ARTICLE

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Expanding the Modular Ester Fermentative Pathways for Combinatorial Biosynthesis of Esters From Volatile Organic Acids

Donovan S. Layton,[1,2] Cong T. Trinh[1,2,3]

[1]Department of Chemical and Biomolecular Engineering, University of Tennessee, Knoxville, Tennessee

[2]BioEnergy Science Center (BESC), Oak Ridge National Laboratory, Oak Ridge, Tennessee

[3]Bredesen Center for Interdisciplinary Research and Graduate Education, University of Tennessee, Knoxville, Tennessee; telephone: +865-974-8121; fax: 865-974-7076; e-mail: ctrinh@utk.edu

ABSTRACT: Volatile organic acids are byproducts of fermentative metabolism, for example, anaerobic digestion of lignocellulosic biomass or organic wastes, and are often times undesired inhibiting cell growth and reducing directed formation of the desired products. Here, we devised a general framework for upgrading these volatile organic acids to high-value esters that can be used as flavors, fragrances, solvents, and biofuels. This framework employs the acid-to-ester modules, consisting of an AAT (alcohol acyltransferase) plus ACT (acyl CoA transferase) submodule and an alcohol submodule, for co-fermentation of sugars and organic acids to acyl CoAs and alcohols to form a combinatorial library of esters. By assembling these modules with the engineered *Escherichia coli* modular chassis cell, we developed microbial manufacturing platforms to perform the following functions: (i) rapid in vivo screening of novel AATs for their catalytic activities; (ii) expanding combinatorial biosynthesis of unique fermentative esters; and (iii) upgrading volatile organic acids to esters using single or mixed cell cultures. To demonstrate this framework, we screened for a set of five unique and divergent AATs from multiple species, and were able to determine their novel activities as well as produce a library of 12 out of the 13 expected esters from co-fermentation of sugars and (C2-C6) volatile organic acids. We envision the developed framework to be valuable for in vivo characterization of a repertoire of not-well-characterized natural AATs, expanding the combinatorial biosynthesis of fermentative esters, and upgrading volatile organic acids to high-value esters.

Correspondence to: C.T. Trinh

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Introduction

Harnessing renewable or waste feedstocks (e.g., switchgrass, corn stover, agricultural residue, or municipal solid waste) can provide an alternative route for sustainable production of chemicals via fermentation while benefiting the environment and reducing our dependence on petroleum sources that are neither renewable nor sustainable (Bokinsky et al., 2011; Dale and Holtzapple, 2015; Silva et al., 2013; Thanakoses et al., 2003). The carboxylate platform is an emerging technology that can directly convert these renewable or waste feedstocks into the carboxylates, dominantly comprised of short-chain (C2-C6) volatile organic acids (VOAs, including acetic, propionic, butyric, pentanoic, and hexanoic acids) (Agler et al., 2011; Chang et al., 2010; Holtzapple et al., 2015). This technology employs robust consortia of microorganisms that are found in cattle rumens, anaerobic digesters, termite hindguts or swamps, and are very efficient for fermentation of renewable or waste feedstocks with minimum requirement of feedstock pre-processing, such as sterilization and biomass pretreatment. The carboxylates, after fermentation, can be upgraded to hydrocarbon fuels or chemicals via traditional chemical conversion (Agler et al., 2011; Holtzapple et al., 2015; Napora-Wijata et al., 2014). However, some current challenges to the carboxylate platform are to alleviate the toxicity of acids inhibiting microbes and carboxylate production, and to minimize the competitive conversion of sugars and/or carboxylates to unwanted byproducts such as methane, hydrogen, and/or carbon dioxide.

Different from the carboxylates, esters can be directly used with broader applications such as flavors, fragrances, solvents, and biofuels. By using alcohol acyl transferases (AATs), cells can

synthesize esters by condensing acyl CoAs and alcohols from biomass-derived fermentable sugars and/or organic acids. Recent studies have successfully engineered recombinant *E. coli* strains to produce ester platforms including acetate esters (Rodriguez et al., 2014; Tai et al., 2015; Tashiro et al., 2015) and butyrate esters (Layton and Trinh, 2014) from fermentable sugars. Anaerobic ester production can be captured by in situ fermentation and extraction, which can minimize product toxicity. In comparison to the carboxylates (Günther et al., 2011), esters constitute a larger space of novel and unique molecules (Layton and Trinh, 2014). Even though there is a great potential to upgrade the carboxylate platform to the ester platform via fermentation, the approach has not yet been demonstrated.

The key to expanding the ester platform from the carboxylate platform is to have novel AATs with desirable catalytic activities. However, the functions of these AATs are poorly understood even though AATs are abundant in nature, as found in fruits and plants. The main limitation is the characterization of these enzymes, which depends on the use of expensive, unstable substrates such as acyl CoAs and low throughput GC/MS screening methods. Currently, most of the AATs are characterized with a small library of substrates, primarily acetyl CoA and alcohols (Beekwilder et al., 2004; El-Sharkawy et al., 2005) to produce acetate esters; however, the activities of these AATs towards the biosynthesis of (>C2) acylates are poorly understood (Balbontín et al., 2010; Beekwilder et al., 2004; El-Sharkawy et al., 2005; Verstrepen et al., 2003). Therefore, the development of new analytical methods to characterize these abundant AATs with their novel potential functions together with assistance of bioinformatics (e.g., protein structure, molecular dynamic simulation Galaz et al., 2013; Morales-Quintana et al., 2011; Morales-Quintana et al., 2012) can provide fundamental understanding of functional roles of these AATs in their natural fruits, plants, and flowers as well as facilitate the upgrading of the carboxylate to ester platform for broad industrial applications.

In this study, we developed a general framework to upgrade the carboxylates to esters via co-fermentation of sugars and (C2-C6) VOAs that are dominant in the carboxylate platform. In this framework, we applied modular pathway engineering to design the acid-to-ester modules, consisting of an AAT (alcohol acyltransferase) plus ACT (acyl CoA transferase) submodule and an alcohol submodule, that generate acyl CoAs and alcohols from sugars and VOAs to form a combinatorial library of esters. By assembling these modules with the engineered *E. coli* modular chassis cell, we developed novel microbial manufacturing platforms and applied them for: (i) rapid in vivo screening of novel AATs for their catalytic activities; (ii) expanding the combinatorial biosynthesis of 12 unique fermentative esters, including acetate esters (e.g., ethyl acetate, acyl acetate) as well as acylate esters (e.g., ethyl acylate, acyl acylate); and (iii) demonstrating the biological upgrading of carboxylates to higher-value esters by using single and/or mixed cell cultures.

Materials and Methods

Strains and Plasmids

Strains

Table I shows a list of strains and plasmids used in this study. Both *Clostridium propionicum* and *Saccharomyces cerevisae* were obtained from the ATCC strain collection, and were used for genomic DNA extraction. *E. coli* TOP10 strain was used for

Table I. A list of strains and plasmids used in this study.

Plasmids/Strains	Genotypes	Sources
Plasmids		
pCP20	*flp, bla, cat, cI857λts*	Yale collection
pCOLA	kan^+	Novagen
pETite C-His	pBR322 *ori*; kan^+	Lucigen
pETite*	kan^R	Layton and Trinh (2014)
pCT24	pETite* P_{T7}::RBS::*pdc*::RBS::*adhB*::T_{T7}; kan^+	Layton and Trinh (2014)
pDL004	pETite* atf1; kan^+	This study
pDL005	pETite* atf2; kan^+	This study
pDL001	pETite* SAAT; kan^+	Layton and Trinh (2014)
pDL006	pETite* VAAT; kan^+	This study
pDL008	pETite* AeAT9; kan^+	This study
pDL009	pETite* P_{T7}::RBS::*pct*::RBS::*atf1*::T_{T7}::P_{T7}::RBS::*pdc*::RBS::*adhB*::T_{T7}; kan^+	This study
pDL010	pETite* P_{T7}::RBS::*pct*::RBS::*atf2*::T_{T7}::P_{T7}::RBS::*pdc*::RBS::*adhB*::T_{T7}; kan^+	This study
pDL011	pETite* P_{T7}::RBS::*pct*::RBS::SAAT::T_{T7}::P_{T7}::RBS:: *pdc*::RBS::*adhB*::T_{T7}; kan^+	This study
pDL012	pETite* P_{T7}::RBS::*pct*::RBS::VAAT::T_{T7}::P_{T7}::RBS:: *pdc*::RBS::*adhB*::T_{T7}; kan^+	This study
pDL013	pETite* P_{T7}::RBS::*pct*::RBS::AeAT9::T_{T7}::P_{T7}::RBS::*pdc*::RBS::*adhB*::T_{T7}; kan^+	This study
Strains		
C. propionicum	wildtype	ATCC 25522
S. cerevisiae	MAT a, *ura3d0, his3-d100, leu2-d0, met15-d0*	ATCC 201388
TOP10	F-*mcrA* Δ(*mrr-hsd*RMS-*mcr*BC)Φ80*lacZ* ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen
EcDL002	TCS083 (λDE3) Δ*fadE*::kan^- (cured)	Layton and Trinh (2014)
EcDL102	EcDL002 carrying pDL009; kan^+	This study
EcDL103	EcDL002 carrying pDL010; kan^+	This study
EcDL104	EcDL002 carrying pDL011; kan^+	This study
EcDL105	EcDL002 carrying pDL012; kan^+	This study
EcDL106	EcDL002 carrying pDL013; kan^+	This study

molecular cloning. For ester production, we deployed the engineered *E. coli* modular chassis cell EcDL002 as the ester production host (Layton and Trinh, 2014; Trinh et al., 2015). By transforming the modules pDL009-pDL013 into EcDL002 via electroporation (Sambrook, 2001), we created the ester production strains EcDL102-106, respectively.

