

Microbial Synthesis of Triacetic Acid Lactone

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Abstract: Native *g2ps1*-encoded 2-pyrone synthase (2-PS) from *Gerbera hybrida*, a mutant *Brevibacterium ammoniagenes* fatty acid synthase B (FAS-B) and two different mutants of *Penicillium patulum* 6-methylsalicylic acid synthase (6-MSAS) are examined to identify the best enzyme to recruit for the microbial synthesis of triacetic acid lactone (TAL). To identify the best microbial host for these evaluations, the native TAL-synthesizing activity of *g2ps1*-encoded 2-PS is expressed in recombinant *Escherichia coli* and *Saccharomyces cerevisiae* constructs. Five-fold higher expression levels of 2-PS are observed in *S. cerevisiae*. Consequently, microbial synthesis of TAL focuses on *S. cerevisiae* constructs. Comparison of different promoters for the expression of *g2ps1* in *S. cerevisiae* indicates that the alcohol dehydrogenase II promoter (P_{ADH2}) affords the highest expression levels of 2-PS. As a result, the genes encoding the various TAL-synthesizing enzyme activities are expressed in *S. cerevisiae* from a P_{ADH2} promoter. To extend TAL-synthesizing activity beyond *g2ps1*-encoded 2-PS, the ketoreductase domains of *fasB*-encoded FAS-B and 6-MSAS-encoded 6-MSAS are modified using a single mutation. Modification of the nicotinamide cofactor-binding site of 6-MSAS with a triple mutation is also examined. Separate *S. cerevisiae* constructs expressing native *g2ps1*, mutant Y2226F *fasB*, mutant Y1572F 6-MSAS, and mutant G1419A-G1421P-G1424A 6-MSAS are cultured under the same fermentor-controlled conditions. The highest concentration (1.8 g/L) and yield (6%) of TAL are synthesized from glucose by *S. cerevisiae* expressing the Y1572F mutant of 6-MSAS. © 2005 Wiley Periodicals, Inc.

Keywords: triacetic acid lactone; pyrone synthase; fatty acid synthase; methylsalicylic acid synthase

INTRODUCTION

4-Hydroxy-6-methyl-2-pyrone, which is also referred to as triacetic acid lactone (TAL, Fig. 1), is a precursor in the chemical synthesis (Hansen and Frost, 2002) of the natural product 1,3,5-trihydroxybenzene (phloroglucinol, Fig. 1). Phloroglucinol, in turn, is a starting material used in the synthesis (Agarwal, 1998; Benziger, 1977; Ott and Benziger,

1990) of the thermally stable energetic material 1,3,5-triamino-2,4,6-trinitrobenzene (TATB, Fig. 1). Reduction of phloroglucinol (Hansen and Frost, 2002) affords resorcinol (Fig. 1), which is used in resin and adhesive formulations. TAL is currently manufactured in five chemical steps starting with the pyrolysis of acetic acid (Abaercherli and Miller, 1991; Weissmermel and Arpe, 1997).

To achieve a direct microbial synthesis of TAL from glucose, one approach pursued in this account relies on recruitment of an enzyme with native TAL-synthesizing activity. This enzyme is 2-pyrone synthase (2-PS) encoded by the *g2ps1* gene isolated from *Gerbera hybrida* (Eckermann et al., 1998). Synthesis of TAL based on heterologous expression of *g2ps1*-encoded 2-PS is examined in both *Escherichia coli* and *Saccharomyces cerevisiae*. The impact of different promoters controlling expression of *g2ps1* in *S. cerevisiae* on the concentration and yield of synthesized TAL is also determined.

Another approach to microbially synthesize TAL directly from glucose is based on rational design to create TAL-synthesizing activity. Genetic modification of 6-MSAS, which encodes *Penicillium patulum* 6-methylsalicylic acid synthase (6-MSAS), and *fasB*, which encodes *Brevibacterium ammoniagenes* fatty acid synthase B (FAS-B), leads to TAL-synthesizing activities that are not found in their native forms. Concentrations and yields of TAL are determined in *S. cerevisiae* constructs expressing native and mutated genes from an alcohol dehydrogenase II promoter (P_{ADH2}). All TAL-synthesizing constructs are cultured under identical fermentor-controlled conditions. Insights are thus offered into whether native or rationally designed TAL-synthesizing activities lead to the highest concentration and yield of TAL.

MATERIALS AND METHODS

Culture Medium

All solutions were prepared in distilled, deionized water. LB medium (1 L) contained Bacto tryptone (10 g), Bacto yeast extract (5 g), and NaCl (10 g). M9 salts (1 L) contained Na₂HPO₄ (6 g), KH₂PO₄ (3 g), NH₄Cl (1 g), and NaCl (0.5 g). M9 medium contained D-glucose (20 g) and MgSO₄ (0.24 g)

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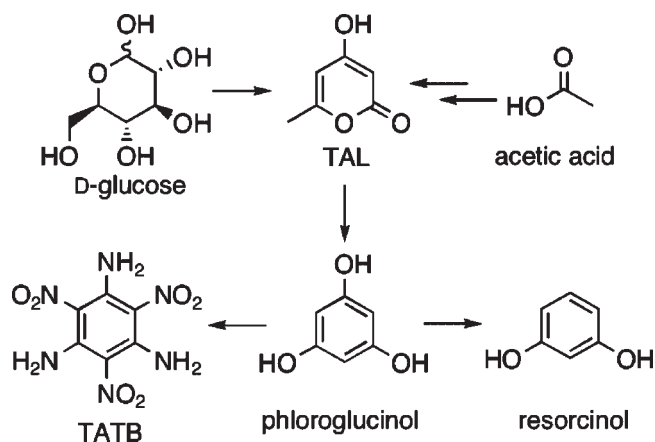


Figure 1. Synthesis of phloroglucinol from D-glucose via intermediacy of triacetic acid lactone (TAL). Both 1,3,5-triamino-2,4,6-trinitrobenzene (TATB) and resorcinol can be synthesized from phloroglucinol.

