Biotechnology Journal

DOI 10.1002/biot.200600194

Biotechnol. J. 2006, 2, 266–274

Research Article

Expression of an acetyl-CoA synthase and a CoA-transferase in *Escherichia coli* to produce modified taxanes *in vivo*

Catherine Loncaric, Amanda F. Ward and Kevin D. Walker

Departments of Chemistry, Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI, USA

Received 26 September 2006
Accepted 15 November 2006

Previous *in vitro* studies revealed that the 10-deacetylbaccatin III 10β-*O*-acetyltransferase (DBAT) from *Taxus* can catalyze the transfer of acetyl, propionyl or *n*-butyryl from CoA to the C10-hydroxyl of 10-deacetylbaccatin III. Accordingly, *Escherichia coli* JM109 were transformed to recombinantly express *dbat*, and this enzyme function was coupled to that of acetyl-CoA synthase (*acs*, EC 6.2.1.1) expressed from and regulated by genes encoded on the bacterial chromosome. Incubation of the bacteria with 10-deacetylbaccatin III and increasing concentrations of acetic acid revealed an *in vivo* conversion (~10%) of substrate to natural product baccatin III (C10-acetylated), which was remarkably similar to the relative conversion without acid supplementation. Incubation of the modified *E. coli* with 5 mM propionic acid, revealed a fivefold increase in the conversion (~13%) of 10-deacetylbaccatin III to 10-deacetyl-10-propionylbaccatin III, compared to ~2% conversion in the absence of exogenous propionate. To produce the butyrylbaccatin III analog *in vivo*, bacteria were engineered to co-express the *dbat* and *atoAD* (EC 2.8.3.8) genes; the latter encodes an acetoacetate:acetyl-CoA CoA-transferase that activates butyrate to butyryl CoA. The bacteria were incubated with 10-deacetylbaccatin III and 25–100 mM butyrate, and a maximum of ~2.6% conversion to 10-butyrylbaccatin III was observed compared to ~0.6% conversion when no exogenous butyrate was supplied.

Keywords: Baccatin III · Paclitaxel · Second-generation taxanes · Acetoacetate:acetyl-CoA CoA-transferase of the *ato* operon · Acetyl-CoA synthase

1 Introduction

The production of acetyl-CoA in *E. coli* is synchronized with the production of primary metabolites and transcriptional regulation of the acetyl-CoA synthase (*acs*) gene (E.C. 6.2.1.1) (Fig. 1). ACS converts acetate to acetyl-CoA, which branches to the tricarboxylic acid cycle and the glyoxylate shunt pathways [1]. Under aerobic conditions on glucose, excess acetyl-CoA dissimilates to acetate, which is excreted, *via* the phosphotransacetylase (PTA) and acetate kinase A (ACKA) pathway in *E. coli* [2, 3]. Upon transition into stationary phase, the acetogenic glycolytic pathways are depleted, and acetate is resorbed by the bacteria and irreversibly converted to acetyl-CoA, through acetyl-adenylate (acetyl-AMP), by elevated ACS activity [3] (Fig. 1). In parallel, propionate is converted purportedly by a propionyl-CoA synthase, PrpE (EC 6.2.1.17), *via* an AMP intermediate to propionyl-CoA on the methylcitrate cycle, and the CoA thioester is ultimately converted to pyruvate to complete propionate metabolism [4] (Fig. 1). However, 2D-gelelectrophoretic analysis of protein isolated from *E. coli* fed propionate minimal medium revealed that ACS was expressed, while PrpE was not detectable, suggesting that ACS likely activated propionate to propionyl-CoA [4, 5]. The substrate flexibility of ACS is further supported by *in vitro* catalysis

Correspondence: Professor Kevin D. Walker, Michigan State University, 320 Chemistry, East Lansing, MI 48824, USA
E-mail: walke284@msu.edu
Fax: +1-517-353-1793

Abbreviations: ACS, acetyl-CoA synthase; **AtoAD**, acetoacetate:acetyl-CoA CoA-transferase of the *ato* operon; **10-DA-10-acylB**, 10-deacetyl-10-acylbaccatin III; **10-DAB**, 10-deacetylbaccatin III; **10-DABB**, 10-deacetyl-10-butyrylbaccatin III; **10-DAPB**, 10-deacetyl-10-propionylbaccatin III; **DBAT**, 10-DAB 10β-*O*-acetyltransferase; **IPTG**, isopropyl β-D-1-thiogalactopyranoside; **LB**, Luria-Bertani; **PrpE**, propionyl-CoA synthase; **PTA-ACKA**, phosphotransacetylase/acetic acid kinase

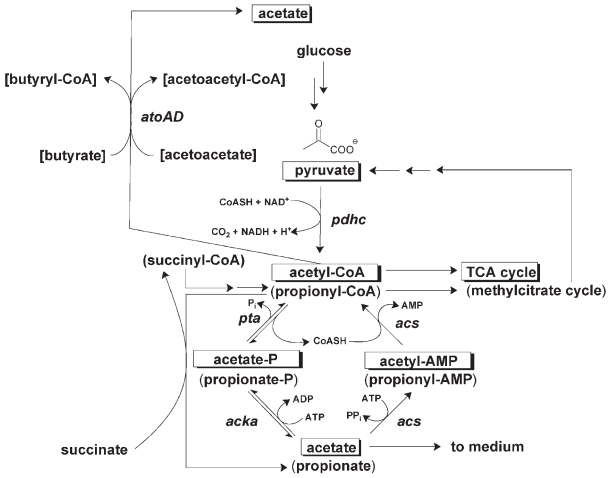

Figure 1. Acetate and propionate activation pathways. The acetogenic metabolites are encircled with shadow boxes, the propionogenic metabolites are enclosed in parentheses, and the fatty acid degradation metabolites on the *atoAD* activation pathway are enclosed in brackets. *acka*, acetate kinase; *acs*, acetyl-CoA synthase; ADP, adenosine diphosphate; AMP, adenosine monophosphate; *atoAD*, acetoacetate:acetyl-CoA CoA-transferase; ATP, adenosine triphosphate; CoASH, coenzyme A; NAD(H), oxidized/reduced forms of nicotinamide adenine dinucleotide; P, phosphate; *pdhc*, pyruvate dehydrogenase complex; *pta*, phosphotransacetylase; TCA, tricarboxylic acid.

that coupled acetate, propionate, and other short-chain acids (but not *n*-butyrate) to coenzyme A [6], although PrpE was demonstrated *in vitro* to convert butyrate to butyryl-CoA in a separate study [7]. Furthermore, the PTA-ACKA pathway in *E. coli* can activate propionic acid to its CoA-thioester [3] (Fig. 1). Collectively, the function of these propionate-activating pathways suggests that *E. coli* are potentially able to supply propionyl-CoA substrate to a recombinantly expressed acyl-CoA-dependent acyltransferase with relaxed specificity, such as the 10-deacetylbaccatin III 10β-*O*-acetyltransferase (DBAT).