Plasmids

In this study, the ester fermentative pathways were designed as exchangeable ester production modules that are assembled from multiple sub-modules (Layton and Trinh, 2014). The acid-to-ester production module consisted of a propionyl-CoA transferase (PCT, belonging to the general class of ACT) plus AAT submodule and the ethanol production submodule (Fig. 1). These submodules were organized in plasmids and transcribed by the T7 promoter. Table I shows a list of plasmids used and generated in this study, and Table II presents a list of primers used for the plasmid construction and validation. All plasmids were derived from the pETite* backbone (Layton and Trinh, 2014) and constructed by using the Gibson gene assembly method (Gibson et al., 2009).

Ethanol Submodule

The ethanol production submodule, pCT24, was designed to convert pyruvate to ethanol and was previously constructed (Layton and Trinh, 2014).

AAT Submodules

Five AAT genes, including ATF1 and ATF2 of *S. cerevisiae* (Verstrepen et al., 2003), SAAT of *Fragaria ananassa* (Aharoni et al., 2000), VAAT

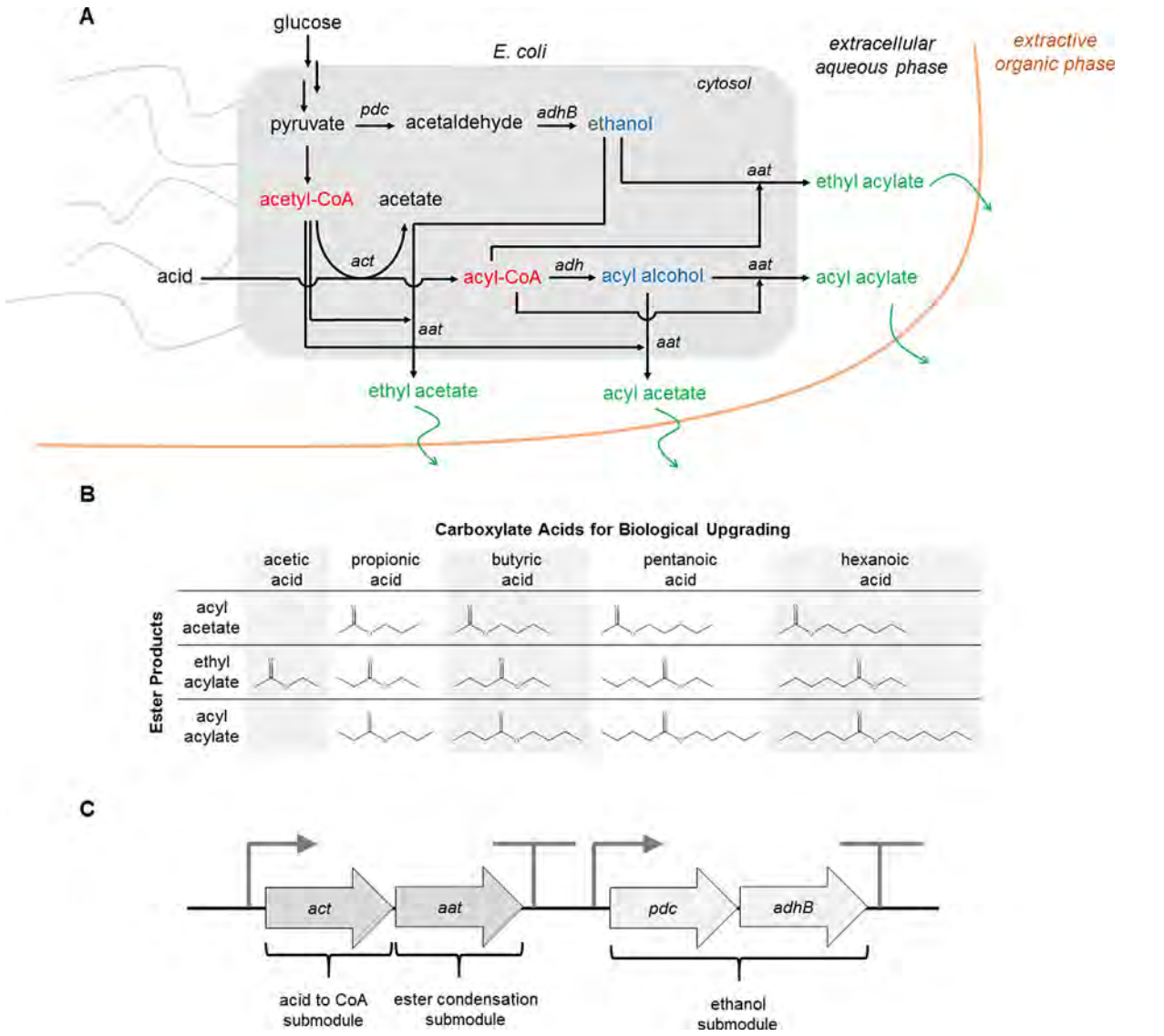

Figure 1. (**A**) Acid-to-ester pathways for combinatorial biosynthesis of esters from co-fermentation of sugars and fatty acids via in situ fermentation and extraction. (**B**) Potential esters produced from the carboxylate platform. (**C**) Design of the acid-to-ester production module.

Table II. A list of primers for plasmid construction and validation.

