

# Cloning and Characterization of the Genes Encoding a Cytochrome P450 (PipA) Involved in Piperidine and Pyrrolidine Utilization and Its Regulatory Protein (PipR) in *Mycobacterium smegmatis* mc<sup>2</sup>155

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**Transposon mutagenesis of *Mycobacterium smegmatis* mc<sup>2</sup>155 enabled the isolation of a mutant strain (called LGM1) altered in the regulation of piperidine and pyrrolidine utilization. The complete nucleotide sequence of the gene inactivated in mutant LGM1 was determined from the wild-type strain. This gene (*pipR*) encoded a member of the GntR family of bacterial regulatory proteins. An insertion element (IS1096), previously described for *M. smegmatis*, was detected downstream of the gene *pipR*. Three additional open reading frames were found downstream of IS1096. The first open reading frame (*pipA*) appeared to encode a protein identified as a cytochrome P450 enzyme. This gene is the first member of a new family, *CYP151*. By a gene replacement experiment, it was demonstrated that the cytochrome P450 *pipA* gene is required for piperidine and pyrrolidine utilization in *M. smegmatis* mc<sup>2</sup>155. Genes homologous to *pipA* were detected by hybridization in several, previously isolated, morpholine-degrading mycobacterial strains. A gene encoding a putative [3Fe-4S] ferredoxin (*orf1*) and a truncated gene encoding a putative glutamine synthetase (*orf2'*) were found downstream of *pipA*.**

Rapidly growing species of the genus *Mycobacterium* play an important role in the metabolism of a variety of recalcitrant organic molecules, including vinyl chloride (20), polycyclic aromatic hydrocarbons (6, 14, 24, 26), halogenated phenols (52, 53), isonicotinate (30), aromatic compounds (10, 45, 48, 50), and secondary amines (8, 11, 28, 29, 40). Morpholine, pyrrolidine, and piperidine are closely related secondary amines used in industry and eventually released in the environment. The latter two compounds can also be synthesized by different organisms. Recently, we isolated several mycobacterial strains able to degrade morpholine and demonstrated that a cytochrome P450 enzyme was involved in the degradation of this amine in all these strains (41). All these bacteria, like nearly all the morpholine degraders described in the literature, belong to the genus *Mycobacterium*. The induction of a heme-containing monooxygenase was also noted when these bacteria were grown on pyrrolidine and, for some of them, on piperidine. It was shown that morpholine-nondegrading mycobacterial strains (*Mycobacterium fortuitum* and *Mycobacterium smegmatis* mc<sup>2</sup>155) produced a cytochrome P450 monooxygenase during growth on piperidine and pyrrolidine (41).

Cytochromes P450 are heme-containing enzymes which play a central role in the oxidative metabolism of organic compounds. These proteins seem to be important for mycobacteria, since 22 genes encoding putative cytochromes P450 have been detected in the genome of *Mycobacterium tuberculosis* (13).

In this study, we have isolated, sequenced, and analyzed the genes encoding the piperidine-inducible cytochrome P450 (PipA) and its regulatory protein (PipR) from the *M. smegma-*

*tis* strain mc<sup>2</sup>155. The isolation of a mutant, in which *pipR* was inactivated by transposition mutagenesis, clearly demonstrated the involvement of the protein PipR in the regulation of piperidine and pyrrolidine metabolism. It was shown, by gene replacement experiment, that *pipA* was required for piperidine and pyrrolidine utilization in strain mc<sup>2</sup>155.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown in liquid medium or on solid Luria-Bertani (L) medium containing ampicillin (50 µg ml<sup>-1</sup>), gentamicin (20 µg ml<sup>-1</sup>), kanamycin (20 µg ml<sup>-1</sup>), or streptomycin (50 µg ml<sup>-1</sup>). *M. smegmatis* mc<sup>2</sup>155 was grown in Middlebrook liquid 7H9 medium or solid 7H10 medium (Difco, Fisher Scientific, Elancourt, France) supplemented with Bacto Middlebrook ADC Enrichment supplement and 0.05% Tween 80, at 37°C except for transposition mutagenesis. With this strain, gentamicin and kanamycin were used at 5 and 20 µg per ml, respectively. Other mycobacterial strains were grown in liquid medium or on solid L medium at 30°C.

In gene replacement experiments, transformants were selected on solid L medium containing gentamicin and kanamycin (LGK). Ten percent sucrose was added to solid L medium containing kanamycin (LKS) to select allelic-exchange events.

Mineral salts (MS) medium which contained (per liter) 1 g of KH<sub>2</sub>PO<sub>4</sub>, 1 g of K<sub>2</sub>HPO<sub>4</sub>, 0.04 g of MgSO<sub>4</sub> · 7H<sub>2</sub>O, 0.004 g of FeCl<sub>3</sub> · 6H<sub>2</sub>O, and 1 g of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (the last compound was omitted when the amines were used as the carbon, nitrogen, and energy sources) was used to grow mycobacterial strains on piperidine, pyrrolidine, or glucose. Noble agar (Difco) was added at 1.5% (wt/vol) to prepare solid MS medium. The pH was adjusted to 7.0 with HCl after the addition of the amine. Piperidine (C<sub>5</sub>H<sub>11</sub>N) and pyrrolidine (C<sub>4</sub>H<sub>9</sub>N) were purchased from Fluka (Sigma Aldrich Sarl, St. Quentin Fallavier, France) and used at 10 mM. Bacterial growth was determined by monitoring the optical density of the cultures at 600 nm.

**Degradation of piperidine and pyrrolidine by *M. smegmatis* resting cells.** Cells growing in 7H9 medium or MS medium amended with piperidine were harvested, at the end of the exponential phase, by centrifugation at 6,000 × g for 10 min at 20°C. The supernatant was discarded, and the pellet was washed twice with MS medium and resuspended in 8 ml of this medium (circa 10<sup>10</sup> cells per ml). Cells were incubated with 5 mM piperidine or pyrrolidine at 37°C with magnetic agitation and aeration (2 liters of air per h). Samples (110 µl) were taken at regular intervals and immediately centrifuged at 20,000 × g for 2 min, and the concentration of the amine was determined.