DBAT expressed in bacteria from a cDNA clone isolated from *Taxus* was earlier assayed *in vitro* and demonstrated to regiospecifically transfer acetyl (the naturally occurring functional group) as well as, but to a lesser degree, *n*-propionyl and *n*-butyryl from corresponding CoA thioesters to the C10 hydroxyl of 10-deacetylbaccatin III (10-DAB) [8]. The so-derived C10-modified baccatins are precursors for the synthesis of Taxol analogs (Paclitaxel is the generic name for Taxol, which is a registered trademark of Bristol-Myers Squibb. Because of the greater familiarity with the word "Taxol", this will be used throughout the manuscript), which were identified in a combinatorial chemistry library and found to be slightly more efficacious than the parent drug Taxol (Fig. 2) [9]. This prolific chemotherapeutic agent is used principally against ovarian and breast cancers, but has recently shown promising application in the treatment of brain tumors [10], interventional heart disease [11], and neurodegenerative disorders, such as Alzheimerís disease [12].

The described substrate specificity of DBAT, combined with the abundant quantities of endogenous acetyl-CoA (>250 μM) in *E. coli*, prompted a previous *in vivo* study in which bacteria engineered to express the *Taxus*-derived acyl-CoA-dependent DBAT enzyme were able to acetylate exogenously supplied 10-DAB to baccatin III, the diterpene substrate of Taxol (Fig. 2) [8]. An extension of this *in vivo* production system is described herein, whereby the engineered bacteria were incubated with up to 5 mM propionic acid and found to produce elevated levels of the modified taxane 10-deacetyl-10-propionylbaccatin III (10-DAPB).

Furthermore, to construct 10-deacetyl-10-butyrylbaccatin III (10-DABB) in *E. coli*, the limitation of ACS (and/or PrpE) for activation of primarily C_2- and unbranched C_3-alkanoic acids required engineering *E. coli* to overexpress the *atoAD* genes of the *ato* operon that encode the operationally soluble tetrameric acetoacetate:acetyl-CoA CoA-transferase. This enzyme activates acetoacetate and *n*-butyrate to their corresponding CoA thioesters on the tricarboxylic acid/glyoxylate [13] and fatty acid degradation [14] pathways. Co-expression of *atoAD* and *dbat* by isopropyl β-D-1-thiogalactopyranoside (IPTG) induction in *E. coli* for the *in vivo* production of 10-deacetyl-10-butyrylbaccatin III is described, thus marking the first attempt

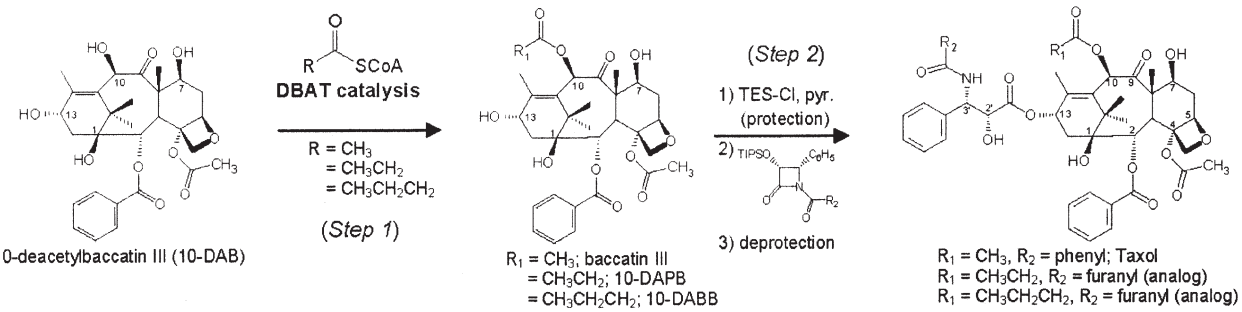

Figure 2. Outline of the biosynthetic reaction catalyzed by DBAT in the presence of acetyl-CoA (R = CH_3), propionyl-CoA (R = CH_3CH_2), and butyryl-CoA (R = $CH_3CH_2CH_2$) (step 1). Outline of a potential semisynthesis of 3'*N*-10-diacyl analogs of Taxol from 10-DA-10-acylB surrogates (step 2). pyr, pyridine; TES-Cl, triethylsilyl chloride; TIPS, triisopropylsilyl.

toward making unnatural 10-deacetyl-10-acylbaccatin III (10-DA-10-acylB) derivatives as potential precursors of second-generation Taxol derivatives in engineered *E. coli*.

2 Materials and methods

2.1 General

A Varian Inova-300 or a Varian UnityPlus500 instrument was used to acquire proton nuclear magnetic resonance (^{1}H-NMR) spectra. A Finnigan LC-electrospray isonization (ESI) LCQ Deca XP tandem mass spectrometer (Thermo Electron Corp.) or a JEOL AX-505 Fast Atom Bombardment ionization double focusing mass spectrometer was used for mass analysis. An Agilent 1100 HPLC system was used for LC with UV detection of the effluent.

2.2 Substrates, reagents and cDNA clones

10-Deacetylbaccatin III was purchased from Natland (Research Triangle Park, NC, USA). Acetic acid, propionic acid, butyric acid and all the other reagents were purchased from Sigma-Aldrich and used without further purification, unless otherwise noted. The *atoAD* from *Escherichia coli*, previously subcloned into an expression vector derived from pET26b, designated pKOS173-091, was acquired from Kosan Biosciences, Inc. (Hayward, CA, USA) as a generous gift. The *dbat* gene clone in vector pCWori$^+$ was acquired from the Washington State University Research Foundation (Pullman, WA, USA).