in 1 L of M9 salts. The corresponding solid medium was prepared by addition of Bacto agar to a final concentration of 1.5% (w/v). SC dropout mix lacking L-tryptophan and uracil contained adenine (2.0 g), L-arginine HCl (2.0 g), L-histidine HCl (2.0 g), L-isoleucine (2.0 g), L-leucine (2.0 g), L-lysine HCl (2.0 g), L-methionine (2.0 g), L-phenylalanine (3.0 g), L-serine (2.0 g), L-threonine (2.0 g), L-tyrosine (3.0 g), and L-valine (9.0 g). SC dropout mix lacking uracil also contained L-tryptophan (3.0 g) in addition to the components listed above. Dry SC dropout components were combined in a 250-mL plastic bottle, 20 glass marbles with a 5-mm diameter were added, and the bottle was shaken vigorously. SC dropout mix was stored at 4°C. SC dropout medium (Burke et al., 2000) lacking L-tryptophan and uracil (1 L) contained yeast nitrogen base without amino acids (6.7 g), SC dropout mix lacking L-tryptophan and uracil (0.8 g), and D-glucose (20 g). The pH of SC dropout medium was adjusted to 5.6 with 10 N NaOH prior to sterilization. YPAD medium (1 L) contained yeast extract (10 g), peptone (10 g), adenine (0.08 g), and D-glucose (20 g). The corresponding solid medium was prepared by addition of Bacto agar to a final concentration of 1.0% (w/v). Fermentation medium (1 L) for *E. coli* strains contained K₂HPO₄ (7.5 g), citric acid monohydrate (2.1 g), ammonium iron (III) citrate (0.3 g), and concentrated H₂SO₄ (1.2 mL). The pH was adjusted to 7.0 using concentrated NH₄OH prior to autoclaving. Before inoculation, the following supplements were added to the fermentation medium (1 L): D-glucose (30 g), MgSO₄ (0.24 g), and trace minerals including (NH₄)₆(Mo₇O₂₄)·4H₂O (0.0037 g), ZnSO₄·7H₂O (0.0029 g), H₃BO₄ (0.0247 g), CuSO₄·5H₂O (0.0025 g), and MnCl₂·4H₂O (0.0158 g). Fermentation medium (1 L) for *S. cerevisiae* strains contained yeast extract (10 g), peptone (10 g), adenine (0.08 g), and D-glucose (2 g). Except where specifically mentioned, antibiotics were added where appropriate to the following final concentrations: ampicillin (Ap), 50 µg/mL; kanamycin (Kan), 50 µg/mL. Isopropyl-β-D-thiogalactopyranoside (IPTG) was prepared as a 25 mg/mL stock solution.

Solutions of antibiotics, trace minerals, and IPTG were sterilized through 0.22-µm membranes. Solutions of MgSO₄ and D-glucose were autoclaved separately.

General Microbiology

Escherichia coli strain DH5α served as the host strain for plasmid constructions unless otherwise stated. Standard protocols were used for construction, purification, and analysis of plasmid DNA as well as for transformation of *E. coli* (Sambrook and Russel, 2001) and *S. cerevisiae* (Burke et al., 2000) strains. PCR amplifications were performed by standard methods using *Pfu Turbo* polymerase (Stratagene, La Jolla, CA). Unless otherwise stated, cultures of *E. coli* and *S. cerevisiae* were grown at 37°C and 30°C, respectively, with agitation at 250 rpm.

Strains and Plasmids

Strains, plasmids, and restriction site maps of plasmids are provided in Supplemental Online Material as Table III and Table IV. *S. cerevisiae* INVSc1 was obtained from Invitrogen. *E. coli* strain JWF1(DE3) (Hansen, 2002) was prepared from *E. coli* JWF1 (Hansen et al., 1999) using the λDE3 Lysogenization Kit (Novagen, Madison, WI) according to the manufacturer's protocol. Shuttle-vectors p426GPD and p426ADH were obtained from ATCC. pMR228 was obtained from C. Khosla (Richardson et al., 1999) and pKOS12-128a was provided by Kosan Biosciences (Burlingame, CA; Kealey et al., 1998). Plasmid pHis8-2PS containing the *G. hybrida* gene *g2ps1* was obtained from J. Schröder (Jez et al., 2000a,b). Plasmid M1 containing the 3.85-kb *SalI/SacI* fragment of the wild-type 6-MSAS gene cloned into pUC19 (Yanisch-Perron et al., 1985) was obtained from E. Schweizer (Beck et al., 1990). pDX3.85, pZS1, and pWZ2 plasmids were derived from pMR228 carrying the gene encoding the triple 6-MSAS mutant (G1419A-G1421P-G1424A) via yeast in vivo homologous recombination (Ma et al., 1987; Prado and Aguilera, 1994) by replacing the 6-MSAS triple mutant with wild-type *g2ps1*, Y1572F 6-MSAS, and Y2226F *fasB*, respectively. A fragment of the 6-MSAS triple mutant insert was purified away from the *BglIII/XhoI*- or *AvrII/BglIII*-digested pMR228 plasmid and the excised vector co-transformed with the appropriate DNA fragment. Transformants were selected on SC medium lacking uracil. pWZ-PPT1 was derived from pKOS12-128a by replacing the gene *sfp* with *ppt1* (Zha et al., 2004). Plasmids were transformed into INVSc1 using the lithium acetate/single stranded carrier DNA/polyethylene glycol (PEG) method (Agatep et al., 1998).

pJA1.147A

A 1.6-kb *serA* locus was excised from plasmid pRC1.55B (Yi et al., 2003) by digestion with *SmaI* and ligated with *SmaI*-digested pHis8-2PS vector, carrying the *g2ps1* gene, to afford

7.8-kb plasmids pJA1.147A/B that differ in insert orientation. Insertion of the *serA* locus disrupted the kanamycin resistance gene. Ligation mixtures were transformed into L-serine auxotroph JC158 and transformants carrying the *serA* insert were selected on M9 medium plates. Both *serA* and *g2ps1* are transcribed in the same direction in pJA1.147A.

pDX3.81

The 1.2-kb *g2ps1* gene was excised from plasmid pHis8-2PS by digestion with *Bam*HI and *Eco*RI and ligated with *Bam*HI/*Eco*RI-digested p426GPD vector to afford the 7.8-kb plasmid pDX3.81.

pDX3.82

The 1.2-kb *g2ps1* gene was excised from plasmid pHis8-2PS by digestion with *Bam*HI and *Eco*RI and ligated with *Bam*HI/*Eco*RI-digested p426ADH vector to afford the 8.7-kb plasmid pDX3.82.

pDX3.85

A 1.2-kb fragment encoding the *G. hybrida g2ps1* gene was amplified by PCR using 5'-CTATCAACTATTAACATATCGTAATACCATATGGGATCTTACTCATCTGATGAT-3' as the forward primer (nucleotides complementary to the ADH2 promoter region of vector pMR228 are in italic, *Nde*I restriction site is underlined, and nucleotides complementary to *g2ps1* are in bold) and 5'-CATCGGTCCGGTCGACTATGAATTCT-AGAAATCAGTTTCCATTGGCAACCGCAGC-3' as the reverse primer (nucleotides complementary to the ADH2 terminator region of vector pMR228 are in italic, *Rsr*II, *Sal*I, and *Eco*RI restriction sites are underlined, and nucleotides complementary to *g2ps1* are in bold) from pHis8-2PS. The PCR product was co-transformed with the gapped *Bgl*II/*Xho*I-digested pMR228 into *S. cerevisiae* INVSc1 and transformants were selected on SC medium lacking uracil for plasmid DNA isolation using the ZymoprepTM Yeast Plasmid Miniprep Kit (Zymo Research, Orange, CA). The resulting plasmid DNA was transformed into *E. coli* DH5 α for identification of clones carrying the *g2ps1* gene.

pZS1

Overlap extension PCR-based site-directed mutagenesis (Horton et al., 1990) was used to convert the codon TAC (encoding tyrosine at position 1572 in 6-MSAS) to TTC (encoding phenylalanine) on a 1.3 kb DNA fragment covering the KR domain in plasmid M1. The resulting DNA fragment of 6-MSAS was subcloned into the corresponding yeast expression plasmid as described above and the desired mutation, Y1572F, was confirmed by DNA sequencing.

