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Branched-chain fatty acid biosynthesis in *Escherichia coli*

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β-Ketoacyl-acyl carrier protein (ACP) synthase III (KASIII) catalyzes the first elongation step in straight-chain fatty acid (SCFA) biosynthesis in *Escherichia coli*. Overproduction of the corresponding KASIII gene, or the *Brassica napus* KASIII gene has previously been observed to lead to an increase in the amount of shorter-chain fatty acids produced by *E. coli*. In this study it is shown that overexpression of the KASIII gene, which initiates branched-chain fatty acid (BCFA) in *Streptomyces glaucescens*, does not lead to a change in the fatty acid profiles of *E. coli*. *E. coli* produces trace levels of BCFAs when grown in the presence of isobutyric acid, but the amounts of these are not significantly altered by expression of the *S. glaucescens* KASIII gene. In contrast, the amounts of BCFAs produced from isobutyryl CoA *in vitro* by *E. coli* cell-free extracts can be increased at least four-fold by the presence of the *S. glaucescens* KASIII. These observations suggest that *in vivo* production of isopalmitate by *E. coli* expressing the *S. glaucescens* KASIII is limited by availability of the appropriate BCFA biosynthetic primers. *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 246–251.

Keywords: fatty acid biosynthesis; ketoacyl ACP synthase III; FabH; branched-chain fatty acids

Introduction

The first step of fatty acid biosynthesis by type II dissociable fatty acid synthases (FASs) is a decarboxylative condensation of malonyl acyl carrier protein (ACP) with an acyl CoA substrate, catalyzed by β-ketoacyl-ACP synthase III (KASIII, FabH) [2,13]. Subsequent elongation steps are catalyzed by different β-ketoacyl-ACP synthases and utilize an acyl ACP rather than acyl CoA substrate. Therefore, KASIII plays a pivotal role in initiation of fatty acid biosynthesis in plants and bacteria. This enzyme has attracted interest as a target for developing new antibiotics [3].

KASIII is also thought to play a role in determining the type of fatty acid made by a type II FAS and has attracted interest as a site for manipulation to create transgenic plants with altered seed oil compositions [14]. *Escherichia coli*, like most bacteria and plants, produces only straight-chain fatty acids (SCFAs) using acetyl CoA and propionyl CoA as the appropriate starter units. The *E. coli* KASIII has been reported to have a substrate preference for acetyl CoA and propionyl CoA [5]. Streptomycetes, in contrast, produce predominantly branched-chain fatty acids (BCFAs) using isobutyryl CoA and methylbutyryl CoA as starter units [9]. The KASIII of *Streptomyces glaucescens in vitro* utilizes a range of acyl CoA substrates, but has a clear binding preference for isobutyryl CoA over acetyl CoA [4].

The KASIII of a type II FAS thus appears to be responsible for both initiating fatty acid biosynthesis and determining the type of fatty acids made, suggesting that manipulation in an organism of the levels or substrate specificity of KASIII could result in changes in the fatty acid profiles. Indeed, overexpression of the bacterial *E. coli* KASIII gene has been shown to increase the amounts of the shorter myristic acid (C14) and decrease amounts of *cis*-vaccenate (C18:1) [13]. A similar change in fatty acid profiles has also been observed with heterologous expression of the plant *Brassica napus* KASIII gene in *E. coli* [14]. In these experiments both the *E. coli* and *B. napus* KASIII gene (*fabH*) originated from systems that produce exclusively SCFAs. The effect on fatty acid profiles of heterologous expression and overexpression of a KASIII gene from a system that produces predominantly BCFAs had not been studied until recently [1].

We report herein that heterologous expression of the *S. glaucescens* KASIII gene in *E. coli* leads neither to shortening of fatty acids, nor increased production of BCFAs. *In vitro* studies of the *E. coli* FAS, however, provide the first unequivocal demonstration that such a system can produce BCFAs, and that the *S. glaucescens* KASIII significantly increases the efficiency of the process.

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Materials and methods

Materials

The following chemicals were purchased from Sigma (St. Louis, MO): *E. coli* FAS ACP, malonyl-CoA, β-NADPH. [1-^{14}C]Butyryl-CoA (specific activity, 4 mCi/mmol) was from Moravek Biochemicals (Brea, CA). Radiolabeled isobutyrate and butyrate were obtained from ICN Radiochemicals (Costa Mesa, CA). Restriction endonucleases and other enzymes were purchased from New England Biolabs (Beverly, MA), Perkin-Elmer (Branchburg, NJ) and Boehringer Mannheim (Bedford, MA). Deuterated acetyl-CoA and isobutyryl-CoA were synthesized as described previously [4,15].

