A Novel Sterol 14α-Demethylase/Ferredoxin Fusion Protein (MCCYP51FX) from Methyllococcus capsulatus Represents a New Class of the Cytochrome P450 Superfamily*

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Sterol 14α-demethylase encoded by CYP51 is a member of the cytochrome P450 (CYP) superfamily of enzymes and has been shown to have an essential role in sterol biosynthesis in eukaryotes, with orthologues recently being described in some bacteria. Examination of the genome sequence data for the proteobacterium Methyllococcus capsulatus, a bacterial species known to produce sterol, revealed the presence of a single CYP with strong homology to CYP51, particularly to a form in Mycobacterium tuberculosis. This M. capsulatus CYP51 protein represents a new class of CYP consisting of the CYP domain naturally fused to a ferredoxin domain at the C terminus via an alanine-rich linker. Expression of the M. capsulatus MCCYP51FX fusion in Escherichia coli yielded a P450, which, when purified to homogeneity, had the predicted molecular mass of 62 kDa on SDS/PAGE and bound lanosterol as a putative substrate. Ste- rol 14α-demethylase activity was shown (0.24 nmol of lanosterol metabolized per minute per nanomole of MCCYP51FX fusion) by gas chromatography/mass spectrometry with the activity dependent upon the presence of ferredoxin reductase and NADPH. Our unique findings describe a new class of naturally existing cytochrome P450, which will provide pivotal information for CYP structure/function in general.

Cytochrome P450 (CYP)* genes are members of one of the most functionally versatile superfamilies of enzymes found in nature. CYP enzymes are involved both in essential endogenous biosynthetic functions, e.g. steroids, hormones, and fatty acids, and in the metabolism of xenobiotic chemicals, e.g. drugs, carcinogens, and pollutants (1–3). The biodiversity of cytochrome P450 genes has been revealed in dramatic fashion by genome sequencing projects, and so much so that many thousands of different forms have been or will be identified shortly. Within bacteria, some species such as Escherichia coli lack cytochrome P450 genes, whereas others contain many, such as the 20 forms of Mycobacterium tuberculosis (4). Recently, genomic information on the methane-utilizing bacterium Methyllococcus capsulatus has become available (www.tigr.org). This bacterium is one of the first and best elucidated of any prokaryotes for the production of steroids (5, 6), a feature generally associated with eukaryotes. These investigations used minimal sterol-free media for growth, and gas chromatography/mass spectrometry/mass spectrometry for identification of 4-demethyl, 4-methyl, and 4-demethyl sterols. This information indicated that a sterol 14α-demethylase was present in the organism that is encoded by a cytochrome P450, CYP51.

The CYP-catalyzed reaction involves the molecular splitting of atmospheric oxygen to produce a molecule of water and a monoxygenated substrate (7). Two reducing equivalents are essential for this reaction. They are supplied by NADH or NADPH, depending on the origin of the CYP, and are transferred in two stages through either one or two redox partners. Different CYPs of eukaryotes and prokaryotes differ in their reductase partners as well as in co-factor requirements for catalytic activity (for review, see Ref. 8). In prokaryotes and mitochondrial CYPs (the general class I system), an iron-sulfur ferredoxin and a ferredoxin reductase are essential for electron transfer to the CYP. Class II eukaryotic membrane-bound CYPs of the endoplasmic reticulum require a FAD/FMN flavoprotein, namely NADPH cytochrome P450 reductase for activity. A similar class III system has also been found. It consists of a fused arrangement of CYP with a cytochrome P450 reductase-like FAD/FMN flavoprotein reductase domain. This has been observed and extensively studied in the case of CYP102A1 (BM3) from Bacillus megaterium (9, 10), and similar proteins are evident in Bacillus subtilis (11) and Fusarium oxysporum (12). Because of interest in the catalytic cycle and protein-protein interactions, many examples of artificial class II fusion protein systems have been constructed (13–15) and, for CYP101, a bacterial class I enzyme, the production and activity of a triple fusion of CYP101 to ferredoxin and ferredoxin reductase has been reported (16). By linking the ferredoxin reductase and the ferredoxin to CYP101 in an amino- to carboxy-terminal arrangement, a self-sufficient protein with catalytic activity was obtained. This suggested the optimal arrangement for subunit interaction in the class I CYP complex.