Fed-Batch Fermentation of *E. coli* Strains

Fed-batch fermentation was performed in a 2.0-L working capacity B. Braun M2 culture vessel under glucose-rich or glucose-limited conditions as previously described (Yi et al., 2002). Temperature, pH, and dissolved oxygen (D.O.) were controlled with proportional-integral-derivative (PID) control loops. Temperature was maintained at 36°C. Addition of concentrated NH₄OH or 2 N H₂SO₄ was used to maintain pH at 7.0. D.O. was monitored using a Mettler-Toledo 12 mm sterilizable O₂ sensor fitted with an Ingold A-type O₂ permeable membrane and maintained at 20% of air saturation. Antifoam (Sigma 204; Sigma, St. Louis, MO) was added as needed. The initial glucose concentration in the fermentation medium was 30 g/L.

Inoculants were prepared by introducing a single colony of JWF1(DE3)/pJA1.147A into 5 mL of M9 medium. Cultures were grown at 37°C with agitation at 250 rpm for 24 h and then transferred to 100 mL of M9 medium, grown at 37°C for an additional 12 h to OD₆₀₀ = 1.0–2.5, and then transferred to the fermentor (1 L) to initiate the fermentation (t = 0). Cell densities were determined by dilution of fermentation broth with water (1:100) followed by measurements of absorption at 600 nm (OD₆₀₀). Beginning 12 h into the fermentation, 25 mg IPTG were added every 6 h until the end of the fermentation. Samples (15–25 mL) of fermentation broth were removed at 6 h intervals and centrifuged (4,300g, 4°C, 6 min). The resulting cell-free supernatant was frozen at –20°C pending analysis.

Glucose-Limited Fed-Batch Fermentation of *S. cerevisiae* Strains

Transformants were selected at 30°C on an SC dropout plate lacking uracil and L-tryptophan. A single colony was inoculated into 5 mL SC dropout medium lacking uracil and L-tryptophan, and the culture was grown with shaking at 30°C for 24 h. An aliquot (1 mL) of the resulting culture was then used to inoculate 100 mL of YPAD medium. The culture was grown with shaking at 30°C until an OD₆₀₀ of approximately 15 was achieved (20–24 h). Fermentation medium (1 L) was then inoculated with 10 mL of this seed culture.

Fermentations employed a 2.0 L working capacity B. Braun M2 culture vessel. Utilities were supplied by a B. Braun Biostat MD controlled by a DCU-3. Data acquisition utilized B. Braun MFCS/Win software (v2.0). Temperature, pH, and glucose feed were controlled by proportional-integral-derivative (PID) control loops. The PID control parameters were set to 0.0 (off) for the derivative control (τ_D), 999.9 s (minimum control action) for the integral control (τ_I), and 200.0% for the proportional band (χ_P). Temperature was maintained at 30°C. pH was maintained at 5.0 for the first 48 h of the fermentation and at 6.0 for the remainder of the run. pH was adjusted by addition of either concentrated NH₄OH or 2 N H₂SO₄. Dissolved oxygen was measured using a Mettler-Toledo 12-mm sterilizable O₂ sensor fitted with an Ingold

A-type O₂ permeable membrane. pO₂ was maintained at 20% of air saturation for the first 48 h of the fermentation and at 5% for the remainder of the run.

Three staged methods were used to maintain pO₂ concentrations at the desired set point throughout the fermentation. With the airflow at an initial setting of 0.15 L/L/min, pO₂ was maintained by increasing the impeller speed from its initial set point of 80 rpm to a preset maximum of 400 rpm. With the impeller speed then constant at 400 rpm, the mass flow controller maintained pO₂ by increasing the airflow rate from 0.15 L/L/min to a preset maximum of 0.5 L/L/min. These two control phases lasted for approximately 12–18 h. At a constant impeller speed of 400 rpm and a constant airflow rate of 0.5 L/L/min, pO₂ was finally maintained at 20% by oxygen sensor-controlled glucose feed. At the beginning of this stage, pO₂ dropped below the set point due to residual glucose in the medium. Glucose (200 g/L) feed commenced automatically when the residual glucose was consumed and pO₂ increased above the set point. The maximum pump speed for the glucose feed was limited to 10 mL/h. After 48 h, pO₂ was maintained at 5% using oxygen sensor-controlled glucose feed.

Carbon Balance Measurements

Analysis of exit gas was performed on-line using an O₂/CO₂ Off-Gas Analyzer (Sartorius/BBI Systems, Bethlehem, PA) in which O₂ and CO₂ measurements rely on paramagnetic and infrared technologies, respectively. The exit gas composition in %O₂ and %CO₂ was recorded every 30 s and transmitted to the DCU-3 controller. Total exhausted CO₂ was calculated by integrating all transient CO₂ formed during fermentation. All calculations assumed a theoretical cellular carbon content at 0.50 g carbon/g dry cell weight for *E. coli* cells and 0.47 g carbon/g dry cell weight for *S. cerevisiae* cells (Shuler and Kargi, 1992). The carbon flow from glucose to biomass was calculated using the final dry cell weight and total glucose consumed. The %C converted to biomass = cellular C content × dry cell weight/total C from glucose consumed × 100%. The %C converted to CO₂ = exit CO₂ C content/total C from glucose consumed × 100%.

Analysis of Fermentation Broth

Samples (5 mL) of fermentation broth were removed at the indicated timed intervals. Cell densities were determined by measurement of absorption at 600 nm (OD₆₀₀). Dry cell weight (g/L) was calculated using a conversion coefficient of 0.43 g/L/OD₆₀₀ for *E. coli* strains and 0.40 g/L/OD₆₀₀ for *S. cerevisiae* strains. The remaining fermentation broth was centrifuged to obtain cell-free broth. Glucose concentrations were measured in cell-free broth using the Glucose Diagnostic Kit (Sigma). TAL concentrations were determined by reversed-phase HPLC using a ZORBAX SB-C18 column (4.6 × 150 mm, 5 μm) and the following elution profile: Solvent A, 1% (v/v) acetic acid in water; solvent B, 1% (v/v) acetic acid in acetonitrile; gradient: 5% B (0–5 min), 5–15%

B (5–18 min), 15–100% B (18–23 min), 100% B (23–30 min); flow rate 1.0 mL/min; wavelength, 280 nm. TAL eluted at 12.9 min. TAL concentrations were determined in cell-free broth by comparison of the integration of the peak at 12.9 min to a standard curve prepared using authentic samples of known concentrations of TAL.