Primer name	Sequences
Primers used to build the AAT submodule (pDL004-006)	
DL_0001	5′-CATCATCACCACCATCACTAA-3′
DL_0002	5′-ATGTATATCTCCTTCTTATAGTTAAAC-3′
DL_0011	5′-GAAATAATTTTGTTTAACTATAAGAAGGAGATATACATATG GAGAAAATTGAGGTCAG-3′
DL_0012	5′-GGCGGCCGCTCTATTAGTGATGGTGGTGATGATGTTAAATT AAGGTCTTTGGAG-3′
DL_0017	5′-GAAATAATTTTGTTTAACTATAAGAAGGAGATATACATATG GAGAAAATTGAGGTCAG-3′
DL_0018	5′-GGCGGCCGCTCTATTAGTGATGGTGGTGATGATGCGGATAA CATACGTAGACCG-3′
DL_0019	5′-GAAATAATTTTGTTTAACTATAAGAAGGAGATATACATATG AATGAAATCGATGAGAA-3′
DL_0020	5′-GCCGCTCTATTAGTGATGGTGGTGATGATGCTAAGGGCCTA AAAGGAGAG-3′
DL_0021	5′-TAGAAATAATTTTGTTTAACTATAAGAAGGAGATATACATA TGGAAGATATAGAAGGATA-3′
DL_0022	5′-GCCGCTCTATTAGTGATGGTGGTGATGATGTTAAAGCGACG CAAATTCGC-3′
Primers used to build the pct_AAT+ethanol module (pDL009-013)	
DL_0023	5′-AAATAATTTTGTTTAACTATAAGAAGGAGATATACATATGA GAAAGGTTCCCATTATTAC-3′
DL_0024	5′-TCAGGACTTCATTTCCTTCAG-3′
DL_0014	5′-ATATCAAGCTTGAATTCGTTACCCGG-3′
DL_0015	5′-GGAGGAACTATATCCGGGTAACGAATTCAAGCTTGATATTA ATACGACTCACTATAGGG-3′
DL_0016	5′-GTCCAGTTACGCTGGAGTCTGAGGCTC-3′
DL_0013	5′-GAGCCTCAGACTCCAGCGTA-3′
DL_0002	5′-ATGTATATCTCCTTCTTATAGTTAAAC-3′
DL_0025	5′-CTGAAGGAAATGAAGTCCTGAAAGGAGATATACATATGAAT GAAATCGATGAGAAAAATC-3′
DL_0026	5′-GGGTCTGAAGGAAATGAAGTCCTGAAAGGAGATATACAT ATGGAAGATATAGAAGGATAC-3′
DL_0027	5′-TGGGTCTGAAGGAAATGAAGTCCTGAAAGGAGATATACAT ATGGAGAAAATTGAGGTCAG-3′
DL_0028	5′-TGGGTCTGAAGGAAATGAAGTCCTGAAAGGAGATATACATATGG AGAAAATTGAGGTCAG-3′
DL_0029	5′-GGGTCTGAAGGAAATGAAGTCCTGAAAGGAGATATACATA TGGCAAGCTCTGTGCGTCTG-3′
gBlock sequences for AeAT9	
AeAT9_1	5′-TAGAAATAATTTTGTTTAACTATAAGAAGGAGATATACATATGGCAAGCTCTGTGCGTCTGGTTAAAAAACCAGTCCTGGTTGCCCCG GTTGATCCGACCCCGAGCACCGTTCTGTCTCTGAGCTCCCTGGACTCTCAGCTGTTCCTGCGTTTCCCAATCGAATACCTGCTGGTTTAT GCTTCTCCGCATGGCGTCGACCGTGCGGTTACCGCGGCACGTGTGAAAGCAGCACTGGCCCGTTCCCTGGTTCCGTACTACCCGCTGGCG GGTCGCGTCAAGACCCGTCCGGATTCCACGGGCCTGGACGTGGTGTGCCAGGCTCAGGGCGCAGGCCTGCTGGAAGCAGTATCCGACT ACACTGCAAGCGACTTCCAGCGTGCGCCGCGTTCCGTAACCGAGTGGCGTAAACTGCTGCTGGTGGAAGTGTTCAAAGTCGTACCGCC GCTGGTTGTCCAGCTGACCTGGCTGAGCGACGGCTGCGTTGCGCTGGGCGTGGGTTTCAGCCACTGCGTGATCGACGGCATTGGTTCCTC TGAATTCCTGAACCTGTTCGCGGAACTGGCAACCGGTCGTGCCCGCCTGAGCGAATTTCAACCGAAGCCGGTTTGGGATCGCCACCTG CTGAACAGCGCAGGCCGTACTAACCTGGGCACCCACCCAGAATTCGGTCGCGTACCGGACCTGAGCGGCTTTGTAACGCGTTTTACCC AGGAGCGTCTGTCCCCTACTAGCATCACTTTTGACAAAAC-3′
AeAT9_2	5′-AGGAGCGTCTGTCCCCTACTAGCATCACTTTTGACAAAACTTG GCTGAAAGAACTGAAGAACATCGCAATGAGCACCAGCCAGCCG GGTGAATTCCCGTATACTTCCTTCGAAGTACTGTCCGGTCATATTTGGCGCTCCTGGGCCCGCTCTCTGAACCTGCCAGCGAAACAGGT GCTGAAACTGCTGTTTTCTATCAACATTCGCAATCGCGTGAAACCGTCCCTGCCAGCAGGTTACTACGGCAATGCTTTCGTGCTGGGCTG CGCGCAAACCAGCGTGAAGGATCTGACCGAAAAAGGCCTGGGTTATTGCGCCGATCTGGTGCGTGGTGCAAAAGAACGTGTTGGCGAC GAATACGCACGTGAAGTGGTTGAATCCGTGAGCTGGCCGCGTCGCGCTAGCCCGGATTCTGTAGGTGTTCTGATCATCTCCCAATGGTC TCGCCTGGGTCTGGACCGTGTTGACTTCGGTCTGGGCCGTCCGGTTCAGGTAGGTCCGATCTGCTGTGACCGTTACTGCCTGTTTCTGCC GGTTCGCGATCGTACTGAATCTGTAAAAGTTATGGTGGCGGTGCCGACCAGCGCAGTCGATCGCTACGAATACTTTATCCGTAGCCCGT ACAGCCATCATCACCACCATCACTAATAGAGCGGCCGCCACC-3′

of *F. vesca* (Beekwilder et al., 2004), and AeAAT of *Actinidia eriantha* (Günther et al., 2011), were harnessed to construct the AAT submodules that condense acyl CoAs and alcohols to produce esters. The SAAT submodule, pDL001, that contained the SAAT gene, was previously constructed (Layton and Trinh, 2014). To create the ATF1 submodule, pDL004, the ATF1 gene was amplified from the genomic DNA of *S. cerevisiae* by using the primers DL_0019/DL_0020, and inserted into the pETite* backbone amplified by using the primers DL_0001/DL_0002. To create the ATF2 submodule, pDL005, the ATF2 gene was amplified from the genomic DNA of *S. cerevisiae* by using the primers DL_0021/DL_0022, and inserted into the pETite* backbone. To create the VAAT submodule, pDL006, the VAAT gene was amplified from the plasmid pRSET-VAAT (Beekwilder et al., 2004) by using DL_0017/DL_0018, and inserted into the pETite* backbone. To create the AeAT9 submodule, pDL007, the AeAT9 gene was assembled from two gBlocks (e.g., AeAT9_1 and AeAT9_2) synthesized via IDTDNA (Coralville, Iowa, USA), and inserted into the pETite* backbone.

Acid-to-Ester Production Modules

Each acid-to-ester production module (including pDL009, pDL010, pDL011, pDL012, or pDL013) was created by assembling 4 DNA fragments including: (i) the PCT gene amplified from the genomic DNA of *C. propionicum* using the primers DL_0023/DL_0024; (ii) the ATF1 gene (amplified from the plasmid pDL004 using primers DL_0025/DL_0014), the ATF2 gene (pDL005, DL_0026/DL_0014), the SAAT gene (pDL001, DL_0027/DL_0014), the VAAT gene (pDL006, DL_0028/DL_0014), or the AeAT9 gene (pDL007, DL_0029/DL_0014); (iii) the ethanol submodule amplified from pCT24 using the primers DL_0015/DL_0016; and (iv) the pETite* backbone amplified using the primers DL_0013/DL_0002.

Media and Cell Culturing Conditions

Culture Media

For molecular cloning, the Luria-Bertani (LB) complex medium, that contained 5 g/L yeast extract, 10 g/L tryptone, 5 g/L NaCl, and 50 μg/mL kanamycin (if applicable), was used. For the acid-to-ester production experiments, the M9 hybrid medium (pH∼7) was used, consisting of 100 mL/L of 10× M9 salts, 1 mL/L of 1 M $MgSO_4$, 100 μL/L of 1 M $CaCl_2$, 1 mL/L of stock thiamine solution (1 g/L), 1 mL/L of stock trace metals solution (Trinh et al., 2008), 5 g/L yeast extract, 20 g/L glucose and 50 μg/mL kanamycin. The stock 10× M9 salt solution contained 67.8 g/L Na_2HPO_4, 30 g/L KH_2PO_4, 5 g/L NaCl, and 10 g/L NH_4Cl. In addition, about 2 g/L of each organic acid was supplemented to the M9 hybrid medium. The VOAs used for this study included acetic, propionic, butyric, valeric, and hexanoic acids that are dominantly found (>95%) in the carboxylate platforms (Holtzapple et al., 2015). For the acid-to-ester production experiments using mixed VOAs, we used the working concentration of 5 g/L with the composition mimicking those found in the countercurrent fermentation of corn stover by pig manure, for example, 40% (w/v) acetic acid, 18% propionic acid, 20% butyric acid, 11% pentanoic acid, and 11% hexanoic acid (Thanakoses et al., 2003).

Cell Culturing Conditions

In situ, high-cell density fermentation and extraction experiments were performed for ester production. Specifically, cells were grown overnight in 15 mL culture tubes containing the M9 hybrid medium, and subcultured the next morning until the exponential phase (OD_{600nm} ∼2.0, 1 OD ∼0.5 g DCW/L) was reached. Next, cells were transferred in a fresh M9 hybrid medium with an initial OD_{600nm} ∼0.05, grown to OD_{600nm} ∼5.0, and then induced with IPTG at a working concentration of 0.5 mM at 37°C for 30 min to activate the ester production modules. To set consistent characterization conditions among strains (e.g., fresh and consistent supply of glucose and other nutrients), cells were then spun down, resuspended in a fresh M9 hybrid medium containing ∼2 g/L organic acid and 0.5 mM IPTG, and transferred into 15 mL glass centrifuge tubes with a working volume of 10 mL. Each tube was overlaid with 1 mL hexadecane for in situ fermentation and extraction. The tubes were wrapped in PTFE tape to seal tube threading and capped to ensure complete anaerobic conditions. The residue oxygen in the medium and head space should be exhausted in less than 1 h.