**Analytical methods.** The piperidine and pyrrolidine concentrations were estimated spectrophotometrically by the method of Stevens and Skov (49) as mod-

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics	Reference or source
<b>Strains</b>		
<i>E. coli</i> XL1-Blue	F <sup>'</sup> ::Tn10 <i>proA</i> <sup>+</sup> <i>B</i> <sup>+</sup> <i>lacI</i> <sup>q</sup> $\Delta$ ( <i>lacZ</i> ) <i>M15</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>hsdR17</i> <i>supE44</i> <i>relA1</i>	Stratagene
<i>M. smegmatis</i> mc <sup>2</sup> 155	Highly transformable mutant	47
<i>M. smegmatis</i> LGM1	Tn611 insertion mutant of strain mc <sup>2</sup> 155	This work
<i>M. aurum</i> MO1	Morpholine-degrading strain	11
<i>Mycobacterium</i> sp.		
BM01	Morpholine-, piperidine-, and pyrrolidine-degrading strain	41
BM04	Morpholine- and pyrrolidine-degrading strain	41
BM05	Morpholine- and pyrrolidine-degrading strain	41
BM06	Morpholine- and pyrrolidine-degrading strain	41
FM10	Morpholine- and pyrrolidine-degrading strain	41
FM30	Morpholine- and pyrrolidine-degrading strain	41
LM20	Morpholine- and pyrrolidine-degrading strain	41
LM32	Morpholine- and pyrrolidine-degrading strain	41
LM40	Morpholine- and pyrrolidine-degrading strain	41
RP1	Morpholine-, piperidine-, and pyrrolidine-degrading strain	40
<b>Plasmids</b>		
pBluescript II KS(+)	<i>E. coli</i> cloning vector; Amp <sup>r</sup>	Stratagene
pJQ200	Cloning vector with <i>sacB</i> and <i>aacC1</i> (Gm <sup>r</sup> ) genes; nonreplicative vector in mycobacteria	42
pCG79	<i>Mycobacterium</i> and <i>E. coli</i> shuttle plasmid, thermosensitive for replication or maintenance in mycobacteria, carries an Str <sup>r</sup> gene expressed in <i>E. coli</i> and the Tn611 transposon containing the Kan <sup>r</sup> gene from Tn903 which is expressed in mycobacteria and in <i>E. coli</i>	19
pLGM20	pBluescript with 5.8-kb <i>EcoRI</i> fragment of <i>M. smegmatis</i> containing <i>pipR</i> , IS1096, <i>pipA</i> , <i>orf1</i> , and truncated <i>orf2</i>	This work
pLGM21	pBluescript with 2,177-bp <i>SacI-EcoRI</i> fragment of pLGM20	This work
pLGM22	pLGM21 with a Kan <sup>r</sup> cassette in the blunt-ended <i>MluI</i> site	This work
pLGM23	pJQ200 with <i>XbaI-PstI</i> fragment of pLGM22 in <i>SmaI</i> site	This work

ified by Knapp et al. (28). The protein concentration was determined by the method of Bradford (7).

**Spectrophotometric analysis of cytochrome P450.** Glucose-grown *M. smegmatis* cells were harvested by centrifugation (6,000  $\times$  g for 10 min) at 4°C. Cells were broken by three passages through a French pressure cell (SLM-Aminco) at 18,000 lb in<sup>-2</sup>. The crude extract was treated as previously described (40).

**DNA manipulations.** Restriction and modification enzymes were purchased from Eurogentec (Eurogentec S.A., Seraing, Belgium). Plasmid DNA isolation was performed with a Qiagen plasmid extraction kit (Qiagen S.A., Courtaboeuf, France). DNA fragments of interest were purified from agarose gel by using the GeneClean III kit (Bio 101, Ozyme, St. Quentin, France). Standard recombinant DNA techniques were carried out as described by Sambrook et al. (43).

**Electroporation of bacteria.** Electrocompetent *E. coli* cells were prepared and electroporated by the method of Dower et al. (17). Cells of *M. smegmatis* mc<sup>2</sup>155 were made electrocompetent and used according to the method described by Pelicic et al. (38).

**Tn611 transposon mutagenesis.** The transposon mutagenesis of *M. smegmatis* mc<sup>2</sup>155 was performed as described by Guilhot et al. (19) by using the thermosensitive plasmid pCG79. This vector, carrying Tn611, was introduced into *M. smegmatis* by electroporation, and transformants were selected on 7H10 medium containing kanamycin at 30°C. A randomly chosen clone was grown for 72 h at 30°C in 5 ml of 7H9 medium supplemented with kanamycin. Antibiotic-free 7H9 medium was then inoculated with this preculture and incubated for 24 h at 39°C. Various dilutions were spread on 7H10 medium supplemented with kanamycin and incubated at 39°C. Eight thousand clones were taken at random and replica plated on solid MS medium containing 10 mM piperidine plus kanamycin and on 7H10 medium containing kanamycin.

**Isolation of genomic DNA, gene cloning, and sequencing.** *M. smegmatis* genomic DNA was isolated from a 10-ml culture (7H9 medium) as follows. Cells were pelleted by centrifugation (6,000  $\times$  g for 10 min), resuspended in 5 ml of 7H9 medium supplemented with 50  $\mu$ g of D-cycloserine ml<sup>-1</sup> and 100  $\mu$ g of lysozyme ml<sup>-1</sup>, and incubated overnight at 37°C. After centrifugation, the cells were resuspended in 500  $\mu$ l of solution I (25% glucose, 50 mM Tris-HCl [pH 8.0], 50 mM EDTA, 500  $\mu$ g of lysozyme ml<sup>-1</sup>) and incubated for 1 h at 37°C. Then, 500  $\mu$ l of solution II (100 mM Tris-HCl [pH 8.0], 50 mM EDTA, 400  $\mu$ g of proteinase K ml<sup>-1</sup>) was added and the mixture was incubated for 3 h at 55°C. DNA was extracted twice with phenol-chloroform and once with chloroform and then was ethanol precipitated. The pellet was dissolved in 1  $\times$  TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) and treated with RNase A (50  $\mu$ g ml<sup>-1</sup>) for 1 h at 37°C. Proteinase K (50  $\mu$ g ml<sup>-1</sup>) was added to the DNA solution, and incubation was continued for 1 h at 37°C. DNA was extracted with phenol-chloroform and chloroform and then concentrated by ethanol precipitation. The genomic DNA of the other mycobacterial strains was isolated as previously described (41).