Enzyme Activity

A 20-mL aliquot of fermentation broth was taken at the indicated intervals, and the cells were collected by centrifugation (4,000g, 10 min, 4°C). Cells were washed twice with 15-mL portions of the appropriate resuspension buffer. For 2-PS and 6-MSAS enzymes the resuspension buffer was HEPES (50 mM, pH 7.5) and for FAS-B, potassium phosphate buffer (50 mM, pH 7.4) containing 1 mM EDTA, 5% glycerol (w/v), and 1 mM PMSF was used. After washing, the collected cells were again resuspended in 5 mL of the appropriate buffer and disrupted by two passages through a French press (16,000 psi). The lysate was clarified by centrifugation (48,000g, 30 min, 4°C) and the insoluble cellular debris was discarded. Protein concentrations were determined using the Bradford dye-binding procedure using protein assay solution purchased from Bio-Rad (Hercules, CA; Bradford, 1976). Protein concentrations were determined by comparison to a standard curve prepared using bovine serum albumin.

The ability of an enzyme to synthesize TAL was quantified by monitoring the corresponding increase in absorbance at 298 nm when incubated with acetyl-CoA and malonyl-CoA. The reaction (1 mL) contained 0.5 mM acetyl-CoA and 1 mM malonyl-CoA in the appropriate assay buffer and was initialized by addition of cell-free lysate. For *E. coli* strains expressing *g2psI*, assays were carried out in HEPES buffer (100 mM, pH 6.0). For all *S. cerevisiae* strains, assays were carried out in potassium phosphate buffer (400 mM, pH 5.3) containing 3 mM DTT. A molar extinction coefficient of 2,540/M/cm was used to quantify TAL. One unit of TAL forming activity was defined as the catalyzed formation of 1 μmol TAL per min at 25°C.

Purification of TAL

Cell-free broth (500 mL) was extracted in a continuous liquid–liquid extraction apparatus with three 600 mL portions of ethyl acetate/acetic acid (99:1, v/v) for a period of 36 h. The organic phases were combined, dried over magnesium sulfate, and filtered through glass wool to remove oily impurities. The solution was then concentrated to dryness under reduced pressure. The resulting material was recrystallized from ethyl acetate, affording pale yellow crystals. ¹H NMR (300 MHz, DMSO-*d*₆): 5.95 (m, 1 H, *J* = 2.2 Hz, 1.1 Hz), 5.19 (d, 1 H, *J* = 2.2 Hz), 2.14 (s, 3 H); ¹³C NMR (125 MHz, DMSO-*d*₆): 172, 165, 164, 101, 89, 20. High resolution EI-MS: calculated for TAL (M⁺) 126.0317, found 126.0320.

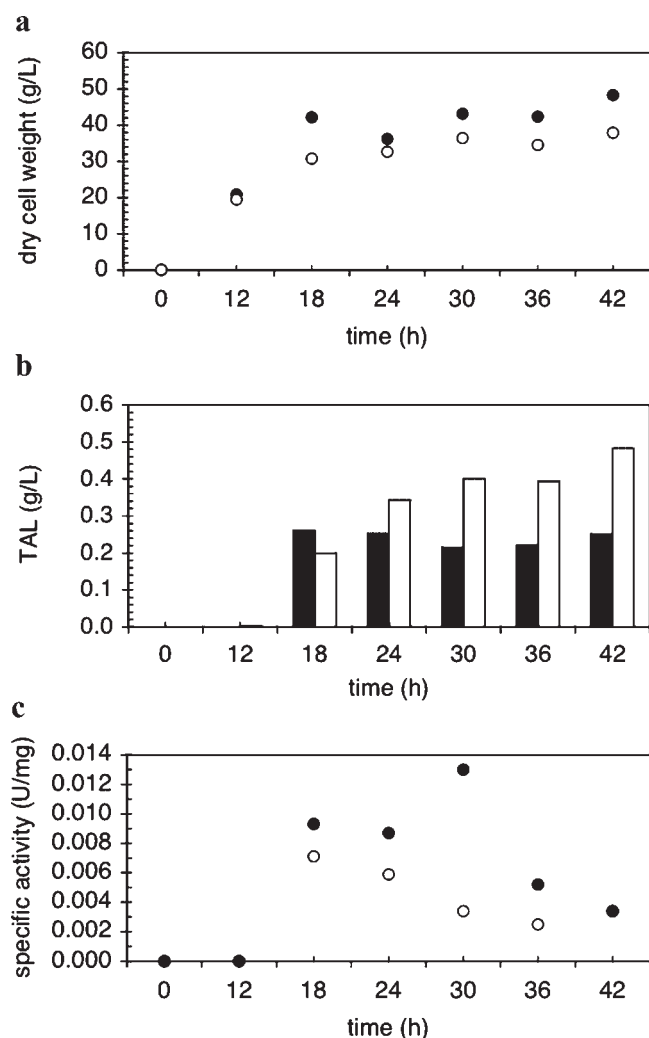


Figure 2. Fermentor-controlled cultivation of *E. coli* JWF1(DE3)/pJA1.147A expressing *g2ps1*: (a) cell mass; (b) TAL concentrations; (c) 2-PS specific activity (1 U = 1 μ mol/min). Legend: open circles and open bars, glucose-limited culture conditions; solid circles and solid bars, glucose-rich culture conditions.

RESULTS

Heterologous Expression of *g2ps1* in *E. coli*

To determine whether heterologous expression of *g2ps1*-encoded 2-PS would result in TAL synthesis in an intact microbe, *E. coli* JWF1(DE3)/pJA1.147A was examined under fermentor-controlled conditions for TAL accumulation in culture supernatants (Fig. 2). Plasmid-localized *g2ps1* was transcribed from a *T7* promoter inducible by addition of isopropyl 1-thio- β -D-galactopyranoside (IPTG). The *serA* insert in plasmid pJA1.147A, which encodes 3-phosphoglycerate dehydrogenase, provided the basis for plasmid maintenance by complementing the inactivated *serA* locus in the chromosome of the L-serine auxotroph *E. coli* JWF1(DE3). Cultivation of *E. coli* JWF1(DE3)/pJA1.147A under fermentor-controlled, glucose-rich conditions (Fig. 2) where glucose concentration is maintained at 10–30 g/L resulted in the synthesis of 0.23 g/L TAL in 0.08% yield (mol/

mol) from glucose. Cultivation of *E. coli* JWF1(DE3)/pJA1.147A under glucose-limited conditions (Fig. 2) resulted in the synthesis of 0.47 g/L of TAL in 0.4% yield (mol/mol) from glucose. Carbon balance experiments during fermentor-controlled cultivation of *E. coli* JWF1(DE3)/pJA1.147A revealed that 20–30% (mol/mol) of glucose was converted to biomass and 60% was lost as CO₂.

