complement MSMEG_2392 mutants. To confirm complementation of the mutant phenotype, complemented strains were streaked on LB agar supplemented with malachite green or methyl violet (0.001, 0.01, or 0.1 mg ml⁻¹). To serve as control strains, *M. smegmatis* mc² 155 mutants and the mutants DLmal1 and DLmal8 were also transformed with pHINT vector alone, creating strains *M. smegmatis* pHINT, DLmal1pHINT and DLmal8pHINT, respectively (Table 1).
Table 1. Strains, plasmids and oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Strain, plasmid, oligonucleotide</th>
<th>Characteristic(s)*</th>
<th>Source or reference</th>
</tr>
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<tbody>
<tr>
<td><strong>Strain</strong></td>
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<td></td>
</tr>
<tr>
<td><em>M. smegmatis</em></td>
<td>mc&lt;sup&gt;2&lt;/sup&gt; 155, parent strain</td>
<td>W.R. Jacobs</td>
</tr>
<tr>
<td>E. coli DH5&lt;sup&gt;x&lt;/sup&gt;</td>
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<td></td>
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<tr>
<td><strong>Plasmid</strong></td>
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<td></td>
</tr>
<tr>
<td>pCR2.1</td>
<td>TA cloning vector, Amp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Invitrogen</td>
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<tr>
<td>pHINT</td>
<td>*M. smegmatis/E. coli shuttle vector, Hyg&lt;sup&gt;+&lt;/sup&gt;</td>
<td>K. Downing (Garbe et al., 1994)</td>
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<td>pHINT&lt;sub&gt;fbiC&lt;/sub&gt;</td>
<td>pHINT with <em>M. smegmatis</em> fbiC and putative promoter</td>
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<td>pHINT&lt;sub&gt;2392&lt;/sub&gt;</td>
<td>pHINT with <em>M. smegmatis</em> MSMEG&lt;sub&gt;2392&lt;/sub&gt; and putative promoter</td>
<td>This study</td>
</tr>
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<td><strong>Oligonucleotide</strong></td>
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<tr>
<td>fbiC-&lt;sup&gt;5&lt;/sup&gt;'</td>
<td>5'-AAGCTTCCGCGACAGACAGACAG-3'&lt;sup&gt;′&lt;/sup&gt;</td>
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</tr>
<tr>
<td>fbiC-&lt;sup&gt;3&lt;/sup&gt;'</td>
<td>5'-CTCGAGACCCTCCGCGTGGAAG-3'&lt;sup&gt;′&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>2392-&lt;sup&gt;5&lt;/sup&gt;'</td>
<td>5'-CTCGAGACGGCAACATCAGCAAG-3'&lt;sup&gt;′&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>2392-&lt;sup&gt;3&lt;/sup&gt;'</td>
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<tr>
<td>Kan-2-Fp-1</td>
<td>5'-ACCTACAAACAGCTCTCAACAC-3'&lt;sup&gt;′&lt;/sup&gt;</td>
<td>Epicenter Technologies</td>
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<tr>
<td>Kan-2-Rp-1</td>
<td>5'-CAATGTAACATCAGATTTTGAG-3'&lt;sup&gt;′&lt;/sup&gt;</td>
<td>Epicenter Technologies</td>
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</table>

*Amp<sup>+</sup>, ampicillin resistance; Hyg<sup>+</sup>, hygromycin resistance.

**Determination of MICs for malachite green and methyl violet.** Minimum inhibitory concentrations (MICs) of malachite green and methyl violet were determined for all mutants and complemented strains. Experiments were performed in 96-well ELISA plates that were prepared by adding LB broth with 0.5 % Tween and appropriate antibiotics along with malachite green or methyl violet to each column of wells in concentrations ranging from 0.05 to 0.55 mg ml<sup>−1</sup> for malachite green and 0.01 to 0.5 mg ml<sup>−1</sup> for methyl violet. Cells grown to mid-exponential phase were used to inoculate prepared ELISA plates at OD<sub>600</sub> 0.05. Plates were incubated at 37 °C for 3 days. The MICs were determined visually and recorded as the lowest concentration with no visible growth. Results from three independent experiments performed in quadruplicate are presented.

