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ScienceDirect
Journal of Biotechnology 131 (2007) 205–208
Journal of BIOTECHNOLOGY
www.elsevier.com/locate/jbiotec

Short communication

Testosterone 15β-hydroxylation by solvent tolerant *Pseudomonas putida* S12

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Received 2 April 2007; accepted 12 June 2007

Abstract

A steroid 15β-hydroxylating whole-cell solvent tolerant biocatalyst was constructed by expressing the *Bacillus megaterium* steroid hydroxylase CYP106A2 in the solvent tolerant *Pseudomonas putida* S12. Testosterone hydroxylation was improved by a factor 16 by co-expressing Fer, a putative Fe-S protein from *Bacillus subtilis*. In addition, the specificity for 15β-hydroxylation was improved by mutating threonine residue 248 of CYP106A2 into valine. These new insights provide the basis for an optimized whole-cell steroid-hydroxylating biocatalyst that can be applied with an organic solvent phase.
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Keywords: CYP106A2; Whole cell biocatalyst; Steroid synthesis; Solvent tolerance; *Pseudomonas putida*; Fer

Stereoselective hydroxylations of steroid precursors are important reactions in the synthesis of steroid drugs. Many wild-type microorganisms, often fungi, have been isolated that are capable of hydroxylating steroids at various positions (Mahato and Garai, 1997). However, the application of biocatalysts in steroid transformations is restricted by the extremely limited water-solubility of the substrates. Therefore, we aimed at constructing new whole-cell biocatalysts for steroid conversions, based on the solvent tolerant bacterium *Pseudomonas putida* S12 (for reviews: De Bont, 1998; Wery and De Bont, 2004). Whole-cell biocatalysts based on this host can be operated in the presence of a second phase of organic solvent (De Bont, 1998; Wierckx et al., 2005), which property can be exploited to alleviate the solubility problems normally encountered in steroid bioconversions.

To obtain a steroid hydroxylating biocatalyst, CYP106A2 of *Bacillus megaterium* ATCC 13368 was selected for expression in *P. putida* S12. CYP106A2 ($P450_{meg}$) is a class I cytochrome P450 (cytP450) and one of the few known bacterial steroid hydroxylases. It hydroxylates 3-keto-4-*ene*-steroids at the 15β-position and, to a lesser extent, at the 6β-position (Berg et al., 1976) (Fig. 1). In the present study, testosterone was selected as the model substrate. The bacterial strains, plasmids and oligonucleotide primers used are listed in Tables 1 and 2.

The *cyp106A2* gene (Rauschenbach et al., 1993) was obtained by PCR from genomic DNA of *B. megaterium* and cloned into expression vector pTn-1, yielding pTn_cyp106A2. *P. putida* S12 was electrotransformed with pTn_cyp106A2 and the testosterone hydroxylating capacity of the transformants was assessed. HPLC-DAD analysis of the expression culture supernatants demonstrated that three products were formed from testosterone (Fig. 2). The major product was identified by ^{1}H NMR as 15β-hydroxytestosterone. 6β-Hydroxytestosterone and androst-4-*ene*-3,17-dione (AD) were present as minor products. Typically, no further increase of hydroxylated testosterone derivatives was observed after 72 h. As expected, no hydroxylated testosterone derivatives were observed in cultures of *P. putida* S12[pTn-1] (empty vector control) or *P. putida* S12[pTn_cyp106A2] without salicylate (uninduced control). AD was also produced in the control cultures, confirming previous findings that the oxidation of testos-

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doi:10.1016/j.jbiotec.2007.06.007

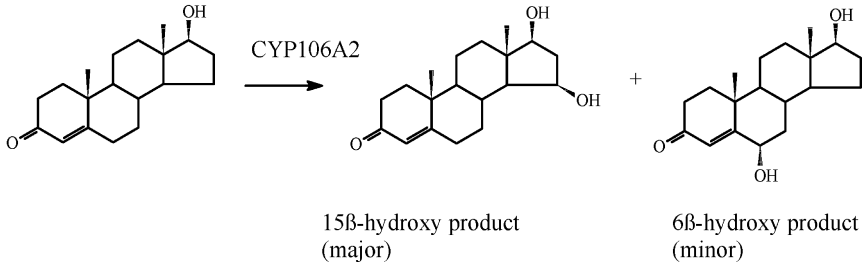

Fig. 1. CYP106A2 catalyzed hydroxylation of testosterone.