Cells were grown on a 75° angled platform in a New Brunswick Excella E25 at 37°C and 175 rpm. Whole-cells and cell supernatants were collected and stored at −20°C for subsequent metabolite analysis while hexadecane overlay was stored at room temperature for ester analysis. All experiments were performed with at least three biological replicates.

Analytical Methods

High Performance Liquid Chromatography (HPLC)

Metabolites (sugars, organic acids, and alcohols) from culture supernatants were quantified by using the Shimadzu HPLC system equipped with the RID and UV-Vis detectors (Shimadzu, Inc., Columbia, MD), and the Aminex HPX-87H cation exchange column (BioRad, Inc., Hercules, CA). Samples were first filtered through 0.2-μm filter units, loaded into the column operated at 50°C, and eluded with the 10 mN H_2SO_4 mobile phase running at a flow rate of 0.8 mL/min.

Gas Chromatography Coupled With Mass Spectroscopy (GC/MS)

To identify and quantify esters being produced, GC/MS samples were processed from both the aqueous and organic phases from the in situ, high-cell density fermentation and extraction experiments. For the aqueous phase, 500 μL of whole-cells and supernatants were transferred to a 2 mL polypropelyene microcentrifuge tube with a screw cap containing 100–200 mg of glass beads (0.25–0.30 mm in diameter), 60 μL of 6 N HCl, and 500 μL of hexadecane solution containing 5.8 mg/L amyl acetate as an internal standard. The cells were lysed by bead bashing for 4 min using a Biospec Mini BeadBeater 16 and then centrifuged at 13,300*g* for 1 min. The GC/MS samples were collected from the organic layer and directly used for GC/MS runs.

For the organic phase from the in situ, high-cell density fermentation and extraction experiments, the extractants were diluted with hexadecane containing internal standard in a 1:1 (v/v) ratio, and then directly used for GC/MS runs.

All esters were analyzed by using the HP6890 GC/MS system equipped with a 30m × 0.25mm i.d., 0.25 μm film thickness column plus an attached 10 m guard column (Zebron ZB-5MS, Phenomenex, Torrance, CA) and a HP 5973 mass selective detector. A selected ion mode (SIM) method was deployed to analyze 1 μL of samples. The GC method was programmed with an initial temperature of 50°C with a 1°C/min ramp up to 58°C then a 25°C/min ramp was deployed to 235°C. The final ramp was then issued to a final temperature of 300°C at a rate of 50°C/min to elute any residual non desired analytes. The injection was performed using a splitless mode with an initial MS source temperature of 200°C. The carrier gas used was helium flowing at a rate of 0.5 mL/min. The detection of the desired products was accomplished using the following SIM parameters: (i) ions 45.00, 61.00, 70.00, and 85.00 detected from 0–5.40 min for ethyl acetate; (ii) ions 57.00, 74.00, and 102.00 detected from 5.40–7.20 min for ethyl propionate and propyl acetate where propyl acetate and ethyl propionate were separated further using their parent ions for quantification if necessary; (iii) ions 71.10, 88.10, and 116.00 detected from 7.20–7.71 min for ethyl butyrate; (iv) ions 57.00, 75.00, and 87.00 detected from 7.71–7.98 min for propyl propionate; (v) ions 56.00, 61.00, 73.00 from 7.98–9.40 min for butyl acetate; (vi) ions 61.00, 70.00, and 87.00 from 9.40–9.90 min for isoamyl acetate; (vii) ions 85.00, 88.00, and 101.00 from 9.90–10.20 min for ethyl pentanotate; (viii) ions 70.10 and 101.00 from 10.20–11.25 min for amyl acetate; (ix) ions 71.10, 89.10, and 101.00 for butyl butyrate from 11.25–11.53 min; (x) ions 60.00, 88.00, and 99.00 from 11.53–11.66 min for ethyl hexanoate; (xi) ions 56.00, 61.00, and 84.00 from 11.66–13.00 min for hexyl acetate; and (xii) ions 70.0, 85.00, and 103.00 from 13.00-t_{final} minutes for pentyl pentanoate.

For our GC/MS analysis, the lower limit for quantifying ester production is within the range of 10 μg/mL with good signal to noise ratio (>3:1).

Bioinformatics

For sequence alignment and phylogenetic analysis, each protein sequence was retrieved from NCBI, and was inputted into MEGA6 (Tamura et al., 2013) and aligned via MUSCLE (Edgar, 2004). The phylogenetic tree was generated using the neighbor-joining algorithm with a 1000 bootstrap value. Template proteins used for the analysis include: ATF1 (AJU14295.1, gene bank ID), ATF2 (AJR99982.1), SAAT (AAG13130.1), VAAT (AAN07090.1), and AeAT9 (HO772635).

Results

Establishing the Acid-to-Ester Fermentative Pathways in *E. coli*

VOAs, including acetic, propionic, butyric, pentanoic, and hexanoic acids, are the dominant byproducts of fermentation, for instance, for the carboxylate platform (Holtzapple et al., 2015). To upgrade these acids into high-value esters, we developed a general framework to engineer microbial cell factories that co-utilize fermentable sugars (e.g, glucose) and fatty acids (e.g., VOAs) for combinatorial biosynthesis of target esters (Fig. 1). Sugars are primarily used as the carbon and energy source for cell growth as well as production of enzymes and the precursor acetyl CoA for ester biosynthesis. We also engineered the heterologous ethanol pathway from *Zymomonas mobilis* for conversion of sugars into the additional precursor ethanol to expand the biosynthesis of the fermentative ester library. Under anaerobic conditions, fatty acids, that have higher degrees of reduction than sugars, are primarily converted to the precursors, acyl CoAs and acyl alcohols, for the ester biosynthesis. This general framework is tailored to generate a fermentative ester library including acetate esters (e.g., ethyl acetate, acyl acetate) and acylate esters (e.g., ethyl acylate, acyl acylate) via co-utilization of sugars and fatty acids. The esters produced were collected in the organic overlay during the in situ fermentation and extraction.

We designed the acid-to-ester pathways as production modules consisting of the ethanol submodule and the PCT plus AAT submodule. The ethanol module, containing PDC and AdhB of *Z. mobilis*, was previously engineered and functional (Layton and Trinh, 2014). The broad substrate specificity of the bi-functional aldehyde/alcohol dehydrogenase AdhB was purposely used in the design to reduce acyl CoAs to acyl alcohols. It should be noted that the endogenous bi-functional alcohol/aldehyde dehydrogenases, such as AdhE, can also reduce acyl CoAs to their respective acyl alcohols. To build the PCT plus AAT submodule, we chose the enzyme PCT that is known to exhibit broad substrate specificity towards various VOAs for producing acyl CoAs (Schweiger and Buckel, 1984). Due to the limited information about the catalytic and specific function of AATs towards production of our target esters, we first mined a library of potential AAT sequences from literatures, NCBI, and Uniprot database (Fig. 2) (Aharoni et al., 2000; Balbontín et al., 2010; Beekwilder et al., 2004; Cumplido-Laso

