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Cloning, expression and characterization of a fast self-sufficient P450: CYP102A5 from *Bacillus cereus*

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Abstract

CYP102s represent a family of natural self-sufficient fusions of cytochrome P450 and cytochrome P450 reductase found in some bacteria. One member of this family, named CYP102A1 or more traditionally P450BM-3, has been widely studied as a model of human P450 cytochromes. Remarkable detail of P450 structure and function has been revealed using this highly efficient enzyme. The recent rapid expansion of microbial genome sequences has revealed many relatives of CYP102A1, but to date only two from *Bacillus subtilis* have been characterized. We report here the cloning and expression of CYP102A5, a new member of this family that is very closely related to CYP102A4 from *Bacillus anthracis*. Characterization of the substrate specificity of CYP102A5 shows that it, like the other CYP102s, will metabolize saturated and unsaturated fatty acids as well as *N*-acylamino acids. CYP102A5 catalyzes very fast substrate oxidation, showing one of the highest turnover rates for any P450 monooxygenase studied so far. It does so with more specificity than other CYP102s, yielding primarily ω-1 and ω-2 hydroxylated products. Measurement of the rate of electron transfer through the reductase domain reveals that it is significantly faster in CYP102A5 than in CYP102A1, providing a likely explanation for the increased monooxygenation rate. The availability of this new, very fast fusion P450 will provide a great tool for comparative structure–function studies between CYP102A5 and the other characterized CYP102s.

Keywords: Cytochrome P450; Cytochrome P450 reductase; Fatty acid metabolism; Enzymology; *Bacillus cereus*; CYP102A1; CYP102A5; Anthrax

Cytochrome P450s (P450 or CYP) constitute the largest superfamilies of monooxygenase enzyme proteins [1]. Discovered in 1958 by Klingenberg, P450 genes are widely distributed in prokaryotes and are present in all eukaryotic organisms [2]. Nearly 7000 P450 genes have been sequenced [3], and genome sequencing projects continue to reveal many new P450 members. Much of the interest in P450 enzymes stems from their extensive roles in Phase I drug metabolism ('xenobiotic' metabolism) and in the metabolism of sterols and other lipid derived hormones.

P450s interact with a wide variety of substrates [4]. They reductively cleave molecular oxygen to produce an oxygenated organic product and water. They perform regiospecific and stereospecific oxidation of non-activated hydrocarbons at physiological temperature. Because of the high reactivity of the active intermediate, P450s have been compared to a blowtorch [4]. Most P450s show monooxygenase activity typically resulting in substrate hydroxylation or olefin epoxidation, although *N*-, *O*-, and *S*-dealkylation, intermolecular oxygen transfer, organic peroxide isomerization and several other less common reactions are also catalyzed by specific P450 enzymes.

One of the most widely studied P450s is CYP102A1 (commonly referred to as P450BM-3), which is found in the gram-positive soil borne bacterium *Bacillus megaterium*. CYP102A1 is catalytically self-sufficient thanks to the natural fusion of the P450 with flavoprotein domains homologous to cytochrome P450 reductase, the electron donor in mammalian endoplasmic reticulum bound

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P450s. CYP102A1 exhibits one the highest turnover numbers (>3000 min^{-1})[1] of any P450 monooxygenase [5–7]. In *B. megaterium* this enzyme is inducible by barbiturates, fatty acids, and nonsteroidal anti-inflammatory drugs [8–12]. Because it contained a reductase homologous to mammalian cytochrome P450 reductase (CPR)[2] and was inducible like mammalian enzymes, CYP102A1 historically made a very good bacterial model for inducible xenobiotic metabolizing P450s in mammals. More recently, its extremely high catalytic efficiency has made it a template for the development of novel catalysts using techniques like random mutagenesis [13–15].

CYP102A1 is generally considered to be a fatty acid hydroxylase. It has been known for quite some time that CYP102A1 is capable of oxidizing saturated and unsaturated fatty acids, saturated fatty amides, and fatty alcohols [16–18]. More recently it has been shown that *N*-acylated amino acids are much better substrates for CYP102A1 [19]. The exact physiological significance of these reactions is unknown although CYP102A1 is believed to be involved in fatty acid homeostasis and detoxification [12,20–22]. The most extensive work *in vivo* in *B. megaterium* suggested that CYP102A1 protects the bacterium from the toxicity of polyunsaturated fatty acids (PUFA), a form of 'xenobiotic' metabolism [23]. This makes it and other members of the CYP102 family potential therapeutic targets for organisms that must thrive in the presence of PUFA.