Bacterial strains, plasmids and cultivation of bacteria

E. coli BL21(DE3) pLysS transformed with pLH14 [4] was used for expression of wild-type *S. glaucescens fabH*. *E. coli* cells carrying plasmid were grown in LB medium at 37°C, supplemented with ampicillin (100 μg/ml). Induction was carried out with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when the optical

density (540 nm) of the culture reached 0.5. The cells were then grown for an additional 3 h at 30°C, harvested and the recombinant His tag-FabH was purified as previously described [2].

General methods

All DNA manipulations and transformation of *E. coli* were performed according to standard protocols [11]. Analysis of KASIII expression was performed by resuspending cells in 200 μl of Laemmli loading buffer. The mixture was boiled for 5 min and 10 μl of the mixture was loaded onto a 15% sodium dodecyl sulfate polyacrylamide gel. Following electrophoresis the gel was stained with Coomassie blue. For protein purification, cells were lysed and purified by metal chelate chromatography according to the methodology supplied by Novagen (Madison, WI). Fatty acid profiles of *E. coli* were determined following standard protocols [16]. The KASIII assay was performed as described previously using [1-^{14}C]butyryl-CoA and malonyl ACP [4].

Fatty acid biosynthesis in cell-free extracts

Fatty acid biosynthesis *in vitro* was performed with cell-free extracts of *E. coli* BL21(DE3)pLysS/pLH14 and *E. coli* BL21(DE3)pLysS/pET15b, following standard methodologies [15]. Briefly, a cell pellet from an *E. coli* culture (20 ml) grown on IPTG and induced for 3 h was resuspended in 1 ml of 50 mM sodium phosphate buffer (pH 7.3) and sonicated for 30 s. After centrifugation at 12,000×*g* the resulting cell-free extract was used for FAS assays. The FAS assays contained 200 μl of cell-free extract, 0.13 μmol of *E. coli* ACP, 0.92 μmol of NADPH, 1 μmol malonyl-CoA, and either 0.5 μmol of isobutyryl-CoA or 0.5 μmol of deuterated acetyl-CoA in a final volume of 500 μl. In the case of cell-free extracts of *E. coli* BL21 (DE3)pLysS/pET15b, FAS assays were also carried cut in the presence of either purified *S. glaucescens* KASIII (1–5 μg per assay) or 10 μl of a cell-free extract from *E. coli* BL21(DE3)pLysS/pLH14, which contained unpurified *S. glaucescens* KASIII. After 2 h of incubation at 37°C the FAS assays were terminated by treatment with 400 μl of methanolic sodium hydroxide (1.2 N NaOH in 50% methanol) at room temperature for 15 min. Subsequently 0.2 ml of 6 N HCl and 0.4 ml of BCl_3 were added to the suspension. The resulting acidified suspension was heated at 85°C for 10 min. Upon cooling to room temperature fatty acid methyl esters were analyzed by GC-MS as described previously [15]. The relative efficiency of BCFA and SCFA biosynthesis within a set of FAS assays was obtained using the palmitoleate present in cell-free extract as a standard. This fatty acid was selected as a standard because the levels present in a cell-free extract did not change even when FAS assays were conducted with deuterated acetyl CoA. The relative efficiency of BCFA biosynthesis from isobutyryl CoA under different conditions was obtained by integration of the methyl isopalmitate and methyl palmitoleate peaks. The relative efficiency of SCFA from deuterated acetyl CoA was obtained by comparison of the amounts of labeled methyl palmitate and methyl palmitoleate in the GC-MS analyses (deuterated acetyl CoA was necessary because of the presence of palmitate in the cell-free extract).