2.3 Alkanoic acid supplementation for production of 10-DA-10-acylB compounds

10-DAB (100 µM, 10 µmol) and acetic acid or propionic acid (1 mM, 100 µmol) were fed to *Escherichia coli* JM109 cells (100 mL, at near-log phase OD_{600}=0.6–0.8) expressing *dbat* from the vector pCWori$^+$ (pCWDBT) for the *in vivo* transfer of the short-chain acyl group to the terpene substrate. Generally, the bacteria with ampicillin resistance were grown at 37°C to $OD_{600} \approx 0.8$, induced with 1 mM IPTG, and then at 31°C for 18 h in the presence of an alkanoic acid. The bacteria were harvested by centrifugation at 5000 × *g* (10 min), and the supernatant was isolated and saved for later. The cells were weighed, then resuspended in brine (10 mL), and lysed by brief sonication (5 × 20-s bursts at 50% power; Misonix sonicator, Farmingdale, NY, USA). Lastly, the lysate was extracted with ethyl acetate (3 × 5 mL) by vigorous mixing, and the colloidal suspension was centrifuged at 5000 × *g* (10 min) to separate the immiscible layers. The ethyl acetate fractions were combined, dried over anhydrous $MgSO_4$, filtered, and the solvent evaporated. The remaining sample residue was resuspended in methanol (200 µL), and a 5-µl aliquot was used for HPLC analyses (described below).

NaCl (~30 g) was added to the previously saved supernatant fraction to minimize emulsification, and the resulting brine solution was extracted with ethyl acetate (3 × 30 mL). The combined organic fractions were dried over anhydrous $MgSO_4$, filtered and the solvent evaporated. The remaining residue was resuspended in methanol (2 mL), and analyzed separately by reverse-phase HPLC with UV monitoring (A_{228}) of the effluent (isocratic elution with 50:50 acetonitrile/water). The retention times of each baccatin product in the sample extracts were compared to those of authentic standards for baccatin III (C10-acetylated) and 10-DAPB. For a negative control experiment, *E. coli* JM109 cells possessing a plasmid expressing a *Taxus*-derived taxane 2α-*O*-benzoyltransferase, designated TAX02, were processed identically to bacteria expressing *dbat*; the level of 10-DA-10-acylB produced by these cells was below the detection limits of the analysis.

To confirm the identity of each 10-DA-10-acylB product made by *in vivo* DBAT catalysis, a preparative-scale (1 L) feeding assay was conducted. The feeding protocol and cell processing of the bacteria were identical to that for the pilot assays described above, except proportionally larger volumes of resuspension buffer and extraction solvents were used to isolate the products. The putative target products isolated from the cells or the associated growth medium were further purified by preparative silica gel TLC (mobile phase of 40:60 hexane/ethyl acetate) to provide a mixture of 10-DA-10-acylB products with retention factors (R_f) at ~0.40. The partially purified mixture isolated from TLC was further partitioned by reverse-phase HPLC that was eluted isocratically with 50:50 acetonitrile/water at 1 mL/min with UV monitoring (A_{228}) of the effluent. The C10-acyl products were separated and isolated based on identical elution times as those for authentic standards [8]. The solvent of each fraction containing purified product was evaporated completely, and each of the remaining residues was dissolved separately in deuterated chloroform as internal reference for analysis by ^{1}H-NMR. For additional confirmation, a fraction of each isolated 10-DA-10-acylB was further analyzed by combined LC-ESI/MS or fast atom bombardment MS (FABMS in positive ion mode). The ^{1}H-NMR spectra and MS fragmentation data for baccatin III [diagnostic ^{1}H-NMR signals, δ: 2.21 (singlet, CH_3 of C10 acetate), 6.29 (singlet, H-10); diagnostic ions: MH^+ at *m/z* 587, *m/z* 527 (M^+–acetic acid at C10), and *m/z* 509 (*m/z* 527–H_2O)] and 10-DAPB [diagnostic ^{1}H-NMR signals, δ: 1.22 (triplet, *J*=4 Hz, CH_3 of C10 propionyl), 2.52 (multiplet, CH_2 of C10 propionyl), 6.32 (singlet, H-10); diagnostic ions: MH^+ at *m/z* 601, *m/z* 527 (M^+–propionic acid at C10), and *m/z* 509 (*m/z* 527–H_2O)] corresponded well with data reported previously for these compounds [8].

The percent conversion of 10-DAB to product was assessed by calculating the ratio of the area under the A_{228} peak corresponding with a 10-DA-10-acylB product, as the numerator, to the total area under the A_{228} peaks

corresponding to all of the acylbaccatin III compounds, as the denominator. The quotient is reported as a percentage that is normalized according to the weight of the corresponding bacterial biomass harvested from the medium.

2.4 Co-expression of the *dbat* and *atoAD* genes for 10-DABB production

The pKOS173-091 plasmid, conferring kanamycin resistance and encoding the acetoacetate:acetyl-CoA CoA-transferase (AtoAD), was used to transform an ampicillin-resistant *E. coli* strain BL21(DE3) possessing vector pCWDBT that expresses *dbat*. Briefly, these BL21(DE3) cells were transferred to LB liquid medium (5 mL) containing ampicillin (100 μg/mL), and cells were made competent by $CaCl_2$ treatment [15] for co-transformation with pKOS173-091. Co-transformants were selected on LB agar plates containing kanamycin (50 μg/mL) and ampicillin (100 μg/mL). These cells were used for the co-expression of the *dbat* and *atoAD* genes in feeding studies incorporating sodium butyrate (25 mM) and 10-DAB (100 μM) in 25 mL LB liquid medium. The bacteria were grown at 37 °C to OD_{600}=0.8, induced with IPTG, and grown for 48 h at 22°C. The cells and medium were partitioned and extracted separately with organic solvent, processed, and analyzed by spectroscopic methods, described above, for identification and characterization of the de novo biosynthetically derived product. The retention time on HPLC and mass spectral fragmentation data were identical to those for authentic 10-DABB [8] [FABMS (positive ion mode), diagnostic ions: MH^+ at *m/z* 615 , *m/z* 527 (M^+–butyric acid at C10), and *m/z* 509 (*m/z* 527–H_2O)]. To confirm of the identity of the butyryl compound by ^{1}H-NMR, large-scale (2 L) cultures of transformed *E. coli* were grown analogous to methods described previously, and the medium was supplemented with 25 mM sodium butyrate, kanamycin (50 μg/mL) and ampicillin (100 μg/mL). After incubation, the cells and medium were partitioned, and suspension medium was extracted with organic solvent, as described before. The isolated taxane mixture was purified by silica gel flash chromatography (50:50 ethyl acetate/hexane), and the fractions containing the baccatin III analogs were combined and the solvent was evaporated. The remaining residue was further purified by preparative TLC (60:40 ethyl acetate/hexane); the band on the silica plate ($R_f \approx 0.4$) visualized by UV_{254} absorbance corresponding to the taxanes was isolated, and the silica gel was extracted with ethyl acetate. The extraction solvent was removed *in vacuo*, and the resulting residue was further purified by HPLC with A_{228} monitoring of the effluent (50:50 acetonitrile/water). The analyte eluting at identical retention time to authentic 10-DABB [8] was isolated, and the solvent was evaporated under vacuum. ^{1}H-NMR analysis of the isolated material dissolved in $CDCl_3$ for reference revealed diagnostic resonances at δ 1.01 ($CH_3CH_2CH_2C$=O), 1.73 ($CH_3CH_2CH_2C$=O) and 2.46 ($CH_3CH_2CH_2C$=O) of the butyryl at C10 and at δ 6.30 (singlet), which is the signature signal for H-10; these data matched those listed in a previous report [8].