Heterologous Expression of *g2ps1* in *S. cerevisiae* From GPD, ADH1, and ADH2 Promoters

In *S. cerevisiae* strains INVSc1/pDX3.81, INVSc1/pDX3.82, and INVSc1/pDX3.85, the *g2ps1* gene was transcribed from promoters derived from the genes encoding for glyceraldehyde-3-phosphate dehydrogenase (*P_{GPD}*), alcohol dehydrogenase I (*P_{ADH1}*), and alcohol dehydrogenase II (*P_{ADH2}*), respectively (Pichuanes et al., 1996; Romanos et al., 1992). Unlike *P_{GPD}* and *P_{ADH1}* (Mumberg et al., 1995), transcription from *P_{ADH2}* is known to be repressed in the presence of glucose (Badziong et al., 1999; Lewin, 1997; Noronha et al., 1999), which required cultivation of *S. cerevisiae* INVSc1/pDX3.85 under glucose-limited conditions. This restriction was not a limiting factor as expression of *g2ps1* from *P_{GPD}* and *P_{ADH1}* promoters under glucose-rich culture conditions (Table I, entries 2 and 4) led to lower concentrations and yields of synthesized TAL.

S. cerevisiae INVSc1/pDX3.82, which relied on a *P_{ADH1}* promoter for *g2ps1* expression, synthesized the lowest concentrations and yields of TAL (Table I, entries 3 and 4) irrespective of the culture conditions employed. Under glucose-limited culture conditions, expression of *g2ps1* from a *P_{GPD}* promoter in *S. cerevisiae* INVSc1/pDX3.81 (Table I, entry 1) resulted in a higher specific activity for 2-PS relative to expression of *g2ps1* from a *P_{ADH2}* promoter in *S. cerevisiae* INVSc1/pDX3.85 (Table I, entry 5). However, *S. cerevisiae* INVSc1/pDX3.85 (Table I, entry 5) synthesized TAL in higher concentration and in a higher yield relative to *S. cerevisiae* INVSc1/pDX3.81 (Table I, entry 1). Examination of TAL accumulation in culture supernatants of *S. cerevisiae* INVSc1/pDX3.85, which expressed *g2ps1* from a *P_{ADH2}* promoter, revealed a steady increase in product concentration over the course of a 168 h fermentor run when cultured under glucose-limited conditions (Fig. 3). Expression of *g2ps1* from a *P_{GPD}* promoter in *S. cerevisiae* INVSc1/pDX3.81 and from a *P_{ADH1}* promoter in *S. cerevisiae* INVSc1/pDX3.82 did not sustain TAL production beyond 144 h and 120 h, respectively, when cultured under glucose-limited conditions (Fig. 3). In view of these results, genes encoding engineered TAL-synthesizing activity in *S. cerevisiae* constructs were expressed from *P_{ADH2}* promoters cultured under glucose-limited conditions.

Comparison of all TAL Producers Under Fed-Batch Fermentor Conditions

Although the *P_{ADH2}* was selected in this study for expression of genes encoding engineered TAL-synthesizing enzymes

Table I. The impact of the promoter employed for the expression of *g2ps1* on the concentrations and yields of TAL synthesized by *S. cerevisiae* constructs under fermentor-controlled conditions.

Entry	INVSc1/plasmid	Promoter	Glucose control	Specific activity ^a	TAL (g/L)	TAL yield ^b
1	pDX3.81	GPD	Limited	0.066	0.17	0.3
2	pDX3.81	GPD	Rich	N.D. ^c	0.11	0.3
3	pDX3.82	ADH1	Limited	0.047	0.08	0.1
4	pDX3.82	ADH1	Rich	N.D. ^c	0.04	0.1
5	pDX3.85	ADH2	Limited	0.036	0.37	0.7

^aHighest specific enzyme activity during the fermentor run in $\mu\text{mol}/\text{min}/\text{mg}$.

^b $\text{mol TAL}/\text{mol glucose consumed} \times 100\%$.

^cNot determined.

in *S. cerevisiae*, this promoter is repressed by glucose (Badziong et al., 1999; Lewin, 1997; Noronha et al., 1999). A strategy for synthesis of TAL was thus developed in which cells were grown in a bench-top fermentor where addition of glucose was regulated by dissolved oxygen concentrations (pO_2) in the medium. Using this method, the glucose concentration in the medium was maintained at a sufficiently low level to avoid repression of P_{ADH2} . The initial glucose concentration in the medium was 2 g/L, and the concentration of the glucose feed was 200 g/L. To ensure that glucose concentrations remained sufficiently low to avoid P_{ADH2} repression, the pump speed for the glucose feed was limited to 10 mL/h. A two-stage control strategy was developed in which pO_2 was maintained at 20% air saturation and pH was maintained at 5.0 for the first 48 h. These conditions resulted in maximum biomass formation (Fig. 4a). From 48 h until the end of the fermentation, pO_2 was maintained at 5% air saturation and pH was adjusted to 6.0 to achieve TAL production during the stationary phase of cell growth. This two-stage control profile for pO_2 and pH was used to examine synthesis of TAL (Fig. 4b) using *S. cerevisiae* strains INVSc1/pDX3.85, INVSc1/pWZ2/pWZ-PPT1, INVSc1/pZS1/pKOS12-128a, and INVSc1/pMR228/pKOS12-128a, which expressed *g2ps1*, the single mutant Y2226F *fasB*, the single mutant Y1572F *6-MSAS*, and the triple mutant G1419A-G1421P-G1424A *6-MSAS*, respectively. HPLC analysis of the culture medium of all four strains indicated that a compound with the same retention time as that of

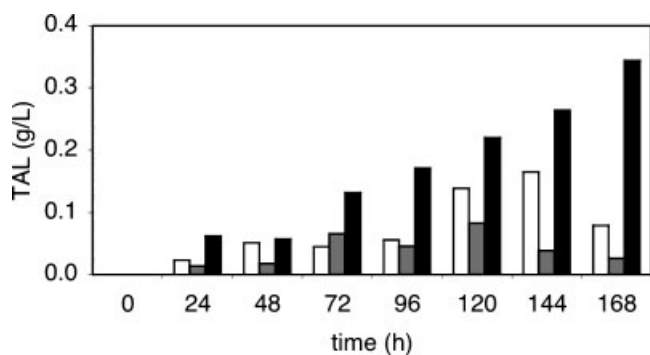


Figure 3. Time course of TAL synthesized under fermentor-controlled conditions by *S. cerevisiae* constructs as a function of the promoter used for expression of *g2ps1*: INVSc1/pDX3.81 (P_{GPD} , open bars); INVSc1/pDX3.82 (P_{ADH1} , gray bars); INVSc1/pDX3.85 (P_{ADH2} , black bars).

