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Identification of a new plasmid-encoded cytochrome P450 CYP107DY1 from *Bacillus megaterium* with a catalytic activity towards mevastatin

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ABSTRACT

In the current work, we describe the identification and characterization of the first plasmid-encoded P450 (CYP107DY1) from a *Bacillus* species. The recombinant CYP107DY1 exhibits characteristic P450 absolute and reduced CO-bound difference spectra. Reconstitution with different redox systems revealed the autologous one, consisting of BmCPR and Fdx2, as the most effective one. Screening of a library of 18 pharmaceutically relevant compounds displayed activity towards mevastatin to produce pravastatin. Pravastatin is an important therapeutic drug to treat hypercholesterolemia, which was described to be produced by oxyfunctionlization of mevastatin (compactin) by members of CYP105 family. The hydroxylation at C6 of mevastatin was also suggested by docking this compound into a computer model created for CYP107DY1. Moreover, in view of the biotechnological application, CYP107DY1 as well as its redox partners (BmCPR and Fdx2) were successfully utilized to establish an *E. coli* based whole-cell system for an efficient biotransformation of mevastatin. The in vitro and in vivo application of the CYP07DY1 also offers the possibility for the screening of more substrates, which could open up further biotechnological usage of this enzyme.

1. Introduction

Selective oxyfunctionalization of nonactivated carbon-hydrogen bonds represents a challenge in the industrial field. In general, the use of chemical methods has many disadvantages such as hazardous conditions, cost-efficiency and the lack of chemo-, stereo-, and regioselectivity. Therefore, in the last years many efforts have been carried out in the search for selective and efficient enzymatic systems that are able to incorporate oxygen into nonactivated carbon-hydrogen bonds. Cytochromes P450 (P450s) [E.C.1.14.-] are heme-iron containing enzymes that catalyze the monooxygenation of various nonactivated hydrocarbons with high regio-, stereo- and enantioselectivity including the biosynthesis of hormones, signal molecules, defense-related chemicals and secondary metabolites in addition to their central role in the metabolism of endogenous (steroids and fatty acids) and exogenous (drugs and toxins) substances (Bernhardt, 2006; Bernhardt and Urlacher, 2014). They are found in all kingdoms of life including mammals, plants, insects, fungi, archaea, and bacteria (except *E. coli*) as well as in viruses. In eukaryotes, they are mostly integral membrane-bound proteins, whereas prokaryotic P450 systems are more likely soluble and located in the cytoplasm. P450s rely for their activities on redox partners. Based on the composition of the redox partner involved in the transfer of electrons, the P450 systems can be categorized into different classes (Hannemann et al., 2007) of which the most researched ones are the classes I and II. Class I contains the bacterial and the eukaryotic mitochondrial P450s, which obtain electrons from NADPH using two proteins, a flavin adenine dinucleotide (FAD)-containing ferredoxin reductase and an iron-sulfur containing ferredoxin. Class II comprises the eukaryotic microsomal P450s, which obtain electrons from NADPH via a FAD and FMN-containing P450 reductase.

The numbers of the newly identified P450s increased drastically over the past few years (Nelson, 2009), but there is nevertheless a still growing demand to exploit novel P450s as a valuable biocatalyst in the industrial field.

Bacillus megaterium is a nonpathogenic, aerobic, Gram-positive rod-shaped bacterium. Due to its high protein production capacity, plasmid stability and the ability to take up a variety of hydrophobic

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substrates, *B. megaterium* gained throughout the last decades a lot of interest in the industrial field for the production of biotechnologically relevant substances (Bunk et al., 2010). The publication of the complete genome sequence of the *B. megaterium* strains QM B1551 and DSM319 in 2011 (Eppinger et al., 2011) enabled the identification of new proteins. Among them are cytochromes P450 and a NADPH dependent diflavin reductase, which have been shown to be very important for biotechnological and pharmaceutical applications (Brill et al., 2013; Milhim et al., 2016). *B. megaterium* encodes for several P450s. The self-sufficient CYP102A1 (also known as BM3) is the most investigated bacterial P450 so far, which has been used and redesigned to catalyze the oxidation of a variety of biotechnologically interesting substances (Whitehouse et al., 2012). In addition, the biotechnologically valuable CYP106 family, CYP106A1 from *B. megaterium* strain DSM319 (Brill et al., 2013) and CYP106A2 from *B. megaterium* strain ATCC 13368 (Berg et al., 1976, 1979), was characterized to be associated with the biotransformation of a diverse array of substrates such as steroids and terpenoic substances (Brill et al., 2013; Schmitz et al., 2012). Furthermore, CYP109E1 was recently identified from *B. megaterium strain* DSM319 as steroid hydroxylase (Jóźwik et al., 2016).

In this study, we report the identification and characterization of a new plasmid-encoded P450 from the *B. megaterium* QM B1551. The bioinformatic analysis of the new P450 showed that it belongs to the CYP107 family. It was successfully cloned and expressed in *E. coli*. Screening of a potential substrates showed that CYP107DY1 possesses hydroxylation activity towards mevastatin.

2. Materials and methods

2.1. Strains, expression vectors, enzymes, and chemicals

E. coli TOP10 from Invitrogen (Karlsruhe, Germany) was used for cloning experiments. *E. coli* C43 (DE3) and the expression vector pET17b, both from Novagen (Darmstadt, Germany), were used for recombinant gene expression. Substrates were obtained from TCI (Eschborn, Germany). Pravastatin lactone was from Santa Cruz Biotechnology (Heidelberg, Germany). All other chemicals were purchased from Sigma–Aldrich (Schnelldorf, Germany).