2.5 Optimal alkanoic acid concentration for *in vivo* production of baccatin III analogs

To assess the effects of varying concentrations of alkanoic acids on the *in vivo* rate of formation of 10-DA-10-acylB analogs, *E. coli* JM109 cultures (100 mL) expressing the *acs* gene, under native genomic regulation and expression, and engineered to overexpress *dbat* from pCWDBT, were grown at 31°C to OD_{600}=0.6–0.8, then induced with IPTG. Separate cultures (in triplicate) were fed acetic acid or propionic acid at 0, 1, 2 and 5 mM for 16 h at 31°C, and each culture flask also contained co-substrate 10-DAB at 100 μM.

Sodium butyrate at 0, 25, 50, 75, and 100 mM was incubated with an *E. coli* BL21(DE3) transformant harboring pCWDBT for *dbat* expression, and pKOS173-091 for expression of the *E. coli*-derived *atoAD*. The growth medium (25 mL) contained 10-DAB at 100 μM, and the bacteria cultures (in triplicate) were grown at 37°C to OD_{600}=0.8, then induced with IPTG, and grown for 48 h at 22°C. In all of the feeding studies, the cells and medium were partitioned, and the aqueous fraction was extracted separately with organic solvent, and the isolated taxane mixtures, from each sample containing different concentrations of alkanoic acid, were analyzed separately by UV-HPLC, as described above.

3 Results

3.1 Effects of C_2- and C_3-alkanoic acids on the production of baccatin III analogs in *E. coli*

When grown aerobically in glucose, *E. coli* push carbon flux through pyruvate directly to acetyl-CoA by the pyruvate dehydrogenase complex (cf. Fig. 1) [3, 16]. Some compensation is made within the bacteria to convert excess acetyl-CoA to acetate *via* the reversible PTA-ACKA pathway [3] (cf. Fig. 1). Several acetogenic pathways in *E. coli* lead to the excretion of acetate, and are central to altering the metabolic profiles of, *e.g.*, fatty acids and polyketide antibiotics [16]. Despite the balancing of acetate and acetyl-CoA concentrations, the availability of the CoA thioester during *E. coli* development is typically within 20–600 μM, and reaches a maximum concentration during exponential growth [2]. However, as the *E. coli* entered stationary phase, it was anticipated that the addition of excess acetic acid to the medium would simultaneously induce the forward ACS and the reverse PTA-ACKA reaction pathways to increase the conversion of acetic acid to acetyl-CoA, which is a necessary metabolite that maintains flux into the tricarboxylic acid

cycle for cellular energy during the lag phase of the growth cycle.

Accordingly, *E. coli* JM109 cultures (at OD_{600} =1.0) expressing *dbat* were grown in the presence of 10-DAB (100 μM) and acetic acid at 1, 2, or 5 mM. The cell biomass and the corresponding growth medium were partitioned by centrifugation, and each fraction was separately extracted with ethyl acetate. After removing the solvent, the remaining residues were analyzed by HPLC with A_{228} monitoring of the effluent; nearly all of the *de novo* taxane was present in the medium, thus indicating that the cells excrete the xenobiotic product after initial entry of the precursor 10-DAB into the cells. The material eluting from the column with identical retention time (4.74 ± 0.01 min) to authentic baccatin III was isolated and analyzed further by ^{1}H-NMR and MS to confirm the identity of the biosynthetic product. Diagnostic ^{1}H-NMR signals appeared at δ 2.21 (singlet, CH_3 of the acetate at C10) and at δ 6.29 (singlet, H-10); diagnostic ions in the FAB (positive ion mode) mass spectrum were assessed as *m/z* 587 (MH^+), m/*z* 527 (M^+ – acetic acid at C10), and *m/z* 509 (*m/z* 527 – H_2O), as seen previously [8]. Interestingly, supplementing the growth medium with increasing concentrations of acetic acid had no significant effects on the overall conversion (~10%) of co-substrates to acetylated product (baccatin III) in separate assays compared to the conversion of substrate to product without acetic acid supplementation (Fig. 3A).

Furthermore, HPLC analysis of extracts of the cell culture medium, for which the effects of acetate on the *in vivo* production of baccatin III were investigated, revealed a peak absorbing at 228 nm, with similar retention time (6.03 ± 0.01 min) to that of authentic 10-DAPB (Fig. 4A); the relative peak area was calculated to be 2.0 ± 0.9% of the total baccatins. FAB ionization (positive ion mode) analysis of the fraction containing the unknown compound revealed diagnostic fragment ions *m/z* 601 (MH^+), 527 [M^+ – $C(O)CH_2CH_3$] and 509 (*m/z* 527 – H_2O) consistent with the fragment ions of authentic material by identical analysis [8]. ^{1}H-NMR analysis revealed diagnostic signals at δ 1.22 and 2.52 (CH_3 and CH_2, respectively, of the propionyl side chain at C10) and at δ 6.32 (singlet, H-10).