**Oxidative stress sensitivity assay.** Disc diffusion assays were performed to determine sensitivity of wild-type parent strain, DLmal6 (a representative mutant that is disrupted in *fbiC*), DLmal600 (the complemented strain of DLmal6), DLmal8 (a representative mutant that is disrupted in MSMEG<sub>2392</sub>) and DLmal800 (the complemented strain of DLmal8) to various oxidants, as described previously (Rawat et al., 2002). Briefly, cells were grown to mid-exponential phase and then diluted to OD<sub>600</sub> 0.5. A lawn of cells was plated onto Middlebrook 7H10 solid medium with 0.5 % glycerol supplemented with 1 % glucose and appropriate antibiotics for mutant and complemented strains. Various amounts of compounds to be tested were added to 6.35 mm diameter paper filter discs in a volume of 5 or 10 μl. The discs were placed onto the lawn of cells and the plates were incubated for 3 days at 37 °C. Two redox-cycling agents, menadione (0.5 μmol) and plumbagin (0.025 μmol), as well as hydrogen peroxide (1 μmol) and cumene hydrogen peroxide (0.5 μmol), were tested. Three independent experiments with five replicates were conducted and results from one representative experiment are presented.

**Determination of MICs and MBCs of sodium nitrite.** MICs and minimum bactericidal concentrations (MBCs) of sodium nitrite were determined for wild-type parent strain, DLmal6, DLmal600, DLmal8.
and DLmal800 in 96-well ELISA plates. ELISA plates were prepared by adding Middlebrook 7H9 broth, pH 5.0, supplemented with 0.05% Tween, 1% glucose and appropriate antibiotics and sodium nitrite to each column of wells. To determine MICs and MBCs, concentrations of sodium nitrite ranging from 1 mM to 11 mM and concentrations from 10 mM to 100 mM, respectively, were tested. Sodium nitrite was prepared in acidified Middlebrook 7H9 medium supplemented with 0.05% Tween 80 and 1% glucose (pH 5.0) immediately before the addition of cells. Cells grown to mid-exponential phase were used to inoculate prepared ELISA plates at OD_{600} 0.15. Plates were incubated at 37°C for 24 h and the MICs were determined visually and by measuring OD_{600} using a microplate spectrophotometer (Molecular Devices). To determine the MBC, 10 µl cells were streaked onto Middlebrook 7H10 solid medium supplemented with 0.5% glycerol and 10% OADC using calibrated inoculating loops. Results from three independent experiments performed in quadruplicate are presented.

**RESULTS**

Production and screening of a *M. smegmatis* transposon mutant library

A *M. smegmatis* transposon mutant library, consisting of 14,000 individual mutants, was constructed using the EZ::TN transposome kit (Epicentre Technologies). Eight mutants unable to decolorize malachite green were isolated. Six of the mutants isolated, DLmal1, DLmal6, DLmal7, DLmal10, DLmal12 and DLmal13, were disrupted in MSMEG_5126, an orthologue of *Mycobacterium bovis* fbiC and *M. tuberculosis* Rv1173. FbiC encodes FO synthase, which is essential for the biosynthesis of the electron carrier coenzyme F_{420}. Two of the mutants isolated, DLmal8 and DLmal9, were disrupted in predicted gene MSMEG_2392, encoding an alanine-rich protein with a DUF121 domain, which is an orthologue of *M. tuberculosis* Rv2983.

