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

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Mycobacteria 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.

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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; Stamatii *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 leucomalachite green after 48 h (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

Abbreviations: CI, confidence interval; FO, 7,8-didemethyl-8-hydroxy-5-deazariboflavin; LPPG, lactyl(2)diphospho-(5')guanosine; MBC, minimum bactericidal concentration.

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 & Falkinham (2003) reported that the decolorization of malachite green by pathogenic mycobacteria such as *Mycobacterium chelonae* and *Mycobacterium avium* 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* DH5 α 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 for *M. smegmatis* mc² 155) and LB solid medium. Antibiotics were added as appropriate [25 μ g kanamycin ml⁻¹ for *M. smegmatis* transposon mutants; 75 μ g hygromycin ml⁻¹ for *M. smegmatis* harbouring pHINT; a *M. smegmatis*/*E. coli* shuttle vector with a hygromycin resistance cassette (Garbe *et al.*, 1994); 100 μ g hygromycin ml⁻¹ for *E. coli* harbouring pHINT; and 100 μ g ampicillin ml⁻¹ for *E. coli* harbouring pCR2.1]. All cultures were incubated at 37 °C. The OD₆₀₀ of the cultures was monitored in 1 ml cuvettes or 96-well ELISA plates using a SpectraMax Plus384 spectrophotometer (Molecular Devices).

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 (Epicenter Technologies). Briefly, 1 μ l of the EZ::TN transposome 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 μ g 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 μ g 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 μ g amount of genomic DNA from each malachite green decolorizing mutant listed in Table 1 was digested with restriction enzymes *Pst*I, *Sall*I, or *Sac*II, and 0.25 μ g 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-2Fp-1 and Kan-2Rp-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 *Xho*I and *Hind*III sites engineered into them for cloning into pHINT, a *M. smegmatis*/*E. coli* 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 *Xho*I and *Hind*III; the *fbiC* insert was gel purified, ligated to the digested pHINT vector, transformed into *E. coli* and plated onto LB agar containing 100 μ g hygromycin ml⁻¹. After confirmation by restriction-digest analysis of the purified recombinant plasmid, the construct, pHINT/*fbiC*, was electroporated into competent cells from the mutant strains and plated onto LB agar supplemented with 25 μ g kanamycin ml⁻¹ and 75 μ g 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 *Xho*I and *Hind*III 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

