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Genetic engineering of Bacillus megaterium for high-yield production of the major teleost progestogens 17α,20β-di- and 17α,20β,21α-trihydroxy-4-pregnen-3-one

Adrian Gerber [a], Mohammed Milhim [a], Philip Hartz [a], Josef Zapp [b], Rita Bernhardt [a,*]

[a] Saarland University, Institute of Biochemistry, Campus B2.2, 66123 Saarbrücken, Germany
[b] Saarland University, Institute of Pharmaceutical Biology, Campus C2.3, 66123 Saarbrücken, Germany

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ABSTRACT

17α,20β-Dihydroxy-4-pregnen-3-one (17α,20βDiOH-P) and 17α,20β,21α-trihydroxy-4-pregnen-3-one (20βOH-RSS) are the critical hormones required for oocyte maturation in fish. We utilized *B. megaterium*'s endogenous 20β-hydroxysteroid dehydrogenase (20βHSD) for the efficient production of both progestogens after genetically modifying the microorganism to reduce side-product formation. First, the gene encoding the autologous cytochrome P450 CYP106A1 was deleted, resulting in a strain devoid of any steroid hydroxylation activity. Cultivation of this strain in the presence of 17α-hydroxyprogesterone (17αOH-P) led to the formation of 17α,20α-dihydroxy-4-pregnen-3-one (17α,20αDiOH-P) as a major and 17α,20βDiOH-P as a minor product. Four enzymes were identified as 20αHSDs and their genes deleted to yield a strain with no 20αHSD activity. The 3-oxoacyl-(acyl-carrier-protein) reductase FabG was found to exhibit 20βHSD-activity and overexpressed to create a biocatalyst yielding 0.22 g/L 17α,20βDiOH-P and 0.34 g/L 20βOH-RSS after 8 h using shake-flask cultivation, thus obtaining products that are at least a thousand times more expensive than their substrates.

1. Introduction

The oocyte maturation inducing hormones (MIHs) 17α,20βDiOH-P and 20βOH-RSS are the main progestogens in teleosts. In female fishes, MIH production in the ovarian follicle layers is stimulated by gonadotropin synthesized in the pituitary. MIH induces the formation of the maturation promoting factor (MPF) leading to further oocyte maturation (Nagahama and Yamashita, 2008). In addition, MIHs are produced in the testis of male and, among other functions, regulate spermiation and sperm motility (Scott et al., 2010). Both MIHs are produced from cholesterol in a series of reaction steps involving the cytochromes P450 CYP11A1, CYP17, CYP21 as well as 3β- and 20βHSD.

The administration of MIHs to different species of aquacultured fish has been extensively investigated and shown to greatly increase spawning rates due to, for instance, overcoming ovulation disorder, improving sperm volume and concentration, attracting fish to spawning sites and induction of ovulation (Yamamoto et al., 2015; Martins Pinheiro et al., 2003; Hong et al., 2006; Haider and Rao, 1994; King and Young, 2001; Ohta et al., 1996; Miwa et al., 2001). Induced spawning is of particular interest for economically significant species of fish that do not reproduce spontaneously under captive cultivation conditions (Lee and Yang, 2002). However, the widespread use of both synthetic MIHs in aquacultures has been hindered by their high cost.

Steroid hormones are produced mainly through a combination of chemical and microbial conversion steps, since single-step stereospecific modifications of the unreactive steroid nucleus by chemical means alone are often not possible (Donova and Egorova, 2012). The chemical synthesis of 17α,20βDiOH-P is tedious, requiring multiple reaction steps to prepare the steroid stereoselectively. One method to produce the progestogen includes the chlorination, dechlorination and reduction of RSS to produce the substance with an overall yield of 64 % (Ouedraogo et al., 2013). Another approach consists of reducing 17OH-P by $NaBH_4$ in