**Determination of transposon insertion site in fbiC and MSMEG_2392 mutants**

To determine the insertion site and the orientation of the transposon in fbiC mutants, PCR amplifications were performed with sets of primers described in Table 1. In DLmal1, DLmal6, DLmal7, DLmal10, DLmal12 and DLmal13, the transposon insertion is 1.8 kb, 2.4 kb, 1.9 kb, 2.2 kb, 0.8 kb and 2.2 kb within the *fbiC* ORF, respectively (Fig. 1b). In MSMEG_2392 mutants DLmal8 and DLmal9 the transposon insertion is 0.4 and 0.45 kb within the MSMEG_2392 gene, respectively (Fig. 1a).

**Phenotype of fbiC mutant and MSMEG_2392 mutant strains on solid medium**

Mutant cultures and complemented strains were streaked on LB solid medium supplemented with 0.001, 0.01, or 0.1 mg ml\(^{-1}\) malachite green or methyl violet to confirm the phenotype on solid medium. After 4 days incubation at 37°C, growth was inhibited by the triphenylmethane dyes at all three concentrations for all mutant strains as compared to parent strain *M. smegmatis* mc\(^{2}\) 155 and as compared to their respective complemented strains (data not shown). The wild-type parent strain was able to decolorize malachite green (Fig. 2a) and methyl violet (Fig. 2f), whereas the representative fbiC mutant, DLmal6 (Fig. 2b, g), and the representative MSMEG_2392 mutant strain, DLmal8 (Fig. 2d, i), were not able to decolorize the dyes to the same extent as wild-type parent strain. In fact, colonies of mutant strains DLmal6 (Fig. 2b) and DLmal8 (Fig. 2d) growing on solid medium supplemented with 0.01 mg malachite green ml\(^{-1}\) were...
intensely green in colour and colonies of DLmal6 (Fig. 2g) and DLmal8 (Fig. 2i) growing on solid medium supplemented with methyl violet were intensely violet in colour, indicating that the dye was being taken up from the medium but could not be decolorized and broken down further. The ability to decolorize both dyes in solid medium was restored in the complemented strains DLmal600 (Fig. 2c, h) and DLmal800 (Fig. 2e, j).

**MICs of malachite green and methyl violet for fbiC mutant and MSMEG_2392 mutant strains**

The MICs for malachite green and methyl violet of the six fbiC mutants, the six fbiC complemented strains, the two MSMEG_2392 mutants and the two MSMEG_2392 complemented strains were determined. The MIC of malachite green for the wild-type parent strain was 0.30 mg ml\(^{-1}\) and the MIC for all fbiC and MSMEG_2392 mutant strains was 0.10 mg ml\(^{-1}\). According to Student’s t-test, the differences between wild-type parent strain and mutant strains were significant at >99 % confidence interval (CI). For the complemented strains, the MIC was 0.30 mg malachite green ml\(^{-1}\) for all fbiC complemented strains and MSMEG_2392 complemented strains with the exception of DLmal1200 (0.32 ± 0.02 mg ml\(^{-1}\); mean ± se) and DLmal1300 (0.36 ± 0.03 mg ml\(^{-1}\)), which had MICs slightly higher than the MIC for the wild-type parent strain. Nonetheless, according to Student’s t-test, the differences between wild-type parent strain and these two complemented strains were not significant. The differences between all mutant strains and their respective complemented strains were significant (>99 % CI). The wild-type parent strain can tolerate and decolorize a higher concentration of malachite green than of the chemically similar compound, methyl violet, since the MIC for this dye was 0.05 mg ml\(^{-1}\), which agrees with similar findings for M. avium strain A5 (Jones & Falkinham, 2003). The MIC was 0.01 mg ml\(^{-1}\) for fbiC and MSMEG_2392 mutant strains, a reduction in MIC to one-fifth. As with malachite green, introduction of the respective functional M. smegmatis gene resulted in a MIC of 0.05 mg ml\(^{-1}\) for methyl violet in all complemented strains. Differences in the MIC for methyl violet between wild-type parent strain and mutants and between mutants and their respective complemented strains were significant (>99 % CI). Since the MIC values of complemented strains for both dyes were identical to those for the wild-type parent strain, the inability to decolorize is a result of the disruption of fbiC or MSMEG_2392 in the mutants, and not a downstream pleiotropic effect.