The site of the Tn611 insertion in the chromosome of mutant LGM1 was

determined by using marker rescue as described by Billman-Jacobe et al. (5). Genomic DNA of strain LGM1 was digested with the restriction enzyme *EcoRI* (a unique site in pCG79), diluted, self-ligated, and transformed into *E. coli*. Transformants were selected on solid L medium supplemented with streptomycin and checked for their ability to grow in kanamycin-containing medium. An *EcoRI-HindIII* DNA fragment (759 bp) of *M. smegmatis* chromosomal origin was subcloned into pBluescript II KS(+) (Stratagene, Ozyme, St. Quentin, France) for sequence analysis. The *HindIII* restriction site is situated in IS6100a (position 3,300) of Tn610 (GenBank accession no. X53635). Tn611 is a derivative of Tn610 in which the sulfonamide resistance (*sul3*) gene has been replaced by the Km resistance (Km<sup>r</sup>) gene of Tn903 (31). The 759-bp DNA fragment was used in hybridization experiments (probe I). Total DNA of wild-type *M. smegmatis* mc<sup>2</sup>155 was digested overnight with different restriction endonucleases and separated by electrophoresis by using a 0.8% agarose gel. Southern blots (neutral membrane; Appligene Oncor, Illkirch, France) were hybridized with the digoxigenin-labeled probes. The labeling method, prehybridization and hybridization steps, and detection procedure were performed as recommended by the manufacturer (Boehringer Mannheim S.A., Meylan, France). Prehybridization and hybridization were performed at 42°C by using 50% formamide in solutions. DNA fragments of interest were purified from the agarose gel and cloned into pBluescript II KS(+). Double-stranded DNA sequencing was carried out with an automated sequencer (Genome Express, Paris, France). The French server BLSANCE (15) was used to perform computer-assisted sequence analyses. The homologies between our sequences and the database sequences were determined by using the FASTA (36) and BLASTP (1) programs.

**Construction of pLGM23.** The plasmid pLGM21 was linearized with the restriction enzyme *MluI* (which acts on a single site in the *pipA* gene), treated with Klenow fragment to fill in the ends of the restriction site, and ligated to a blunt-ended *PstI* fragment (approximately 1.1 kb) containing the Tn903 Km<sup>r</sup> cassette (*aph*) to obtain the pLGM22 vector. A 2.8-kb *XbaI-PstI* fragment containing the *pipA*::Km region of plasmid pLGM22 was blunt ended and ligated into the *SmaI* site in pJQ200 to generate the suicide vector pLGM23 (Table 1).

**Nucleotide sequence accession numbers.** The nucleotide sequences presented in this study have been assigned accession no. AF102509 and AF102510 by GenBank.

## RESULTS AND DISCUSSION

**Pyrrolidine and piperidine utilization by *M. smegmatis* mc<sup>2</sup>155.** *M. smegmatis* mc<sup>2</sup>155 is able to grow in liquid MS medium containing piperidine or pyrrolidine as the sole sources of carbon, nitrogen, and energy. Piperidine and pyrrolidine were

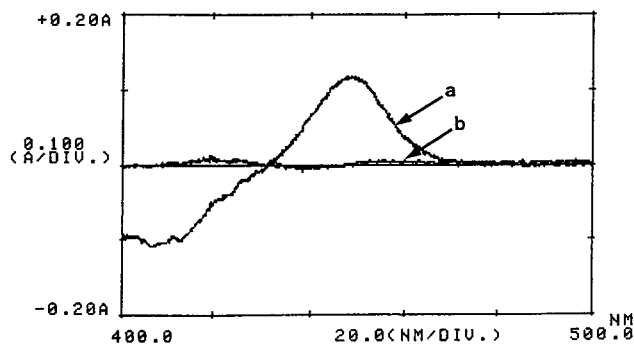


FIG. 1. Reduced CO difference spectra of crude extracts of glucose-grown *M. smegmatis* cells. Spectrum a, Tn611 insertion mutant LGM1; spectrum b, wild-type, *M. smegmatis* mc<sup>2155</sup> strain. The protein concentrations were 10 mg ml<sup>-1</sup>.

degraded quite rapidly, and after 80 h of incubation, no secondary amines could be detected in the culture medium. The values for turbidity of the cultures increased from 0.02 to 1.00 and 0.84 in piperidine-containing medium and pyrrolidine-containing medium, respectively (data not shown).

**Isolation and characterization of a transposon-induced piperidine utilization mutant of *M. smegmatis* mc<sup>2155</sup>.** In order to isolate *M. smegmatis* mutants altered in piperidine metabolism, Tn611 transposon mutagenesis was performed as described by Guilhot et al. (19). A total of 8,000 thermoresistant clones randomly selected from the library were replica plated on MS agar medium containing 10 mM piperidine and on 7H10 medium. Both types of medium were supplemented with kanamycin. After five days of incubation at 39°C on piperidine-containing MS agar medium, a colony growing faster than other ones was obtained. This clone (called strain LGM1) was isolated for further analysis.

The kinetics of piperidine and pyrrolidine degradation by *M. smegmatis* mc<sup>2155</sup> and strain LGM1 resting cells were determined (data not shown). When mc<sup>2155</sup> cells were grown in MS medium containing piperidine, the degradation of this amine began rapidly. All of this compound disappeared from the medium within 60 min. Over the same period, the degradation of piperidine by *M. smegmatis* mc<sup>2155</sup> cells grown in 7H9 medium was very slow. The results are consistent with the fact that the piperidine degradation pathway of strain mc<sup>2155</sup> is inducible. In contrast, cells of strain LGM1 pregrown in 7H9 medium could degrade piperidine and pyrrolidine with the same efficiency as piperidine-grown mc<sup>2155</sup> cells. The enzymes associated with the ability to degrade piperidine and pyrrolidine in *M. smegmatis* mc<sup>2155</sup> need not be induced in strain LGM1.

