Metabolic Engineering 35 (2016) 9–20

Contents lists available at ScienceDirect

Metabolic Engineering

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

Regular Article

Microbial production of bi-functional molecules by diversification of the fatty acid pathway

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ARTICLE INFO

Article history:
Received 27 November 2015
Accepted 7 January 2016
Available online 28 January 2016

Keywords:
3-Ketoacyl-ACP Synthase III
Fatty acid synthesis
Bio-based chemicals
Omega-functionalized fatty acids
Substrate diversity
Microbial engineering

ABSTRACT

Fatty acids that are chemically functionalized at their ω-ends are rare in nature yet offer unique chemical and physical properties with wide ranging industrial applications as feedstocks for bio-based polymers, lubricants and surfactants. Two enzymatic determinants control this ω-group functionality, the availability of an appropriate acyl-CoA substrate for initiating fatty acid biosynthesis, and a fatty acid synthase (FAS) variant that can accommodate that substrate in the initial condensation reaction of the process. In Type II FAS, 3-ketoacyl-ACP synthase III (KASIII) catalyses this initial condensation reaction. We characterized KASIIIs from diverse bacterial sources, and identified variants with novel substrate specificities towards atypical acyl-CoA substrates, including 3-hydroxybutyryl-CoA. Using *Alicyclobacillus acidocaldarius* KASIII, we demonstrate the *in vivo* diversion of FAS to produce novel ω-1 hydroxy-branched fatty acids from glucose in two bioengineered microbial hosts. This study unveils the biocatalytic potential of KASIII for synthesizing diverse ω-functionalized fatty acids.

1. Introduction

The increasing concerns with climate change and price volatility of petroleum feedstocks have prompted a growing search for sustainable sources of chemicals and fuels (Nikolau et al., 2008; Steen et al., 2010). Fatty acids are chemically the most similar biological molecules to petroleum hydrocarbons, and are therefore the most readily reachable bioengineering targets for developing sustainable replacements of petroleum-derived fuels and chemicals (Handke et al., 2011; Metzger and Bornscheuer, 2006). The processes of fatty acid metabolism can generate diverse metabolites with different chemical functionalities, and considerable research efforts have been dedicated to identify the genetic elements, and dissect the enzymology within these metabolic pathways (Steen et al., 2010; Janßen and Steinbüchel, 2014; Rock and Cronan, 1996). Much of this success has been facilitated by the modular nature of the enzymatic machinery that underlies the process of fatty acid synthesis, and the more general process of polyketide biosynthesis (Stewart et al., 2013). These processes are initiated by the condensation of an acyl-CoA substrate with a 2-carbon donor (malonyl-CoA or malonyl-ACP); and this condensation process is iterated in fatty acid/polyketide biosynthesis cycle to elongate the acyl-chain. Whereas fatty acid synthesis follows each condensation reaction by a 3-reaction process (reduction–dehydration–reduction) that generates

Abbreviations: KASIII, 3-Ketoacyl-ACP-Synthase III; HBFA, ω-1 Hydroxy Branched Fatty Acid; FAS, Fatty Acid Synthase; PHB, Polyhydroxybutyrate

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[1] Conceived and designed experiments; identified and characterized KASIII enzymes using enzyme activity assays, and thermal shift assays; acquired, interpreted and analysed data, and led the effort to write the manuscript.
[2] Assembled the *E. coli* constructs for expressing novel KASIIIs; conducted and interpreted the fatty acid analyses; contributed to writing the manuscript.
[3] Constructed the *B. subtilis* and *R. rubrum* strains and analysed fatty acid profiles; contributed to writing the manuscript.
[4] Optimized fermentation conditions of *E. coli* strains, and contributed to writing the manuscript.
[5] Provided the construct encoding the acyl-ACP thioesterase enzyme used in this study.
[6] Oversaw experimental design, data interpretation, and contributed to writing the manuscript.
[7] Conceived the scientific idea, participated in experimental design, data interpretation, and revision of the manuscript for important intellectual content.