There was also no difference in MICs between M. smegmatis mc155 and the control strain M. smegmatis pHINT, between DLmal1 and control strain DLmal1pHINT, or between DLmal8 and control strain DLmal8pHINT, for any of the dyes tested. Furthermore, the MIC values were not due to a difference in growth rates since the growth rates of mutant strains and complemented strains in medium without malachite green or methyl violet were similar (data not shown). The MICs were also determined for the azo dye methyl red, for wild-type parent strain, DLmal6, DLmal8 and their respective complemented strains. MIC values were equivalent for all strains tested (data not shown), indicating that the effect of the mutation is specific to triphenylmethane dyes.

**Sensitivity of fbiC and MSMEG_2392 mutant strains to oxidative and nitrosative stress**

Disc diffusion assays were performed with DLmal6, DLmal600, DLmal8 and DLmal800 to determine sensitivity
to oxidative stress and nitrosative stress. Both fbiC and MSMEG_2392 mutant strains exhibited a greater than twofold increase in sensitivity to 0.5 μmol menadione compared to the wild-type parent strain (Table 2). Both mutant strains also showed a greater than twofold sensitivity to 0.025 μmol plumbagin compared to the wild-type parent strain. The differences in zones of clearing were significant between wild-type parent strain and mutant strains and between mutant strains and respective complemented strains (>99 % CI). There was also a significant difference between the zones of clearing in menadione for the parent strain and DLmal800 at 99 % CI, although the zone of clearing for this strain was smaller, indicating that introduction of the wild-type MSMEG_2392 results in the complementation of the mutation. There is no significant difference in menadione sensitivity for the parent strain and DLmal600 even at 90 % CI. For plumbagin, the zones of clearing of both complemented strains are larger than those of the parent strain and this difference is significant (99 % CI), suggesting that the mutants' sensitivity to plumbagin is only partially reversed by complementation.

In contrast to the sensitivity of the mutants to the redox-cycling agents plumbagin and menadione, there was no difference in sensitivity between the wild-type parent strain and DLmal6 to hydrogen peroxide even at 90 % CI. There was a difference in sensitivity between wild-type parent strain and DLmal8 at a CI of 95 %; however, the difference between DLmal8 and its complemented strain, DLmal800, was not significant at 90 % CI (Table 2). There was also no difference in sensitivity to cumene hydroperoxide amongst any of the strains tested (data not shown).

As for nitrosative stress, neither DLmal6 nor DLmal8 showed a statistically significant increase in sensitivity to sodium nitrite as compared to the wild-type parent strain. In all strains, the MIC values ranged between 3 and 5 mM and the MBC values were approximately 25 mM.

### Table 2. Sensitivity of fbiC and MSMEG_2392 mutants to oxidative stress

Results shown are the means ± SE of measurements from five replicates. According to Student’s t-test results, there is a significant difference between the zones of clearing for parent strain M. smegmatis mc² 155 and both mutant strains, and between both mutant strains and their respective complemented strains (>99 % CI) for both menadione and plumbagin. There was not a significant difference between any of the strains tested for sensitivity to hydrogen peroxide, even at 90 % CI, except between the parent strain and DLmal8 (95 % CI). However, there was not a significant difference between the mutant, DLmal8, and DLmal800, the corresponding complemented strain, even at 90 % CI.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Zone of clearing (mm)</th>
<th>0.5 μmol menadione</th>
<th>0.025 μmol plumbagin</th>
<th>1 μmol H₂O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. smegmatis mc² 155</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>DLmal6</td>
<td>37.2 ± 0.8</td>
<td>33.2 ± 1.4</td>
<td>12.5 ± 0.5</td>
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<tr>
<td>DLmal600</td>
<td>17.2 ± 0.8</td>
<td>20.8 ± 0.8</td>
<td>15.2 ± 1.0</td>
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<tr>
<td>DLmal8</td>
<td>40.0 ± 1.6</td>
<td>38.0 ± 3.4</td>
<td>14.8 ± 1.5</td>
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<tr>
<td>DLmal800</td>
<td>14.4 ± 0.4</td>
<td>26.8 ± 0.5</td>
<td>11.3 ± 0.7</td>
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</tr>
</tbody>
</table>

