Metabolic Engineering 33 (2016) 98–108

Contents lists available at ScienceDirect

Metabolic Engineering

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

Original Research Article

Metabolic engineering of *Pseudomonas putida* for production of docosahexaenoic acid based on a myxobacterial PUFA synthase

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ARTICLE INFO

Article history:
Received 9 August 2015
Received in revised form
22 September 2015
Accepted 17 November 2015
Available online 24 November 2015

Keywords:
Polyunsaturated fatty acids
Polyketide synthase
PUFA synthase
Heterologous expression
Synthetic gene cluster
Pathway engineering

ABSTRACT

Long-chain polyunsaturated fatty acids (LC-PUFAs) can be produced *de novo* via polyketide synthase-like enzymes known as PUFA synthases, which are encoded by *pfa* biosynthetic gene clusters originally discovered from marine microorganisms. Recently similar gene clusters were detected and characterized in terrestrial myxobacteria revealing several striking differences. As the identified myxobacterial producers are difficult to handle genetically and grow very slowly we aimed to establish heterologous expression platforms for myxobacterial PUFA synthases. Here we report the heterologous expression of the *pfa* gene cluster from *Aetherobacter fasciculatus* (SBSr002) in the phylogenetically distant model host bacteria *Escherichia coli* and *Pseudomonas putida*. The latter host turned out to be the more promising PUFA producer revealing higher production rates of *n*-6 docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA). After several rounds of genetic engineering of expression plasmids combined with metabolic engineering of *P. putida*, DHA production yields were eventually increased more than threefold. Additionally, we applied synthetic biology approaches to redesign and construct artificial versions of the *A. fasciculatus pfa* gene cluster, which to the best of our knowledge represents the first example of a polyketide-like biosynthetic gene cluster modulated and synthesized for *P. putida*. Combination with the engineering efforts described above led to a further increase in LC-PUFA production yields. The established production platform based on synthetic DNA now sets the stage for flexible engineering of the complex PUFA synthase.

1. Introduction

Long-chain polyunsaturated fatty acids (LC-PUFAs), especially eicosapentaenoic acid (EPA, 20:5, *n*-3) and docosahexaenoic acid (DHA, 22:6, *n*-3), show advantageous effects on human health, like prevention and treatment of cardiovascular diseases, obesity, and diabetes (Lorente-Cebrian et al., 2013). As continuously more people strive to benefit from these positive effects, the demand of *n*-3 LC-PUFAs as dietary supplements has increased intensely over the past years-with upward tendency for the future. Nowadays, most of EPA and DHA are obtained from oceanic fish and fish oil, but these natural sources are depleting and often contaminated with environmental toxins. In order to satisfy the demand for high-quality LC-PUFAs, the quest for alternative, sustainable sources is indispensable (Lenihan-Geels et al., 2013). Fermentation of prokaryotic and eukaryotic microorganisms capable of producing EPA or DHA in high amounts might have the potential to permit industrial-scale production of LC-PUFAs (Ratledge, 2004). Until recently, it was thought that PUFA-producing microbes are exclusively of marine origin. Some of them synthesize LC-PUFAs *de novo* from acyl-CoA precursors by iteratively acting polyketide synthase (PKS)-like enzymes known as PUFA synthases. These multienzyme complexes are encoded by PUFA (*pfa*) biosynthetic gene clusters (Kaulmann and Hertweck, 2002; Napier, 2002; Wallis et al., 2002) (Fig. 1). Initially, the occurrence of *pfa* gene clusters was described in the marine γ-Proteobacteria *Shewanella pneumatophori* SCRC-2738 and *Photobacterium profundum* SS9 (EPA producers) (Allen and Bartlett, 2002; Metz et al., 2001), *Moritella marina* MP-1 (DHA producer) (Morita et al., 2000), and in the marine microalga *Schizochytrium* sp. ATCC 20888 (DHA and *n*-6 docosapentaenoic acid (DPA, 22:5) producer) (Hauvermale et al., 2006; Metz et al., 2001). Establishment of optimal fermentation conditions for these marine microorganisms (Chang et al., 2013; Ren et al., 2009, 2014), treatment with the fatty acid synthase inhibitor cerulenin (Fang et al., 2004; Morita et al., 2005),