Uptake studies

In feeding experiments either perdeuterated butyric acid or isobutyric acid were added to *E. coli* growth medium to a final concentration of 10 mM. *E. coli* cells with pLH14 were grown in LB to an optical density of 0.5 at 540 nm. IPTG, and either perdeuterated butyric acid, or isobutyric acid (from pH-neutralized stock solutions) were then added to the growth medium. Cells were allowed to grow for 4 h then harvested for fatty acid analysis. In two additional sets of experiments, *E. coli* cells were grown in LB to an optical density of 0.4 at 30°C, and IPTG was added to final concentration 1 mM. After 1 h of growth, perdeuterated butyric acid or isobutyric acid was added to the culture causing a decrease in the pH of the medium to 5.0. The cells were then either incubated overnight under these conditions, or adjusted to pH 7.0 after 1 h of incubation and incubated for an additional 3 h. For uptake of radiolabeled butyrate and isobutyrate into *E. coli* and *Streptomyces*, cells were grown in the presence of radiolabeled materials for 6 and 24 h, respectively. The cells were then harvested and the pellet washed twice with 50 mM phosphate buffer at pH 7.0. The fermentation medium pellet washings and lysed cell pellet were all separately combined with 5 ml of a scintillation cocktail and analyzed using a scintillation counter.

Results

Effect of expression of the S. glaucescens KASIII gene on fatty acid profiles of E. coli

Heterologous expression of the *B. napus* KASIII gene, and overexpression of the *E. coli* KASIII gene, results in the production of larger amounts of the shorter chain fatty acid myristate (C14:0) and decreased amounts of the longer chain unsaturated fatty acid *cis*-vaccenate [13,14]. Surprisingly, however, the levels of these and the other fatty acids in an *E. coli* profile remain essentially unchanged when the *S. glaucescens* KASIII gene was expressed (Table 1). Analysis by SDS-PAGE of the *E. coli* cell-free extract clearly demonstrated that the expressed KASIII was present in soluble form at high levels (Figure 1A) and had no significant effect on the growth of *E. coli* (Figure 1B). Affinity column chromatography was used to purify the N-terminal His tag-KASIII and assays using butyryl CoA and malonyl ACP confirmed that the protein was active [4]. Low level expression of the *S. glaucescens* KASIII was observed without addition of IPTG (Figure 1) and also resulted in no significant changes in the fatty acid profiles.

The ability of *E. coli* to generate BCFAs and the effect of expression of the *S. glaucescens* KASIII gene on this process was also examined. Under standard growth conditions *E. coli* BL21(DE3)pLysS was shown by GC-MS to produce no detectable BCFAs. However, when isobutyric acid was added to the fermentation medium low levels of BCFA isopalmitate (0.25–

Table 1 Fatty acid composition of *E. coli fabH* expression clones

Clone	% Fatty acid				
BL21(DE3)pLysS carrying:	C14:0	C16:1	C16:0	C18:1	C18:0
pET14	9.4	21.8	46.4	21.0	1.4
pLH14	10.5	20.5	46.0	20.3	2.6

Amounts of each fatty acid are expressed as a percentage of the major fatty acids observed in the GC-MS analyses. Cells were grown in LB medium to stationary phase and harvested by centrifugation. Results are from an analysis of the three strains grown simultaneously under the same conditions. Values for C16:1 and C18:1 include contributions from their cyclopropane derivatives. Fatty acids present at less that 1% of the total fatty acids are not reported.