Encouraged by the finite production of 10-DAPB in transformed *E. coli* and by studies where propionate supplementation of the medium increased the flux of propionyl-CoA-dependent pathways in bacteria [4], bacterial cultures were fed 10-DAB (100 μM) and propionic acid at 0, 1, 2, or 5 mM, at the time of induction by IPTG. After 16 h, the baccatins isolated from the medium were surveyed by HPLC with A_{228} monitoring of the mobile phase. A maximum conversion of 10-DAB to 10-DAPB at 13 ± 1.0% was observed in extracts from the medium of cells grown in 5 mM propionic acid (Fig. 3B), compared to the 2.0 ± 0.9% conversion observed in organic extracts of the medium in which cells were grown without added propi-

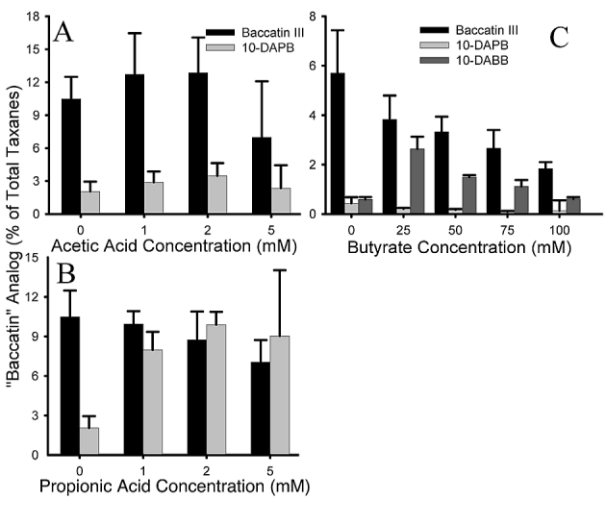

Figure 3. Diagrammed is the percentage *in vivo* conversion of substrate 10-DAB to "baccatin" analogs isolated from the LB medium in which engineered *E. coli* JM109 were grown to express the *dbat* gene; the growth medium was supplemented with 0, 1, 2, and 5 mM of acetic acid (A) or propionic acid (B). *E. coli* BL21(DE3) were engineered to co-express *dbat* and the acetoacetate:acetyl-CoA CoA-transferase (*atoAD*) genes, and the growth medium for the bacteria was supplemented with 0, 25, 50, 75, and 100 mM sodium butyrate (C). The values and error bars represent the average conversion (± SE) of 10-DAB to a corresponding "baccatin" analog in triplicate experiments. The percent conversion was calculated as described in the text.

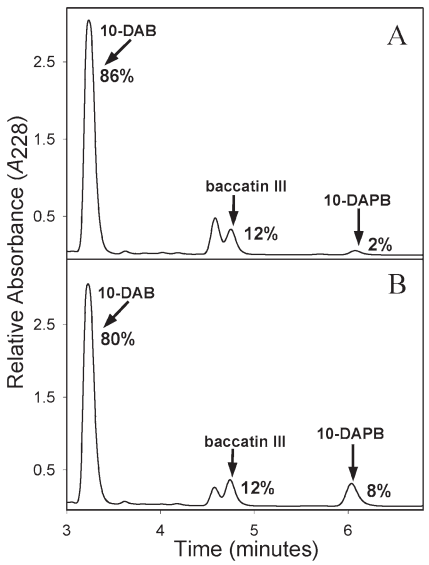

Figure 4. (A) Partial HPLC UV-trace (A_{228}) of 10-DAB and biosynthetically derived baccatin III and 10-DAPB products (retention times at 3.24, 4.72 and 6.03±0.01 min, respectively) isolated from the supernatant in which *E. coli* transformed to express the *dbat* gene, were grown in medium containing 10-DAB (100 μM). (B) Partial UV-HPLC trace (A_{228}) of baccatin products, isolated from growth medium, as above, in which the modified *E. coli* were grown in the presence of 10-DAB (100 μM) and 1 M propionic acid. The relative quantities, as percent conversion, were calculated as described in the text. HPLC conditions: 30:70 acetonitrile/water.

onic acid (Fig. 3A); less than 1% of the baccatin metabolites was associated with the cells under all conditions evaluated. The relative conversion at 1 and 2 mM propionic acid were also significantly increased at 8.0 ± 1.4% (Fig. 4B) and 9.9 ± 1.0%, respectively (Figs. 3B and 4B). The conversion of 10-DAB to 10-DAPB in *E. coli* was likely attributed to an increase in the conversion of propionic acid to endogenous propionyl-CoA by the native ACS and PTA-ACKA catalysts. At higher concentrations of propionic acid (2 and 5 mM), the conversion of 10-DAB to 10-DAPB (9.9 ± 1.0% and 13 ± 1.0%, respectively) by the engineered bacteria was found to be on average slightly greater than the conversion of native acetyl-CoA and 10-DAB substrates to baccatin III (8.7 ± 2.0% and 7.0 ± 1.7%, respectively) in the corresponding sample extracted from the cell medium (Fig. 3B).

To assess that the recombinantly expressed *dbat* product was indeed catalyzing the acyl group transfers *in vivo*, *E. coli* JM109 cells transformed with a plasmid expressing the *Taxus*-derived taxane 2α-*O*-benzoyltransferase, designated TAX02 [17], were incubated identically with 10-DAB and alkanoic acids, as were bacteria expressing *dbat* alone; the level of any 10-DA-10-acylB metabolite produced by these cells was below the detection limits of the analyses (data not shown). This result confirmed that acyl coupling to 10-DAB was due to the expression of *dbat*.