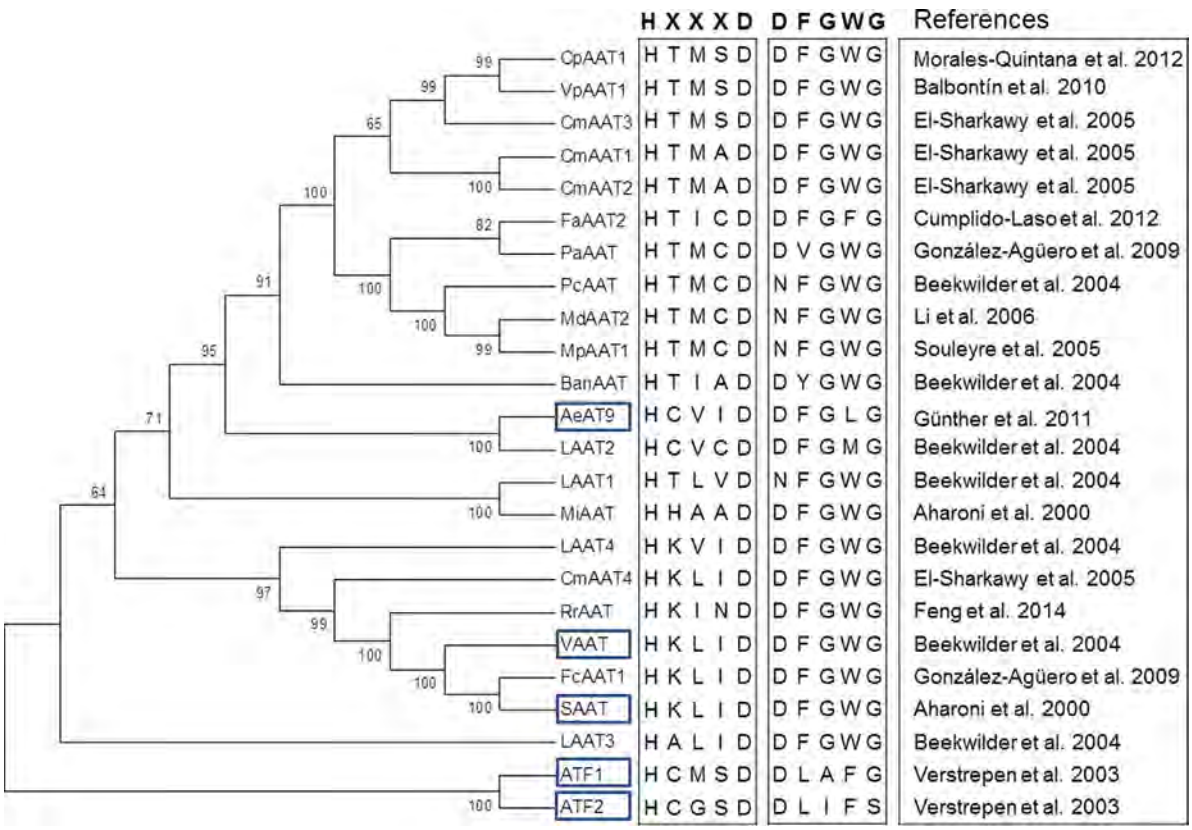

Figure 2. Phylogenetic analysis and structural and catalytic motifs of AATs. The conserved motifs are H-X-X-X-D for catalytic functions and D-F-G-W-G for structural integrity.

et al., 2012; El-Sharkawy et al., 2005; Feng et al., 2014; González-Agüero et al., 2009; Harada et al., 1985; Li et al., 2006; Lucchetta et al., 2007; Olías et al., 2002; Park et al., 2009; Perez et al., 1993, 1996; Shalit et al., 2001; Souleyre et al., 2005; Yahyaoui et al., 2002). From this library, we chose 5 AATs that were both similar in sequence and/or organism but divergent in substrate preference to test production of our target esters. These AATs belong to the BAHD acyltransferase superfamily (St-Pierre and Luca, 2000) and possess the conserved BAHD acyltransferase motifs, for example, H-X-X-X-D for catalytic functions and D-F-G-W-G for structural integrity (Galaz et al., 2013; Günther et al., 2011; Hansson et al., 2002; Ma et al., 2005). In this study, we chose ATF1 and ATF2 of *S. cerevisiae* because they are the most divergent AATs that have only 37% sequence identity between each other, and encompass the capability to convert C2-C6 alcohols to their respective acetate counterpart (Verstrepen et al., 2003). Both the SAAT and VAAT enzymes, derived from the same strawberry genus but different species, were chosen because they have relatively high protein sequence similarity (88% identity) and exhibit broad substrate specificity (Beekwilder et al., 2004). Finally, AeAT9 has divergent sequence identity from each of the other sequences used, and has also been shown to have broad substrate specificity (Günther et al., 2011).

We constructed a total of 5 acid-to-ester production modules, pDL009, pDL010, pDL011, pDL012, and pDL013, each of which contains the PCT plus AAT submodule and the ethanol submodule. All of these modules have the identical structural genes except AATs. Specifically, pDL009, pDL010, pDL011, pDL012, and pDL013, carry the ATF1, ATF2, SAAT, VAAT, and AeAT9 genes, respectively. By transforming these modules into the previously engineered modular chassis cell EcDL002, we created 5 microbial cell factories, EcDL102, EcDL103, EcDL104, EcDL105, and EcDL106, to evaluate the acid to ester conversion as well as specificity of AATs.

Exploring Combinatorial Biosynthesis of Esters

We characterized the strains EcDL102, EcDL103, EcDL104, EcDL105, and EcDL106 by the in situ, high-cell density fermentation and extraction method with co-fermentation of sugars and individual VOAs, including acetic, propionic, butyric, pentanoic, and hexanoic acids, that are dominant fermentative products of the carboxylate platform. This characterization method is designed for rapid in vivo screening of the acid-to-ester production modules for combinatorial biosynthesis of target esters and evaluation of the specificity of the AATs under the same physiological conditions. Throughout this section, we reported only the production of esters that are in the hexadecane overlay used for the in situ fermentation and extraction. For a reference to calculate the total ester production, one could estimate 80% of the esters were secreted extracellularly based on our previous study (Layton and Trinh, 2014), and $>$80% of these extracellular esters were extracted in the hexadecane overlay (Supplementary Fig. S1).

Ester Production From Co-Fermentation of Acetic Acid and Glucose

Strain characterization shows that most of the engineered strains produced ethyl acetate as the sole fermentative ester with different efficiency (Fig. 3A). Among the strains, EcDL105 produced ethyl acetate with the highest level of 19.64 ± 3.20 mg/L after 24 h while EcDL102, EcDL103, EcDL104, and EcDL106 generated 5.88 ± 0.58, 3.01 ± 0.56, 10.58 ± 3.61, and 1.19 ± 0.19 mg/L ethyl acetate, respectively. From these results, we arranged the AAT preference of ethanol and acetyl-CoA to produce ethyl acetate in the decreasing order as follows: VAAT (highest), SAAT, ATF1, ATF2, and AeAT9 (lowest). It should be noted that since we observed insignificant change in growth (OD$\sim$5–7) in our experiments, either ester titers (mg/L) or ester specific productivities (μg/g DCW/h) can be used to compare the AAT activities from the strains characterized. Beyond the presented figure, these values were also reported in the Supplementary Tables S1, S2.

Ester Production From Co-Fermentation of Glucose and Propionic Acid

Our acid-to-ester module design can potentially generate 2 acetate esters (e.g., ethyl acetate and propionyl acetate) and 2 propionate esters (e.g., ethyl propionate and propyl propionate) from co-fermentation of glucose and propionic acid. Strain characterization confirms production of these esters (Fig. 3B–E). However, none of the engineered strains alone were capable of producing all of the target esters likely because these strains expressed AATs with different specificities. Among the strains, EcDL105 produced the highest amount of total esters (86.81 ± 15.30 mg/L) whereas the total ester production were 5.12 ± 2.00 mg/L for EcDL102, 1.15 ± 0.29 mg/L for EcDL103, 22.62 ± 5.42 mg/L for EcDL104, and 1.87 ± 0.61 mg/L for EcDL106 (Fig. 3B–E, Supplementary Table S1). Consistent with the exogenous addition of acetic acid, most of the strains produced ethyl acetate, and EcDL105 produced the highest level of 18.69 ± 4.58 mg/L (Fig. 3B). For propyl acetate, EcDL104 produced the highest amount of 6.07 ± 1.87 mg/L while EcDL102, EcDL103, and EcDL105 generated 2.11 ± 0.71, 0.49 ± 0.09, and 0.88 ± 0.30 mg/L, respectively. EcDL106 did not produce propyl acetate (Fig. 3C).

The results show distinct substrate specificity among AATs for production of propionate esters. For ethyl propionate, EcDL105 produced the highest amount of 67.24 ± 10.41 mg/L, about 10.1 times higher than EcDL104 (5.60 ± 1.06 mg/L), 56.0 times higher than EcDL106 (1.20 ± 0.38 mg/L), and 395.5 times higher than EcDL103 (0.17 ± 0.14 mg/L) (Fig. 3D). EcDL102 did not produce any ethyl propionate. Like the propyl acetate production, EcDL104 generated propyl propionate at the highest level of 4.72 ± 0.87 mg/L while EcDL106 produced much less, 0.68 ± 0.23 mg/L. EcDL102 and EcDL105 did not produce any significant amount of propyl propionate (Fig. 3E).

Taken altogether, the results exhibit some notable trends of AAT specificity from co-fermentation of glucose and propionic acid. VAAT of EcDL105 has substrate specificity for ethanol and acetyl CoA/propionyl CoA to produce ethyl acetate and ethyl propionate while SAAT of EcDL104 has substrate specificity for propanol and acetyl CoA/propionyl CoA to produce propyl acetate and propyl propionate. In contrast, ATF1 shows substrate preference for ethanol/propanol and acetyl CoA to produce ethyl acetate and propyl acetate.