http://dx.doi.org/10.1016/j.ymben.2016.01.003
1096-7176/

a fully reduced alkyl chain, the more general polyketide synthesis mechanism skips one or two of these latter reactions prior to the next condensation iteration, leaving a functional group in the alkyl chain (Austin and Noel, 2003). Examples of such alternative products being generated by fatty acid synthase (FAS) are the mechanisms by which prokaryotes produce unsaturated fatty acids (Metz et al., 2001), and the biosynthesis of methylketones by tomato trichomes (Ben-Israel et al., 2009). Both these processes intercept the 3-reaction reduction–dehydration–reduction cycle and maintain a chemical functional group in the alkyl-chain. The first of these is *via* an isomerization reaction that moves the carbon–carbon double bond, 1-bond further from the carboxylic acid end of the acyl-chain, ultimately generating an unsaturated fatty acid. In the second example, an acyl-ACP thioesterase that is specific to the 3-ketoacyl-ACP intermediate of FAS intercepts the cycle and releases a 3-ketoacid, which undergoes decarboxylation to generate a methylketone.

We evaluated the feasibility of introducing chemical functional groups near the ω-end of fatty acids by exploring the substrate flexibility offered by natural variants of 3-ketoacyl-ACP synthase III (KASIII) (Lai and Cronan, 2003), the enzyme that catalyses the initial condensation reaction of Type II FAS systems. Most well characterized KASIII enzymes, including the enzyme from *Escherichia coli* and most plants, use acetyl-CoA as the substrate in this reaction, and thus the ω-end of the final product is an unreactive methyl-group (Choi et al., 2000). However, KASIII from some bacteria, such as *Bacillus subtilis* and *Staphylococcus aureus* can utilize substituted acyl-CoAs (*i.e.*, methyl branches at the ω-1 (isobutyryl-CoA) and ω-2 (anteisovaleryl-CoA) positions of the acyl-chain, see Supplementary Table 1 for chemical structures of acyl-CoA molecules) (Choi et al., 2000; Gajiwala et al., 2009). These organisms therefore produce fatty acids with methyl branches at or near the ω-ends of the molecules. Therefore, we projected that by providing chemically substituted acyl-CoA substrates to novel KASIII enzymes, one can envision biosynthesizing ω-functionalized fatty acids. Moreover, KASIII are archetypal for many Type III polyketide synthases which catalyse similar Claisen condensation reactions between an acyl-CoA and a malonyl-CoA/ACP thioester substrate (Heath and Rock, 2002) and also show great flexibility in being able to use a variety of acyl-CoA substrates, generating a large collection of polyketide specialized metabolites.

In this study, we specifically tested this hypothesis and demonstrated the ability to biologically produce ω-1 Hydroxy Branched Fatty Acids (HBFAs) using the novel KASIII enzyme from *Alicyclobacillus acidocaldarius* coupled with a system to generate 3-hydroxyacyl-CoA as a substrate for this novel KASIII enzyme. The utility of such bifunctional hydroxy acids is in such applications as polymers (*e.g.*, polyesters) and specialized natural surfactants (*e.g.*, sophorolipids) (Ashby et al., 2008). The system that we have developed has more general applications in that it illustrates the flexibility of Type II FAS systems. These systems can be bioengineered to accept novel initiating acyl-CoA substrates by exploiting the variant enzymes that catalyse the initial condensation reaction and define the ω-end of the final product (*i.e.*, KASIII). A cursory examination of sequence databases (Cantu et al., 2011) currently identifies 14,000 potential KASIII enzymes that could be used to generate a variety of different bifunctional molecules (Chen et al., 2011).

2. Results

2.1. Computational identification of KASIIIs from diverse bacterial sources

Putative KASIII-coding genes were computationally identified by sequence homology, by searching genomic sequences from diverse bacteria that are known to produce large amounts of either terminally branched chain fatty acids or ω-cyclic fatty acids. We projected that organisms that produce large quantities of branched chain or ω-cyclic fatty acids should express novel KASIII enzymes that are capable of utilizing branched-chain acyl-CoAs or ω-cyclic acyl-CoAs as substrates to initiate fatty acid biosynthesis. We further hypothesized that such KASIII enzymes will have relatively larger substrate binding pockets that can not only accommodate branched or ω-cyclic acyl-CoA substrates, but could also accommodate other bulky substrates, such as aromatic, hydroxylated or unsaturated acyl-CoAs.