### Table 3. Analysis of M. smegmatis strains for F₄₂₀ and FO content

<table>
<thead>
<tr>
<th>Strain</th>
<th>F₄₂₀ 4.5.6 (nmol mg⁻¹)</th>
<th>FO (nmol mg⁻¹)</th>
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<tbody>
<tr>
<td>M. smegmatis mc² 155</td>
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<td>0.00074</td>
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<tr>
<td>DLmal6</td>
<td>0</td>
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</tr>
<tr>
<td>DLmal600</td>
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<td>0.0023</td>
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<tr>
<td>DLmal8</td>
<td>0</td>
<td>0.0037</td>
</tr>
<tr>
<td>DLmal800</td>
<td>0.182</td>
<td>0.0022</td>
</tr>
</tbody>
</table>

*Dry cell weight.

### Coenzyme F₄₂₀ and FO levels in fbiC and MSMEG_2392 mutant strains

Coenzyme F₄₂₀ and FO levels were measured in DLmal6, DLmal600, DLmal8 and DLmal800 since FbiC catalyses the synthesis of FO, an intermediate in the coenzyme F₄₂₀ biosynthesis pathway. Greater relative fluorescence was observed in wild-type parent strain cells (100 ± 5.0) relative to DLmal6 (23.9 ± 3.5) and DLmal8 (28.2 ± 4.3) cells, and this difference was significant (>99 % CI). Furthermore, the relative fluorescence for the complemented strains DLmal600 (58.5 ± 3.2) and DLmal800 (61.2 ± 16.5) indicates that there was partial recovery in coenzyme F₄₂₀ and/ or FO levels in these strains since the difference between the values for mutant strains and their complement was significant (>99 % CI). Interestingly, the fluorescence values of DLmal6 and DLmal8 did not appear to be different even at 50 % CI, suggesting the possibility that MSMEG_2392 may also be involved in the biosynthesis of coenzyme F₄₂₀ and/or FO.

Since it is not possible to distinguish between coenzyme F₄₂₀ and FO with the fluorescence measured in this assay,
HPLC analysis of coenzyme F420 and FO was performed (Table 3). Neither coenzyme F420 nor FO was detected in DLmal6 and introduction of the native fbiC into DLmal6 resulted in coenzyme F420 and FO levels near those of the wild-type parent strain. Coinjection of purified FO from a methanogen with the DLmal6 sample confirmed that none of the small peaks in the DLmal6 sample was FO. Coenzyme F420 was not detected in DLmal8, but FO was present, indicating that MSMEG_2392 is involved in the biosynthesis of coenzyme F420 from FO. Indeed, the levels of FO were fivefold higher in DLmal8 as compared to the wild-type parent strain (Table 3). Complementation of DLmal8 with the native MSMEG_2392 returned coenzyme F420 to near wild-type levels.

**DISCUSSION**

It is well established that mycobacteria are able to tolerate malachite green since the Middlebrook 7H10 culture medium, which contains malachite green at 0.00025 mg ml\(^{-1}\), is routinely used for culturing this genus to decrease contamination. We screened a transposon library of *M. smegmatis* for mutants unable to decolorize 0.1 mg malachite green ml\(^{-1}\) to determine how this bacterium is able to decolorize triphenylmethane dyes. From the 14,000 mutants, which represented twofold coverage of the genome, we obtained eight mutants that were unable to decolorize malachite green or methyl violet in liquid or on solid media (Fig. 2).