A spectrophotometric analysis of reduced extracts of mycobacterial cells (strains mc<sup>2155</sup> and LGM1) grown in MS medium supplemented with glucose was recorded. In the spectrum of CO-treated minus nontreated reduced extracts of strain LGM1 cells, an absorption maximum at about 450 nm was observed, indicating the presence of a cytochrome P450 (Fig. 1). This peak was not detected in strain mc<sup>2155</sup> cell extracts. Extracts prepared from glucose-grown LGM1 cells, piperidine-grown LGM1 cells, and piperidine-grown strain mc<sup>2155</sup> cells all contained similar amounts of cytochrome P450 (125 pmol per mg of protein). Thus, a constitutive expression of a cytochrome P450 in mutant LGM1 was noted. This monooxygenase could correspond to the cytochrome P450 involved in the metabolism of piperidine in *M. smegmatis* mc<sup>2155</sup>. Therefore, a gene encoding a protein involved in the negative

regulation of the synthesis of this cytochrome P450 was probably disrupted by the insertion of Tn611.

**Site of insertion of transposon Tn611 in strain LGM1.** Tn611 has been shown to transpose by a replicative mechanism (31). During this transposition event, plasmid pCG79 containing Tn611 was integrated into the chromosome of the bacteria and one of the two copies of IS6100 was duplicated. Thus, by digesting the genomic DNA of the Tn611 insertional mutant bacteria by *Eco*RI (which acts on a unique site in pCG79) it was possible to rescue a plasmid containing the origin of replication for pUC18 and the Str<sup>r</sup> and/or the Km<sup>r</sup> markers (depending on which IS6100 copy was duplicated). The rescued plasmid obtained after strain LGM1 genomic DNA digestion with the restriction endonuclease *Eco*RI was introduced into *E. coli*. This plasmid conferred only Str<sup>r</sup> to *E. coli*. Consequently, during the process of transposition of Tn611 in *M. smegmatis* IS6100a was duplicated since the rescued plasmid obtained after IS6100b duplication would have conferred Str<sup>r</sup> plus Km<sup>r</sup> to *E. coli*. The DNA sequence of chromosomal origin was subcloned from the rescued plasmid and sequenced on one strand (data not shown). In this nucleotide sequence, 737 bp belong to *M. smegmatis* genomic DNA and 22 bp belong to IS6100a of transposon Tn611. The deduced polypeptide had similarity to proteins of the GntR family of bacterial regulatory proteins (21).

**Cloning of the gene encoding a regulatory protein of the piperidine metabolism pathway.** The 737-bp fragment from *M. smegmatis* LGM1 chromosome (probe I) was used to probe Southern blots of completely digested *M. smegmatis* mc<sup>2155</sup> DNA in order to isolate the entire gene. An *Eco*RI fragment of about 5.8 kb was detected, cloned in pBluescript II KS(+) (pLGM20), and selected by colony hybridization. Southern blot hybridization analysis of plasmid pLGM20 DNA, digested with several restriction endonucleases, was carried out with probe I (data not shown). The gene encoding the regulatory protein was located on one end of the 5.8-kb *Eco*RI fragment (Fig. 2). Starting from this end, a 1,215-bp region was sequenced on both strands. Sequence analysis of this DNA fragment revealed the presence of an open reading frame (ORF) (designated *pipR*) encoding a protein of 245 amino acids with a calculated molecular mass of 26,331 Da (Fig. 3). This ORF begins with a GUG initiation codon, is preceded by the sequence AAGGAG, a putative ribosome binding site, and is followed by a sequence potentially forming a stable transcription termination hairpin structure. The use of GUG as the translational initiation codon of PipR could allow the autoregulation of the expression of this protein as demonstrated for

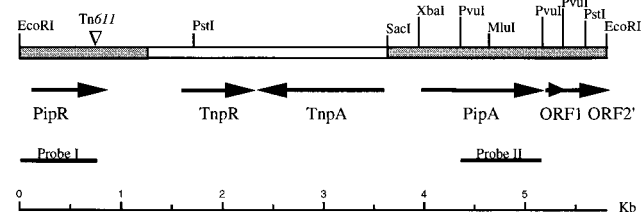


FIG. 2. Map of the 5.8-kb *Eco*RI DNA region of *M. smegmatis* mc<sup>2155</sup> containing the piperidine-inducible cytochrome P450 (*pipA*) and its regulatory gene (*pipR*). The DNA regions sequenced on both strands are indicated in shaded boxes. The other ORFs shown correspond to the putative resolvase (TnpR) and transposase (TnpA) of IS1096 insertion element and to the putative ferredoxin (ORF1) and glutamine synthetase (truncated ORF2') of *M. smegmatis* mc<sup>2155</sup>. Only the restriction sites mentioned in the text are indicated. The position of Tn611 insertion (triangle) into the *pipR* gene, determined from sequence analysis, is shown.

