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EPA ENVIRONMENTAL RESEARCH BRIEF

Gene Engineering of Yeasts for the Degradation of Hazardous Waste

John C. Loper*

Abstract

Environmentally recalcitrant compounds including 2,3,7,8-tetrachlorodibenzo-p-dioxin and hexachlorobenzene are metabolized in mammalian liver via reactions characteristic of cytochrome P-450 monooxygenase systems. This research examined the structure and function of cytochrome P-450 genes in yeast as a model for gene engineering such eukaryotic P-450 enzymes for biodegradation of hazardous waste by yeasts. *Saccharomyces cerevisiae* and *Candida tropicalis* are two yeasts known to produce major P-450 enzymes. These enzymes were purified and antibodies produced in rabbits were then used in the isolation or characterization of clones containing a P-450 gene from each organism. DNA sequence was determined for the gene isolated from *S. cerevisiae* and for several hundred bases of chromosomal DNA on each side of the gene, and deletion experiments in the promoter region were conducted. The deduced protein sequence from this gene was compared to those of the other known subfamilies of eukaryotic P-450 genes; the several features identified included a new homology region for these protein sequences. The *C. tropicalis* gene and its promoter region has been partially sequenced.

Introduction

This is a report of the research conducted under a Cooperative Agreement between the U.S. Environmental Protection Agency (EPA) and the University of Cincinnati. The work examined the yeasts *Saccharomyces cerevisiae* and *Candida tropicalis*, eukaryotic microorganisms known to produce major cytochrome P-450 enzymes. Cytochrome P-450 monooxygenases are the only class of enzymes known to catalyze the specific degradation of certain highly recalcitrant chlorinated aromatic hydrocarbons. A long-range goal of this research direction is to obtain yeasts which combine the uptake and degradation of these target compounds with the capacity to survive or to survive and grow in toxic environments. This result would provide a system for controlled, low-cost biodegradation of such environmentally stable toxic wastes. The work under this Cooperative Agreement sought the characterization of yeast cytochrome P-450 genes, as a model for the gene engineering of eukaryotic cytochromes P-450 in yeast.

The Role of Gene Engineered Eukaryotic Cytochrome P-450 Monooxygenases

Among the targets for engineered biodegradation are compounds which by definition are recalcitrant, that is, not observed to be degraded by natural processes. In general, the most toxic and most stable recalcitrant organic compounds are those with a high degree of chlorination. Of particular concern are polychlorinated aromatic hydrocarbons having no adjacent unsubstituted carbons, for example, hexachlorobenzene, highly chlorinated biphenyls, and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Such compounds are not attacked by bacterial dioxygenases, which are enzymes whose aromatic substrates contain adjacent carbons free of constituents other than hydrogen. However, there is evidence for the hydroxylation/dechlorination of such recalcitrant compounds in some bacteria and particularly in eukaryotic organisms via cytochrome P-450 monooxygenases. These enzymes can modify a substrate one carbon at a time, and because of their number and their occasional overlapping specificity, they provide for a broad range of oxidative and reductive reactions.

Such P-450 catalyzed reactions play essential roles in mammalian metabolism in (a) the synthesis of the major cell constituents cholesterol and fatty acids and of

*Department of Microbiology and Molecular Genetics, University of Cincinnati College of Medicine, Cincinnati, OH 45221.

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hormones including glucocorticoids, sex hormones and prostaglandins, and (b) the metabolism of therapeutic drugs and other xenobiotic compounds. Knowledge of P-450s has been gained primarily from mammalian systems, and the data for the P-450 catalyzed metabolism of TCDD and similarly recalcitrant compounds are from *in vivo* and *in vitro* studies of mammalian liver. Knowledge of yeast P-450 systems is less advanced. Nevertheless, it has been demonstrated that P-450 monooxygenase pathways are constituted similarly among higher and lower eukaryotes and that mammalian P-450 gene products can function in yeasts. Thus, it is likely that P-450 genes for degradation of recalcitrant compounds either can be identified in yeasts or can be introduced for expression in yeasts.

