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CYP51-Like Gene of *Mycobacterium tuberculosis* Actually Encodes a P450 Similar to Eukaryotic CYP51

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A *CYP51*-like gene (Z80226) of *Mycobacterium tuberculosis* was expressed in *Escherichia coli*. The product exhibited absorption spectra characteristic of P450. The expressed P450 formed a stoichiometric complex with ketoconazole, one of the specific ligands of CYP51. These findings indicate that the *CYP51*-like gene of *M. tuberculosis* actually encodes a P450 having active-site environments similar to those of CYP51, confirming the predicted orthologous nature of this gene to eukaryotic *CYP51*. Although eukaryotic CYP51s are membrane-binding proteins, the expressed product was accumulated only in the soluble fraction of the host cells.

Key words: CYP51, expression, molecular evolution, *Mycobacterium tuberculosis*, P450.

The *CYP51* gene encodes a P450 monooxygenase (CYP51) catalyzing the 14-demethylation of sterol precursors (*1*). This P450 is distributed extensively in eukaryotic kingdoms in which *de novo* sterol synthesis occurs, and is the only known example of a P450 found widely in eukaryotes with a conserved metabolic role (*1, 2*). Recently, a gene (Z80226) encoding a protein having an amino acid sequence homologous to that of eukaryotic *CYP51* was found in *Mycobacterium tuberculosis* (*3*). We conducted a molecular evolutionary analysis of the amino acid sequences encoded by eukaryotic *CYP51* and the *CYP51*-like gene of *M. tuberculosis* and suggested that all eukaryotic *CYP51*s and the *CYP51*-like gene of *M. tuberculosis* are orthologous (*4*). We also showed that the branch topology of CYP51 phylogenetic tree, (bacteria, (plants, (fungi, mammals))), was comparable to the phylogeny of the major kingdoms of living matter (*4*). Based on these facts, we proposed that the CYP51 family may have originated in the prokaryotic era and been distributed into eukaryotic kingdoms almost concomitant with their establishment (*4*). However, no information is available on the product of the *CYP51*-like gene of *M. tuberculosis*. Identification and characterization of the product of this gene is essential to confirm our prediction on the evolution of the CYP51 family (*4*).

The *CYP51*-like gene of *M. tuberculosis* was prepared by PCR. The sense primer M-5-Nde (5′-GCCATATGAGCGCTGTTGCACTACCC-3′) and antisense primer M-3-Hind (5′-ATAAGCTTGCTTCGACTCGATAGCCCATG-3′) were used to amplify the *CYP51*-like gene in the genomic DNA of *M. tuberculosis*, which was kindly supplied by Dr. Y. Kashiwabara of the National Institute of Infectious Diseases, Japan. The PCR product was cloned with the vector pGEM-T (Promega) and the sequence was verified by sequencing its restriction fragments subcloned with pBluescript SK− plasmid. The *CYP51*-like gene of *M. tuberculosis* thus obtained was ligated into the *Nde*I-*Hin*dIII site of the expression vector pCWori+ (*5*) and introduced into *Escherichia coli* JM109 competent cells (*5*). Dr. M.R. Waterman of Vanderbilt University kindly supplied the host-vector system. The transformed host cells were cultivated overnight, and 3 ml of the pre-cultivated cell-suspension was inoculated into 300 ml of Terrific-broth containing ampicillin (100 μg/ml). The cultivation was carried out aerobically at 37°C. When the optical density at 550 nm reached 0.8, isopropylthiogalactoside (1 mM) was added to the culture, and cultivation was continued at a lower temperature (30°C). Twenty-four hours after the start of cultivation the cells were collected by centrifugation, suspended with 50 mM potassium phosphate buffer, pH 7.5, containing 0.25 M sucrose and 0.5 mM EDTA, and disrupted with French press. After removal of undisrupted cells by centrifugation, the cell-free suspension was separated into membrane and soluble fractions by centrifugation at 100,000 × g for 150 min.

Figure 1 shows the dithionite-reduced CO-difference spectra of the soluble fraction obtained from the transformed cells. A difference spectrum with a peak at 448 nm was observed immediately after the addition of dithionite into a CO-saturated specimen (solid line A in Fig. 1). This spectrum is typical of the reduced CO-difference spectrum of P450, indicating the presence of P450 in the soluble fraction. On the other hand, under the same conditions, the membrane fraction prepared from the same cells showed only a small difference spectrum, with a peak and trough at around 420 and 445 nm, respectively (data not shown). Neither the soluble nor the membrane fraction prepared from control cells harboring the native pCWori+ vector