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gaattcgtcgcgaaatcgggtfagattgcccacatctccagacgccacttcgcggaatcgggtccccgccgggattgcccactgta 84
      PipR > V S P S P L V A P E A P V G R A D E I V Q 21
tctcggaaaggagaacdcgcg GTGTCGCCGTCGCCGCTCGTCGCGCCGAGGCTCCCGTGGGTGCGCCGACGAGATCGTGACG 167
R I T E A I H L G L L D D G E R L P V E V D L A A Q F G 49
CGCATCACCGAAAGCGATCCATCTGGGTCTGCTGGATGACGGTGAACGCCTGCCGGTGGAGGTGACCTGGCCGCGCAGTTCGCG 251
V A P M T V R E A L A T L R E L E L V E T R R G R S G G 77
GTCGCCCCGATGACCGTGC GCGAGGCGCTCGCGACGCTGCGTGAACTCGAGCTGGTGGAGACCCGGCGCGGGCGCAGCAGCGCGG 335
S F V R R P A G P P V E K L T A R L A A M S A S D L R D 105
TCGTTCGTCCGACGAGCCCGCGGGTCCCCCGGTGCGAGAAGTCGACCGCCCGCTGCGCCATGAGCGCCTCTGACCTGCGAGAT 419
L F D E H T A I A G Q A A R L A A E R A A P S T V R R L 133
CTGTTGACGAGACACACCGCATCGCCGGTCAAGCCCGCCGATTGGCCGCGCAGCGGGCCGCTCCGTGACCGTGCAGCGCGGTTG 503
F A L T D Q L D T A T S L R D R I R A D S R F H I Q V A 161
TTCGCGCTGACCGATCAACTCGACACCGCGACGTCGCTGCGCGACCGGATCCGGGCCGACAGCCGGTTCACACATCCAGGTCGCG 587
V A A Q S A R L A R R E A N L Q A E V S G L I W L P I G 189
GTGCGGCCCGAGTCGGCCCGCTCGCGCGCCGCGAGGCGAACCTGCAGGCCGAGGTGTCGGGCTGATCTGGCTACCCATCGGG 671
P P I D V A A Y V E E K H A I S A A I A A E N A Q E A R 217
CCACCCATCGATGTGGCCGCTATGTGGAGGAGAAGCAACGCGATCTCTGCTGCCATTGCTGCCGAAAACGCACAGGAGGCCCGC 755
Q L A E A H V M G Q L A R L T Q I N L D L T T K E A G R 245
CAGTTGGCCGAGGCTCACGTATGCGGCGAGCTCGCGCGCTGACCCAGATCAACCTGGATCTCAACGACGAAGGAGGCCCGGCCGA 839
*
TGAgtggaatcagctggccgagctcttggccaaggaagcctcggacgctcttgagccgctggtcgggctgctcgagagcattcc 923
gtgaggacgtgctcgcacatcaggccgcccgcgcgcgcgcgctgaccgaagcgcacttcagtgggctgcagcgcggctcgcgcagc 1007
tgctgcaggcccccacgacgtatggggcatggggttcatcgccgcaccgcttctggtgctgagggcaaggagcgtatctggcct 1091
gggtggcagcgtcccggcgcaccaggtggccggctgcggctcaacttcgaccgcaccagcgtggacgctgtacgactactctgca 1175
gatggactcttaccagctggcgcagcgtggccagggcccg 1215

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FIG. 3. Nucleotide sequence of the 1,215-bp fragment containing the regulatory protein (PipR) gene. The presumptive ribosome binding site aaggaaga (italic), the stop codon (asterisk), and the nucleotides potentially involved in the formation of a stem-loop structure (underlined) are indicated. The GenBank accession number is AF102509. Bases shown in lowercase letters are noncoding sequences.

the *cam* repressor (CamR) of the cytochrome P-450cam hydrolase operon (2). The *pipR*-encoded protein shows significant similarity to members of the GntR bacterial regulatory family, as follows: a probable transcriptional regulator (SCI35\_36) of *Streptomyces coelicolor* (35) (31% identity in 234 amino acids), a putative lactate operon regulator (LctR) of *E. coli* (16) (30% identity in 227 amino acids), a pyruvate dehydrogenase complex repressor (PdhR) of *Rhodobacter capsulatus* (54) (28% identity in 218 amino acids), and an Uxu operon regulator (UxuR) of *Haemophilus influenzae* (18) (27% identity in 238 amino acids). In members of this family (21), a conserved helix-turn-helix DNA-binding domain was found in the N-terminal part of the protein ([LIVAPKR]-[PILV]-X-[EQTIVMR]-X<sub>2</sub>-[LIVM]-X<sub>3</sub>-[LIVFT]-[DNGSTK]-[RGT LV]-X-[STAIVP]-[LIVA]-X<sub>2</sub>-[STAGV]-[LIVMFYH]-X<sub>2</sub>-[LMA]). This PROSITE (3) consensus sequence (PROSITE accession no. PS00043) was detected in the N-terminal region of PipR protein, between residues 38 and 62, but with two differences, at positions 51 (A instead of [DNGSTK]) and 52 (P instead of [RGTLV]). Many members of this family act as transcriptional repressors and possess, in addition to the DNA-binding domain, an effector-binding domain.

**Identification of putative functional ORFs.** The DNA sequence downstream of *pipR* was partially sequenced (data not shown). It is identical to the nucleic acid sequence encoding the putative resolvase of the insertion element *IS1096* (Gen-

Bank accession no. M76495) isolated from *M. smegmatis* mc<sup>26</sup> by Cirillo et al. (12). This insertion element is 2,276 bp in length. Strain mc<sup>2155</sup> is an efficient plasmid transformation mutant of *M. smegmatis* mc<sup>26</sup>.

The 2,177-bp *SacI-EcoRI* fragment located downstream of *IS1096* was subcloned (pLGM21), and its nucleotide sequence was determined (Fig. 4). In this sequence, three ORFs (designated *pipA*, *orf1*, and *orf2'*) were found in the same orientation. The last ORF (*orf2'*) was truncated. The deduced amino acid sequence of *pipA* (400 amino acids; *M<sub>r</sub>*, 44,747) matches the PROSITE consensus motif, F-[SGNH]-X-[GD]-X-[RHPT]-X-C-[LIMVFAP]-[GAD], for cytochromes P450 (PROSITE accession no. PS00086). PipA shows similarity to bacterial cytochromes P450, as follows: 31% identity to a putative cytochrome P450 (MTV023\_25) of *M. tuberculosis* (13), 31% identity to a cytochrome P450-like protein governing hydroxylation and epoxidation in mycinamicin II biosynthesis of *Micromonospora griseorubida* (22), and 36% identity (but in a 291-amino-acid overlap only) to a cytochrome P450 of *Streptomyces hygroscopicus* (32). PipA clearly has less than 40% identity with known P450 proteins, which indicates that it represents the first member of a new family, CYP151 (33). Upstream of *pipA*, two imperfect repeats, which could constitute a DNA-binding motif, were detected. An imperfect 9-bp inverted repeat sequence spanned the putative -35 region (TTGACA) of *pipA*, and a 7-bp imperfect inverted repeat was localized between the pu-