The importance of sterols in nature is well known, but not their role in bacteria. Because of our interest in such compounds in bacterial systems, the genome data for the Gram-negative bacterium M. capsulatus was examined for the presence of a sterol 14α-demethylase. A single CYP was detected with strong homology to CYP51, particularly to a form in

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Definitive evidence of sterol production in this latter organism in sterol-free minimal medium is still to be demonstrated. Interestingly, this *M. capsulatus* CYP51 protein represented a new class of CYP consisting of the CYP domain fused to a ferredoxin domain at the C terminus via an alanine-rich linker. This new CYP class is related to class I CYPs and suggests an optimal or at least a functional arrangement selected through evolution, as reflected by this order of protein domains. Herein, we describe the discovery of this new CYP form and the biochemical evaluation of the novel protein.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Media, and Growth Conditions**—The *Methylococcus capsulatus* type strain ATCC 19069 was obtained from the National Collection of Industrial and Marine Bacteria (NCIMB Ltd., Aberdeen, Scotland). Cells were cultured in 250 ml of minimal salts medium (18) at 37 °C in an atmosphere of methane/air, 1:1 (v/v). After 3 days of incubation, cells were harvested by centrifugation and washed once in 10 ml of 0.1 mM Tris-HCl, pH 7.5.

**Identification of the *M. capsulatus* MCCYP51FX ORF—**Sequence data for the genome of *Methylococcus capsulatus* was obtained from The Institute for Genomic Research website at www.tigr.org. A BLASTX search predicted from both the alignment with the Mtb CYP51 amino acid sequence and from FramePlot codon usage analysis (www.nih.go.jp/H11011/jun/research/frameplot/index.html), although the upstream Shine-Dalgarno sequence is weak. The alanine-rich linker region between the P450 and ferredoxin domains is shown underlined and is expanded in panel B. This diagram also indicates the location of two ORFs that flank *mccyp51fx*: upstream is a 1875-bp putative *thiC* homologue, and downstream is a 714-bp ORF (*orf1*) of unknown function.

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**M. tuberculosis** (17). Definitive evidence of sterol production in this latter organism in sterol-free minimal medium is still to be demonstrated. Interestingly, this *M. capsulatus* CYP51 protein represented a new class of CYP consisting of the CYP domain fused to a ferredoxin domain at the C terminus via an alanine-rich linker. This new CYP class is related to class I CYPs and suggests an optimal or at least a functional arrangement selected through evolution, as reflected by this order of protein domains. Herein, we describe the discovery of this new CYP form and the biochemical evaluation of the novel protein.
(19) homology search of the translated M. capsulatus genome was made using the protein sequence of the M. tuberculosis (Mt) cytochrome P450 sterol 14 α-demethylase protein (CYP51) (20). DNA Extraction and PCR Amplification of mccyp51fx—Fifty milligrams of cells were resuspended in 500 μl of sterile, double-distilled water and boiled for 10 min. The cell debris was pelleted by centrifugation at 13,000 rpm for 5 min, and the supernatant was aspirated and snap-cooled on ice. Primers MC51F (5'-ACGCTATCCAGGGCATCCACCCCTCC-3') and MC51R (5'-GCAATAGCTCATTGGTAGTTAGGGTGATGTTAGGAGGGTTGATGTTAGGAGGC-3') were designed to amplify the M. capsulatus cyp51fx ORF. The primers incorporate unique Ndel (underlined) and HindIII (double underlined) cloning sites and a C-terminal polyhistidine tag (bold) for downstream purification of the expressed protein. The PCR reaction was assembled using 4 μl of 10× PCR buffer, 2 μl of magnesium chloride (25 mM), 6 μl of dNTPs (25 mM each dATP, dGTP, dCTP, and dTTP), 6 μl of each primer (10 pmol), 253 μl of PCR-grade water, 28 μl of dimethyl sulfoxide (Sigma), 10 units of Taq DNA polymerase, and 32 μl of DNA template (all PCR reagents and restriction enzymes supplied by Promega). The PCR mix was divided into four 100-μl aliquots. Amplification was carried out by denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 60 s. A terminal extension of 10 min at 72 °C completed the reaction.