3.2 Engineering *E. coli* for the production of 10-DABB

The commercially available BL21 strain was used for overexpression of the *atoAD* genes in pKOS173-091, since this vector requires the viral T7 RNA polymerase, which is engineered into the genome of the host strain and expressed under the control of IPTG induction. Transformed bacteria were grown in LB medium supplemented with 10-DAB (100 μM) and butyrate (0–100 mM). After induction with IPTG and growth at 22 °C for 48 h, the cell biomass and the corresponding growth medium were partitioned by centrifugation, and each fraction was separately extracted with ethyl acetate. After removing the solvent, the remaining residues from each fraction were analyzed separately by HPLC with A_{228} monitoring of the effluent. The butyrylbaccatin analog partitioned nearly exclusively into the medium during the feeding study, as did the other baccatins. The material eluting from the column with identical retention time (7.74 ± 0.01 min) of authentic 10-DABB [8] was isolated and analyzed further by ^{1}H-NMR and MS to confirm the identity of the biosynthetic product. Diagnostic ^{1}H-NMR signals appeared at δ 1.01 ($CH_3CH_2CH_2C$=O), 1.73 ($CH_3CH_2CH_2C$=O) and 2.46 ($CH_3CH_2CH_2C$=O) for the butyryl side chain at C10, and at δ 6.30 (singlet, H-10). Diagnostic ions in the FABMS spectrum (positive ion mode) were observed at *m/z* 615 (MH^+), *m/z* 527 (M^+ – butyric acid at C10), and *m/z* 509 (*m/z* 527 – H_2O). These data matched those previously reported [8] and confirmed the identity of the biosynthetic product as 10-DABB, derived apparently *via* butyryl-CoA production by AtoAD catalysis [18]. Interestingly, 0.6% of the total baccatin compounds (Fig. 3C) was identified by LC-ESI/MS analysis as butyrylbaccatin in extracts of the medium (not supplemented with butyrate) in which engineered *E. coli* BL21(DE3) were grown to co-express *atoAD* and *dbat*. In contrast, the metabolite was absent in extracts of the medium (supplemented with 2 mM butyrate) in which cells were grown to express only the *dbat* gene, as stated previously. Therefore, overexpression of the *atoAD* likely activated the fatty acid degradation pathway [19] that proceeded consecutively through butyrate and butyryl-CoA, and provided enough thioester for acyl group transfer to the diterpene by DBAT catalysis without adding butyrate to the culture broth. However, a greater than fourfold increase in the level of the isolated butyrylbaccatin analog was observed in extracts of medium in which the "co-expression" bacteria were grown for 2 days at 22 °C in LB broth supplemented with 25 mM butyrate (Fig. 3C). The organic solvent extract of the growth medium contained 2.6 ± 0.5% butyrylbaccatin relative to the other baccatin III compounds. Similar organic extracts of the medium in which cells were grown for 6 days under similar conditions, *i.e.*, with 25 mM butyrate added, yielded butylated product at 4.5 ± 0.5% (data not shown). At higher butyrate concentration, cell growth was obviously impaired, and the conversion to product was diminished (Fig. 3C).

4 Discussion

In a previous study, the expression of *dbat* in bacteria and isolation of the catalyst demonstrated that DBAT possessed modest substrate specificity for short-chain acyl-CoA thioesters (acetyl-, *n*-propionyl- and *n*-butyryl-CoAs). The enzyme transferred the natural acetyl group from the corresponding thioester to the C10-hydroxyl of 10-DAB with greatest efficiency, and the *n*-propionyl- and *n*-butyryl side chains were transferred less effectively [8]. Furthermore, these bacteria engineered for *dbat* expression were grown in culture medium supplemented with 10-DAB [8]. Using endogenous acetyl CoA as the acyl group donor, the bacteria were able to produce baccatin III *in vivo*. This method established a basic strategy to engineer *E. coli* to increase the production of baccatin III and "unnatural" analogs 10-DAPB and 10-DABB *in vivo*.

Engineered bacteria were grown in LB medium to co-express the *dbat* clone either with the *acs* (under native regulation of expression from the *E. coli* chromosome) or the recombinantly expressed activator (*atoAD*) of the *ato* operon encoding the short-chain fatty acid CoA transferase [18]. The medium was supplemented with 10-DAB along with acetic acid, propionic acid, or sodium butyrate to increase the corresponding CoA esters of these acids

to expand the production of baccatin III and its C10-acyl analogs. However, exogenously supplied acetic acid was shown, surprisingly, to not perturb the possibly already steady-state concentration of acetyl-CoA available to DBAT ($K_m \approx 8$ μM for acetyl-CoA [20]) in the cells. However, at higher concentration (5 mM) of acetic acid, bacterial cell growth (data not shown) and the overall production of baccatin III were reduced (cf. Fig. 3A), likely due to acid toxicity, as described elsewhere [3].

The small amount of propionyl analog, 10-DAPB, produced in *E. coli* engineered to express *dbat* indicated that an endogenous pool of propionyl-CoA was available for DBAT, and this short-chain CoA was likely derived through the ACS and PTA-ACKA enzymes that also interchange acetate and acetyl-CoA. These acetogenic catalysts have been shown to also regulate, in part, the equilibrium between propionate and propionyl-CoA [21, 22] (cf. Fig. 1), which is central to several pathways in *E. coli*, including α- and β-oxidation to pyruvate and malonyl-CoA, respectively, carboxylation to 2-oxobutyrate, and Claisen condensations with oxaloacetate to methylcitrate [23]. Propionyl-CoA is obligatory to *E. coli* metabolism as evidenced previously [4] by an increase in the flux of propionic acid to methylcitrate, *via* propionyl-CoA, in bacteria grown in nutritional medium containing propionic acid.

Thus, based on the utility of exogenous propionic acid to bacteria, 1–5 mM of this acid was added to the medium of *E. coli* engineered to express *dbat*, and the *de novo* production of 10-DAPB increased to levels greater than those for baccatin III, the C10 acetylated product (cf. Fig. 3B). This observation was intriguing since the relative *in vitro* catalytic efficiency (V_{max}/K_m) of DBAT for acetyl-CoA is nearly twice that for propionyl-CoA [8], which suggests that at equal and saturating concentration of both CoA thioesters, baccatin III should be more efficiently produced than the propionylbaccatin analog. However, in this *in vivo* study, the transport of exogenous propionate (at 5 mM concentration) into the cells, at or near the stationary phase, may likely be greater than the conveyance of free acetate (at high μM concentration) excreted earlier by the bacteria during aerobic growth [24]. Consequently, the ACS enzyme (cf. Fig. 1) may be occupied with propionate over acetate; thus, an increase in propionyl-CoA concentration could conceivably dilute the natural supply of acetyl-CoA by approximately twofold, and balance the effects of the twofold difference in catalytic efficiency of DBAT for the CoA substrates. Additionally, the utility of coenzyme A for propionate metabolism *via* propionyl-CoA could diminish the availability of this prosthetic group thiol to other pathways, including those leading to acetyl-CoA, that are independent of the ACS and PTA-ACKA routes, and consequently decrease the rate of baccatin III production.

10-Butyrylbaccatin III derivatives were not detectable in extracts of *E. coli* JM109 or BL21(DE3) engineered to overexpress only the *dbat* gene and grown in medium containing 2 mM *n*-butyric acid and 10-DAB at 100 μM. This observation implied that butyric acid metabolism, *via* butyryl-CoA, is not required in *E. coli*, or that the pertinent acyl-CoA synthase is unavailable or has low specificity for the activation of butyrate. Moreover, the catalytic efficiency of DBAT for butyryl-CoA is nearly 20 times lower than for acetyl-CoA as previously reported, and thus further attenuates the overall *in vivo* production of the butyryl taxane analog.