gagctcgtcǎaggcggcagǎaagtcgtcǎǎgtcagcgcǎǎgccaagcccǎcccǎaccggtagcgtcagǎǎacgtcǎgǎgǎtcttt 84  
 cagatǎgatggcgtǎǎgaacctcǎatcǎtcggaagǎacctcǎaccǎtǎtcccggcǎccgǎcgcgcǎǎgǎcǎcctǎcǎaccctc 168  
 aǎctgcaǎagǎgǎcctgcaatcǎcatgatttctaccggtgagtaaccgagtcǎtaacagggcgtǎatggagccǎtaacǎaaaacgc 252  
cgaaaatǎgacagttgtǎgacctattgǎcttǎaacaatcǎacttctǎǎgattttagtǎgaccggtcǎǎgǎtcgcaaccǎtǎggag 336  
*PipA* > M S S T T L D S V P S F D V T D P A F S I T S D E 25  
 gtagcactgATGTCGTCGACCAcCCTCGACTCAGTCCCAGTTTGGATGTACGGACCCGGCATTCTCGATCACCTCGGACGAG 420  
 V H E A R E K S W Y A T T P Y G L A V L R Y E Q V N R L 53  
 GTCCAGAAǎcccgcgagǎagǎgctggtaǎcǎacǎgacǎgcccǎtǎcgggctggccgattǎgǎcgtǎcǎgǎcǎggtǎcǎaccǎcctg 504  
 I K H P K L R Q G S A A W P A H N G V T E G P F A E W F 81  
 ATCAǎgcaǎcccaǎaǎctgǎcǎcǎggǎcǎgǎcǎgǎcǎcǎgǎcǎcǎgǎc 588  
 A S W I L N K E G E E H H R L R R L M N P A F S P K L I 109  
 GcCAGCTGGATcCTCAACAAgǎAGGCGAGǎAACACCAcCGcCTGCGcCGGcCTCATGAACcCCGCGTTCcTcGCCCAAGCTgǎTc 672  
 G S L V P R F Q A L A N E L I D N F A E P D R C E F V S 137  
 GGGTCGcTGGTGCCcCGǎTTCAGGcGcTGgCAAcGǎACTCATCGǎAAcTTcGcCǎGAACCcGATCgǎTǎTGcGAGTTCgTcGAGc 756  
 E F A E P Y A A R V I A I M L G L P E E E W K V I S T E 165  
 GAGTTCGcCGAAcCgTACGcGGcGcGGGTcATcGcGATCATGcTcGcCCTGcCǎGAAGAGGAGTǎGAAGGTcATcTcGACCGAG 840  
 S A T I G L A L G V T L R E D L P K I E A A V Q R L Y E 193  
 TCCGcGACcATcCGcCCTGcGcCCTGGGcGTcACGcTcGcGcǎGGAcCTGcCǎAAgATCGǎǎGcCGcCGTgǎcǎGcGcTTCgǎTcGAGc 924  
 Y S D E L I A D R R A N P R D D F M T T L V N A S R P D 221  
 TACAGcGACGAGTTCgǎTcGcGGAcCCcCGGcCAAcCCcGcGcGAcGǎACTTCATGAcCAGcCTGGTcAAcGcGTCcGcGTcCGGAc 1008  
 D G R L S D K E L R D A I L L L I F G G F D T T R N Q L 249  
 GǎCGcGAGGcTcGAGcGAcAAǎGcTcGcGǎTcGcCATcCTcGcTcGATǎTTCGGGGcCTTCGAcACCACcCCGAcACCAGcTg 1092  
 G L A M Q T F M K H P D Q W R L L G E R P D L G G K A V 277  
 GGcTGGcGATGcAGAcGǎTTCATGAAGcATCCGAcCǎGcTGGcTcTcTcCGcGAGcGTCcCGATcCTcCGcCGcCAAGcCGTC 1176  
 E E V M R V N P T V R W V T R E V V E D F E Y E G V T L 305  
 GAGǎAGGTcATGcGcCGTCAAcCCcCACcGTGcGGTgGGTgACCcGcGAGGTGGTcGAGGAcTTCgǎGTAcGAAGcCGTgACGcTg 1260  
 K A G T T V H L Y S E S A G T D P R V F E P G F D I T A 333  
 AAGcCGcCǎCACcCGTgǎCCTGTACAGcGAGTcGGcAGcCACcGACCcCGcCGTgTTcGAGcCCGcGcTTCGAcATcAcCGcA 1344  
 E R K P H F G F G G G V H H C L G H F V A R S D M S E A 361  
 GAAGcCAAGcCGcAcTTCGgTTTTcGcCGGTGGcGTcCACcACTGcCCTcGgTCAcTTCGTGGcCCGcTCGgAcATgǎGcGAGcGA 1428  
 L P L L A R R L R D P H E L P G A T W L P D S G N T G P 389  
 CTGCCCTGCTgGCcCGcCGcCTcGcGcGAcCǎGcATGAGTTCcCGcGcGcAcCTGGcTGCcCGAcTCGGGcAAcCACcGGAcCC 1512  
 N T L P I G F T P A P \* ORF1 > M 1  
 AACACTGcCGATCGgTTTCACCCcGGcCCCCcTGAacǎgagcccccǎgaaǎcgctccaǎacǎgccccǎaaǎaggattǎgatcATG 1596  
 R V S V D L N R C Q D H G Q C V I A A P S V F S M T D D 29  
 CGAGTcTCCGTTGǎTCTCAAcCGcTGTcAGGAcCAGcCGcCAGTgCGTcATcGcCgCCCCcTcCGGTGTTcTcGATgACCgAGcGǎC 1680  
 G V L E Y I S T P S E S E R F A V E E A A D V C P L Q A 57  
 GGcTcCTcGAATAcATcAGcAcGcCCTTCgǎAATcCGAcGgTTCGcGGTcGAGGAGcCGcCGAcGTCcTcCCcGCTcCǎGGcC 1764  
 I T I E D \* ORF2' > M T I T P L D A H R Q A N A T S P D L A 20  
 ATcAGATcGAGGAcTgA tcaaccATgACCATcACAcCCCTTgAcGcGcAcCGGcAGcGcCAAcCGcAcGAGccccǎcCTcCGT 1847  
 A V L E T I A E R G V E F V Y F Q A V T I T G R V V G K 48  
 GcCGTACTcGAGAcCATcGcCCgǎACGGGAGTcGAGTTCGTcTACTTCcAGGcCGTcACCATcACGGGcCGGgTcGTGGGcAAG 1931  
 V A P A K H F E R L A V R G V Q Q H Q T A V A N L Q G T 76  
 GTCGCTcCCcCGcCAAGcAcTTCGAcAGcCTcGcCGTcAGcGGTGTGcAGcǎGAcCAGAcCGcGcGTCcAAcCTcGAcGǎGCcACC 2015  
 R E G V L L A G G V N A P E Y T A I P D L E T F A V L P 104  
 CGcǎGGGTGTGCTgCTcGcCGGTgCGcTGAACcGcCCcTGAgtǎAcCGcGATcCCGgATCTGGAGAcCTTCGcGcGTGCTGcCG 2099  
 W D T S F A R V F C R L Y E P D H L A E R A G A E F 130  
 TGGGAcCACcǎCTTCGcCGcǎGTGTTcTGCcGGTGTAcGǎAcCCGATcAcCTGGcCGAcǎGTGcAGGcGcCGAcATTc 2177