Engineering the gene expression of these appropriate P-450 monooxygenases in yeasts would make possible the testing of these microorganisms for biodegradation of recalcitrant hazardous compounds (8). In some cases the establishment of the expressed P-450 gene may be the only additional trait required to equip the cell for the desired degradation; in others it is likely that additional properties will be important, for compound uptake and for survival, etc. The primary effort of this study was to characterize cytochrome P-450 genes in *S. cerevisiae*, the yeast organism most amenable for such molecular genetic studies. Genes engineered in this organism subsequently can be transferred to other strains. Since different species of yeast are likely to tolerate better the ecological conditions presented by various noxious environments, strains isolated from those environments could be used as recipients of functional P-450 gene sets to establish the preferred detoxicating variants. *Candida tropicalis* is a likely candidate as one of these ecologically suitable organisms. This yeast already contains an elaborate machinery for the assimilation of hydrophobic long chain hydrocarbons enabling its growth on crude oil. Secondary to the studies in *S. cerevisiae*, P-450 genes in this organism were also examined.

Characterization of Yeast P-450 Genes: Isolation and Sequence Determination

When this study started, considerable data were available on a few P-450 enzymes of yeast but no yeast P-450 genes were available. In *S. cerevisiae* the best characterized P-450 enzyme was known to demethylate lanosterol and the same or a quite similar form was known to hydroxylate benzo(a)pyrene. The gene for this demethylase was chosen for isolation as a model system and as a gene of potential use in general biodegradation.

Another well studied yeast P-450 enzyme is alkane ω-hydroxylase. This enzyme catalyzes the first step in the metabolism of long chain n-alkanes as a carbon and energy source. It is found in a variety of yeasts other than *S. cerevisiae; Candida tropicalis* is a major example. The *C. tropicalis* gene for this enzyme was chosen for isolation as the second model. This choice was based upon the likelihood that this P-450 may be of direct use in biodegradation, and because utilization of alkanes involves processes for cell uptake of hydrophobic substrates which are likely to be of use in the uptake of hydrophobic hazardous wastes.

As a useful step toward P-450 gene isolation and identification, microsomal preparations were first isolated from these two microorganisms and the *S. cerevisiae lanosterol demethylase, and the C. tropicalis* ω-hydroxylase were purified. Antibodies in rabbits were prepared and physical and immunological similarities of the two enzymes were examined (1,2). Then using a recently described method based upon gene dosage effects for isolation of genes in yeast, we isolated a lanosterol demethylase gene from *S. cerevisiae* (3).

Using a cloned subfragment of DNA containing the entire gene, overlapping deletions were produced which then were used as templates for DNA sequencing of one DNA strand of the gene by the M13 dideoxy method. The complementary strand was also sequenced by the dideoxy method, using oligonucleotide primers synthesized in the Department. As a result, the DNA sequence of this gene has been determined together with the sequence of several hundred nucleotide base pairs upstream and downstream of the protein coding region (4).

When genes are sequenced, a useful procedure is to use the universal genetic code to identify the predicted amino acid sequence of the protein product. Since the isolation and sequence characterization of mammalian P-450 genes is a major area of research among numerous laboratories, several P-450 genes have now been examined in this procedure and their protein sequence determined. When the protein sequence of the yeast P-450 was compared to those of higher eukaryotes, many similar features were identified (4). Chief among these are (a) the presence of a strongly hydrophobic region near the amino terminal end, which could serve as a typical anchor peptide for attachment of the protein to intracellular membranes, and (b) a 21 amino acid segment near the C terminal region which conforms to a homologous region detected for all other P-450 genes. This homologous region apparently is required for the binding of heme to the apoprotein.

These features of the deduced protein sequence identify this *S. cerevisiae* gene as a cytochrome P-450 gene. Based on these and upon overall DNA homology properties, this yeast P-450 protein comprises an additional subfamily among the superfamily of eukaryotic P-450s. It is clear also that this *S. cerevisiae* P-450 gene has more in common with other subfamilies of eukaryotic P-450 genes than it does with the structure of the known bacterial P-450 gene (4,5). Thus, the sequence characterization of a yeast P-450 gene has provided unique information on evolutionary relationships among the P-450 superfamily.