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http://dx.doi.org/10.1016/j.ymben.2015.11.001
1096-7176/

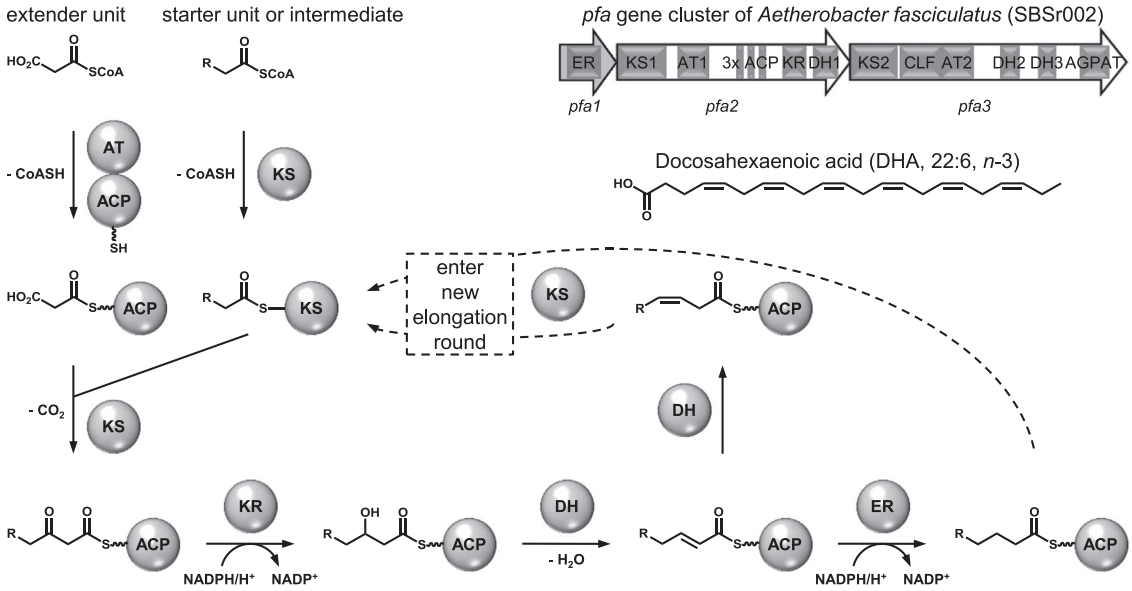

Fig. 1. Anaerobic biosynthesis of polyunsaturated fatty acids (PUFAs) by iterative type I fatty acid synthase (FAS)/polyketide synthase (PKS)-like PUFA synthases encoded by a *pfa* gene cluster. The primer molecule (acetyl-CoA) is extended by several rounds of decarboxylative Claisen condensation reactions, resulting in the elongation of the fatty acyl chain by two carbons (derived from malonyl-CoA) per cycle. Following each round of elongation, the β-keto group is either fully reduced or only reduced to the *trans* double bond which is then isomerized. Finally, an acyl chain with methylene-interrupted *cis* double bonds is synthesized. AT=acyltransferase, ACP=acyl carrier protein, KS=ketosynthase, KR=ketoreductase, DH=dehydratase/isomerase, ER=enoylreductase.