Abbreviations: AKR, aldo-keto reductase; 17OH-P, 17α-hydroxyprogesterone; 17α,20αDiOH-P, 17α,20α-dihydroxy-4-pregnen-3-one; 17α,20βDiOH-P, 17α,20β-dihydroxy-4-pregnen-3-one; 20βOH-RSS, 17α,20α,21α-trihydroxy-4-pregnen-3-one; DOC, 11-deoxycorticosterone; FabG, 3-oxoacyl-(acyl-carrier-protein) reductase; HSD, hydroxysteroid dehydrogenase; LB, lysogeny broth; MIH, maturation inducing hormone; MPF, maturation promoting factor; NMR, nuclear magnetic resonance; ORF, open reading frame; PHB, poly(3-hydroxybutyrate); RP-HPLC, reverse phase high performance liquid chromatography; RSS, 11-deoxycortisol; SOE-PCR, splicing by overlapping polymerase chain reaction; TB, terrific broth; Upp, uracil phosphoribosyltransferase

* Corresponding author.
E-mail address: ritabern@mx.uni-saarland.de (R. Bernhardt).

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Table 1
List of *B. megaterium* strains and plasmids used in this study.

Strains or plasmids	Description	Reference
Strains		
MS941	Mutant of DSM319, Δ*nprM* (extracellular protease)	Wittchen and Meinhardt (1995)
GHH1	Mutant of MS941, Δ*upp* (selection marker)	Gerber et al. (2015)
GHH2	Mutant of GHH1, Δ*cyp106A1*	This study
GHH5	Mutant of GHH2, Δ*BMD_0912* (20αHSD)	This study
GHH6	Mutant of GHH5, Δ*BMD_1068* (20αHSD)	This study
GHH7	Mutant of GHH6, Δ*BMD_3715* (20αHSD)	This study
GHH8	Mutant of GHH7, Δ*BMD_1595* (20αHSD)	This study
Plasmids		
pSMF2.1	Backbone vector for protein overexpression	Bleif et al. (2012)
pSMF2.1_0912	Expression of *BMD_0912* (20α HSD)	This study
pSMF2.1_1595	Expression of *BMD_1595* (20α HSD)	This study
pSMF2.1_1068	Expression of *BMD_1068* (20αHSD)	This study
pSMF2.1_3715	Expression of *BMD_3715* (20αHSD)	This study
pSMF2.1_FabG	Expression of FabG (20βHSD)	This study
pUCTV2_Upp	Backbone vector for gene deletion	Gerber et al. (2015), based on Wittchen and Meinhardt (1995)
pUCTV2_Upp_Δ*106A1*	Genomic deletion of *cyp106A1*	This study
pUCTV2_Upp_Δ*0912*	Genomic deletion of *BMD_0912*	This study
pUCTV2_Upp_Δ*1595*	Genomic deletion of *BMD_1595*	This study
pUCTV2_Upp_Δ*1068*	Genomic deletion of *BMD_1068*	This study
pUCTV2_Upp_Δ*3715*	Genomic deletion of *BMD_3715*	This study

methanol, resulting in a ~30 % yield of 17α,20βDiOH-P (Kovganko et al., 2001). In microbial fermentations with strains of *B. megaterium*, *Yarrowia lipolytica*, *Saccharomyces cerevisiae* or *Bifidobacterium adolescentis* 17α,20βDiOH-P appeared only as a minor side-product (Shkumatov et al., 2003; Winter et al., 1982; Koshcheenko et al., 1976). Genetically engineered microorganisms are often used to increase the accumulation of desired products by, for instance, deletion of genes to avoid by-product formation (Yao et al., 2014), augmenting the activity of endogenous enzymes by overexpression (Yao et al., 2013) or mutagenesis (Hunter et al., 2011), or increasing the tolerance of the organism towards metabolite and substrate stress (Nicolaou et al., 2010).