For isolation of the ω-hydroxylase P-450 gene from *C. tropicalis*, direct use was made of the anti-enzyme antibody for gene detection in a gene expression library. This procedure led to the isolation of the complete ω-hydroxylase gene (6). Total sequence characterization of this gene is in progress, using a double strand plasmid sequencing procedure that employs templates from sets of deletions extending across either end of the isolated gene.

A New Region of Sequence Similarity Among All Eukaryotic P-450s

As noted, the *S. cerevisiae* P-450 comprises a new P-450 subfamily. With the advantage of this new information a detailed comparison of homology among all seven of the subfamilies in this sytem was conducted. The analysis identified a new region of sequence similarity among all the eukaryotic P-450s (5,7). This advance is important to research on the structure and function relationships of all P-450s since it indicates protein sites where site-specific mutagenesis would be particularly informative.

Regulation of P-450 Expression in Yeast

Understanding and gene engineering of regulatory signals are important for the controlled expression of a P-450 gene for degradation reactions. Considerable progress has been made on several aspects of this question. For *S. cerevisiae*, the DNA sequence analysis has included over 800 nucleotide bases proximal to the structural gene coding region. Deletion analysis of this region has shown that the major upstream portion can be removed without preventing gene expression (9). Also, additional genes can alter the level of lanosterol demethylase maintained in this organism. Recombinant strains have been constructed that provide for stable high cytoplasmic levels of this enzyme (3).

The signals for induction of ω-hydroxylase in *C. tropicalis* are of particular interest for gene engineering the biodegradation of toxic hydrophobic compounds. This work indicates that transcription of this enzyme is induced one hundred-fold or more as the cells adapt to assimilation of mineral oil alkanes. It appears that over 24 additional genes are involved in this assimilation process. Expression of these genes provides for changes in surfactant products, lipophilic changes in cell wall structure and production of intracellular organelles involved in catabolism of the alkanes. The result is a yeast extensively adapted for utilization of these hydrophobic compounds, and it is likely that they then can take up more hazardous hydrophobic compounds as well. A strategy has been outlined for engineering P-450 enzymes for monooxygenation of specific, hazardous hydrocarbons into such yeasts, with the inserted P-450 genes under the inductive control of the ω-hydroxylase P-450 gene promoter. This promoter has been isolated as part of the *C. tropicalis* DNA segment containing the ω-hydroxylase gene (6). Sequencing of this promoter was in progress in this laboratory as this Cooperative Agreement ended.

Publications

The following publications describe research supported by this Cooperative Agreement.

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3. Kalb, V. F., Loper, J. C., Dey, C. R., Woods, C. W. and Sutter, T. R. Isolation of a cytochrome P-450 structural gene from *S. cerevisiae*. Gene, 45(3):237-245, 1986.
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Abstracts

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1. Loper, J. C., Kalb, V. F. and Chen, C. Cytochrome P-450 genes in *Candida tropicalis*, 6th International Symposium Microsomes and Drug Oxidations, Brighton, England, 5-10 August, 1984.
2. Chen, C., Dey, C. R. and Loper, J. C. Multiple cytochrome P-450 proteins from *S. cerevisiae* and *C. tropicalis*: immunological comparisons, American Society for Microbiology Genetics and Molecular Biology of Industrial Microorganisms Conference, Bloomington, IN, Sept. 30-Oct. 3, 1984.
3. Sutter, T. R., Kalb, V. F., Woods, C., Dey, C. R. and Loper, J. C. Yeast cytochrome P-450 genes. 1986. Annual Meeting Society of Toxicology, New Orleans, LA, March, 1986.

4. Kalb, V. K., Woods, C. W., Dey, C. R., Sutter, T. R. and Loper, J. C. Isolation and sequence of a cytochrome P-450 structural gene from *Saccharomyces cerevisiae* 55th Annual Meeting Genetics Society of America, Urbana, IL, June, 1986.

5. Sanglard, D., Chen, C. and Loper, J. C. Isolation of the genes for the cytochrome P-450 ω-hydroxylase system of the alkane assimilating yeast *Candida tropicalis*. 55th Annual Meeting Genetics Society of America, Urbana, IL, June, 1986.