Typically, the short-chain fatty acid, *n*-butyrate, is biosynthesized and activated as butyryl-CoA [25] by anaerobic bacteria, such as *Clostridium difficile* [26] and *Faecalibacterium* (previously *Fusobacterium*) *nucleatum* [27]. Reports suggest that aerobic bacteria, like *E. coli* BL21, have limited utility for butyryl-CoA [18], which is undetectable in these cells [18]. Therefore, to produce 10-DABB in *E. coli*, a two-vector, IPTG-induced co-expression system was constructed, wherein one plasmid (pKOS173-091) expressed the *atoAD* genes, and the other (pCWDBT) expressed the *dbat* cDNA. *AtoAD* of the *ato* operon (regulated naturally by AtoC) on the *E. coli* genome encodes a tetrameric acetoacetate:acetyl-CoA CoA-transferase (subunit composition $[(AtoA)_2][(AtoD)_2]$) that activates acetoacetate, butyrate and other short-chain fatty acids to their CoA thioesters [18, 28, 29]. Expression of *atoAD* in BL21 cells has been shown to increase the production of butyryl-CoA from 3–50% of the total thioesters isolated [18]. Although the concentration of butyryl-CoA was not measured directly in this investigation, there was an apparent increase in the endogenous concentration of this acyl donor in the engineered bacteria as evidenced by the significant increase in the production of 10-DABB *in vivo* (cf. Fig. 3C).

Furthermore, the average conversion of the 10-DAB substrate to baccatin III is noticeably lower in transformed *E. coli* cultures expressing the *atoAD* and *dbat* genes, and likely occurs as a consequence of the *atoAD* expression product, which uses acetyl-CoA to convert acetoacetate and butyrate to the corresponding coenzyme A thioesters (cf. Fig. 3C). Siphoning of the acetyl-CoA pool into the fatty acid degradation pathway, *via* IPTG-induced overexpression of the CoA transferase, presumably diminishes baccatin III biosynthesis in the cultures. Baccatin III production is reduced further at higher concentrations of butyrate (cf. Fig. 3C); however, this decrease coincided with slower cell growth (data not shown) possibly resulting from the toxicity of the added carboxylate.

In conclusion, the described approach provides an alternative, biochemical method to produce precursors of second-generation Taxol molecules. Foreseeably, acyl group modification at C10 or elsewhere in Taxol may facilitate drug application in the treatment of cancer, heart disease, and neurodegenerative ailments. Biocatalysis-based transformations are highly attractive over current synthetic methods that rely on protecting group manipu-

lation, heavy metal catalysis, and organic solvent usage. In general, the specificity of additional putative biocatalysts derived from various organisms can be characterized by substrate screening [30, 31], and functional enzymes can then be strategically incorporated into an *in vivo* system or *in vitro* synthetic process for potential biosynthesis of pharmaceutically important molecules, such as Taxol and its analogs, artemisinin [32], and polyketide antibiotics [18].

In vivo production of baccatin III and analogs in engineered *E. coli* has been demonstrated by functional expression of *dbat*, derived from *Taxus*, and acyl-CoA synthases/transferases, derived from the *E. coli* genome. Although moderate transformation rates were observed in this preliminary *in vivo* study, rational improvements to the system are potentially gained through optimization and stabilization of cell culture conditions by growth in regulated fermentors, selection of culture type, or expanded genetic engineering targets. For instance, engineering *E. coli* to overexpress *panK* (pantothenate kinase) [16] to increase the conversion of pantothenic acid to coenzyme A provides substrate for acyl-CoA synthase-dependent synthesis of thioesters. In parallel, the overexpression of the pyruvate dehydrogenase to increase the flux of pyruvate to acetyl CoA [16] could theoretically enhance the *in vivo* production of baccatin III.

Optimizing the described semi-biosynthetic method in bacteria potentially provides a practical means of developing commercial-scale production of baccatin III analogs, which serve as key intermediates in the semi-synthesis of second-generation Taxols. Moreover, application of this *in vivo* method with the other acyltransferases on the Taxol biosynthetic pathway could likely provide alternative methods to attach modified acyl groups throughout the molecule.

We acknowledge Sumati Murli (Kosan Biosciences, Inc.) for providing the vector pKOS173-091, for atoAD expression, as a generous gift, and the Washington State University Research Foundation for supply the vector pCWDBT. We also thank undergraduates Allyson Boers, and Christopher Johnson for their technical assistance. This research was funded by the Michigan Agricultural Experiment Station and Michigan State University College of Natural Science.

5 References

[1] Kumari, S., Simel, E. J., Wolfe, A. J., s70 is the principal sigma factor responsible for transcription of *acs*, which encodes acetyl coenzyme A synthetase in *Escherichia coli*. *J. Bacteriol.* 2000, *182*, 551–554.

[2] Takamura, Y., Nomura, G., Changes in the intracellular concentration of acetyl-CoA and malonyl-CoA in relation to the carbon and energy metabolism of *Escherichia coli* K12. *J. Gen. Microbiol.* 1988, *134*, 2249–2253.

[3] Wolfe, A. J., The acetate switch. *Microbiol. Mol. Biol. Rev.* 2005, *69*, 12–50.

[4] Brock, M., Maerker, C., Schutz, A., Volker, U., *et al.,* Oxidation of propionate to pyruvate in *Escherichia coli*: Involvement of methylcitrate dehydratase and aconitase. *Eur. J. Biochem.* 2002, *269*, 6184–6194.

[5] Polen, T., Rittmann, D., Wendisch, V. F., Sahm, H., DNA microarray analyses of the long-term adaptive response of *Escherichia coli* to acetate and propionate. *Appl. Environ. Microbiol.* 2003, *69*, 1759–1774.

[6] Patel, S. S., Walt, D. R., Substrate specificity of acetyl coenzyme A synthetase. *J. Biol. Chem.* 1987, *262*, 7132–7134.

[7] Valentin, H. E., Mitsky, T. A., Mahadeo, D. A., Tran, M. *et al.,* Application of a propionyl coenzyme A synthetase for poly(3-hydroxypropionate-co-3-hydroxybutyrate) accumulation in recombinant *Escherichia coli*. *Appl. Environ. Microbiol.* 2000, *66*, 5253–5258.