FIG. 4. Nucleotide sequence of the 2,177-bp *SacI-EcoRI* region encoding a cytochrome P450 (PipA), a putative ferredoxin (ORF1), and a putative glutamine synthetase (truncated ORF2'). The two sets of possible inverted repeats (underlined) in the putative promoter region of *pipA*, the putative ribosome binding sites ggagg (italic), and stop codons (asterisks) are indicated. The first 182 nucleotides of this sequence are identical to the end of the *IS1096* insertion element (positions 2086 to 2268) except for one nucleotide: the base A, in position 2147 in *IS1096*, is missing. The GenBank accession number is AF102510. Bases shown in lowercase letters are noncoding sequences.

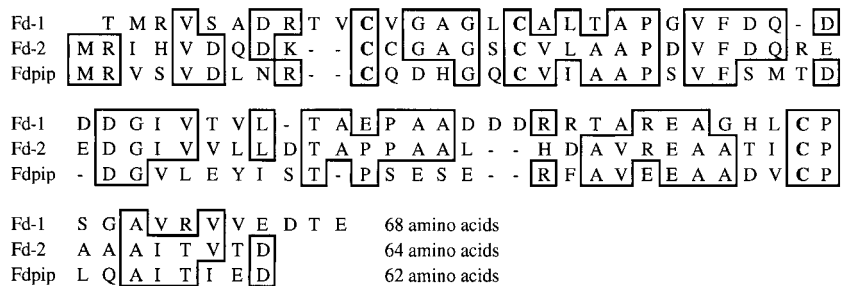


FIG. 5. Amino acid sequence comparisons of *S. griseolus* (Fd-1 and Fd-2) and *M. smegmatis* (Fdpip) ferredoxins. The three cysteine residues, which are supposed to be involved in the attachment of a [3Fe-4S] cluster and are conserved in all three proteins, are indicated in bold. Hyphens indicate gaps introduced to optimize alignments. Residues that are identical in at least two of the three sequences are boxed.

tative -10 region (TAGAGT) and the putative ribosome binding site (GGAGG) of *pipA*. Bacterial cytochromes P450 are often substrate inducible, and the expression of some of them has been shown to be negatively regulated at a transcriptional level. The expression of the cytochrome P450<sub>BM-3</sub> gene of *Bacillus megaterium* ATCC 14581 (46) and of the cytochrome P-450cam of *Pseudomonas putida* PpG1 ATCC 17543 (2) were demonstrated to be negatively regulated through the interaction of a repressor with an operator (inverted repeats) located upstream of these genes. In both these cases, the genes encoding cytochrome P450 and their regulatory proteins were adjacent and divergently oriented.

The presence of sequences homologous to the *M. smegmatis pipA* gene was checked, with the internal *PvuI* fragment of *pipA* (788 bp) used as a probe (probe II), in the genomes of the following mycobacterial strains: *Mycobacterium* sp. strains BM01, BM04, BM05, BM06, FM10, FM30, LM20, LM32, LM40, and RP1 and *Mycobacterium aurum* MO1. These different strains were isolated from activated sludge, soils, or sediments for their ability to degrade morpholine (11, 40, 41). They are also able to degrade pyrrolidine and eventually piperidine. The degradation of these cyclic amines required the involvement of a cytochrome P450 in all these bacteria. A positive signal was obtained with all the DNAs tested in the experiment, indicating that closely related cytochrome P450 genes are present in these strains (data not shown). The bacteria tested in this study are not representative of all the rapidly growing mycobacterial strains but represent several clusters as defined by the phylogenetic analysis of Pitulle et al. (39). Thus, it is tempting to conclude that a homologous gene encoding a cytochrome P450 involved in the metabolism of cyclic amines is present in rapidly growing mycobacteria.

An ORF encoding a protein of 62 amino acids with an  $M_r$  of 6,760 (*orf1*) was identified in the region downstream of *pipA*. This ORF begins 46 bp after the stop codon (UGA) of *pipA*. The two genes are in the same reading frame. The amino acid sequence of the protein was 44, 41, and 40% identical to the reported primary structures of ferredoxin 2 and ferredoxin 1 (Fd-1 and Fd-2) from *Streptomyces griseolus* (34) and a putative ferredoxin (AE001094\_1) from *Archaeoglobus fulgidus* (27), respectively. As shown in Fig. 5, the three cysteine residues (Cys-11, Cys-17, and Cys-56 of Fd-1 and Cys-10, Cys-16, and Cys-55 of Fd-2) supposed to be involved in ligating a [3Fe-4S] cluster in each of Fd-1 and Fd-2 (34) are conserved in the protein encoded by *orf1* (Cys-10, Cys-16, and Cys-53). Type II cytochrome P450 systems, which are found in bacteria, use a flavin-containing reductase and a small iron- and sulfur-containing redox protein (ferredoxin) to transfer electrons to the terminal cytochrome P450. However, no ferredoxin reductase gene was detected downstream of *pipA*. In the genome of

*M. tuberculosis* (13), two genes encoding putative ferredoxin proteins (MTCY369\_08c and MTV049\_08) linked to cytochrome P450 genes (MTCY369\_09c and MTV049\_07c) were found. The sequences of these ferredoxins are similar to that of the ferredoxin of *M. smegmatis*. The cytochrome P450 systems cloned from *S. griseolus* (34) and *Streptomyces griseus* (51) contain a cytochrome P450 gene and, downstream, the ferredoxin one. Cytochrome P450 systems present in *Streptomyces* and *Mycobacterium* strains are similarly organized.