2.2. Cloning of the gene encoding CYP107DY1

For protein purification purposes, the DNA fragment encoding the full length CYP107DY1 (Supplementary Fig. S1) was synthesized (Genart, Regensburg, Germany) and cloned into the expression vector pET17b with the *Nde*I/*Kpn*I restriction sites. For purification with IMAC, the 3′end of the gene was extended with a sequence coding for six histidines. Plasmid was verified by sequencing.

2.3. Heterologous gene expression and purification of CYP107DY1, reductases and ferredoxins

For heterologous gene expression, *E. coli* C43 (DE3) cells were co-transformed with the expression vector pET17b congaing the sequence for CYP107DY1 and the chaperone GroEL/GroES-encoding plasmid pGro12, which has a kanamycin resistance gene (Brixius-Anderko et al., 2015; Nishihara et al., 1998). Cultures were grown at 37 °C to an optical density of 0.6 in 200 ml TB medium containing the suitable antibiotics. The expression of the protein was induced by adding 1 mM IPTG, the synthesis of heme was enhanced by addition of 1 mM heme precursor δ-ALA. The cells were grown at 28 °C and 180 rpm for 24 h.

For purification, cell pellets were sonicated in 50 ml lysis buffer (50 mM potassium phosphate pH 7.4, 20% glycerol, 0.1 mM DTE, 500 mM sodium acetate, and 0.1 mM PMSF). After centrifugation at 30,000*g* for 30 min at 4 °C, the supernatant was applied on a Ni–NTA agarose column equilibrated with lysis buffer. The column was washed with 100 ml equilibration buffer supplemented with 40 mM imidazole followed by 20 ml elution buffer supplemented with 200 mM imidazole (50 mM potassium phosphate pH 7.4, 20% glycerol, 0.1 mM DTE, and 0.1 mM PMSF). The eluted protein was dialyzed against elution buffer without imidazole, concentrated and stored at −80 °C.

The *B. megaterium* redox system BmCPR and Fdx2 were purified as reported previously (Brill et al., 2013; Milhim et al., 2016). The purification of the redox system from the fission yeast *Schizosaccharomyces pombe* Arh1 and Etp1^{fd} was carried out as described before (Bureik et al., 2002; Ewen et al., 2008). Recombinant bovine AdR and the Adx$_{4\text{-}108}$ (truncated form of Adx comprising amino acids 4–108) were purified as mentioned elsewhere (Sagara et al., 1993; Uhlmann et al., 1992).

The concentration of recombinant P450 was estimated using the CO-difference spectral assay as described previously with $\varepsilon_{450-490} = 91\ mM^{-1}\ cm^{-1}$ (Omura and Sato, 1964). The concentration of BmCPR was quantified by measuring the flavin absorbance at 456 nm with $\varepsilon_{456} = 21\ mM^{-1}\ cm^{-1}$ for the oxidized enzyme (Milhim et al., 2016). The concentrations of the AdR and Arh1 were measured using the extinction coefficient $\varepsilon_{450} = 11.3\ mM^{-1}\ cm^{-1}$ (Ewen et al., 2008; Hiwatashi et al., 1976). The concentrations of Fdx2 and Etp1^{fd} were measured using the extinction coefficient $\varepsilon_{390} = 6.671\ mM^{-1}\ cm^{-1}$ and $\varepsilon_{414} = 9.8\ mM^{-1}\ cm^{-1}$, respectively (Brill et al., 2013; Schiffler et al., 2004).

2.4. Investigation of electron transfer partners

The functional interaction of the electron transfer partners for a particular P450 can be determined by recording the NADPH reduced CO-complex peak at 450 nm when P450 was coupled with the different ferredoxins/ferredoxin reductases in the absence of substrate. For this, CYP107DY1 was mixed with ferredoxins (Fdx2, Etp1^{fd} or Adx$_{4\text{-}108}$) and ferredoxin reductases (BmCPR, Arh1 or AdR) with ratios of 1:40:5 μM [CYP107DY1:ferredoxin:ferredoxin reductase] in 50 mM HEPES buffer pH 7.4 and NADPH was added to a final concentration of 1 mM. The spectrum of NADPH-reduced samples was recorded after bubbling the sample with carbon monoxide (CO) gas. The reduction efficiency of the redox partners was then evaluated by comparing the peak at 450 nm of the CO-complexed CYP107DY1 reduced with the different redox systems and the peak at 450 nm of the CO-complexed CYP107DY1 reduced with sodium-dithionite.

2.5. In vitro conversion and HPLC analysis

The in vitro conversion of the substrates was carried out with a reconstituted system at 30 °C in conversion buffer (50 mM HEPES, pH 7.4, 20% glycerol). The reconstituted system contained 0.5 μM CYP107DY1, 2.5 μM BmCPR, 20 μM Fdx2, 1 mM $MgCl_2$, 5 mM glucose-6-phosphate, 1 U glucose-6-phosphate dehydrogenase for NADPH regeneration and 100 μM substrate. The reaction was started by adding NADPH (200 μM) and stopped after 15 min by the addition of 1 vol ethyl acetate and extracted twice. The organic phase was evaporated under vacuum. Residuum was dissolved in 20% acetonitrile/water mixture and subjected to HPLC analysis. HPLC analysis was performed using a Jasco system. A reversed-phase ec MN Nucleodur C18 (4.0 × 125 mm) column (Macherey-Nagel) was used for all experiments at an oven temperature of 40 °C. Mevastatin and its metabolite pravastatin were eluted from the column using a gradient of acetonitrile from 20 to 100% in water over 20 min. The detection wavelength of mevastatin and its metabolite pravastatin was 236 nm.