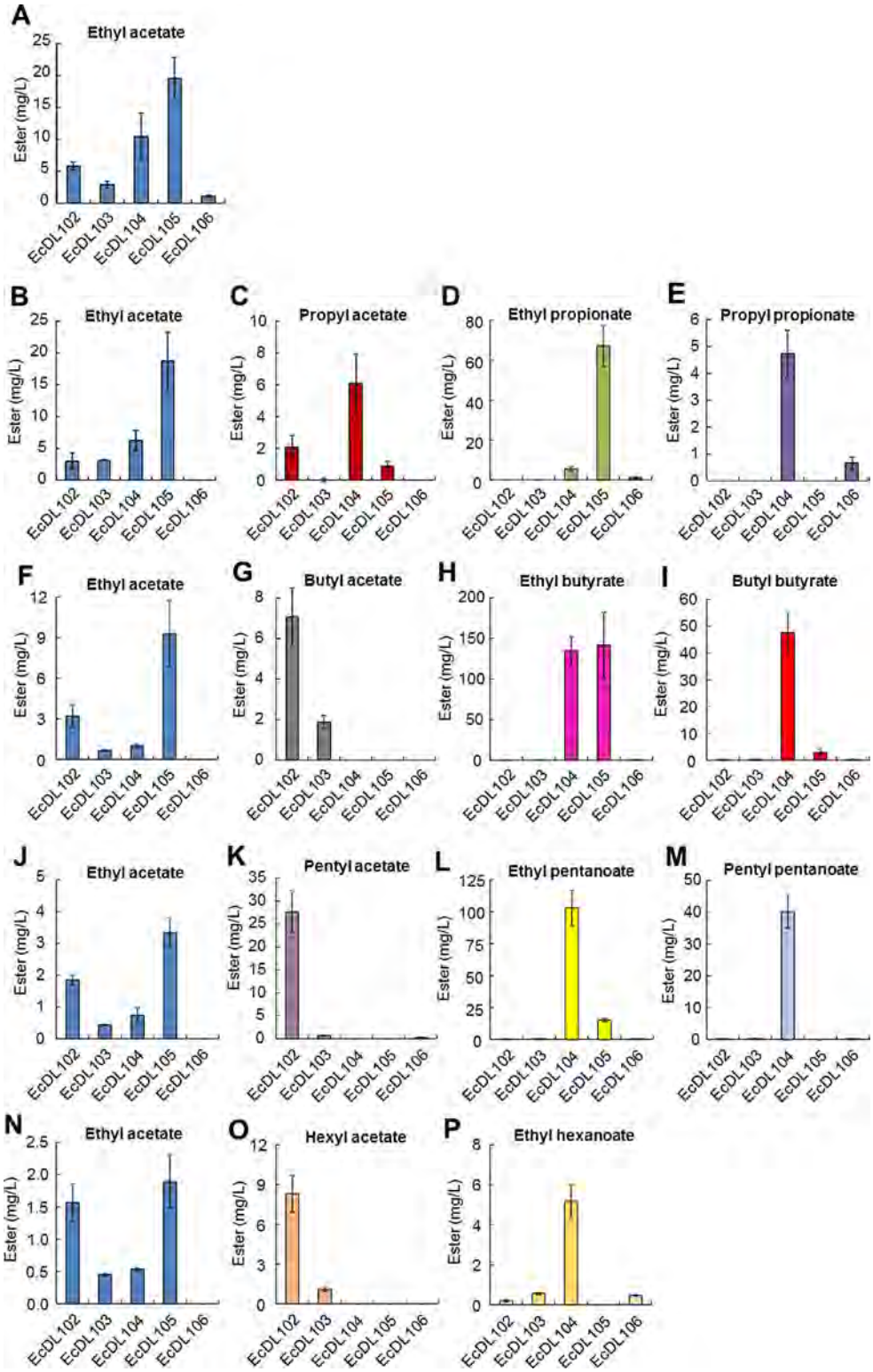

Figure 3. Ester production from exogeneous addition of (**A**) acetic acid, (**B**–**E**) propionic acid, (**F**–**I**) butyric acid, (**J**–**M**) pentanoic acid, and (**N**–**P**) hexanoic acid after 24 h. Each panel represents an ester that is produced by 5 microbial cell factories, EcDL102, EcDL103, EcDL104, EcDL105, and EcDL106 that express ATF1, AFT2, SAAT, VAAT, and AeAT9, respectively.

Ester Production From Co-Fermentation of Glucose and Butyric Acid

Strain characterization confirms the production of two acetate esters (e.g., ethyl acetate, butyl acetate) and two butyrate esters (e.g., ethyl butyrate and butyl butyrate) from co-fermentation of glucose and butyric acid (Fig. 3F–I). Both EcDL104 and EcDL105 produced the highest amount of total esters, for example, 183.09 ± 25.46 and 153.65 ± 44.00 mg/L, respectively while the total ester production were 10.69 ± 2.30 mg/L for EcDL102, 3.29 ± 0.46 mg/L for EcDL103, and 0.61 ± 0.55 mg/L for EcDL106 (Fig. 3F–I, Supplementary Table S1). Like the exogenous addition of propionic acid, none of the engineered strains alone was capable of producing all of these target esters. For the ethyl acetate production, the same trend of AAT specificity was observed, where EcDL105 produced the highest amount of 9.29 ± 2.45 mg/L among the strains characterized (Fig. 3F). For butyl acetate, only EcDL102 and EcDL103 produced at the levels of 7.08 ± 1.44 and 1.90 ± 0.31 mg/L, respectively (Fig. 3G). Clearly, ATF1 of EcDL102 has higher activity than ATF2 of EcDL103. In comparison with SAAT, VAAT, and AeAT9, both ATF1 and ATF2 have substrate preference for longer chain alcohol and acetyl CoA to produce acyl acetate.

In addition, the results show very distinct AAT specificities for butyrate ester production. Only EcDL104 and EcDL105 produced ethyl butyrate at relatively high levels of 134.43 ± 17.66 and 141.60 ± 40.20 mg/L, respectively (Fig. 3H). In contrast, all of the strains produced butyl butyrate (Fig. 3I). EcDL104 made butyl butyrate at the highest level of 47.63 ± 7.63 mg/L while EcDL102, EcDL103, EcDL105, and EcDL106 produced much less, 0.19 ± 0.03, 0.28 ± 0.03, 2.76 ± 1.35, and 0.17 ± 0.15 mg/L, respectively.

Taken altogether, we can draw some notable trends of substrate preference among AATs from co-fermentation of glucose and butyric acid. VAAT of EcDL105 again has substrate specificity for ethanol and acyl CoA/butyryl CoA to produce ethyl acetate and ethyl butyrate while SAAT of EcDL104 has substrate preferences for ethanol/butanol and butyryl CoA to produce ethyl butyrate and butyl butyrate. Contrastingly, both ATF1 and ATF2 have distinct substrate preference for butanol and acetyl CoA to produce butyl acetate.

Ester Production from Co-Fermentation of Glucose and Pentanoic Acid

Strain characterization shows that all four of the expected esters, including two acetate esters (e.g., ethyl acetate, pentyl acetate) and two pentanoate esters (e.g., ethyl pentanoate, pentyl pentanoate), were produced (Fig. 3J–M). EcDL104 produced the highest amount of total esters (143.85 ± 19.44 mg/L) while the total production were 29.85 ± 4.77 mg/L for EcDL102, 2.04 ± 0.25 mg/L for EcDL103, 18.76 ± 1.83 mg/L for EcDL105, and 1.12 ± 0.14 mg/L for EcDL106 (Fig. 3J–M, Supplementary Table S1). Even though the production of ethyl acetate was low, we observed the same trend of AAT specificity for ethyl acetate among the engineered strains (Fig. 3J). Like the exogenous addition of propionic and butyric acids, EcDL102 produced pentyl acetate at the highest level of 27.64 ± 4.57 mg/L while EcDL103 and EcDL106 produced this ester at significantly lower levels of 0.70 ± 0.09 and 0.30 ± 0.05 mg/L (Fig. 3K).