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Abbreviations: CYP51, sterol 14-demethylase P450 (14DM) and its orthologues; *CYP51*, CYP51 gene.

exhibited the difference spectrum characteristic of P450; the difference spectra of these fractions were similar to that of the membrane fraction of the transformed cells. These spectral observations thus indicated that P450 occurred only in the soluble fraction of the transformed cells. A protein band having an apparent M_r of 51,000, which is comparable to the theoretical M_r value of the protein (50,877 with 451 amino acid residues) encoded by the *CYP51*-like gene of *M. tuberculosis* (Z80226), was observed on SDS-polyacrylamide gel electrophoresis of the soluble fraction from the transformed cells. This protein band was not observed in the membrane fraction of the transformed cells or in the soluble and membrane fractions of the control cells. It can thus be concluded that the product of the *CYP51*-like gene of *M. tuberculosis* is a soluble P450.

The *M. tuberculosis* P450 showed the reduced CO-difference spectrum of P450 immediately after the reduction (solid line A in Fig. 1). However, the absorption peak at 448 nm disappeared within a few minutes after the reduction, and a new absorption peak appeared at 420 nm (solid line B in Fig. 1), indicating the rapid denaturation of the P450.

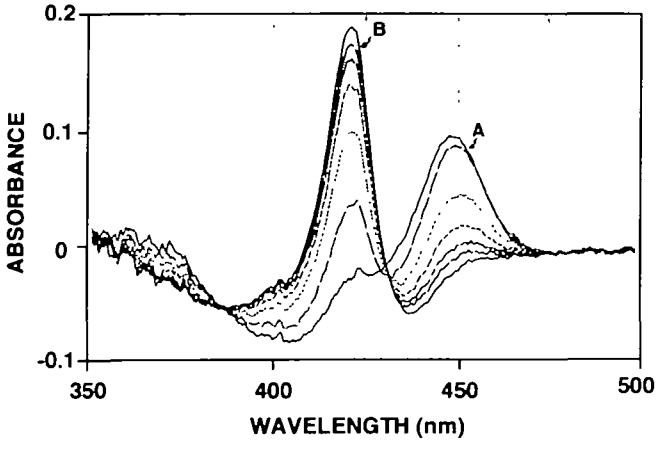

Fig. 1. **Reduced CO-difference spectra of the soluble fraction prepared from transformed cells.** An aliquot (4 ml) of the soluble fraction (6.7 mg protein/ml) isolated from the transformed cells was divided into sample and reference cuvettes. CO was bubbled into the sample cuvette for 1 min. A small amount of solid dithionite was then added to both the sample and reference cuvettes. Difference spectra were recorded repeatedly at about 1-min intervals from immediately after the reduction (solid line A). About 5 to 6 min after the reduction, P450 was completely denatured into P420 (solid line B).

The P450 expressed from the *CYP51*-like gene of *M. tuberculosis* was therefore quite unstable to the reduction under the experimental conditions used.

Figure 2A represents the absolute absorption spectrum of the expressed P450 (solid line) and the spectral change caused by the addition of ketoconazole (broken line). Ketoconazole is one of the specific and potent ligands of eukaryotic CYP51 (*6*), and the spectral change of the P450 caused by ketoconazole (Fig. 2A) was identical to that observed on yeast CYP51 (*6*). The binding of ketoconazole to the P450 was determined spectrophotometrically (Fig. 2B). The expressed P450 was linearly titrated with ketoconazole and the titration reached saturation at 5 to 6 μM of ketoconazole. The P450 concentration of the specimen estimated from the intensity of the 418 nm peak shown in Fig. 2A and the absorbancy index of the corresponding absorption peak of oxidized CYP51 (113 $mM^{-1} \cdot cm^{-1}$) (*7*) was 5 μM. Therefore, the expressed P450 formed the ketoconazole complex stoichiometrically with very high affinity. Formation of the stoichiometric ketoconazole complex with very high affinity is characteristic of CYP51 (*6*).

Taking these lines of evidence together, it can be concluded that the *CYP51*-like gene of *M. tuberculosis* encodes a P450 having active-site environments similar to those of CYP51, as expected from its primary structure. In our previous paper, we inferred that the *CYP51*-like gene of *M. tuberculosis* is a prokaryotic orthologue of *CYP51* and that CYP51 is a unique P450 family conserved through biological evolution from the prokaryotic era to the present day (*4*). The results presented here firmly support this prediction and confirm the evolutionary continuity of the *CYP51* gene.