Strain, plasmid, oligonucleotide	Characteristic(s)*	Source or reference
Strain		
<i>M. smegmatis</i>	mc ² 155, parent strain	W.R. Jacobs
<i>E. coli</i> DH5 α	F ⁻ <i>recA1 hsdR17 thi-1 gyrA96 supE44 endA1 relA1 recA1 deoR</i> Δ (<i>lacZYA-argF</i>)U169 (ϕ 80 <i>lacZ</i> Δ M15)	
DLmal1	<i>M. smegmatis</i> mutant disrupted in <i>fbiC</i>	This study
DLmal6	<i>M. smegmatis</i> mutant disrupted in <i>fbiC</i>	This study
DLmal7	<i>M. smegmatis</i> mutant disrupted in <i>fbiC</i>	This study
DLmal10	<i>M. smegmatis</i> mutant disrupted in <i>fbiC</i>	This study
DLmal12	<i>M. smegmatis</i> mutant disrupted in <i>fbiC</i>	This study
DLmal13	<i>M. smegmatis</i> mutant disrupted in <i>fbiC</i>	This study
DLmal100	DLmal1 complemented with <i>M. smegmatis fbiC</i>	This study
DLmal600	DLmal6 complemented with <i>M. smegmatis fbiC</i>	This study
DLmal700	DLmal7 complemented with <i>M. smegmatis fbiC</i>	This study
DLmal1000	DLmal10 complemented with <i>M. smegmatis fbiC</i>	This study
DLmal1200	DLmal12 complemented with <i>M. smegmatis fbiC</i>	This study
DLmal1300	DLmal13 complemented with <i>M. smegmatis fbiC</i>	This study
DLmal8	<i>M. smegmatis</i> mutant disrupted in predicted gene MSMEG_2392	This study
DLmal9	<i>M. smegmatis</i> mutant disrupted in predicted gene MSMEG_2392	This study
DLmal800	DLmal8 complemented with <i>M. smegmatis</i> MSMEG_2392	This study
DLmal900	DLmal9 complemented with <i>M. smegmatis</i> MSMEG_2392	This study
pHINT	<i>M. smegmatis</i> harbouring empty pHINT vector	This study
DLmal1pHINT	DLmal1 harbouring empty pHINT vector	This study
DLmal8pHINT	DLmal8 harbouring empty pHINT vector	This study
Plasmid		
pCR2.1	TA cloning vector, Amp ^r	Invitrogen
pHINT	<i>M. smegmatis</i> / <i>E. coli</i> shuttle vector, Hyg ^r	K. Downing (Garbe <i>et al.</i> , 1994)
pHINT <i>fbiC</i>	pHINT with <i>M. smegmatis fbiC</i> and putative promoter	This study
pHINT2392	pHINT with <i>M. smegmatis</i> MSMEG_2392 and putative promoter	This study
Oligonucleotide		
<i>fbiC</i> -5'	5'-AAGCTTCGCGGACAGAGACAG-3'	This study
<i>fbiC</i> -3'	5'-CTCGAGGACCTCGCGGTGAAG-3'	This study
2392-5'	5'-CTCGAGACGGCCAACATCACG-3'	This study
2392-3'	5'-AAGCTTCTTCGCTCACGTGTC-3'	This study
Kan-2-Fp-1	5'-ACCTACAACAAAGCTCTCATCAACC-3'	Epicer Technologies
Kan-2-Rp-1	5'-GCAATGTAACATCAGAGATTTTGAG-3'	Epicer Technologies

*Amp^r, ampicillin resistance; Hyg^r, 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⁻¹ for malachite green and 0.01 to 0.5 mg ml⁻¹ for methyl violet. Cells grown to mid-exponential phase were used to inoculate prepared ELISA plates at OD₆₀₀ 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_2392) 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 OD₆₀₀ 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 μ l. 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 μ mol) and plumbagin (0.025 μ mol), as well as hydrogen peroxide (1 μ mol) and cumene hydrogen peroxide (0.5 μ mol), 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 OD₆₀₀ 0.15. Plates were incubated at 37 °C for 24 h and the MICs were determined visually and by measuring OD₆₀₀ 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.

Coenzyme F₄₂₀/FO determination. Initially, relative coenzyme F₄₂₀/FO (7,8-didemethyl-8-hydroxy-5-deazariboflavin) levels were determined for wild-type parent strain, DLmal6, DLmal600, DLmal8 and DLmal800 using methods adapted from Heine-Dobbernack *et al.* (1988). Briefly, 100 ml cells were grown to late-exponential or stationary phase, harvested by centrifugation at 16 000 g for 30 min, and washed twice with 0.9 % (w/v) NaCl at 4 °C. Pellets were resuspended in 3 ml extraction buffer (Na₂HPO₄, KH₂PO₄, 50 mM each, pH 5.7), incubated in a boiling water bath for 10 min, and then centrifuged at 47 600 g for 20 min at 4 °C. Two millilitres of the resulting supernatant was mixed with an equal volume of 2-propanol at −20 °C and centrifuged at 47 600 g for 20 min at 4 °C. Relative fluorescence of these samples was measured using a Bio-Rad VersaFluor spectrofluorometer with an excitation wavelength of 390 ± 22 nm and an emission wavelength of 460 ± 10 nm in 3 ml cuvettes. The fluorescence of the wild-type parent strain extract was set at 100 and the relative fluorescence was recorded for the remaining strains. The experiment was performed in quadruplicate.