[8] Loncaric, C., Merriweather, E., Walker, K. D., Profiling a Taxol pathway 10β-acetyltransferase: Assessment of the specificity and the production of baccatin III by *in vivo* acetylation in *E. coli*. *Chem. Biol.* 2006, *13*, 1–9.

[9] Baloglu, E., Hoch, J. M., Chatterjee, S. K., Ravindra, R. *et al.,* Synthesis and biological evaluation of C-3'NH/C-10 and C-2/C-10 modified paclitaxel analogues. *Bioorg. Med. Chem.* 2003, *11*, 1557–1568.

[10] Rice, A., Liu, Y., Michaelis, M. L., Himes, R. H. *et al.,* Chemical modification of paclitaxel (Taxol) reduces p-glycoprotein interactions and increases permeation across the blood-brain barrier *in vitro* and *in situ*. *J. Med. Chem.* 2005, *48*, 832–838.

[11] Choong Cliff, K., Phan, L., Massetti, P., Haddad Fabio, J. *et al.,* Prolongation of patency of airway bypass stents with use of drug-eluting stents. *J. Thorac. Cardiovasc. Surg.* 2006, *131*, 60–64.

[12] Boutte, A. M., Neely, M. D., Bird, T. D., Montine, K. S. *et al.,* Diminished Taxol/GTP-stimulated tubulin polymerization in diseased region of brain from patients with late-onset or inherited Alzheimer's disease or frontotemporal dementia with parkinsonism linked to chromosome-17 but not individuals with mild cognitive impairment. *J. Alzheimers Dis.* 2005, *8*, 1–6.

[13] Turner, J. E., Greville, K., Murphy, E. C., Hooks, M. A., Characterization of Arabidopsis fluoroacetate-resistant mutants reveals the principal mechanism of acetate activation for entry into the glyoxylate cycle. *J. Biol. Chem.* 2005, *280*, 2780–2787.

[14] Campbell, J. W., Morgan-Kiss, R. M., Cronan, J. E., A new *Escherichia coli* metabolic competency: Growth on fatty acids by a novel anaerobic β-oxidation pathway. *Mol. Microbiol.* 2003, *47*, 793–805.

[15] Dagert, M., Ehrlich, S. D., Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. *Gene* 1979, *6*, 23–38.

[16] Dittrich, C. R., Vadali, R. V., Bennett, G. N., San, K.-Y., Redistribution of metabolic fluxes in the central aerobic metabolic pathway of *E. coli* mutant strains with deletion of the acka-pta and poxb pathways for the synthesis of isoamyl acetate. *Biotechnol. Prog.* 2005, *21*, 627–631.

[17] Walker, K., Croteau, R., Taxol biosynthesis: Molecular cloning of a benzoyl-CoA:taxane 2α-*O*-benzoyltransferase cDNA from *Taxus* and functional expression in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 2000, *97*, 13591–13596.

[18] Kennedy, J., Murli, S., Kealey, J. T., 6-Deoxyerythronolide B analogue production in *Escherichia coli* through metabolic pathway engineering. *Biochemistry* 2003, *42*, 14342–14348.

[19] Pauli, G., Overath, P., *ato* operon: a highly inducible system for acetoacetate and butyrate degradation in *Escherichia coli*. *Eur. J. Biochem.* 1972, *29*, 553–562.

[20] Walker, K., Croteau, R., Molecular cloning of a 10-deacetylbaccatin III-10-*O*-acetyl transferase cDNA from *Taxus* and functional expression in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 2000, *97*, 583–587.

[21] Kumari, S., Tishel, R., Eisenbach, M., Wolfe, A. J., Cloning, characterization, and functional expression of acs, the gene which encodes acetyl coenzyme A synthetase in *Escherichia coli*. *J. Bacteriol.* 1995, *177*, 2878–2886.

[22] Kumari, S., Beatty, C. M., Browning, D. F., Busby, S. J. W. *et al.,* Regulation of acetyl coenzyme A synthetase in *Escherichia coli*. *J. Bacteriol.* 2000, *182*, 4173–4179.

[23] Caspi, R., Foerster, H., Fulcher, C. A., Hopkinson, R. *et al.,* MetaCyc: A multiorganism database of metabolic pathways and enzymes. *Nucleic Acids Res.* 2006, *34*, D511–516.

[24] Diez-Gonzalez, F., Russell, J., The ability of *Escherichia coli* O157:H7 to decrease its intracellular pH and resist the toxicity of acetic acid. *Microbiology* 1997, *143*, 1175–1180.

[25] Duncan, S. H., Barcenilla, A., Stewart, C. S., Pryde, S. E. *et al.,* Acetate utilization and butyryl coenzyme A (CoA):acetate-CoA transferase in butyrate-producing bacteria from the human large intestine. *Appl. Environ. Microbiol.* 2002, *68*, 5186–5190.

[26] Karlsson, S., Lindberg, A., Norin, E., Burman, L. G. *et al.,* Toxins, butyric acid, and other short-chain fatty acids are coordinately expressed and down-regulated by cysteine in *Clostridium difficile*. *Infect. Immun.* 2000, *68*, 5881–5888.

[27] Kapatral, V., Anderson, I., Ivanova, N., Reznik, G. *et al.,* Genome sequence and analysis of the oral bacterium *Fusobacterium nucleatum* strain ATCC 25586. *J. Bacteriol.* 2002, *184*, 2005–2018.

[28] Reed, J., Vo, T., Schilling, C., Palsson, B., An expanded genome-scale model of *Escherichia coli* K-12 (iJR904 GSM/GPR). *Genome Biol.* 2003, *4*, R54.

[29] Sramek, S. J., Frerman, F. E., *Escherichia coli* coenzyme A-transferase: kinetics, catalytic pathway and structure. *Arch. Biochem. Biophys.* 1975, *171*, 27–35.

[30] Bommarus, A. S., Polizzi, K. M., Novel biocatalysts: Recent developments. *Chem. Eng. Sci.* 2005, *61*, 1004–1016.

[31] Xie, D., Shao, Z., Achkar, J., Zha, W. *et al.,* Microbial synthesis of triacetic acid lactone. *Biotechnol. Bioeng.* 2006, *93*, 727–736.

[32] Smolke, C. D., Martin, V. J. J., Keasling, J. D., Tools for metabolic engineering in *Escherichia coli*, in: Baneyx, F. (Ed.), *Protein Expression Technologies*. Horizon Scientific Press, Norwich 2004, pp. 149–197.