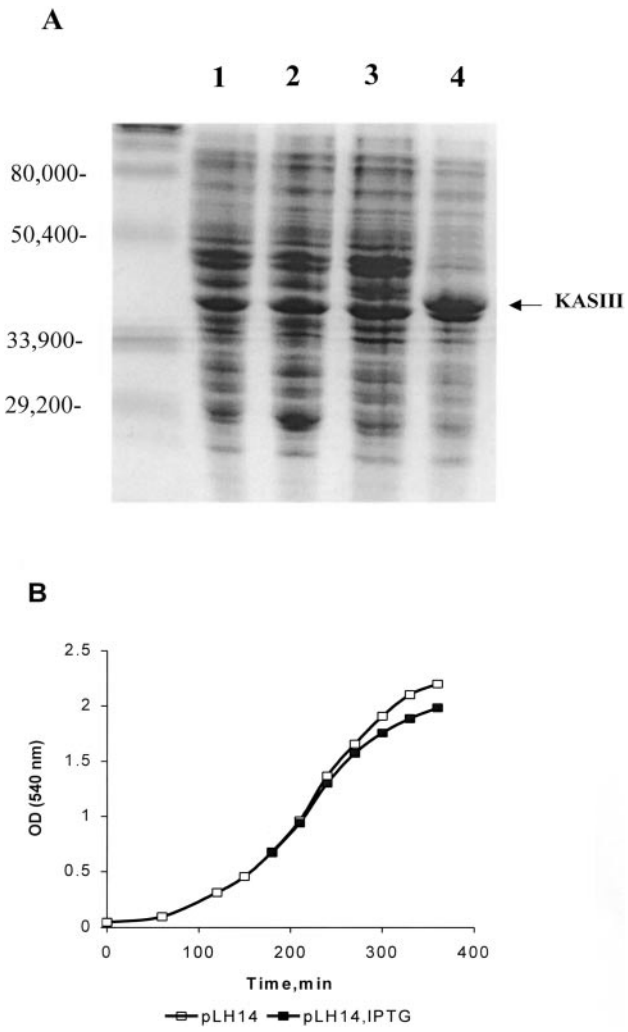

Figure 1 Expression of *S. glaucescens* KASIII in *E. coli*. (A) SDS-PAGE analysis of *E. coli* cell-free extracts. Extracts were prepared from *E. coli* BL21(DE3)pLysS carrying pET15 (1 and 2) of pLH14 (3 and 4). Cells were harvested 3 h after IPTG induction at 30°C (2 and 4). Control experiments without IPTG induction were also carried out (1 and 3). (B) Growth of *E. coli* carrying pLH14. The optical density (OD) at 540 nm was measured at 30-min intervals and IPTG was added to 1 mM at an OD of 0.5.

0.35% of the major SCFA palmitate) were consistently observed. Production of BCFAs by an *E. coli* FAS has not previously been reported and this observation clearly demonstrates that the enzymes of this system can process both BCFA and SCFA biosynthetic pathway intermediates. When the experiment was repeated using *E. coli* BL21(DE3)pLysS/pLH14 (expressing the *S. glaucescens* KASIII gene), similar low levels of BCFA were observed with isobutyrate addition (no detectable levels of BCFAs were produced in the absence of exogenous isobutyrate).

A series of incorporation studies with perdeuterated butyrate was also conducted with *E. coli* BL21(DE3)pLysS and *E. coli* BL21(DE3)pLysS/pLH14. The *S. glaucescens* KASIII has been shown *in vitro* to be able to utilize butyryl CoA as a building block and *in vivo* studies with *S. glaucescens* have shown that perdeuterated butyrate in the fermentation medium can be utilized as a starter unit for SCFA biosynthesis, presumably using KASIII [4,16]. No intact incorporation of the perdeuterated butyrate into the fatty acids produced by either *E. coli* strain was observed. Furthermore, in these experiments with perdeuterated butyrate, incorporation of deuterated acetate or malonate was not detected. Analogous experiments conducted in *S. glaucescens* GC-MS analyses have revealed such labeling, presumably as a result of β-oxidation of perdeuterated butyrate once it is incorporated into the cell and activated to the corresponding coenzyme A thioester [15]. When *E. coli* was grown in the presence of perdeuterated acetate, significant incorporation of deuterium label into the fatty acids was observed (data not shown). These observations suggest that *E. coli* is less efficient than *S. glaucescens* at uptake of the exogenously supplied butyrate. Incorporation experiments with radiolabeled butyrate and isobutyrate were carried out to confirm this hypothesis. Addition of ^{14}C-labeled butyrate and isobutyrate to *E. coli* resulted in no significant incorporation into the cells. In *S. glaucescens*, 13% and 34% of the labeled butyrate and isobutyrate, respectively, were taken up by cells.

In an attempt to improve uptake of both perdeuterated butyrate and unlabeled isobutyrate into *E. coli*, fermentations were carried out at pH 5. Previous analyses of *E. coli* have shown that short-chain fatty acids can penetrate better at a lower pH as the undissociated acid than at neutral pH in ionic form [10]. Thus, cells were treated with isobutyric acid or perdeuterated butyric acid at pH 5, 1 h after induction with IPTG. In one set of experiments cells were then incubated overnight without further pH adjustment. Under these conditions cells did not continue to grow. Nonetheless, BCFAs were produced specifically from *E. coli* treated with isobutyric acid, demonstrating that fatty acid biosynthesis was active at this acidic pH and that some uptake of isobutyric acid had taken place. There were no detectable differences, however, in the amounts of BCFAs produced by *E. coli* BL21(DE3)pLysS and *E. coli* BL21(DE3)pLysS/pLH14. In a second set of experiments the fermentation medium was readjusted to pH 7.0, 1 h after treatment with the short-chain fatty acid. Under these conditions the cells continued to grow but no BCFAs were detected. In both sets of experiments no labeling of the palmitate pool from labeled butyrate was observed.