In this study, we present a genetically modified *B. megaterium* strain allowing the high yield production of both 17,20βDiOH-P and 20βOH-RSS from cheap steroid precursors. This Gram-positive, rod-shaped bacterium has gained considerable interest in recent years as a recombinant expression host due to its high protein production capacity, plasmid stability, ability to take up a variety of hydrophobic substrates as well as its large cell size, which allows detailed microscopic analyses (Vary et al., 2007). Like other *Bacillus* species, *B. megaterium* can be genetically engineered through homologous recombination with exogenous DNA, with or without applying a marker gene (Dong and Zhang, 2014). The classic procedure consists of protoplast transforming cells with the deletion vector, the integration of parts of the vector into the chromosome, screening the target locus for the mutation by PCR and finally curing the vector from the cells (Biedendieck et al., 2011). A more recent approach allows the one-step deletion of genes by using a transconjugation protocol with *Escherichia coli* as plasmid-donor cells (Richhardt et al., 2010). *B. megaterium* has been shown to be particularly suitable for the bioconversion of hydrophobic steroidal compounds, including cholesterol and analogs (Gerber et al., 2015), natural steroid hormones derived from cholesterol such as pregnenolone and dehydroepiandrosterone (Schmitz et al., 2014), synthetic steroid hormones such as prednisolone and dexamethasone (Kiss et al., 2015) and the secosteroid vitamin D3 (Ehrhardt et al., 2016). The genome of *B. megaterium* DSM319, the precursor of the strain used in this study, contains the gene for the cytochrome P450 CYP106A1, resulting in an endogenous steroid hydroxylation activity. Cytochromes P450 form a superfamily of heme-thiolate proteins, which are, in bacteria, mainly involved in the metabolism of xenobiotics and the production of secondary metabolites (Bernhardt, 2006; Bernhardt and Urlacher, 2014). CYP106A1 is able to convert a wide variety of steroids, including testosterone, progesterone, 17-hydroxyprogesterone, RSS, DOC and cortisol (Kiss et al., 2015).

The aim of this study was to construct a biocatalyst for the production of the fish progestogens 17α,20βDiOH-P and 20βOH-RSS by utilizing an endogenous 20βHSD and abolishing side-product formation from the likewise endogenous CYP106A1 and four 20αHSDs through genetic engineering.

2. Materials and methods

2.1. Materials

20βOH-RSS (4-pregnen-17α,20β,21-triol-3-one, catalog ID: Q4080-000), 20βOH-cortisol (4-pregnen-11β, 17, 20β, 21-tetrol-3-one, Q3790-000) and 20βOH-cortisone (4-pregnen-17, 20β, 21-triol-3, 11-dione, Q3960-000) were purchased from Steraloids. 17OH-P (4-pregnen-17α-ol-3,20-dione, H5752) RSS (4-Pregnene-17α,21-diol-3,20-dione, R0500), cortisol (4-pregnen-11β,17α,21-triol-3,20-dione, H4001), cortisone (pregnen-17α,21-diol-3,11,20-trione, C2755) and 17α,20αDiOH-P (4-pregnen-17, 20β-diol-3-one, P6285), xylose (95729), tetracycline (T7660), ethyl acetate (34858) and acetonitrile (34851) were from Sigma-Aldrich. LB broth (244610), yeast extract (212750) and tryptone (211705) were obtained from Becton Dickinson.

2.2. Molecular cloning and gene deletion

All plasmids were constructed by conventional cloning using restriction enzyme digestion and ligation. *E. coli* strain TOP10 (Invitrogen) was used for the propagation of plasmids. *B. megaterium* was transformed according to the PEG-mediated protoplast transformation method (Biedendieck et al., 2011). Table 1 lists all plasmids used in this study. Plasmid maps displaying backbone vectors and the restriction sites used for cloning are depicted in Supplemental Fig. 1. The xylose-inducible vector pSMF2.1 was used as a backbone for the overexpression of genes. ORFs of *BMD_0912*, *BMD_1595*, *BMD_1068*, *BMD_3715* and *FabG* were amplified from genomic DNA of *B. megaterium* MS941 and cloned into pSMF2.1. Genomic DNA was prepared using a genomic DNA

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