Cloning and Expression of the MCCYP51FX Protein—The PCR product was purified from an agarose gel and cloned into the pGEM-T Easy vector (Promega). The insert was excised by double-digestion with Ndel and HindIII restriction enzymes (Promega), cloned into the pET17b expression vector (Novagen), and transformed into E. coli BL21-pLysE cells (Invitrogen). For protein expression, transformed cells were grown in Terrific broth (12 g/liter tryptone, 24 g/liter yeast extract, 4 ml/liter sodium chloride, and MCCYP51FX was eluted with buffer A containing 0.5 M potassium phosphate and 20% (v/v) glycerol, pH 7.4 (Buffer A). The column was washed with Buffer A containing 50 mM glycine and 0.5 M potassium phosphate buffer, 24 μl of magnesium chloride (25 mM), 6 μl of dNTPs (25 mM each dATP, dGTP, dCTP, and dTTP), 6 μl of each primer (10 pmol), 253 μl of PCR-grade water, 28 μl of dimethyl sulfoxide (Sigma), 10 units of Taq DNA polymerase, and 32 μl of DNA template (all PCR reagents and restriction enzymes supplied by Promega). The PCR mix was divided into four 100-μl aliquots. Amplification was carried out by denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 60 s. A terminal extension of 10 min at 72 °C completed the reaction.

Purification of MCCYP51FX—Eight liters of MCCYP51FX culture was pelleted by centrifugation at 1,500 × g and resuspended in 200 ml of potassium phosphate buffer, pH 7.4. Cells were broken following two passages through a C5 homogenizer (Avestin) using an operating pressure of 15,000 p.s.i. The lysed cells were centrifuged at 10,000 × g to remove unbroken cells and cell debris. The cytosolic fraction was separated from the membrane fraction by ultracentrifugation at 100,000 × g for 45 min. MCCYP51FX was isolated using a nickel-nitritotriacetic acid (Ni2+-NTA) affinity column (Qiagen) equilibrated with 50 mM potassium phosphate and 20% (v/v) glycerol, pH 7.4 (Buffer A). The column was washed with Buffer A containing 50 mM glycine and 0.5 M sodium chloride, and MCCYP51FX was eluted with Buffer A containing 100 mM imidazole. MCCYP51FX was dialyzed overnight against 50 mM Tris-HCl, pH 7.4, containing 10% (v/v) glycerol.

Reconstituted Sterol 14α-Demethylase Catalytic Activity—Activities of class I CYP enzymes require the support of a ferredoxin and a ferredoxin reductase for catalysis. Although the MCCYP51FX contained the CYP and the ferredoxin domains fused, the third essential component of a functional endogenous ferredoxin reductase for MCCYP51FX activity was unknown. The capacity of spinach ferredoxin reductase (Sigma) to drive MCCYP51FX-mediated sterol 14α-demethylation was determined. Furthermore, the addition of saturating amounts of spinach ferredoxin and ferredoxin reductase were assessed in their contribution to stimulate MCCYP51FX activity and hence determine inter- or intramolecular electron transfer within the MCCYP51FX fusion complex. Reaction mixtures contained 1 nmol/ml purified MCCYP51FX and 1 nmol of spinach ferredoxin reductase. Lanosterol dispersed in Triton WR-1339 was resuspended in MOPS buffer (50 mM). The reaction volume was adjusted to 950 μl, and NADPH was added to a 1 mm final concentration to start the reaction. All reactions were incubated at 37 °C for 20 min with gentle agitation. Reactions were stopped by the addition of 3 ml of methanol, and sterols were extracted by incubation with 90% (v/v) ethanolic potassium hydroxide at 80 °C for 1 h in a preheated water bath. Following silylation for 1 h at 60 °C with bis(trimethylsilyl) trifluoroacetamide (BSTFA) (50 μl) in 50 μl of toluene, sterol substrates and metabolites were clearly separated and identified by gas chromatography/mass spectrometry (VG 12–250; VG Biotech). The activity (nanomole of demethylated product formed per minute per nanomole of P450) was calculated using the amount of substrate added and the conversion ratio (calculated from the

FIG. 2. Protein purification analysis showing the isolation of MCCYP51FX as monitored by Coomassie Brilliant Blue staining. MCCYP51FX was purified to electrophoretic homogeneity through His6 tag purification technology using Ni2+-NTA affinity chromatography. Lane 1, isolated E. coli P450 folating heterologous expression of MCCYP51FX; lane 2, MCCYP51FX eluate obtained following two passes over Ni2+-NTA.