Effect of S. glaucescens KASIII on BCFA biosynthesis with an E. coli cell-free extract

Radioactivity-based assays of cell-free extracts of *E. coli* have previously shown that acetyl CoA is utilized more efficiently than propionyl CoA or butyryl CoA as a starter unit for SCFA biosynthesis [7]. These observations are consistent with the observations that butyryl CoA is a poorer substrate than acetyl CoA or propionyl CoA for the *E. coli* KASIII [5]. It has also been shown that BCFAs are produced from L-α-keto-β-methylvalerate (presumably converted to a methylbutyryl CoA starter unit) in cell-free extracts of bacterial systems that produce BCFAs, but not by *E. coli* cell-free extracts [7].

From these observations it has been argued that organisms that produce SCFAs cannot produce the BCFA equivalents as they have neither enzymes active toward primers with more than three carbons, nor a suitable system for producing these primers [7].

In this study we used a GC-MS-based assay and an *E. coli* cell-free extract to determine the effect of the *S. glaucescens* KASIII on efficiency of BCFA biosynthesis from isobutyryl CoA. This GC-MS-based assay is useful for comparing relative efficiencies of BCFA and SCFA biosynthesis *in vitro* [15]. In the current study an *E. coli* cell-free extract produced the BCFA isopalmitate when