A truncated ORF (*orf2'*) was detected, on the *SacI-EcoRI* DNA sequence, 6 bp downstream of the UGA stop codon of *orf1*. The polypeptide deduced from this incomplete gene shows significant homology with different glutamine synthetases (GlnA) from *Archaea* and *Eubacteria*. It is 28, 31, and 24% identical to the putative GlnA (U67574\_10) of *Methanococcus jannaschii* (9), the putative GlnA (MTV003\_4) of *M. tuberculosis* (13), and the GlnA (SSGLNA\_2) of *Sulfolobus solfataricus* (44), respectively. Glutamine synthetase catalyzes the formation of glutamine by condensation of glutamate and ammonia and therefore plays a key role in the metabolism of nitrogen. A consensus pattern, [FYWL]-D-G-S-S-X<sub>6,8</sub>-[DENQSTAK]-[SA]-[DE]-X<sub>2</sub>-[LIVMFY], is usually present in the N-terminal section of these enzymes (PROSITE accession no. PS00180). The putative GlnA of *M. jannaschii* and the GlnA of *S. solfataricus* contain this consensus sequence, but it is missing in the putative GlnA of *M. tuberculosis* and *M. smegmatis* mc<sup>2</sup>155. The three ORFs (*pipA*, *orf1*, and *orf2'*) are closely linked and could form part of an operon.

**Disruption of the *pipA* gene.** Experiments were performed to replace the functional allele *pipA* with an inactivated copy (*pipA::Km*) in *M. smegmatis* mc<sup>2</sup>155. A two-step method for gene replacement, as described by Pelicic et al. (38), was performed by using the suicide vector pLGM23 and *M. smegmatis* mc<sup>2</sup>155. This plasmid carries the *aacC1* and *sacB* genes, from the pJQ200 vector (42), which confer to mycobacteria gentamicin resistance and sucrose sensitivity, respectively (37). An individual clone was randomly chosen from those obtained on LGK plates, was propagated overnight in 7H9 medium containing kanamycin, and was spread on LKS plates. The clones resulting from this experiment were expected to contain the interrupted allele of the *pipA* gene. A Southern blot analysis was performed on 10 randomly chosen clones by using probe II (data not shown). The internal *pipA* probe hybridized to a 3.9-kb fragment of the *PstI*-digested DNA of *M. smegmatis* mc<sup>2</sup>155, as expected. All the clones selected on medium containing kanamycin plus sucrose showed hybridization to a 5-kb fragment. An additional hybridization fragment was observed in one clone (clone 4) which could have been the result of a single recombination event in the *pipA* gene. The plasmid pLGM23 was still present in the chromosome of this bacte-

rium, since this clone was resistant to gentamicin. The resistance to sucrose could be due to a mutation in *sacB* gene. All these clones showed a shift in the size of the fragment hybridizing to the internal *pipA* probe (from 3.9 to 5 kb) corresponding to the length of the *aph* cassette. The ability of these clones to grow on piperidine- or pyrrolidine-containing MS medium or to degrade these compounds was tested. None of these bacteria could grow on these amines or degrade them (data not shown). These results clearly indicated the involvement of CYP151 in the piperidine and pyrrolidine degradative pathway. However, growth was noted in piperidine- and pyrrolidine-containing medium with clone 4 when incubation had been prolonged for several days. The interrupted allele of the *pipA* gene could have been eliminated by a deletion-recombination event in some bacteria of this clone.

The cytochrome P450 PipA could catalyze the C-N bond cleavage of piperidine and pyrrolidine rings, as has been suggested for morpholine metabolism by *Mycobacterium* sp. strain RP1 (40), but further experiments have to be done to confirm this. Jacoby and Fredericks (23) have studied the metabolism of pyrrolidine by *Pseudomonas fluorescens* ATCC 13430. No pyrrolidine-inducible cytochrome P450 was described for this strain, but it is worth noting that their experiments were done in 1959. The authors proposed a pathway for pyrrolidine degradation (pyrrolidine  $\rightarrow$   $\Delta^1$ -pyrrolidine  $\rightarrow$   $\gamma$ -aminobutyraldehyde  $\rightarrow$   $\gamma$ -aminobutyric acid  $\rightarrow$  succinic acid semialdehyde  $\rightarrow$  succinate) by this strain. They demonstrated that the enzymes induced by pyrrolidine metabolism were also detected during putrescine utilization. The linearized compounds formed during piperidine or pyrrolidine metabolism by *M. smegmatis* mc<sup>2</sup>155 could be further transformed by enzymes involved in the cadaverine or putrescine utilization pathways.

Cytochromes P450 have been shown to play a central role in many anabolic and catabolic reactions performed by bacteria, particularly by actinomycetes. Among rapidly growing mycobacteria, the cytochrome P450 systems induced in *Mycobacterium chlorophenolicum* by pentachlorophenol (53), in *M. fortuitum* CG-2 by halogenated phenols (52), and in *M. fortuitum* KCTC 1062 by steroids (25) have not yet been genetically characterized. At least 20 cytochrome P-450 genes were found in the genome of *M. tuberculosis* (13). The physiological function of these proteins is still unknown, but one of them (CYP51-like P450) was able to convert, in vitro, dihydrolanosterol to its 14 $\alpha$ -demethylated product (4). Some of these cytochrome P450 enzymes could be involved in the synthesis of the complex cell wall components and therefore could constitute potential targets for antimycobacterial drugs.

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