or transposon mutagenesis (Amiri-Jami et al., 2006) gave rise to an improved production of LC-PUFAs under laboratory conditions. In order to correlate the biosynthetic pathways with their products, to reduce the cultivation time, and/or to study LC-PUFA biosynthesis, several marine PUFA biosynthetic gene clusters were transferred and expressed into suitable host organisms. Thereby, recombinant PUFA production could be accomplished with the *pfa* gene clusters from *Shewanella* sp. in *Escherichia coli* (3.3 ± 0.7 mg EPA/g cell dry weight (CDW), *Synechococcus* sp. (0.6 ± 0.3 mg EPA/g CDW) (Takeyama et al., 1997), and in *Lactococcus lactis* ssp. *cremoris* (0.1 ± 0.04 mg EPA/g CDW and 1.4 ± 0.5 mg DHA/g CDW) (Amiri-Jami et al., 2014), from *M. marina* MP-1 in *E. coli* (5% DHA of total fatty acids) (Orikasa et al., 2006) or from *Schizochytrium* sp. in *E. coli* (10% *n*-6 DPA+DHA of total fatty acids) (Hauvermale et al., 2006). Besides intracellular accumulation of a high-performance catalase in *E. coli* expressing the *pfa* gene clusters from *Shewanella* sp., which resulted in up to 7.3 mg EPA/g CDW (Orikasa et al., 2007), recombinant EPA production could be enhanced by optimizing the cultivation conditions for the latter host harboring the *pfa* gene cluster from *Shewanella* to 18 mg/g CDW (Amiri-Jami and Griffiths, 2010).

Recently, *pfa* gene clusters for *de novo* LC-PUFA biosynthesis were also identified and characterized from several terrestrial myxobacteria. Intriguingly, the myxobacterial pathways differ significantly from the marine systems in terms of gene organization, catalytic domain arrangement, and sequence identity of the encoded PUFA synthases. Two types of *pfa* gene clusters were deciphered from genomes of linoleic acid (LA, 18:2, *n*-6) producing *Sorangium cellulosum* species and of novel myxobacterial isolates described as *Aetherobacter* spp., the latter of which turned out to be prolific producers of EPA and DHA (Garcia et al., 2011; Gemperlein et al., 2014). However, these native producer strains grow very slowly, are not easy to handle, and genetic modifications are difficult to implement. Cloning, transfer and heterologous expression of the *pfa* genes in the myxobacterial model strain *Myxococcus xanthus* allowed to reduce cultivation times compared to the slow growing native producer, but production rates still have to be improved to yield a commercially viable production system (Gemperlein et al., 2014).

In the present work, we aimed to evaluate alternative host strains for the expression of myxobacterial PUFA synthases. Besides *E. coli*, already used as expression strain for marine *pfa* gene clusters, we focussed on *Pseudomonas putida* KT2440, which has several advantageous features including a higher GC content and a codon usage similar to the myxobacteria. Due to these reasons, this strain has already been used for heterologous expression of several complex myxobacterial natural product pathways directing the production of polyketide/nonribosomal peptide hybrid metabolites (Chai et al., 2012; Fu et al., 2008; Gross et al., 2006; Wenzel et al., 2005). As *P. putida* is generally recognized as safe (GRAS) for industrial production, highly robust under extreme environmental conditions, genetically well accessible, and grows rapidly, it represents an attractive host for recombinant LC-PUFA production.

Here, we describe our work on establishing the heterologous expression of the native *pfa* gene cluster from *A. fasciculatus* (SBSr002) in both hosts, *E. coli* and *P. putida*. We also report on the successful application of metabolic engineering and synthetic biology approaches to further improve LC-PUFA production in *P. putida*.

2. Materials and methods

2.1. Strains, culture conditions and transformation procedures

E. coli DH10B (Grant et al., 1990) or SCS110 (Stratagene) were used for cloning experiments. *E. coli* HB101/pRK2013 (Figurski and Helinski, 1979) was used as helper strain for conjugation experiments. *E. coli* BL21(DE3) (Studier and Moffatt, 1986) was used for heterologous expression experiments. *E. coli* HS996/pSC101-BAD-gbaA (tet^R) (Wang et al., 2006) and GB05-red (Fu et al., 2012) were used for plasmid modification via Red/ET recombineering. The cells were grown in LB medium or on LB agar (1% tryptone, 0.5% yeast extract, 0.5% NaCl, (1.5% agar)) at 30–37 °C (and 200 rpm) overnight. Antibiotics were used at the following concentrations: 100 μg/ml ampicillin, 50 μg/ml kanamycin, 34 μg/ml chloramphenicol, 20 μg/ml gentamicin, and 6–12.5 μg/ml tetracycline.