To obtain a more accurate measure of coenzyme F₄₂₀ and FO levels, HPLC analysis was performed. Extracts of 100 ml flask cultures of *M. smegmatis* strains, grown for 4 days to the stationary phase, were made by heating equal volumes of packed cell pellets and water at 95 °C for 30 min, followed by vortex mixing and centrifugation to remove the pellet. Coenzyme F₄₂₀ and FO were separated by HPLC using a Varian ProStar HPLC system with a PS-363 fluorescence detector (excitation at 400 nm, emission at 470 nm). An alpha-Bond C-18 column (Supelco; 3.9 mm by 300 mm) was eluted at 1 ml min^{−1}. 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: 0–2 min, 0 %; 2–6 min, a linear gradient of 0–2 %; 6–15 min, 2–10 %; 15–22 min, 10–28 %; 22–27 min, 28–0 %. Identities of peaks were confirmed by comparison with F₄₂₀ purified from *M. smegmatis* (F₄₂₀-4,5,6) and F₄₂₀-2 and FO purified from *Methanothermobacter thermoautotrophicus*. Coenzyme F₄₂₀ and FO levels were calculated based on arbitrary fluorescence units in a standard curve created with purified FO.

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 F₄₂₀. 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* mc² 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

Fig. 1. Transposon insertion sites in (a) two MSMEG_2392 mutants, DLmal8 and DLmal9, and (b) six *fbiC* mutants, DLmal1, DLmal6, DLmal7, DLmal10, DLmal12 and DLmal13.

Fig. 2. Decolorization ability of *M. smegmatis* mc² 155, DLmal6 (a *fbiC* mutant), DLmal600 (complemented strain of DLmal6), DLmal8 (MSMEG_2392 mutant), and DLmal800 (complemented strain of DLmal8) on LB agar medium supplemented with malachite green (0.01 mg ml⁻¹) or methyl violet (0.01 mg ml⁻¹). (a–e) Growth on malachite green of *M. smegmatis* mc² 155 (a), DLmal6 (b), DLmal600 (c), DLmal8 (d) and DLmal800 (e). (f–j) Growth on methyl violet of *M. smegmatis* mc² 155 (f), DLmal6 (g), DLmal600 (h), DLmal8 (i) and DLmal800 (j).

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* mc²155 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 triphenyl-methane 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 *fbtC* 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 hydrogen peroxide 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 3. Analysis of *M. smegmatis* strains for F_{420} and FO content

Strain	$F_{420-4,5,6}$ (nmol mg ⁻¹ *)	FO (nmol mg ⁻¹ *)
<i>M. smegmatis</i> mc ² 155	0.184	0.00074
DLmal6	0	0
DLmal600	0.255	0.0023
DLmal8	0	0.0037
DLmal800	0.182	0.0022

*Dry cell weight.

Coenzyme F_{420} and FO levels in *fbtC* and MSMEG_2392 mutant strains

Coenzyme F_{420} and FO levels were measured in DLmal6, DLmal600, DLmal8 and DLmal800 since *FbtC* catalyses the synthesis of FO, an intermediate in the coenzyme F_{420} 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 F_{420} 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 F_{420} and/or FO.

Since it is not possible to distinguish between coenzyme F_{420} and FO with the fluorescence measured in this assay,

Table 2. Sensitivity of *fbtC* and MSMEG_2392 mutants to oxidative stress

Results shown are the means $\pm$ 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.

