Journal of Biotechnology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Enhanced production of *n*-alkanes in *Escherichia coli* by spatial organization of biosynthetic pathway enzymes

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ARTICLE INFO

Article history:
Received 28 May 2014
Received in revised form 10 October 2014
Accepted 13 October 2014
Available online xxx

Keywords:
Alkanes
Synthetic biology
DNA scaffold
Chimeric expression
Biofuel

ABSTRACT

Alkanes chemically mimic hydrocarbons found in petroleum, and their demand as biofuels is steadily increasing. Biologically, *n*-alkanes are produced from fatty acyl-ACPs by acyl-ACP reductases (AARs) and aldehyde deformylating oxygenases (ADOs). One of the major impediments in *n*-alkane biosynthesis is the low catalytic turnover rates of ADOs. Here, we studied *n*-alkane biosynthesis in *Escherichia coli* using a chimeric ADO-AAR fusion protein or zinc finger protein-guided ADO/AAR assembly on DNA scaffolds to control their stoichiometric ratios and spatial arrangements. Bacterial production of *n*-alkanes with the ADO-AAR fusion protein was increased 4.8-fold (24 mg/L) over a control strain expressing ADO and AAR separately. Optimal *n*-alkane biosynthesis was achieved when the ADO:AAR binding site ratio on a DNA scaffold was 3:1, yielding an 8.8-fold increase (44 mg/mL) over the control strain. Our findings indicate that the spatial organization of alkane-producing enzymes is critical for efficient *n*-alkane biosynthesis in *E. coli*.

1. Introduction

Bio-based alkanes are a potentially valuable source of biofuels because of their chemical and structural similarities to hydrocarbons present in petroleum. Production of *n*-alkanes has been reported in *Arabidopsis*, cyanobacteria, and most recently in engineered *Escherichia coli.* Enzymes encoding cyanobacteria biosynthetic pathway components have been introduced into *E. coli* for the production of *n*-alkanes (Schirmer et al., 2010). This heterologous pathway in *E. coli* employs 2 sequential enzymatic steps in *n*-alkane production (Fig. 1a). The first step involves the reduction of *E. coli* fatty acyl-ACPs to fatty aldehydes by acyl-ACP reductase (AAR). This step is followed by the conversion of fatty aldehydes to *n*-alkanes by aldehyde decarbonylase (ADO) (Zhang et al., 2013).

The limited production (2–5 mg/L) of *n*-alkanes in *E. coli* may be caused by the low catalytic turnover rate of ADO and the toxicity of aldehyde intermediates (Akhtar et al., 2013; Andre et al., 2013; Warui et al., 2011).

In multi-step metabolic pathways, the yields of final products are highly dependent upon rate-limiting enzymes and the accumulation of detrimental metabolic intermediates (Dueber et al., 2009; Lee et al., 2012). To improve pathway efficiencies, researchers frequently pursue strategies related to enzyme property enhancement, such as mutagenesis, the use of alternate isozymes, or engineered enzyme complex formation. Enzyme complexes formed by the use of chimeric proteins or synthetic scaffolds have been used successfully for the efficient production of sesquiterpene, mevalonate, glucaric acid, or L-threonine (Albertsen et al., 2010; Conrado et al., 2012; Dueber et al., 2009; Lee et al., 2012).

Based on these promising results, we investigated the potential of enzyme complex formation in enhancing the production of *n*-alkanes. In one set of experiments, we evaluated *n*-alkane production in *E. coli* expressing a chimeric enzyme between ADO and AAR (Fig. 1b). Alternatively, these enzymes were juxtaposed on DNA scaffolds with DNA binding modules (Fig. 1c). This study demonstrates that by controlling the spatial organization of ADO and AAR, enhanced biosynthesis of *n*-alkanes in *E. coli* may be achieved.

Abbreviations: ACP, acyl carrier protein; LB, Luria Bertani; RM, Reisenberg medium; IPTG, isopropyl β-D-1-thiogalactopyranoside; CoA, coenzyme A; ABD, artificial DNA-binding domain; NADPH, nicotinamide adenine dinucleotide phosphate (reduced).

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http://dx.doi.org/10.1016/j.jbiotec.2014.10.014
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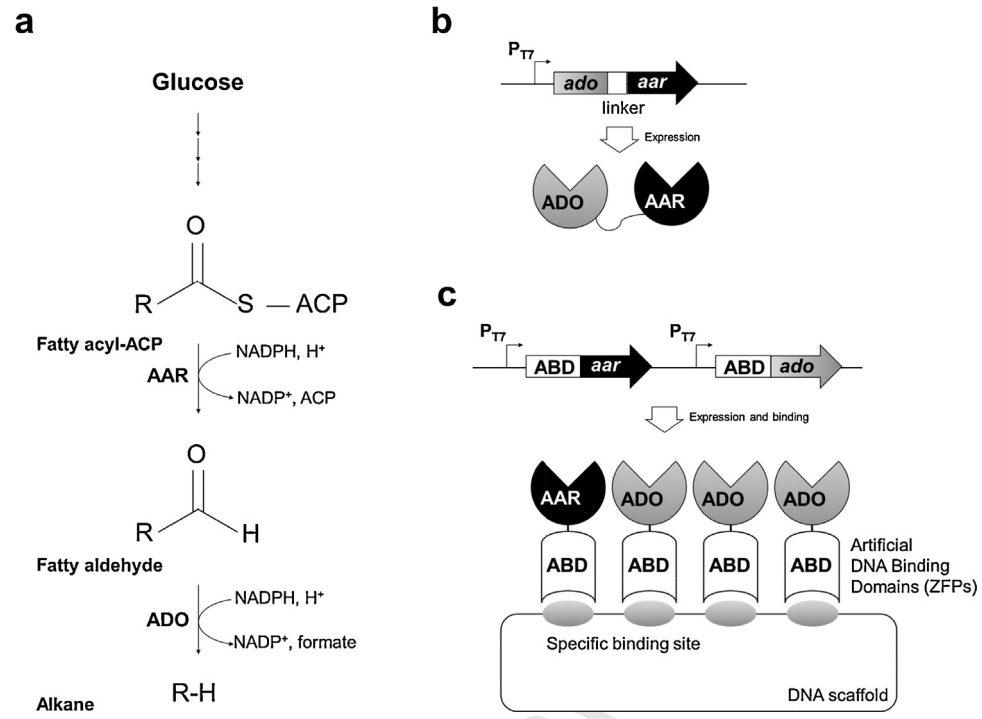