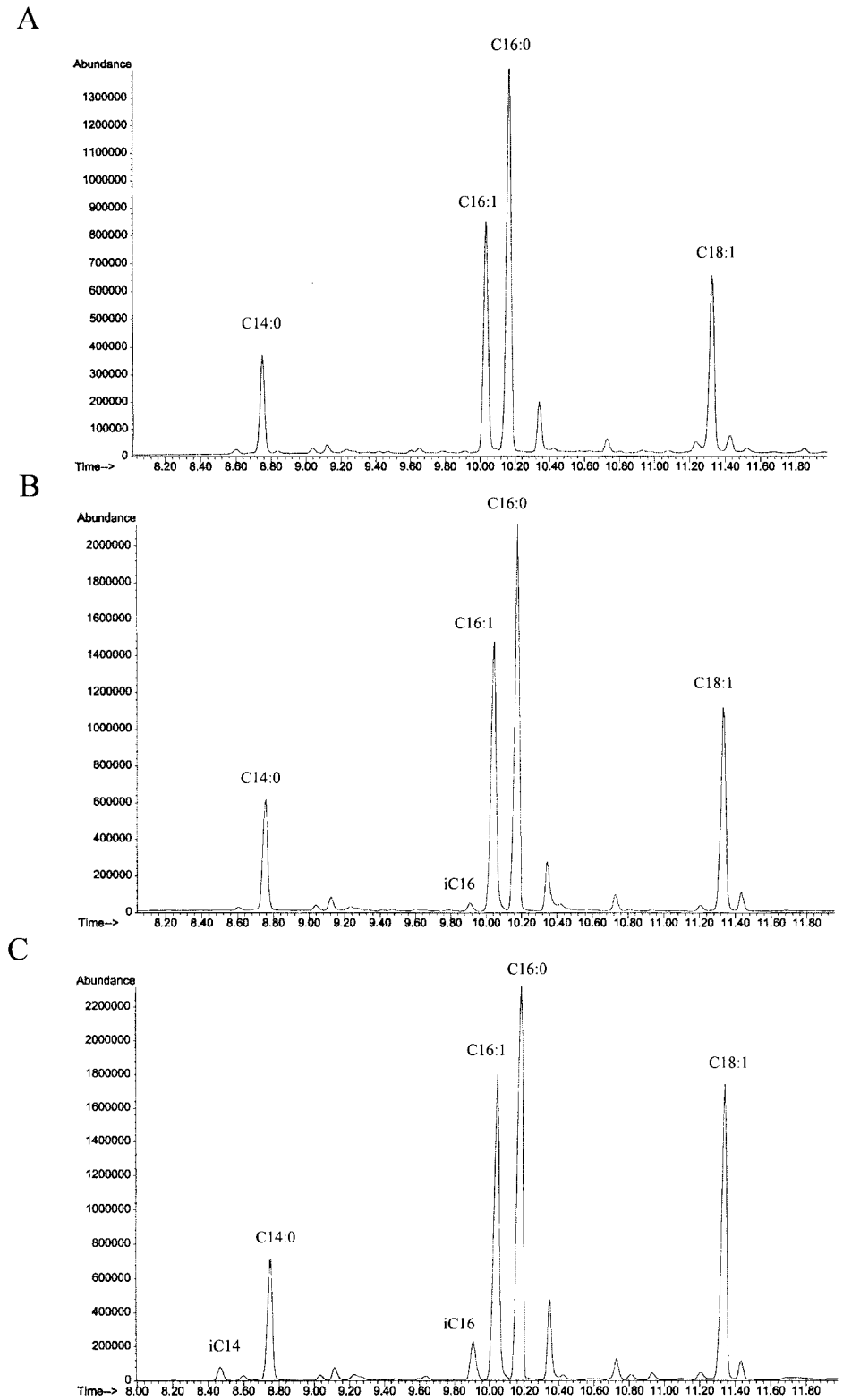

Figure 2 Fatty acid profiles of FAS assays with *E. coli* cell-free extracts. FAS assays were performed as described in Materials and Methods, using strain BL21(DE3)pLysS/pET15b in the following combinations: control (without isobutyryl CoA or purified *S. glaucescens* FabH) (A), with isobutyryl CoA (0.5 μmol) (B), with isobutyryl CoA (0.5 μmol) and purified *S. glaucescens* FabH (5 μg) (C). Fatty acid abbreviations: iC14, isomyristate; C14:0, myristate; iC16, isopalmitate; C16:1, palmitoleate; C16: palmitate; C18:1, *cis*-vaccenate.

presented with the appropriate primer, isobutyryl CoA (Figure 2B). No isopalmitate was observed when isobutyryl CoA was omitted from the assay (Figure 2A). The efficiency of isopalmitate biosynthesis was compared with the efficiency of labeled palmitate

Table 2 Isopalmitate and palmitate biosynthesis in cell-free extracts of *E. coli*

Experiment	Isopalmitate produced (%)	Palmitate (%)
I. *E. coli* BL21 (DE3)pLysS	7	73
II. *E. coli* BL21 (DE3)pLysS	5	86
III. *E. coli* BL21 (DE3)pLysS/pLH14	22	30
IV. *E. coli* BL21 (DE3)pLysS (100 μl)	3	N/D
+0.1 μg KASIII	10	N/D
+1 μg KASIII	13	N/D
+5 μg KASIII	14	N/D

The amount of each fatty acid is expressed as a percentage of palmitoleate (C16:1) present in the cell-free extract. I–III represent data collected from separate cell-free preparations. Data within experiments IV were obtained from FAS assays conducted simultaneously with the same cell-free extract. N/D; not determined.

biosynthesis from a perdeuterated acetyl CoA starter unit (the amounts of isopalmitate and palmitate obtained in the FAS assay were compared to the unsaturated fatty acid, palmitoleate) (Table 2). These results show that the production of SCFA *in vitro* was typically 10- to 15-fold higher than BCFA using the starter units acetyl CoA and isobutyryl CoA, respectively. By comparison, analyses of the fatty acid profiles of *E. coli* grown in the presence of isobutyrate suggest that SCFA biosynthesis *in vivo* is at least 200-fold higher than BCFA. The difference between the *in vivo* and *in vitro* observations is likely a reflection of poor transportation of isobutyrate into *E. coli*.

A cell-free extract of *E. coli* BL21(DE3)pLysS/pLH14 generated under similar conditions demonstrated less than a two-fold difference between BCFA and SCFA biosynthesis, suggesting that the presence of *S. glaucescens* KASIII may have increased the efficiency of the BCFA biosynthetic process (Table 2). Support for this hypothesis was obtained by showing that the amount of BCFA biosynthesis in an *E. coli* cell-free extract increased two-fold when a much smaller quantity of an *E. coli* BL21(DE3)pLysS/pLH14 cell-free extract was added. Finally, addition of a purified *S. glaucescens* KASIII to an *E. coli* BL21(DE3)pLysS extract led to over a four-fold increase in the amount of BCFA biosynthesis (Figure 2C, Table 2).