Eukaryotic CYP51s are membrane-binding proteins. As described above, the CYP51 of *M. tuberculosis* was accumulated only in the soluble fraction. In accord with this finding, the hydrophobic membrane-binding domain, which exists in the N-terminal of eukaryotic CYP51s, was not found in the *M. tuberculosis* CYP51 (Fig. 3), and the latter CYP51 was smaller (451 amino acids) than the eukaryotic orthologues (503 amino acids for rat CYP51) (*2*). This is a conspicuous difference between the structures of *M. tuberculosis* CYP51 and the eukaryotic orthologues, and seems to suggest the possibility that the N-terminal domain and the core of P450 evolved independently to adapt to the intracellular environments of eukaryotes and prokaryotes.

A gene encoding a ferredoxin-type protein similar to

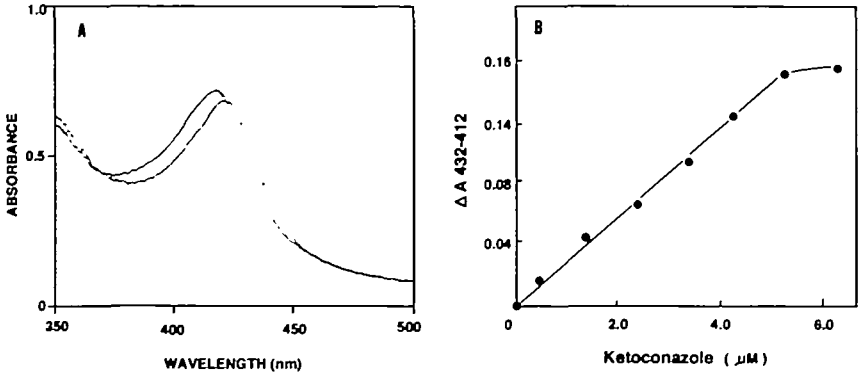

Fig. 2. **Ketoconazole-induced spectral change in the soluble fraction prepared from transformed cells and its titration.** A: Absolute absorption spectra of the soluble fraction (13.3 mg protein/ml) prepared from the transformed cells were recorded before (solid line) and after (broken line) the addition of ketoconazole (6.5 μM). B: The indicated concentrations of ketoconazole were added to the soluble fraction (13.3 mg protein/ml) prepared from the transformed cells, and intensity of the ketoconazole-induced difference spectrum (432-412 nm) was plotted against the ketoconazole concentration.

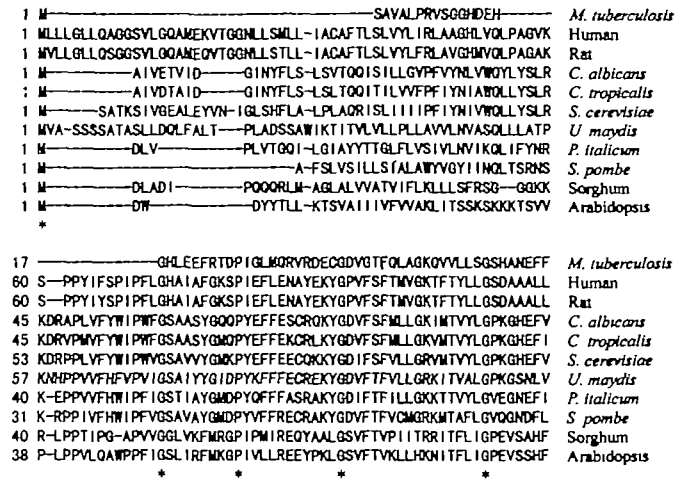

Fig. 3. **Multiple amino acid sequence alignment of CYP51 family.** Amino acid sequences of the CYP51s of the indicated organisms were aligned using a recent algorithm (*11*). Amino acid sequences were obtained from public sequence databases. Alignment of the N-terminal part (1st to 120th alignment positions) showing the characteristic differences between *M. tuberculosis* CYP51 and eukaryotic CYP51s is shown. The amino acid residues that are common to all sequences are asterisked.

ferredoxin$_{soy}$ of *Streptomyces griseus* (*8*) exists closely downstream of the CYP51 gene, and genes encoding a ferredoxin reductase-like protein have also been identified in *M. tuberculosis*. However, no gene encoding the eukaryote-type P450 reductase was found in *M. tuberculosis*. These facts suggest that the electron-transport system for the CYP51 of *M. tuberculosis* may be the prokaryote type. If this is the case, the domain interacting with the electron donor may also have evolved independently in eukaryotes and prokaryotes, even in the orthologous CYP51.