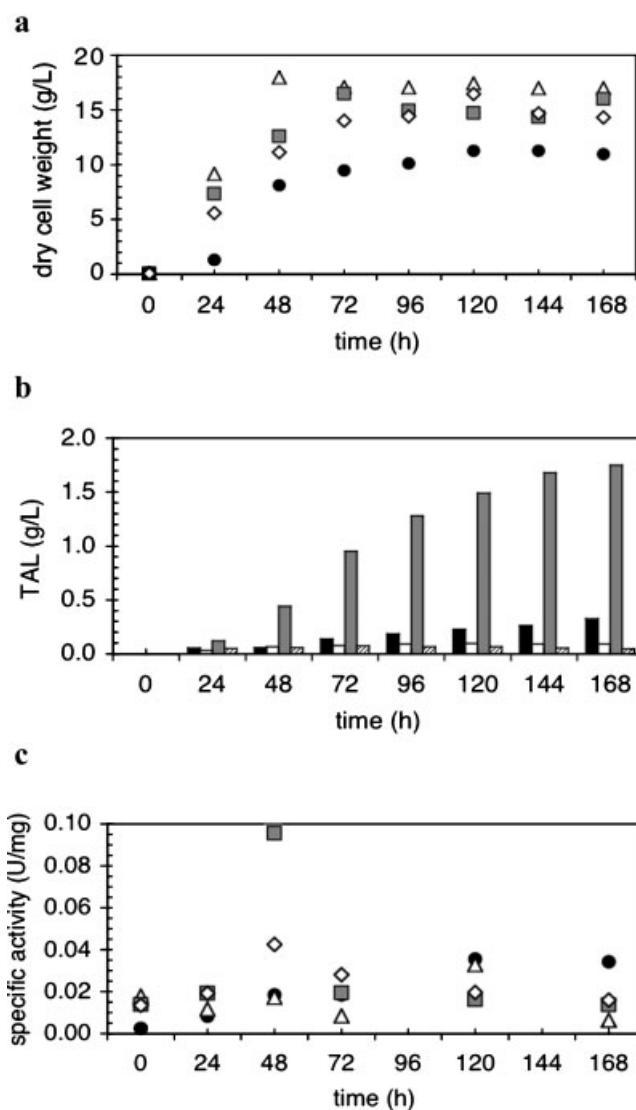


Figure 4. (a) Cell growth, (b) TAL synthesized, and (c) TAL-synthesizing specific activities (1 U = 1 $\mu\text{mol}/\text{min}$) for the fermentor-controlled cultivation of *S. cerevisiae* constructs: INVSc1/pDX3.85 (2-PS, black circles and bars); INVSc1/pWZ2/pWZ-PPT1 (Y2226F FAS-B, open triangles and bars); INVSc1/pZS1/pKOS12-128a (Y1572F *6-MSAS*, dark gray squares and bars) and INVSc1/pMR228/pKOS12-128a (G1419A-G1421P-G1424A *6-MSAS*, open diamonds and hashed bars).

authentic TAL was present. The compound was isolated from the cell-free broth of the INVSc1/pZS1/pKOS12-128a culture by continuous extraction into ethyl acetate and was subsequently crystallized from ethyl acetate to afford a 4% isolated yield of TAL. ¹H and ¹³C NMR spectra of this product were identical to those of authentic TAL.

Comparing *g2ps1* Expression in *E. coli* and *S. cerevisiae*

Similar concentrations of TAL were synthesized when *g2ps1* was expressed in *E. coli* JWF(DE3)/pJA1.147A (Table II, entry 1) and *S. cerevisiae* INVSc1/pDX3.85 (Table II, entry 2). Whereas *E. coli* JWF(DE3)/pJA1.147A synthesized a slightly higher TAL concentration relative to *S. cerevisiae* INVSc1/pDX3.85, the latter construct synthesized TAL in a better yield (Table II, entry 2 vs. entry 1). However, the yield of TAL synthesized from glucose was below 1% (mol/mol) irrespective of the host chosen for expression of *g2ps1*. The chief factor that ultimately favored use of *S. cerevisiae* for expression of genes encoding engineered TAL-synthesizing activity followed from the five-fold higher specific activity for 2-PS in *S. cerevisiae* INVSc1/pDX3.85 relative to *E. coli* JWF(DE3)/pJA1.147A (Table II, entry 2 vs. entry 1).

TAL-Synthesizing Activity

Enzymatic activity resulting in the synthesis of TAL was measured periodically throughout each fermentor run (Table II, and Fig. 4c). For the *S. cerevisiae* INVSc1/pZS1/pKOS12-128a and *S. cerevisiae* INVSc1/pMR228/pKOS12-128a, which expressed mutants of 6-MSAS, TAL-synthesizing activity reached a maximum approximately 48 h after initiation of the fermentor run (Fig. 4c). *S. cerevisiae* INVSc1/pZS1/pKOS12-128a, which expressed the Y1572F mutant of 6-MSAS, had more than twofold higher TAL-forming activity at 48 h relative to *S. cerevisiae* INVSc1/pMR228/pKOS12-128a, which expressed the triple mutant of 6-MSAS. At subsequent time points, however, these two constructs were found to have comparable levels of TAL-

synthesizing activity (Fig. 4c). Both *S. cerevisiae* INVSc1/pDX3.85, which expressed 2-PS, and *S. cerevisiae* INVSc1/pWZ2/pWZ-PPT1, which expressed the Y2226F mutant of FAS-B, showed similar low levels of TAL-synthesizing activity. Interestingly, the TAL-synthesizing activity associated with the 2-PS expressed by *S. cerevisiae* INVSc1/pDX3.85 remained stable while the TAL-synthesizing activity associated with the Y2226F mutant of FAS-B expressed by *S. cerevisiae* INVSc1/pWZ2/pWZ-PPT1 declined by the end of the fermentor run (Fig. 4c).

Product Concentrations and Yields

The concentrations and yields of TAL synthesized by the four *S. cerevisiae* strains expressing wild-type 2-PS, the Y2226F mutant of FAS-B, the Y1572F mutant of 6-MSAS, and the G1419A-G1421P-G1424A triple mutant of 6-MSAS are shown in Figure 4 and summarized in Table II. *S. cerevisiae* INVSc1/pZS1/pKOS12-128a, which expressed the Y1572F mutant of 6-MSAS (Table II, entry 4), synthesized the highest concentration (1.8 g/L) and the highest yield (6%) of TAL from glucose. *S. cerevisiae* INVSc1/pZS1/pKOS12-128a, which expressed the Y1572F mutant of 6-MSAS (Table II, entry 4), synthesized over 20-fold more TAL than did *S. cerevisiae* INVSc1/pMR228/pKOS12-128a, which expressed the triple mutant of 6-MSAS (Table II, entry 5). *S. cerevisiae* INVSc1/pZS1/pKOS12-128a also synthesized fivefold higher concentrations of TAL in 10-fold better yield than did *S. cerevisiae* INVSc1/pDX3.85, which was the strain expressing wild-type 2-PS (Table II, entry 2). The single mutation in the active site tyrosine of FAS-B expressed by *S. cerevisiae* INVSc1/pWZ2/pWZ-PPT1 (Table II, entry 3) did not lead to the same level of TAL synthesis as was realized with the comparable mutation in 6-MSAS in *S. cerevisiae* INVSc1/pZS1/pKOS12-128a (Table II, entry 4). Indeed, *S. cerevisiae* INVSc1/pWZ2/pWZ-PPT1 (Table II, entry 3), which expressed the FAS-B with the single mutation, along with *S. cerevisiae* INVSc1/pMR228/pKOS12-128a (Table II, entry 5), which expressed the triple mutant 6-MSAS, synthesized the lowest concentrations of TAL of the strains evaluated under fermentor-controlled conditions.