Six of the eight mutants were disrupted in fbiC (Fig. 1b), which codes for an FO synthase that is essential for the biosynthesis of the electron-transfer agent coenzyme F420 (Choi et al., 2002). The enzyme catalyses the transfer of the hydroxybenzyl group from 4-hydroxyphenylpyruvate, a precursor to tyrosine, to 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione, an intermediate in riboflavin synthesis, resulting in FO (Graham et al., 2003). FO is eventually converted to coenzyme F420 through a series of reactions (Choi et al., 2001). Interestingly, coenzyme F420 has been found exclusively in certain archaea, cyanobacteria and actinomycetes (Purwantini et al., 1997). HPLC analysis confirmed that a representative fbiC mutant lacked both coenzyme F420 and FO (Table 3).

The remaining two mutants were disrupted in MSMEG_2392 (Fig. 1a), encoding a protein in the DUF121 superfamily of proteins with an unknown function (http://www.tigr.org, *M. smegmatis* mc\(^2\) 155 annotated genome database). Proteins containing this domain average approximately 208 residues in length (http://www.sanger.ac.uk) and in some strains, including *M. smegmatis* mc\(^2\) 155, the protein is alanine-rich (18.1%). Like fbiC, genes encoding proteins with the DUF121 domain have been found mainly in archaea and actinomycetes. Intriguingly, like the fbiC mutant, the MSMEG_2392 mutant lacks coenzyme F420 but, unlike the fbiC mutant, it contains FO, indicating that a step subsequent to that catalysed by FbiC is affected in the MSMEG_2392 mutant (Table 3). The pathway after the synthesis of FO has not been elucidated in complete detail in mycobacteria, although both FbiA and FbiB are known to be involved (Choi et al., 2001). In methanogens, the latter part of the biosynthetic pathway consists of the transfer of a 2-phosphoalactate moiety from lactyl(2)diphospho-(5′)guanosine (LPPG) to FO to form coenzyme F420-0, a reaction catalysed by a LPPG:FO 2-phospho-1-lactate transferase (Graupner & White, 2001). This is followed by polyglutamylation, initially, by a F420-0-γ-glutamyl ligase, which adds two glutamate residues to the coenzyme F420-0 (Li et al., 2003a), and then by an enzyme, with sequence similarity to glutathione synthetases, that adds a single α-linked terminal glutamate residue (Li et al., 2003b). In mycobacteria, there are five and six glutamyl forms of γ-linked glutamates (Bair et al., 2001). The MSMEG_2392-encoded protein may be involved by itself or as part of a multisubunit enzyme in the transferase reaction, the glutamylation reactions, or the synthesis of 2-phosphoalactate or LPPG.

We did not obtain any transposon mutants disrupted in fbiA (MSMEG_1830) or fbiB (MSMEG_1829) in our screen. Even though mutants of fbiA and fbiB from *M. bovis* BCG have been reported (Choi et al., 2001), it is still possible that pleiotropic effects of mutations in these two genes in *M. smegmatis* may have made it difficult to isolate transposon mutants in our screen. The genes in the operon containing fbiA and fbiB in these two *Mycobacterium* species are not identical; in both operons, fbiA is located immediately upstream of fbiB but the downstream gene is different. In *M. smegmatis* mc\(^2\) 155, the gene immediately downstream of fbiB is MSMEG_1827, which encodes a protein that is 182 residues in length with a NUDIX domain involved in DNA metabolism, while in *M. bovis* BCG, the gene immediately downstream of fbiB is Mb3291, a probable DNA methylase that is 553 residues in length (http://genolist.pasteur.fr/BoviList/). An alternative explanation for the lack of fbiA and fbiB mutants in the screen is that, instead of coenzyme F420, an intermediate such as FO, the product of the reaction catalysed by fbiC, can serve adequately as the cofactor. In cyanobacteria, for example, a DNA photolyase uses FO instead of coenzyme F420 (de Wit & Eker, 1987; Eker et al., 1990; Lin & White, 1986). However, since the levels of FO are five times higher in DLmal8 and the mutant is still unable to decolorize malachite green, it appears that coenzyme F420 is responsible for the triphenylmethane dye decolorization.