Fig. 1. Schematic representation of *n*-alkane production in *E coli*. (a) Fatty acyl-ACPs are converted to fatty aldehydes and *n*-alkanes by the enzymes AAR and ADO, respectively. (b) The ALKF chimera encodes the AAR and ADO enzymes fused together with Gly_4Ser (G_4S) linker. (c) The enzymes AAR and/or ADO are fused with ABDs (artificial DNA binding domain), facilitating their juxtaposition on DNA scaffolds.

2. Materials and methods

2.1. Bacterial strains, enzymes, and chemicals

The bacterial strains, plasmids, and primers used in this study are listed in Table 1. *E. coli* strain BL21 (DE3) was used for enzyme expression and as an *n*-alkane-producing host. Primers and genes used in this study were synthesized by GenoTech Corp. (Daejeon, Korea). Enzymes were purchased from New England Biolabs (Beverly, MA). All chemicals were obtained from Sigma–Aldrich (St. Louis, MO).

2.2. Construction of expression vectors encoding AAR, ADO, and an ADO-AAR chimera

The *AAR* and *ADO* genes were PCR-amplified from genomic DNA of *Synechococcus elongatus* PCC 7942 (ATCC 33912), using forward and reverse primers (Table 1). PCR products encompassing the *AAR* and *ADO* genes were digested with NcoI/BamHI or NdeI/BglII, respectively, and cloned into pETDuet-1 (Novagen, Darmstadt, Germany) to obtain pET-AAR, pET-ADO, and pET-ALK. The plasmid used to express the ADO-AAR fusion protein (pET-ALKF) was constructed by ligating the XmaI/BamHI-digested AAR DNA fragment into the XmaI/BglII site of pET-ADO.

2.3. Construction of plasmids encoding ABDs fused to AAR or ADO

Plasmids containing artificial DNA-binding domains (ABDs) were obtained from J.H.L. (Lee et al., 2012). Four zinc finger domains were fused in a modular fashion to obtain the ADBs that recognize the unique 12-bp DNA sequences (Tables S1 and S2). Plasmids pABD2 and pABD4 respectively encode ABD2 (RSHR-RSHR-RSHR-QAHR) and ABD4 (QSNI-CSNR-QSSR-QSHT), which recognize DNA sequences, 5′-GGAGGGGGGGG-3′ and 5′-AGAGTAGAACAA-3′, respectively. The ABD2 and ABD4 DNA fragments were obtained by digesting pABD2 and pABD4 with XmaI/AgeI and subcloned into the XmaI/AgeI site of pET-AAR and pET-ADO plasmids to generate pET-ABD_2AAR and pET-ABD_4ADO, respectively. The ABD_2-AAR fragment from pET-ABD_2AAR was then subcloned into the NcoI/BamHI site of pET-ABD_4ADO to generate the pET-ZFALK plasmid. Scaffold DNA templates encoding binding site sequences for ABD_2AAR and ABD_4ADO were synthesized and ligated into pSC (Lee et al., 2012) with 8-bp spacer DNA sequences to construct the scaffold plasmids pS-2224, pS-2244, pS-2424, and pS-2444.

2.4. Media, growth conditions, and alkane production analysis

LB medium (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) and Reisenberg medium (RM; 20 g/L glucose, 1.4 g/L $MgSO_4$, 4 g/L $(NH_4)_2HPO_4$, 13.5 g/L KH_2PO_4, and 1.8 g/L citric acid) were modified by the addition of 0.1% triton X100, 1 mg/L thiamine, and trace metals as described previously (Schirmer et al., 2010). The antibiotics ampicillin and kanamycin were used at final concentrations of 50 μg/mL and 25 μg/mL, respectively. Bacterial strains were grown at 30 °C and/or 37 °C in 500 mL flasks containing 100 mL media supplemented with either 2% glucose or 2% glycerol and induced with 0.1 mM IPTG at an optical density at 600 nm of 0.4. Bacteria were incubated for 24 h following induction. Western blot analysis was performed as described before (Conrado et al., 2012). For *n*-alkane analyses, 0.5 mL of bacterial cultures were extracted with 0.5 mL of chloroform and analyzed with a gas chromatograph–mass spectrometer (GC–MS) as described previously (Schirmer et al., 2010). Data was obtained from three independent experiments. The masses of *n*-alkanes were compared with commercial standards from Sigma–Aldrich.

3. Results and discussion

3.1. Expression of a chimeric ADO-AAR enzyme in E. coli

To study the importance of spatial organization of alkane-producing enzymes, an ADO-AAR chimera was constructed by