Eukaryotic CYP51 contributes to sterol synthesis, which is indispensable for eukaryotes, and in mammals CYP51 is known to be a housekeeping enzyme (*9, 10*). However, sterol synthesis is not an essential function for prokaryotes, and sterols have not yet been identified in *M. tuberculosis*. Accordingly, the function of the CYP51 of *M. tuberculosis* at present remains an enigma. Expression of the electron-transferring components mentioned above and reconstitution of the P450 monooxygenase system with them are necessary to reveal the electron-transfer mechanism and the function of *M. tuberculosis* CYP51.

Note Added in Proof: After the submission of this manuscript, the expression of *M. tuberculosis* CYP51 in *E. coli* and characterization of the expressed protein were reported independently by Bellamine *et al.* in a poster at the 4th International Symposium on P450 Biodiversity and Biotechnology (July 12-16, 1998, Strasbourg) and by Vaz *et al.* in a poster at the 12th International Symposium on Microsomes and Drug Oxidations (July 20-24, 1998, Montpellier). The properties of the expressed CYP51 of *M. tuberculosis* reported in these two studies were essentially identical to those described in this paper. Furthermore, Bellamine *et al.* showed that the expressed *M. tuberculosis* CYP51 could catalyze the 14-demethylation of 24,25-dihydrolanosterol and obtusifoliol, although it could not metabolize lanosterol. This finding proves the functional similarity of *M. tuberculosis* CYP51 to the eukaryotic CYP51, a hypothesis based on the sequence similarity of the gene encoding a CYP51-like protein to the other known CYP51s (*4*).

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REFERENCES

1. Yoshida, Y. (1993) Sterol biosynthesis in *Cytochrome P-450*, 2nd ed. (Omura, T., Ishimura, Y., and Fujii-Kuriyama, Y., eds.) pp. 93-101, Kodansha, Tokyo and VCH, Weinheim
2. Aoyama, Y., Noshiro, M., Gotoh, O., Imaoka, S., Funae, Y., Kurosawa, N., Horiuchi, T., and Yoshida, Y. (1996) Sterol 14-demethylase P450 (P45014DM) is one of the most ancient and conserved P450 species. *J. Biochem.* **119**, 926-933
3. Cole, S.T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S.V., Eiglmeier, K., Gas, S., Barry, C.E. III., Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, K., Osborne, J., Quail, M.A., Rajandream, M.-A., Rogers, J., Rutter, S., Seeger, K., Skelton, J., Squares, R., Squares, S., Sulston, J.E., Taylor, K., Whitehead, S., and Barrell, B.G. (1998) Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**, 537-544
4. Yoshida, Y., Noshiro, M., Aoyama, Y., Kawamoto, T., Horiuchi, T., and Gotoh, O. (1997) Structural and evolutionary studies on sterol 14-demethylase P450 (CYP51), the most conserved P450 monooxygenase: II. Evolutionary analysis of protein and gene structures. *J. Biochem.* **122**, 1122-1128
5. Barnes, H.J., Arlotto, M.P., and Waterman, M.R. (1991) Expression and enzymatic activity of recombinant cytochrome P450 17α-hydroxylase in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **88**, 5597-5601
6. Yoshida, Y. and Aoyama, Y. (1987) Interaction of azole antifungal agents with cytochrome P-45014DM purified from *Saccharomyces cerevisiae* microsomes. *Biochem. Pharmacol.* **36**, 229-235
7. Yoshida, Y., Aoyama, Y., Kumaoka, H., and Kubota, S. (1977) A highly purified preparation of cytochrome P-450 from microsomes of anaerobically grown yeast. *Biochem. Biophys. Res. Commun.* **78**, 1005-1010
8. Trower, M.K., Lenstra, R., Omer, C., Buchholz, S.E., and Sariaslani, F.S. (1992) Cloning, nucleotide sequence determination and expression of the genes encoding cytochrome P-450soy (soyC) and ferredoxin$_{soy}$ (soyB) from *Streptomyces griseus*. *Mol. Microbiol.* **6**, 2125-2134
9. Noshiro, M., Aoyama, Y., Kawamoto, T., Gotoh, O., Horiuchi, T., and Yoshida, Y. (1997) Structural and evolutionary studies on sterol 14-demethylase P450 (CYP51), the most conserved P450 monooxygenase: I. Structural analyses of the gene and multiple sizes of mRNA. *J. Biochem.* **122**, 1114-1121
10. Stromstedt, M., Rozman, D., and Waterman, M.R. (1996) The ubiquitously expressed human CYP51 encodes lanosterol 14α-demethylase, a cytochrome P450 whose expression is regulated by oxysterols. *Arch. Biochem. Biophys.* **329**, 73-81
11. Gotoh, O. (1996) Significant improvement in accuracy of multiple protein sequence alignments by iterative refinement as assessed by reference to structural alignments. *J. Mol. Biol.* **264**, 823-838