DISCOVERY AND CHARACTERIZATION OF A CYP51/FERREDOXIN PROTEIN

RESULTS AND DISCUSSION

Discovery of mccyp51fx Fusion through Bioinformatic Analysis—BLASTX searching using the protein sequence of the M. tuberculosis cytochrome P450 sterol 14 α-demethyle protein (CYP51) (20) as a query against the M. capsulatus genome identified a single CYP with homology to CYP51s from a range of organisms (Figs. 1A and 5A). Using the Pfam motif of CYP (heme-binding domain FXGXXRXXX) in a BLASTX search of the genome, this putative CYP51 was shown to be the only CYP present in the genome of this organism. The M. capsulatus CYP51 protein is 551 amino acids in length and shows the strongest homology to CYP51 from M. tuberculosis (17) (49% identical, 219/444; 64% similar, 289/444). The closest eukaryotic homologue is CYP51 from Sorghum bicolor (24) (38% identical, 172/449). However, the putative M. capsulatus CYP51 is 100 residues longer than its closest CYP51 homologue from M. tuberculosis. This additional downstream sequence was proposed to comprise a fused putative ferredoxin,
Fig. 3. Absolute, carbon monoxide difference spectrum, and substrate binding spectra of purified MCCYP51FX. A, absorbance of purified MCCYP51FX, the absolute oxidized form (b) and sodium dithionite-reduced form (c), demonstrating the α- and β-bands for the oxidized and reduced forms. α represents the differential CO-reduced CYP spectrum of MCCYP51FX. The spectrum was recorded with sodium dithionite-reduced MCCYP51FX (0.2 nmol) in each cuvette after bubbling the sample cuvette with CO for 1 min. B, MCCYP51FX type II binding spectra for
with the best homology to a ferredoxin immediately downstream of CYP51 from *M. tuberculosis* (Mtb Rv0763c, 42% identical, 26/61), but this 100-amino acid extension did not include a linker region and a C-terminal domain not found in Mtb Rv0763c. An alanine rich region (7 of 14 residues) is found between the end of the CYP domain and the beginning of the ferredoxin domain, and we propose that this region may act as a flexible hinge allowing interaction between CYP and ferredoxin (Fig. 1B). Such structural architecture is unique to the MCCYP51FX and is not seen in other CYP fusion proteins described to date such as CYP102A1, the most extensively studied CYP fusion. However, an alanine-rich linker region has been described in other bacterial fusion proteins, notably in the pyruvate dehydrogenase complex from *E. coli* (25). A purine rich region is located 10 bp upstream from the start codon and is a potential Shine-Delgarno sequence.

**Cloning, Expression, and Purification of MCCYP51FX**—The *mccyp51fx* 1656-bp open reading frame encoding CYP51 fused with a ferredoxin was cloned, and the sequence was shown to be identical to that revealed from the genome sequencing project. MCCYP51FX was produced as a recombinant protein in *E. coli* with levels of expression exceeding >2000 nmol of hemoprotein per liter of culture as quantified from the reduced carbon monoxide cytochrome P450 difference spectrum. Cell fractionation revealed MCCYP51FX to be a soluble protein, being located in the cytosolic fraction following ultracentrifugation at 100,000 × *g*. No CYP was detected in the isolated *E. coli* membranes. After two consecutive Ni²⁺-affinity chromatography purification steps, the specific content of MCCYP51FX was calculated to be 17.2 nmol of hemoprotein per milligram of protein. A single homogeneous band was observed on SDS-PAGE at ~62 kDa (Fig. 2), being comparable with the predicted molecular mass of the fusion protein (62.4 kDa). Other experiments using a tetrahistidine tag instead of the decahistidine tag at the C terminus of MCCYP51FX showed poor affinity in similar purification attempts, although this is successful for most other soluble bacterial CYP proteins. Purified MCCYP51FX has a typical CYP-reduced CO spectrum, with the spectral maximum located at 448 nm (Fig. 3A). The absolute spectrum revealed the Soret maximum to be located at 419 nm with the g-values of 2.26 and 1.91. This confirms a 3Fe-4S ferredoxin component to the fusion protein with one molecule of iron associated with the heme of the P450. Sephacryl S-400HR column chromatography was used to estimate the oligomeric status of the protein. Compared with molecular mass standards, it gave an apparent molecular mass of 706 kDa. (data not shown). Therefore, MCCYP51FX had a molecular mass in excess of 443 kDa and was probably a 12-mer.