To the best of our knowledge, the only coenzyme F420-dependent enzyme in mycobacteria that has been characterized is an F420-dependent glucose-6-phosphate dehydrogenase (Purwantini et al., 1997; Purwantini & Daniels, 1998), which catalyses a reaction similar to the first enzymic step in the pentose phosphate cycle. The pentose phosphate pathway provides NADPH that is used for reductive biosynthetic reactions and for the maintenance of the cellular redox state. Indeed, a *M. tuberculosis* fbiC mutant is known to be sensitive to nitrosative stress (Darwin et al., 2003) and a *Salmonella*
typhimurium zwf mutant, disrupted in glucose-6-phosphate dehydrogenase, is sensitive to oxidative stress (Lundberg et al., 1999). Thus, we tested the sensitivity of M. smegmatis fbiC and MSMEG_2392 mutant strains, DLmal6 and DLmal8, to oxidative and nitrosative stress. We found that both DLmal6 and DLmal8 were twofold more sensitive to plumbagin and medinadione, redox-cycling agents which increase the superoxide concentration within the cell, as compared to the wild-type parent strain. However, unlike the S. typhimurium zwf mutant, these mutants were not more sensitive to hydrogen peroxide (Table 2). Furthermore, unlike the M. tuberculosis fbiC mutant that was isolated in a screen for mutants sensitive to nitrosative stress (Darwin et al., 2003), neither the fbiC nor MSMEG_2392 transposon mutants displayed increased sensitivity to nitrosative stress as compared to the wild-type parent strain. Other genes involved in resistance to nitrosative stress in M. smegmatis mc2 155 that are not found in M. tuberculosis may compensate for the mutations.

A variety of enzymes in archaea are coenzyme F420-dependent, including alcohol dehydrogenase (Widdel & Wolfe, 1989), hydrogenase (Jacobson et al., 1982), methyltetrahydromethanopterin dehydrogenase (Hartzell et al., 1985), methylenetetrahydromethanopterin reductase (Ma & Thauer, 1990), NADP+-reductase (Jones & Stadtman, 1980), sulfite reductase (Johnson & Mukhopadhyay, 2005) and quinone oxidoreductase (Kunow et al., 1994). An NADPH-dependent F420-reductase and coenzyme F420-dependent hydride transferase are involved in the mineralization of 2,4-dinitrophenol in the actinomycete Nocardioides simplex FJ2-1A (Ebert et al., 1999). In M. smegmatis, certain genes and their gene products have been annotated as possibly requiring coenzyme F420 based on homology (http://www.tigr.org, M. smegmatis mc2 155 annotated genome database). Purification of a triphenylmethane reductase that requires NADH (and NADPH to a lesser extent) as an electron donor to decolorize malachite green to leucomalachite green has been reported from a Citrobacter sp. (Jang et al., 2005). It is possible that a triphenylmethane reductase that catalyses the decolorization of malachite green and that uses coenzyme F420H2 instead of NADH or NADPH exists in mycobacteria. Screening more transposon mutants may result in the identification of the gene encoding the M. smegmatis triphenylmethane reductase unless it is essential or upstream of an essential gene. It is also possible that coenzyme F420H2 serves as an electron donor for the reduction of malachite green directly without the involvement of a reductase. In either case, it may be possible to use malachite green to identify other genes involved in the biosynthesis and regulation of coenzyme F420 and FO in other organisms that use this method to decolorize the dye as we have done here. Further experiments on MSMEG_2392 are under way to determine its exact function in coenzyme F420 biosynthesis and the precise role of coenzyme F420 in triphenylmethane decolorization.

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