The genomes of other sequenced *Bacilli* reveal that all have a fusion P450 sharing sequence similarity to CYP102A1, and *B. subtilis* has two. The *Bacilli* include medically important bacteria. *B. subtilis* was actually discovered for its ability to prevent debilitating diarrhea by outgrowing pathogenic organisms [24–26], and is used to this day in some cultures as a pro-biotic supplement. Pathogenic bacteria are also represented amongst *Bacilli*. The most extreme is *B. anthracis,* a biowarfare agent responsible for the infection commonly referred to as anthrax. Closely related to *anthracis*, *B. cereus* is a common cause of food poisoning in dishes containing mixtures of meat and rice.

Whether beneficial or pathogenic, these organisms are able to thrive in the intestine and/or lung. Both environments are major sites of infection by pathogenic *Bacilli*. Both are rich in lipids, especially fatty acids including polyunsaturated fatty acids. To better understand the role CYP102s may play in the survival and competition of these organisms, we wish to compare the properties of CYP102s. Cloning and characterization of CYP102A2 and CYP102A3 from *B. subtilis* have previously been reported [27–29]. Both of these P450's show differing substrate specificity from each other as well as from the highly studied CYP102A1. In this paper, we report the cloning, expression, and characterization of the substrate specificity of CYP102A5 from the food poisoning agent *B. cereus* to help us better understand the role of CYP102 P450s in the survival of their host organisms in lipid rich environments. The fundamental characterization of the specificity of the enzyme is an important first step to the study of the role of CYP102s *in vivo* in the *B. cereus* model for anthrax. The data obtained show that although the substrate profile of CYP102A5 varies slightly from CYP102A1 (as well as CYP102A2 and CYP102A3), CYP102A5 shows highest activity for oxidation of long-chain unsaturated fatty acids like its previously characterized relatives. It is one of the fastest and most regiospecific members of this class of efficient oxidizing enzymes.

Materials and methods

Materials

pTrcHis-TOPO TA expression kits were purchased from Invitrogen Corporation (Frederick, MD). Quick Ligation Kit, Standard DNA 2-Log Ladder (0.1–10 kb), and all restriction enzymes were purchased from New England Biolabs, Inc. (Beverly, MA). Wizard Plus SV Minipreps DNA purification system and 1 kb DNA standards were purchased from Promega Corporation (Madison, MI). Expand High Fidelity PCR System was purchased from Roche (Mannheim, Germany). The Amicon concentrator and the YM10 ultrafiltration membranes were obtained from Millipore Corp. (Bedford, MA). Prepacked PD-10 Sephadex G-25 columns were purchased from Biosciences AB (Uppsala, Sweden). Oligonucleotides for DNA amplification and sequencing were synthesized by Genosys (The Woodlands, TX) or Invitrogen. CYP102A2 protein with hexahistidine affinity tag removed was provided by Dr. Julian Peterson, University of Texas Southwestern Medical Center at Dallas (Dallas, TX).

Phylogenetic analysis

Alignment of amino acid sequences was done with the ClustalW program via the European Bioinformatics Institute website at http://www.ebi.ac.uk/clustalw/. Alignments were visualized with the program Bioedit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

Substrate synthesis

Acyl glycines were synthesized in good yield from the corresponding commercially available glycine methyl ester and respective acid chloride by methods previously established [19]. Purity and identity were confirmed by thin layer chromatography and ^{1}H NMR spectroscopy.

Bacterial strains and growth conditions

B. cereus strain ATCC14579 was obtained from American Type Culture Collection, (Manassas, VA) and grown on LB-agar plates at 30 °C. Genomic DNA was extracted from a single colony by heating it to 95 °C for 20 min. *E. coli* strain DH5αF'IQ obtained from Invitrogen Corporation, (Frederick, MD) was used for general DNA manipulation and expression.

[1] A single report of a CYP102A1 rate of 15,000 min^{-1} for the oxidation of arachidonic acid exists in the literature [66]. We have been entirely unable to reproduce this rate, however, getting the value reported elsewhere [7,46,60] of around 2000–3000 min^{-1}. The authors of the study suggest that this is due to the use of carbonate instead of ethanol for the dissolution of substrate, but the two methods result in essentially identical rates in our hands.

[2] *Abbreviations used:* PUFA, polyunsaturated fatty acids; CPR, cytochrome P450 reductase; PMSF, phenylmethanesulfonyl chloride; IMAC, Immobilized metal affinity chromatography.