**Substrate Binding**—In the absence of substrates, most CYP enzymes are low-spin with a water molecule hexacoordinated to the CYP heme iron. Substrate addition shifts the heme to the high-spin state, which gives rise to a substrate-induced difference spectrum (22). In the case of many CYPs, the addition of a substrate leads to a peak at 390 nm and a trough at 420 nm. The addition of lanosterol to MCCYP51FX was characterized with a spectral maximum at 438 nm and a trough at 420 nm (Fig. 3B). This is indicative of a type II spectrum, which is usually characterized by molecules interacting with the heme iron as a sixth ligand. However, complementary control experiments showed poor affinity in similar purification attempts, although this is successful for most other soluble bacterial CYP proteins. Purified MCCYP51FX has a typical CYP-reduced CO spectrum, with the spectral maximum located at 448 nm (Fig. 3A). The absolute spectrum revealed the Soret maximum to be located at 419 nm with the α and β-bands for the oxidized form located at 565 and 534 nm, respectively, which is typical for a low-spin cytochrome P450 (Fig. 3A). Quantification of the iron content of MCCYP51FX (3.92 ± 0.14 molecules of iron per molecule of MCCYP51FX indicated that there were four molecules of iron per molecule of MCCYP51FX. This confirms a 3Fe-4S ferredoxin component to the fusion protein with one molecule of iron associated with the heme of the P450. Sephacryl S-400HR column chromatography was used to estimate the oligomeric status of the protein. Compared with molecular mass standards, it gave an apparent molecular mass of 706 kDa. (data not shown). Therefore, MCCYP51FX had a molecular mass in excess of 443 kDa and was probably a 12-mer.

**Comparison of MCCYP51FX supported lanosterol 14α-demethylase activities**

Table I

| Protein combination | Lanosterol 14α-demethylase activity |
|---------------------|-----------------------------------|
| MCCYP51FX alone     | N.D.                             |
| MCCYP51FX + NADPH   | N.D.                             |
| MCCYP51FX + ferredoxin reductase + NADPH | N.D. |
| MCCYP51FX + ferredoxin reductase + NADPH | 0.24 |
| MCCYP51FX + ferredoxin + NADPH | 0.26 |

a Lanosterol 14α-demethylase activity is expressed as a nanomole of product produced per minute per nanomole of MCCYP51FX fusion protein.

b N.D., no detectable activity.

Lanosterol. Equal concentrations of purified MCCYP51FX (500 µl, 1 nmol) were placed in one sample and one reference chamber of two split cuvettes, and an equal volume of buffer was placed in the other chambers. Lanosterol was added to the P450 chamber of the sample cuvette and to the buffer chamber of the reference cuvette (0.1–10 µM). Lanosterol was dispersed in Triton WR-1339 for use in these studies, and the detergent did not produce a spectral change at equivalent concentrations to those used here or in separate experiments with purified *M. tuberculosis* CYP51 wherein a Type I spectrum with lanosterol was observed. The sterol binding spectra were recorded at a scan speed of 1 nm/s. All spectra were corrected for the absorbance contribution of the substrate.
iments utilizing purified M. tuberculosis CYP51 showed that lanosterol induced a type I spectrum (peak at 390 nm, trough at 420 nm). In the case of the results obtained here for MCCYP51FX, the CYP conformational change on association with substrate may be altered because of the ferredoxin domain, giving rise to a substrate-bound, low-spin complex and the corresponding Type II spectrum, but this remains speculation. This result was unusual, and further work will be needed to establish the events causing it.

**EPR Spectra of MCCYP51FX**—Fig. 4 shows the EPR spectrum of purified MCCYP51FX in the oxidized form. The clear features are at g values 2.43, 2.26, and 1.91 in the spectrum of MCCYP51FX; these are very similar to those reported for low-spin ferric iron in various cytochrome P450 genes and, in particular, are essentially identical to the g values of 2.45, 2.27, and 1.92 from the purified lanosterol 14α-demethylating enzymes isolated from *Saccharomyces cerevisiae* and *Candida albicans* (26, 27). The spectrum also shows a derivative signal at g = 2.0, which is presumably from a sum of free radical and 3FeS centers. Because EPR is a sensitive probe of the electronic structure of paramagnetic centers, the low-spin ferric EPR spectrum of Fig. 4 shows that fusion of the CYP51 domain with the ferredoxin domain has no major effect on the CYP heme environment through gross conformational change in the protein architecture.