Likewise, EcDL104 and EcDL105 are the main producers of acylate esters. EcDL104 produced ethyl pentanoate at the highest level of 102.86 ± 13.92 mg/L, about 6.67 higher than EcDL105 (15.42 ± 1.38 mg/L) (Fig. 3L). It should be noted that this trend has switched from exogenous addition of butyric to pentanoic acids. EcDL102, EcDL103, and EcDL106 produced ethyl pentanoate at much lower levels of 0.18 ± 0.01, 0.62 ± 0.03, and 0.49 ± 0.05 mg/L, respectively. Besides high production of ethyl pentanoate, EcDL104 also made pentyl pentanoate at the highest level of 40.25 ± 5.28 mg/L (Fig. 3M).

Taken altogether, the results clearly show that VAAT of EcDL105 has substrate preference for ethanol and acetyl CoA/pentyl CoA to produce ethyl acetate and ethyl pentanoate for co-fermentation of glucose and pentanoic acid while SAAT of EcDL104 has distinct substrate preference for ethanol/pentanol and pentyl CoA to produce ethyl pentanoate and pentyl pentanoate. Different from both VAAT and SAAT, ATF1 of EcDL102 has high preference for pentanol and acetyl CoA to produce pentyl acetate.

Ester Production From Co-Fermentation of Glucose and Hexanoic Acid

Unlike exogenous addition of propionic, butyric, and pentanoic acids, only three out of 4 expected esters, including ethyl acetate, hexyl acetate, and ethyl hexanoate, were produced by co-fermentation of glucose and hexanoic acid (Fig. 3N-P). In comparison to other exogenous addition of acids, the total ester production was relatively low, 10.08 ± 1.73 mg/L for EcDL102 (highest), 2.14 ± 0.23 mg/L for EcDL103, 5.71 ± 0.86 mg/L for EcDL104, 1.89 ± 0.42 mg/L for EcDL105, and 0.47 ± 0.03 mg/L for EcDL106 (Fig. 3N–P, Supplementary Table S1). Production of ethyl acetate exhibited the same trend as exogenous addition of other VOAs (Fig. 3N). EcDL102 was the main producer of hexyl acetate, generating 8.32 ± 1.39 mg/L, about 7.43 times higher than EcDL103 (Fig. 3O).

Like exogenous addition of longer chain VOAs, EcDL104 was the main producer of ethyl hexanoate, 5.17 ± 0.84 mg/L while EcDL102, EcDL103, and EcDL106 produced this ester at significantly lower levels of 0.19 ± 0.05, 0.56 ± 0.04, and 0.47 ± 0.03 mg/L (Fig. 3P). However, we did not observe any significant production of ethyl hexanoate by EcDL105.

In summary, it is possible to produce 12 out of the 13 possible esters with exogenous addition of C2-C6 VOAs using the designed acid-to-ester production modules. The engineered strains EcDL102, EcDL103, EcDL104, EcDL105, and EcDL106 that carry these modules with different AAT specificities, generated 10, 11, 9, 6, and 9 unique esters, respectively. ATF1 of EcDL102, SAAT of EcDL104, and VAAT of EcDL105 were the most active among the five AATs tested for ester production, and exhibited distinct substrate specificity. ATF1 preferred acetyl CoA and acyl alcohols to produce acyl acetate with the highest activity toward pentanol (Supplementary Fig. S2A). Both SAAT and VAAT prefer acyl CoAs and ethanol to produce ethyl acylate (Supplementary Fig. S2A, 2B). However, VAAT is more specific to C2-C4 acyl CoAs while SAAT is more specific to C4-C6 acyl CoAs. Among the AATs characterized, SAAT is the only one that has dominant substrate preference towards acyl CoAs and acyl alcohols to produce acyl acylate with the highest activities

towards butyl butyrate and pentyl pentanoate (Supplementary Fig. S2C). In addition, it was observed that the production of esters was reduced for the fermentation of longer chain acids (e.g., hexanoic acid). This reduction might be caused by toxicity of the exogenous acids, converted intermediates, and/or substrate availability. Further studies beyond our proof of concept would need to be conducted to understand the causes.

Ester Production From Co-Fermentation of Glucose and Mixed VOAs

Use of Single Cultures for Upgrading Mixed VOAs to Target Esters

Anaerobic digestion, such as the carboxylate platform, is very robust for converting lignocellulosic biomass or biomass waste into C2-C6 VOAs that dominantly contain acetic, propionic, butyric, pentanoic, and hexanoic acids (Holtzapple et al., 2015). Since strains EcDL102, EcDL104, and EcDL105 have the most active AATs for production of ethyl acetate, acyl (>C2) acetate, ethyl acylate, and acyl (C>2) acylate, we investigated these acid-to-ester production platforms for their capabilities to upgrade mixed VOAs to high-value esters. Strain characterization shows that EcDL105 produced the highest amount of total esters (138.76 ± 35.42 mg/L), about 1.25 times slightly higher than EcDL104 (110.66 ± 14.10 mg/L) and 4.68 times higher than EcDL102 (29.67 ± 6.13 mg/L) after 24 h (Fig. 4, Supplementary Table S3).

EcDL102 produced 7 out of 10 esters that were produced from exogenous addition of individual VOAs. Consistently, acyl acetates were the most dominant products with high production levels of ethyl acetate (10.45 ± 2.58 mg/L) and pentyl acetate (9.46 ± 1.65 mg/L) (Fig. 4A and B, Supplementary Table S3). EcDL104 produced all 9 esters that were observed from single addition of VOAs. Different from EcDL102, EcDL104 primarily produced ethyl acylate with the highest production of ethyl butyrate (56.80 ± 8.42 mg/L) followed by ethyl pentanoate (40.86 ± 3.85 mg/L) (Fig. 4A and C, Supplementary Table S3). EcDL104 also produced acyl acylate, for example, butyl butyrate at the highest level of 2.32 ± 0.14 mg/L among significantly lower levels of propyl propionate and pentyl pentanoate. Likewise, EcDL105 produced a total of 8 esters. Interestingly, two additional esters, propyl propionate and ethyl hexanoate, that were not observed from single addition of VOAs, were also produced in small quantities, possibly due to the less inhibition of acids and availability of ester precursors. Similar to EcDL104, EcDL105 primarily produced ethyl butyrate (101.77 ± 25.16 mg/L), followed by ethyl propionate (15.88 ± 4.93 mg/L) and ethyl pentanoate (7.10 ± 1.17 mg/L) (Fig. 4A and D, Supplementary Table S3). Consistent with single exogenous addition, EcDL105 produced more ethyl acetate than EcDL102 and EcDL104.

Overall, the total ester production levels and the distribution of ester products from the co-fermentation of glucose and mixed VOAs are very consistent with the substrate specificities of ATF1 (of EcDL102), SAAT (of EcDL104), and VAAT (of EcDL05) that were identified from exogenous addition of individual VOAs.

Use of Mixed Cultures for Upgrading Mixed VOAs to Target Esters

To produce the entire spectrum of target esters from co-fermentation of glucose and mixed VOAs, we used the mixed cultures of EcDL102, EcDL104, and EcDL105 in equal amount for the in situ fermentation and extraction. Strain characterization, indeed, confirms the production of 12 out of 13 expected esters except hexyl hexanoate, which is consistent with the exogenous addition of individual VOAs (Fig. 4A and E, Supplementary Table S3). The total ester production of the mixed cultures was 83.64 ± 13.43 mg/L, lower than the single cultures of EcDL104 and EcDL105 but higher than the single culture of EcDL102.

Among the esters, ethyl butyrate was produced at the highest amount (44.34 ± 5.02 mg/L) followed by ethyl pentanoate (17.49 ± 1.24 mg/L). The results are expected because both SAAT (of EcDL104) and VAAT (of EcDL105) are specific to ethyl acylate production and have higher activities than ATF1 (of EcDL105) that is specific to acyl acetate production.

Discussion

In this study, we established a general framework for upgrading VOAs to high-value esters that have broad industrial applications such as flavors, fragrances, solvents, and biofuels. By assembling the acid-to-ester modules with the established *E. coli* modular chassis cell, we developed microbial manufacturing platforms to perform the following functions: (i) rapid in vivo screening of novel AATs for their catalytic activities; (ii) expanding the combinatorial biosynthesis of unique fermentative esters; and (iii) upgrading carboxylates to higher-value esters using single or mixed cell cultures.