Discussion

The reasons why shorter fatty acids in *E. coli* have previously been seen with increased levels of KASIII are unclear. One suggestion is that the presence of these fatty acids reflects inhibition of cell-growth caused by the overexpression [14]. Accumulation of C14:0 in *E. coli* is an established metabolic effect due to arrest in cell division [14]. These changes in fatty acid profiles have also been suggested as being consistent with a role of KASIII in governing a balance between the supply of malonyl ACP and the rate of fatty acid initiation [13]. The increase in condensation of malonyl ACP with acetyl CoA resulting from increased levels of KASIII may decrease the amount of this substrate available for subsequent elongation steps, leading to production of shorter-chain fatty acids. In this current study, however, high-level expression of the *S. glaucescens* KASIII gene had no significant effect on growth of *E. coli* and did not result in changes in the fatty acid profiles. A similar lack of accumulation of shorter chain fatty acids has recently been observed with overexpression of two *Bacillus subtilis* KASIII isozymes in *E. coli* (these enzymes like the *S. glaucescens* KASIII are thought to initiate BCFA biosynthesis) [1]. These results differ significantly from those obtained using either the *E. coli* or *B. napus* KASIII (both obtained from organisms that produce SCFAs) [13,14].

Evidence for BCFA biosynthesis has not previously been reported for an *E. coli* system. This study unequivocally demonstrated that such a process can occur *in vitro* when the appropriate precursors are provided. It was recently reported that a reconstituted system of purified *E. coli* fatty acid biosynthetic enzymes cannot generate BCFAs from methylbutyryl CoA [1]. These differences may reflect the sensitivity of the two different assay methods, and/or the presence of additional enzymes in cell extracts. The efficiency of BCFA biosynthesis in an *E. coli* cell extract increased in the presence of *S. glaucescens* KASIII, suggesting that the rate-limiting factor under these conditions is initiation from the appropriate precursor. A similar conclusion was drawn from work with the reconstituted *E. coli* fatty acid biosynthetic system where addition of a *B. subtilis* KASIII is required for detectable formation of BCFAs from methylbutyryl CoA [1].

In these current studies the ability of *E. coli* to generate BCFAs *in vivo* was limited by substrate availability. Starter units (isobutyryl CoA and methylbutyryl CoA) for BCFA biosynthesis are obtained in streptomycetes and bacilli from efficient degradation of branched-chain amino acids [6,16]. *E. coli* is unable to metabolize such amino acids as it does not appear to have the appropriate functional branched-chain 2-oxo acid dehydrogenase complex [8,12]. Thus, neither *E coli* nor *E. coli* BL21(DE3)-pLysS/pLH14 (expressing the *S. glaucescens* KASIII) produced BCFA. Addition of isobutyric acid to these *E. coli* strains resulted in the same low levels of production of the corresponding BCFAs. The levels of these fatty BCFAs did not increase despite the presence of high levels of the *S. glaucescens* KASIII, indicating that substrate availability (poor uptake/activation of isobutyric acid) rather than KASIII substrate specificity was a limiting factor. These results contrast with a recent claim that expression of a *B. subtilis* KASIII isozyme in *E. coli* results in production of C-17 BCFAs [1]. The reproducibility of this surprising result was not provided, and determination of the presence of these fatty acids was accomplished solely on the basis of retention time in a GC analysis. The source of the methylbutyryl CoA substrate used for the apparent biosynthesis of the C17 BCFA (*E. coli* cannot obtain this substrate from degradation of branched-chain amino acids and methylbutyric acid was not added to these fermentations), and the reason why isobutyryl CoA-derived C16 BCFAs were not made, was not addressed.

In conclusion, we have observed the first unequivocal demonstration of *in vivo* and *in vitro* production of BCFAs by *E. coli*. This production is critically dependent on availability of the appropriate BCFA acyl CoA precursors. Under *in vitro* conditions systems where these acyl CoA precursors can be provided at saturating conditions. The efficiency of BCFA biosynthesis appears to be limited either by the levels or specificity of the initiation enzyme (KASIII). Finally, the accumulation of shorter chain fatty acids through high-level expression of KASIII is not a generalized phenomenon, and appears to be dependent on the source of the KASIII.

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