Strain	Zone of clearing (mm)		
	0.5 μmol menadione	0.025 μmol plumbagin	1 μmol H ₂ O ₂
<i>M. smegmatis</i> mc ² 155	17.0 $\pm$ 0.6	16.5 $\pm$ 0.5	13.3 $\pm$ 0.7
DLmal6	37.2 $\pm$ 0.8	33.2 $\pm$ 1.4	12.5 $\pm$ 0.5
DLmal600	17.2 $\pm$ 0.8	20.8 $\pm$ 0.8	15.2 $\pm$ 1.0
DLmal8	40.0 $\pm$ 1.6	38.0 $\pm$ 3.4	14.8 $\pm$ 1.5
DLmal800	14.4 $\pm$ 0.4	26.8 $\pm$ 0.5	11.3 $\pm$ 0.7

HPLC analysis of coenzyme F_{420} and FO was performed (Table 3). Neither coenzyme F_{420} nor FO was detected in DLmal6 and introduction of the native *fbtC* into DLmal6 resulted in coenzyme F_{420} 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 F_{420} was not detected in DLmal8, but FO was present, indicating that MSMEG_2392 is involved in the biosynthesis of coenzyme F_{420} 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 F_{420} 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 $0.00025 \text{ mg ml}^{-1}$, 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 ml^{-1} malachite green 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 *fbtC* (Fig. 1b), which codes for an FO synthase that is essential for the biosynthesis of the electron-transfer agent coenzyme F_{420} (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(1*H*,3*H*)-pyrimidinedione, an intermediate in riboflavin synthesis, resulting in FO (Graham *et al.*, 2003). FO is eventually converted to coenzyme F_{420} through a series of reactions (Choi *et al.*, 2001). Interestingly, coenzyme F_{420} has been found exclusively in certain archaea, cyanobacteria and actinomycetes (Purwantini *et al.*, 1997). HPLC analysis confirmed that a representative *fbtC* mutant lacked both coenzyme F_{420} 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* mc² 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* mc² 155, the protein is alanine-rich (18.1%). Like *fbtC*, genes encoding proteins with the DUF121 domain have been found mainly in archaea and actinomycetes. Intriguingly, like the *fbtC* mutant, the MSMEG_2392 mutant lacks coenzyme F_{420} but, unlike the *fbtC* mutant, it contains FO, indicating that a step subsequent to that catalysed by FbtC 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 FbtA and FbtB 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-phospholactate moiety from lactyl(2)diphospho-(5')guanosine (LPPG) to FO to form coenzyme F_{420} -O, a reaction catalysed by a LPPG:FO 2-phospho-L-lactate transferase (Graupner & White, 2001). This is followed by polyglutamylation, initially, by a F_{420} -O: γ -glutamyl ligase, which adds two glutamate residues to the coenzyme F_{420} -O (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-phospholactate or LPPG.

We did not obtain any transposon mutants disrupted in *fbtA* (MSMEG_1830) or *fbtB* (MSMEG_1829) in our screen. Even though mutants of *fbtA* and *fbtB* 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 *fbtA* and *fbtB* in these two *Mycobacterium* species are not identical; in both operons, *fbtA* is located immediately upstream of *fbtB* but the downstream gene is different. In *M. smegmatis* mc² 155, the gene immediately downstream of *fbtB* 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 *fbtB* is Mb3291, a probable DNA methylase that is 553 residues in length (<http://genolist.pasteur.fr/BovList/>). An alternative explanation for the lack of *fbtA* and *fbtB* mutants in the screen is that, instead of coenzyme F_{420} , an intermediate such as FO, the product of the reaction catalysed by *fbtC*, can serve adequately as the cofactor. In cyanobacteria, for example, a DNA photolyase uses FO instead of coenzyme F_{420} (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 F_{420} is responsible for the triphenylmethane dye decolorization.

To the best of our knowledge, the only coenzyme F_{420} -dependent enzyme in mycobacteria that has been characterized is an F_{420} -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* *fbtC* 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, DLmal 6 and DLmal8, to oxidative and nitrosative stress. We found that both DLmal6 and DLmal8 were twofold more sensitive to plumbagin and menadione, 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 mc*² 155 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), methylenetetrahydromethanopterin 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 *Nocardioideis 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 mc*² 155 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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