Table 1
Strains, plasmids and oligonucleotides used in this study

Strain	Relevant characteristics	Source/reference
E. coli DH5α	General cloning host	Invitrogen
P. putida S12	Wild type	Hartmans et al. (1990)
P. putida DSM50198	CAM plasmid; *camCAB* source	DSMZ
Bacillus megaterium DSM2987	*cyp106A2* source	DSMZ
Bacillus subtilis strain 168	*ykuN*, *ykuP*, *fer* source	DSMZ
Plasmid		
pTn-1	*E. coli - P. putida* shuttle vector; P_{nag}, Gm^R, Ap^R, pUCP22 backbone	Nijkamp et al. (2005)
pTn_cyp106A2	pTn-1 containing *cyp106A2*	This study
pTn_camCAB	pTn-1 containing *camCAB*	This study
pTn_cyp106A2camAB	pTn_camCAB; *camC* replaced by *cyp106A2*	This study
pTn_cyp106A2ykuN	pTn_cyp106A2camAB; *camAB* replaced by *ykuN*	This study
pTn_cyp106A2ykuP	pTn_cyp106A2camAB; *camAB* replaced by *ykuP*	This study
pTn_cyp106A2fer	pTn_cyp106A2camAB; *camAB* replaced by *fer*	This study
pTn_cyp106A2T248Vfer	pTn_cyp106A2fer; *cyp106A2* replaced by *cyp106A2T248V*	This study

terone to AD is endogenous to *P. putida* S12 (unpublished data).

CytP450s usually act as the terminal oxidases in multicomponent electron transfer chains that receive reducing equivalents from NAD(P)H. The electron transfer chains associated with class I cytP450s consist of an FAD-containing reductase and an electron shuttle protein, ususally a 2Fe-2S protein of the adrenodoxin/putidaredoxin family (Hannemann et al., 2007). The observation that *E. coli* expressing CYP106A2 does not hydroxylate steroids implies that *E. coli* is devoid of such electron transfer proteins (Rauschenbach et al., 1993; Hannemann et al., 2006). Although *P. putida* S12 did hydroxylate testosterone when expressing CYP106A2, the attained testosterone 15β-hydroxylation was rather modest: 0.7% of the substrate was hydroxylated after 72 h. Therefore, it was decided to construct a more efficient steroid hydroxylating whole-cell biocatalyst by co-expressing exogenous electron transfer proteins and CYP106A2 in *P. putida* S12.

Since no genetic information is available for the native *B. megaterium* CYP106A2 redox partners (megaredoxin and megaredoxin reductase (Berg, 1982)), the CYP101 supporting electron transfer chain from *P. putida* DSM50198 was selected,

Table 2
Oligonucleotides used in this study

Oligonucleotide primer	Target gene	Sequence (5′–3′)[a]	Cohesive end
Primer 1	*cyp106A2*	GCATGAATTCATGAAAGAAGTTATTGCAGTAAAAG	EcoRI
Primer 2	*cyp106A2*	GTCTGCGGCCGCTTACATGCGGCTTGCCTTAAGCG	NotI
Primer 3	*cyp106A2*	GCTTAATTAATTACATGCGGCTTGCCTTAAGCG	PacI
Primer 4	*camC*	GCATGAATTCATGACGACTGAAACCATACAAAGC	EcoRI
Primer 5	*camC*	GCTTAATTAAACCGCTTTGGTAGTCGCCGG	PacI
Primer 6	*camA*	GCTTAATTAAATGGGAGTGCGTGCTAAGTGAACG	PacI
Primer 7	*camB*	GCATGCGGCCGCTTACCATTGCCTATCGGGAACATCG	NotI
Primer 8	*fer*	GCTTAATTAAATCTGGGAGGTTTTATTCATGGC	PacI
Primer 9	*fer*	GCATGCGGCCGCGATTGAGCAGCAGCGAAGTGC	NotI
Primer 10	*ykuN*	GCTTAATTAACAACTAATGGGGTGATAACATGGC	PacI
Primer 11	*ykuN*	GCATGCGGCCGCCCGATGCCGTAACAGCGGTTGC	NotI
Primer 12	*ykuP*	GCTTAATTAAAGAGGAGGAACAAGGAAaTGGCGAAG	PacI
Primer 13	*ykuP*	GCATGCGGCCGCCCCAGTGAGTGATCAGACAGCG	NotI
Primer 14	*cyp106A2*	GGAGTCGAGACA**GTC**AGTCATTTATTGG	Mutagenic primer ACC > GTC
Primer 15	*cyp106A2*	CCAATAAATGACT**GAC**TGTCTCGACTCC	Mutagenic primer GGT > GAC

[a] The restriction sites used for cloning have been underlined.

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