**MCCYP51FX Reconstitution and Activity**—Reconstitution of recombinant MCCYP51FX with spinach ferredoxin reductase was carried out using lanosterol as substrate. After 20 min incubation, the reconstitution reaction mixture was extracted using ethanolic potassium hydroxide, and the sterols were partitioned into hexane, silylated, and analyzed by gas chromatography/mass spectrometry. Following reconstitution with lanosterol, the extracted reaction mixture contained two principal sterols (Table I). The compound with a retention time of 92 min was silylated lanosterol with a molecular ion at 498 m/z. A compound, the formation of which was dependent upon the presence of MCCYP51FX, had a retention time of 87 min and a molecular ion at 484 m/z. This compound was identified as the silylated 14α-demethylated 4α-methyl-5α-ergosta-8,14,24(28)-trien-3β-ol. In control experiments, the fragmentation pattern of the molecular ion of the silylated product catalyzed by MCCYP51FX is identical to the mass spectrometry fragmentation pattern observed for the 14α-demethylated product from lanosterol generated in a purified, reconstituted *C. albicans* CYP51/NADPH cytochrome P450 reductase enzyme system (28). The activity was calculated to be 0.24 nmol of demethylated product formed per minute per nanomole of MCCYP51FX, with no metabolism detected in the absence of MCCYP51FX or NADPH. Furthermore, MCCYP51FX activity was wholly dependent upon the presence of spinach ferredoxin reductase with no sterol demethylase activity being observed when ferredoxin reductase was omitted from the reaction mixture. The addition of 18 nmol of spinach ferredoxin to the reaction in the presence of 1 nmol of MCCYP51FX and 1 nmol of spinach ferredoxin reductase had no significant effect on demethylase activity, with a turnover of 0.26 nmol of demethylated product formed per minute per nanomole of MCCYP51FX being obtained. Observations, particularly within eukaryotic CYP systems (29–31), indicate that one reductase molecule is able to transfer reducing equivalents to multiple CYP molecules, and the situation for MCCYP51FX is complicated by the oligomeric nature of the purified protein. It may transfer electrons directly to the CYP to which it is attached, or it may equally reduce the CYP attached to a separate molecule. Further work is required to separate the possibilities as for other examples of fusion proteins.

**Conclusion**—Herein, we describe a new class of cytochrome P450 comprising a fusion of the hemoprotein at the C terminus to a ferredoxin via an alanine-rich linker. The role of this CYP in sterol biosynthesis was confirmed, and activity was measured showing the functional role of the ferredoxin in...
MCCYP51FX. The measurement of the hemoprotein by UV-visible and EPR confirmed the characteristics of the purified hemoprotein domain as a low-spin type CYP, and the ferredoxin was considered to be a 3Fe-4S type of ferredoxin. The requirement of sterol biosynthesis in the bacterium and the evolutionary origin of this CYP, normally associated with eu-
ontorary, is open to much conjecture. The similarity to plant evolutionary origin of this CYP, normally associated with eu-
Currently, the presence of such genes in bacteria is extremely rare. The relationship of MCCYP51FX to Mtb Rv0744c and Mtb Rv0764c in the genome of an orphan ORF. Neither flanking genes are found adjacent to Mtb Rv0764c, and mutation and selection may have given rise to this new class of CYP protein. The flanking genes for MCCYP51Fx are, from the mutation of a CYP51 nonsense codon allowing subse-
quent translational read-through to an adjacent ferredoxin, and downstream, an orphan ferredoxin open reading frame followed by ferredoxin Mtb Rv0763 are areas open to conjecture (Fig. 5, A and B). Possibly, the bacterial genes arose by horizontal transfer; but this transfer, together with other genes that are not genetically linked, seems difficult to understand. The possibility exists that MCCYP51Fx arose from the mutation of a CYP51 nonsense codon allowing subsequent translational read-through to an adjacent ferredoxin, and mutation and selection may have given rise to this new class of CYP protein. The flanking genes for MCCYP51Fx are, upstream, a potential thic-type gene, which in B. subtilis is involved in thiamine biosynthesis (32), and, downstream, an orphan ORF. Neither flanking genes are found adjacent to Mtb Rv0764c in the genome of M. tuberculosis. Further work toward understanding the evolution of CYP51 is important also for understanding the origins of the superfamily, and this novel fusion protein adds additional interest to these ongoing considerations.

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