Table II. TAL synthesized under fermentor-controlled conditions by microbes expressing wild-type 2-PS and engineered mutants of FAS-B and 6-MSAS.

Entry	Host/plasmid(s)	Enzyme	Specific activity ^a	TAL (g/L)	TAL yield ^b
1 ^c	<i>E. coli</i> JWF1(DE3)/pJA1.147A	2-PS	0.007	0.47	0.4
2 ^d	<i>S. cerevisiae</i> INVSc1/pDX3.85	2-PS	0.036	0.37	0.7
3 ^d	<i>S. cerevisiae</i> INVSc1/pWZ2/pWZ-PPT1	FAS-B ^e	0.033	0.10	0.6
4 ^d	<i>S. cerevisiae</i> INVSc1/pZS1/pKOS12-128a	6-MSAS ^f	0.096	1.8	6
5 ^d	<i>S. cerevisiae</i> INVSc1/pMR228/pKOS12-128a	6-MSAS ^g	0.045	0.08	0.5

^aHighest specific activity during the fermentor run in $\mu\text{mol}/\text{min}/\text{mg}$.

^bmol TAL/mol glucose consumed $\times$ 100%.

^cT7 promoter.

^dADH2 promoter.

^eSingle (Y2226F) mutant.

^fSingle (Y1572F) mutant.

^gTriple (G1419A-G1421P-G1424A) mutant.

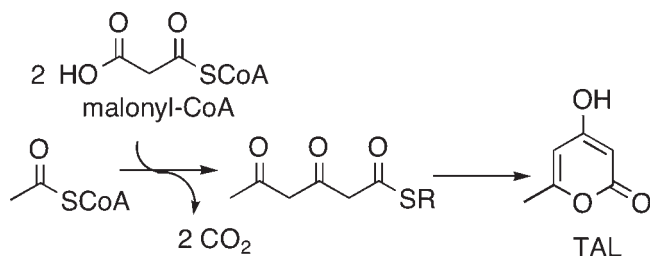


Figure 5. Biosynthesis of TAL by wild-type 2-PS.

DISCUSSION

Enzyme-catalyzed formation of a 3,5-diketohexanoate thioester (Figs. 5–7) and subsequent cyclization of this intermediate provides the basis for microbial syntheses of TAL. Formation and cyclization of a 3,5-diketohexanoate thioester (Fig. 5) is part of the reaction coordinate catalyzed by native 2-pyrone synthase. Cyclization of the 3,5-diketohexanoate thioester may be catalyzed by 2-PS active site residues or occur spontaneously. The *g2ps1* gene encoding 2-PS was isolated from the flowering plant *Gerbera hybrida* (Eckermann et al., 1998) and belongs to the chalcone synthase (CHS) superfamily of type III polyketide synthases (PKS) (Austin and Noel, 2003). Its native function is to catalyze TAL synthesis from acetyl-CoA via two sequential condensations with malonyl-CoA molecules (Fig. 5). In the absence of acetyl-CoA, 2-PS catalyzes the decarboxylation of malonyl-CoA to produce acetyl-CoA and proceeds with TAL biosynthesis, albeit at a slower rate (Eckermann et al., 1998; Jez et al., 2000a,b).

In this account, the heterologous expression of *g2ps1* in *E. coli* and *S. cerevisiae* cultured under fermentor-controlled conditions was demonstrated to lead to the synthesis and accumulation of TAL in culture supernatants. Such in vivo synthesis of TAL by an intact microbe expressing *g2ps1*-encoded 2-PS had not previously been demonstrated.

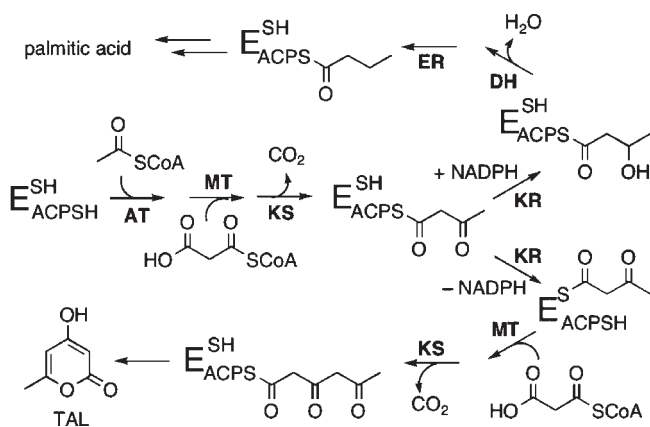


Figure 6. Biosynthesis of palmitic acid and TAL by FAS-B. Abbreviations: AT, acyl transferase; DH, dehydratase; ER, enoyl reductase; KR, ketoacyl reductase; KS, ketoacyl synthase; MT, malonyl transferase. +NADH, active KR; –NADH, inactive KR.

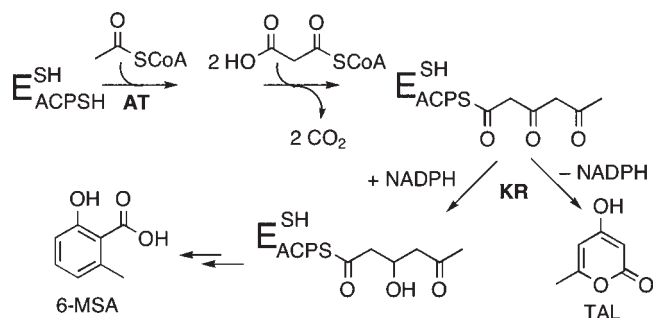


Figure 7. Biosynthesis of TAL and 6-methylsalicylic acid (6-MSA) by 6-MSAS. Abbreviations: AT, acyl transferase; KR, ketoacyl reductase. +NADH, active KR; –NADH, inactive KR.

Fermentor-controlled cultivation of *E. coli* JWF1(DE3)/pJA1.147A and *S. cerevisiae* INVSc1/pDX3.85 under glucose-limited conditions gave comparable concentrations (Table II) of TAL. However, the higher specific activity observed for 2-PS with expression of *g2ps1* in yeast (Table II) resulted in use of *S. cerevisiae* INVSc1 as the host for expression of all TAL-synthesizing enzyme activities. Previous examination of mutant G1419A-G1421P-G1424A 6-MSAS resulted in the observation of higher TAL concentrations synthesized by a *S. cerevisiae* construct relative to an *E. coli* construct when cultured under shake-flask conditions (Richardson et al., 1999). Higher concentrations (9,000-fold) of TAL were also synthesized under shake-flask conditions when Y2226F *fasB* was expressed in *S. cerevisiae* relative to *E. coli* (Zha et al., 2004). Comparison of the concentrations and yields of TAL synthesized by *S. cerevisiae* INVSc1 constructs expressing *g2ps1* from different promoters (Fig. 3, Table I) led to selection of an alcohol dehydrogenase II promoter (*P_{ADH2}*) for expression of all TAL-synthesizing enzymes in *S. cerevisiae* INVSc1.