Based on these presuppositions, we characterized two KASIII enzymes, one from the thermophile *Thermus aquaticus*, an organism that can produce 95% branched chain fatty acids (Jackson et al., 1973) and another from the acidothermophile *A. acidocaldarius*, which can produce a large proportion of ω-alicyclic fatty acids (59%) and branched chain fatty acids (36%) (Moore et al., 1993). We compared the properties of the *A. acidocaldarius* (aaKASIII) and *T. aquaticus* (taKASIII) enzymes to two functionally well-characterized KASIIIs, one from *E. coli* (ecKASIII, encoded by the *fabH* gene), and the second from *B. subtilis* (bsKASIIIb, encoded by the *yhfB* gene). These latter two enzymes were selected because they are known to display different substrate specificity; ecKASIII is specific for acetyl-CoA and is unable to use branched chain acyl-CoA substrates (Choi et al., 2000; Heath and Rock, 1996), whereas bsKASIIIb can utilize both acetyl-CoA and branched-chain acyl-CoA substrates (Choi et al., 2000).

Primary sequence analyses of aaKASIII and taKASIII revealed that each possesses the conserved catalytic triad residues (Cys, His and Asn; Supplementary Fig. 1) typical of KASIII enzymes, suggesting that both belong to the family of decarboxylating thiolase enzymes (Heath and Rock, 2002). Moreover, both aaKASIII and taKASIII contain the substrate binding residues that are conserved among well-characterized KASIII enzymes (Gajiwala et al., 2009) (see Supplementary Fig. 1).

2.2. In vivo analysis of KASIII function in a B. subtilis ΔyjaX ΔyhfB strain

The bacterium *B. subtilis* primarily synthesizes branched chain fatty acids (95%) (Kaneda, 1991), and possesses two KASIII homologs, bsKASIIIa (encoded by the *yjaX* gene) and bsKASIIIb (encoded by the *yhfB* gene); both possess high specificity for branched chain acyl-CoA substrates (Choi et al., 2000). Deletion of these two endogenous KASIII genes from *B. subtilis* results in a lethal phenotype, which can be rescued by growing the double mutant strain in the presence of branched-chain fatty acids (Jin and Nikolau, unpublished data). This *B. subtilis* Δ*yjaX* Δ*yhfB* mutant strain was used as a vehicle to screen for KASIII enzymes that can utilize branched chain acyl-CoA substrates and can therefore synthesize branched chain fatty acids, thereby rescuing the lethal condition. The selected aaKASIII and taKASIII were assessed for their ability to support the production of branched chain fatty acids, by integrating each of them into the genome of the *B. subtilis* Δ*yjaX* Δ*yhfB* mutant strain. In parallel, the ecKASIII that is unable to utilize branched-chain substrates (Choi et al., 2000) was also integrated into the genome of this strain. The resulting three *B. subtilis* Δ*yjaX* Δ*yhfB* mutant strains, each expressing one of the recombinant KASIII genes, were grown in the presence and absence of exogenously supplied branched-chain fatty acids. Of the three recombinant KASIII genes that were tested, *aaKASIII* and *taKASIII* could rescue the lethal phenotype of the Δ*yjaX* Δ*yhfB* mutant strain but as expected the *ecKASIII* could not, and this strain could not grow in media that does not contain branched-chain fatty acids.

Fatty acid analysis of the *B. subtilis* Δ*yjaX* Δ*yhfB* strains harboring either aaKASIII or taKASIII revealed that both strains could produce similar branched chain fatty acid profiles, with anteiso-branched chain fatty acids accounting for the largest portion of the fatty acids (48–52%), followed by iso-branched chain fatty acids (27–34%) (Fig. 1). These data establish that aaKASIII and taKASIII have the ability to use