Among the five AATs screened, ATF1 of *S. cerevisiae*, SAAT of *F. ananassa*, and VAAT of *F. vesca* were the most active towards the carboxylate library, and exhibited activities for 10, 9, and 9 out of 13 unique fermentative esters, respectively. ATF1, SAAT, and VAAT had substrate preference for biosynthesis of acyl (C4-C6) acetate, ethyl (C2-C4) acylate, and ethyl (C4-C6) acylate, respectively. Different from ATF1 and VAAT, SAAT also exhibited high substrate preference for biosynthesis of acyl acylates (e.g., propyl propionate, butyl butyrate, and pentyl pentanoate). Based on the total ester production from exogenous addition of single or mixed VOAs, both VAAT and SAAT had higher activities than ATF1. Examination of the highly conserved catalytic (H-X-X-X-D) and structural (D-F-G-W-G) motifs (Fig. 2) offered some hypothesis of substrate specificity for the characterized AATs. However, there are more factors influencing substrate specificity than simply the catalytic and structural motifs; for instance, SAAT and VAAT have both identical catalytic and structural motifs but different substrate specificity. Using molecular dynamics simulations should help elucidate modes of action for substrate specificity, and guide protein engineering efforts of the characterized, and other, AATs for in vivo engineering of ester formation (Galaz et al., 2013; Morales-Quintana et al., 2012, 2013).

The conventional method to characterize AAT activities is time-consuming and expensive because this method: (i) requires protein expression, purification, and characterization; (ii) uses expensive substrates (e.g., acyl CoAs) that sometimes are not available and

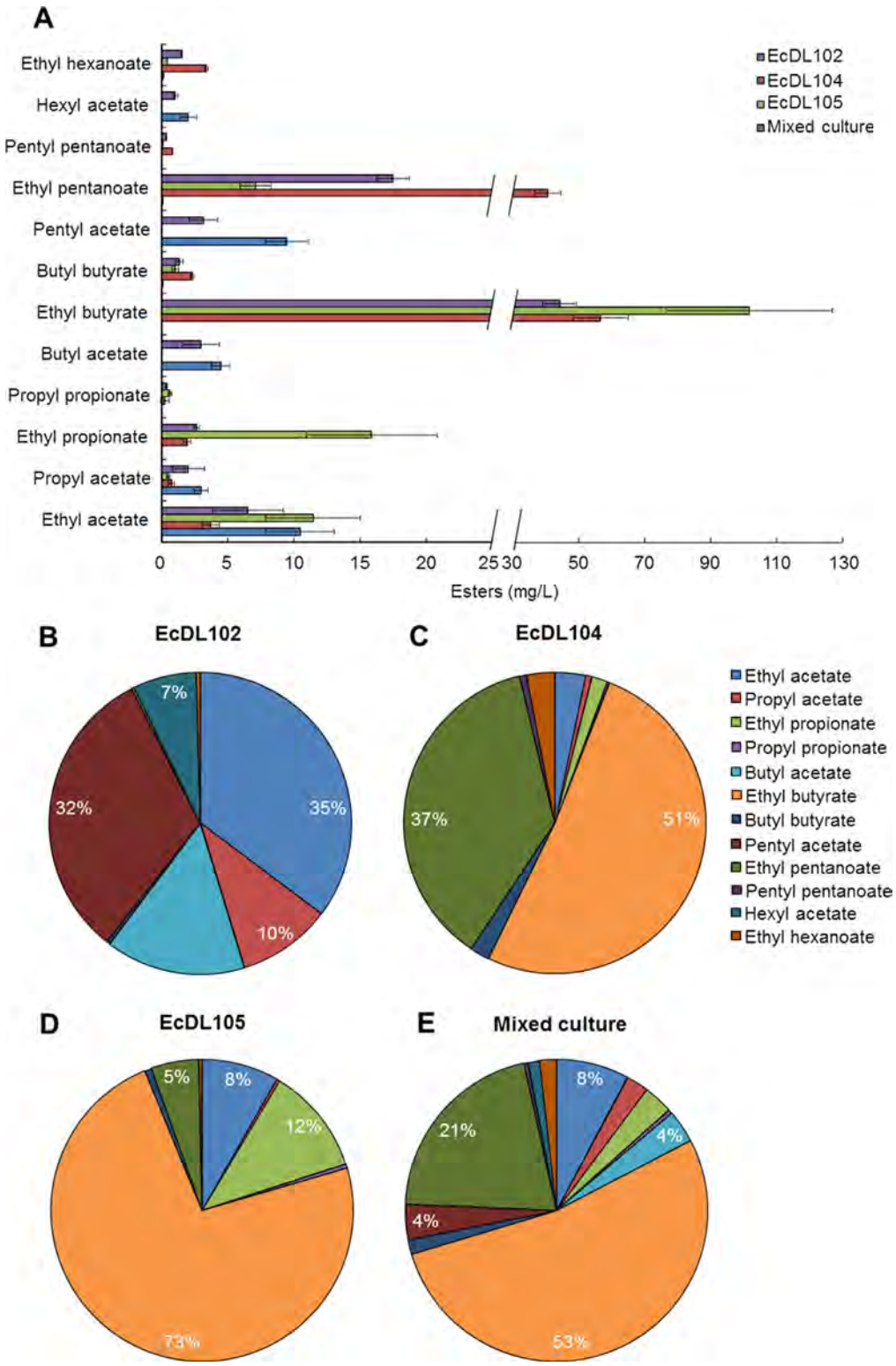

Figure 4. (**A**) Ester production and (**B-E**) fraction of ester profiles of EcDL102, EcDL104, EcDL105, and mixed cultures of EcDL102, EcDL104, and EcDL105 for co-fermentation of glucose and 5 g/L total mixed organic acids after 24h. A color version of this figure is available online.

hence limit the space of substrates tested; and (iii) relies on the low throughput GC/MS method to quantify esters produced. In contrast, our in vivo screening method developed can address some of these limitations. By using cheap substrates such as mixed VOAs, the in vivo method can screen for AAT activities for combinatorial biosynthesis of more than 10 unique esters simultaneously, and access their relative substrate preferences as determined for ATF1 of EcDL102, SAAT of EcDL104, and VAAT of EcDL105 in our studies. In addition, the in situ high cell density fermentation and extraction eliminate many steps of protein expression, purification, enzyme assay, and ester extraction for GC/MS while evaluating the functional in vivo activities of AATs in heterologous hosts. In our study, the in vivo method not only confirmed the known activities of ATF1, SAAT, and VAAT for the biosynthesis of acetate esters but also discovered their new catalytic functions towards the biosynthesis of ethyl acylate, acyl acylate, and perhaps more. The knowledge of AAT activities will be useful for fundamental understanding of their functional roles in natural environments such as fruits and flowers as well as for elucidation of protein structures and catalytic functions for rational novel protein engineering.

The design of the acid-to-ester modules is quite flexible to enable expansion of the combinatorial biosynthesis of esters. The acid-to-ester module of this study can potentially generate a set of 13 unique esters by using a mixture of short, linear (C2-C6) VOAs that are dominantly found in the anaerobic digestion of lignocellulosic biomass or biomass wastes. By replacing the ethanol submodule with other branched, short-chain alcohol modules (e.g., isopropanol, isobutanol, isopentanol, and isopentenol), we can potentially generate a library of 24 additional unique esters. By manipulating AATs with controllable specificities as well as employing single and/or mixed cell cultures for fermentation, it is possible to tailor the acid-to-ester modules to produce desirable esters as high-purity chemicals or mixed, designer bioesters.

VOAs investigated in this study are the dominant fermentative products of the carboxylate platform (e.g., anaerobic digestion of lignocellulosic biomass or biomass wastes) and have great potential for downstream upgrading to fuels and high-value chemicals via chemical conversion (Agler et al., 2011; Holtzapple et al., 2015). Our study shows the alternative microbial conversion route that can be deployed for directly upgrading these VOAs to higher-value esters. Since VOAs are toxic to cells, upgrading these VOAs to esters for convenient extraction helps alleviate the acid toxicity while producing higher-value chemicals. This approach can be potentially realized by deploying engineered, compatible acid-to-ester strains to the microbial communities of the anaerobic digester. Even though the proof-of-concept for upgrading VOAs to esters is demonstrated, the yields, titers, and productivities for the acid-to-ester conversion are low (Supplementary Tables S1–S5) likely due to VOA toxicity, non-optimized cultivation conditions, non-optimized acid-to-ester production modules, and/or appropriate choice of the production hosts. In our study, relatively high amount of ethanol secreted to the medium (see Supplementary Table S5) implies that carbon fluxes through ACT and/or AAT submodules might have been limiting potentially due to low catalytic efficiency and poor protein expression/folding of ACT and AATs (Zhu et al., 2015). Addressing these limitations is necessary for enhanced acid-to-ester conversion in the future study.

In summary, we envision that our developed framework is valuable and powerful for in vivo characterization of a repertoire of not-well-characterized natural AATs, for expanding the combinatorial biosynthesis of fermentative esters, and for upgrading volatile organic acids to higher-value esters.

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