Creation of TAL-synthesizing activity focused on genetic modification of enzymes that do not normally catalyze the formation along their reaction coordinate of a 3,5-diketohexanoate thioester. The bacterial type I fatty acid synthase B (FAS-B) from *B. ammoniagenes* (Meurer et al., 1992) is a large single multifunctional enzyme consisting of multiple functional domains. The ketoacyl synthase, dehydratase, enoyl reductase, ketoacyl-reductase, acyl transferase, and acyl carrier protein domains catalyze the sequential reactions leading to the biosynthesis of fatty acids such as palmitic acid (Fig. 6). Similarly, the fungal type I PKS 6-methylsalicylic acid synthase (6-MSAS) from *Penicillium patulum* (Beck et al., 1990; Schorr et al., 1994; Spencer and Jordan, 1992) is a large multidomain protein that catalyzes the multi-step biosynthesis of a partially reduced tetraketide in route to 6-methylsalicylic acid (Fig. 7). Although the exact boundary for each of the 6-MSAS functional domains had not previously been determined, a ketosynthase, an acyltransferase, a dehydratase, acyl carrier protein, and a ketoreductase domain were identified (Beck et al., 1990). Both *B. ammoniagenes* FAS-B (Hansen, 2002) and *P. patulum* 6-MSAS (Richardson et al., 1999) were previously shown to produce the unreduced

triketide TAL in vitro in the absence of NADPH (Figs. 6 and 7). This led to the hypothesis that elimination of the ketoreductase activity of FAS-B and 6-MSAS would result in TAL biosynthesis when expressed in an intact microbe.

Recently, we outlined a strategy for the rational design of a TAL-producing FAS-B mutant (Zha et al., 2004). Briefly, the ketoreductase domain and related key catalytic residues for *B. ammoniagenes* FAS-B were identified using bioinformatics tools. The FAS-B ketoreductase domain was shown to share signature features with other members of the short-chain dehydrogenase/reductase (SDR) superfamily of NAD(P)/NAD(P)H-dependent enzymes (Joernvall et al., 1995; Price et al., 2001). Shared features included the well-known Rossman fold motif (Chen et al., 1990; Rescigno and Perham, 1994), necessary for cofactor binding, and highly conserved Y²²²⁶XXXX catalytic residues (Oppermann et al., 1997), involved in hydrogen bonding and proton transfer. Replacement of catalytically active tyrosine with chemically inert phenylalanine in the Y2226F FAS-B mutant eliminated ketoreductase activity (Zha et al., 2004). A similar modification of the KR domain of modular PKS 6-deoxyerythronolide B (DEBS) had been demonstrated to result in the biosynthesis of unreduced products (Reid et al., 2003). Heterologous coexpression of Y2226F *fasB* and *ppt1*-encoded *B. ammoniagenes* phosphopantetheine transferase (Stuible et al., 1997, 1998), which was required for conversion of the expressed mutant apo-FAS-B to its functional holo form, resulted in the synthesis of 6 µg/L and 52 mg/L of TAL using *E. coli* and *S. cerevisiae* hosts, respectively, cultured under shake-flask conditions (Zha et al., 2004).

In parallel with the successful strategy employed to create a TAL-synthesizing mutant of FAS-B, a Y1572F mutant of 6-MSAS was constructed, which resulted in the inactivation of 6-MSAS ketoreductase activity. The KR domain of 6-MSAS was identified to be a member of the SDR superfamily of enzymes with a key Y¹⁵⁷²XXXN catalytic motif similar to that identified for FAS-B. Synthesis of TAL was evaluated upon coexpression in *S. cerevisiae* of the Y1572F mutant 6-MSAS and *sfp*, which encodes surfactin phosphopantetheinyl transferase from *Bacillus subtilis* for the apo-to-holo activation of the expressed mutant 6-MSAS (Kealey et al., 1998). A previous attempt to eliminate 6-MSAS ketoreductase activity focused on altering the NADPH-binding domain by mutating all three highly conserved glycine residues (G¹⁴¹⁹XGXXG) within its Rossman fold (Richardson et al., 1999). The resulting G1419A-G1421P-G1424A triple mutant 6-MSAS (A¹⁴¹⁹XPXXA) and the surfactin phosphopantetheinyl transferase (*sfp*) gene from *Bacillus subtilis* were coexpressed in *E. coli* and *S. cerevisiae*. TAL was reportedly detected in culture supernatants, although neither concentrations nor yields were reported. To effectively compare the two different strategies for transforming 6-MSAS into a TAL-synthesizing enzyme both Y1572F 6-MSAS and G1419A-G1421P-G1424A 6-MSAS were expressed from *P_{ADH2}* promoters in identical *S. cerevisiae* INVSc1 hosts and cultured under the same fermentor controlled conditions (Fig. 4, Table II).

CONCLUSION

2-PS encoded by *Gerbera hybrida* *g2ps1* provided the native TAL-synthesizing enzyme activity needed to establish the best microbial host (*S. cerevisiae*) and promoter (*P_{ADH2}*) for microbial synthesis of TAL from glucose (Fig. 2, Table II). This, in turn, provided the experimental benchmarks needed to compare strategies for creating TAL-synthesizing enzyme activity. The previously demonstrated strategy involving mutation (Y2226F) of the tyrosine active site residue of *B. ammoniagenes* FAS-B to a catalytically passive phenylalanine residue proved to be applicable to *P. patulum* 6-MSAS. *S. cerevisiae* INVSc1/pZS1/pKOS12-128a, which expressed the Y1572F 6-MSAS, synthesized the highest concentration and yield of TAL (Fig. 4, Table II). By contrast, *S. cerevisiae* INVSc1/pMR228/pKOS12-128a, which expressed G1419A-G1421P-G1424A 6-MSAS, synthesized the lowest concentrations of TAL (Fig. 4, Table II). Mutating the tyrosine catalytic residue of the KR domain is thus a more effective method than modifying the Rossman fold to inactivate 6-MSAS ketoreductase activity. *S. cerevisiae* INVSc1/pZS1/pKOS12-128a, which expressed the Y1572F 6-MSAS, also synthesized a higher concentration and yield of TAL relative to *S. cerevisiae* INVSc1/pDX3.85, which expressed *g2ps1* (Fig. 4, Table II). Created TAL-synthesizing enzyme activity thus proved to be more effective relative to native TAL-synthesizing enzyme activity in formulating an intact microbe capable of converting glucose into TAL under fermentor-controlled conditions.

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