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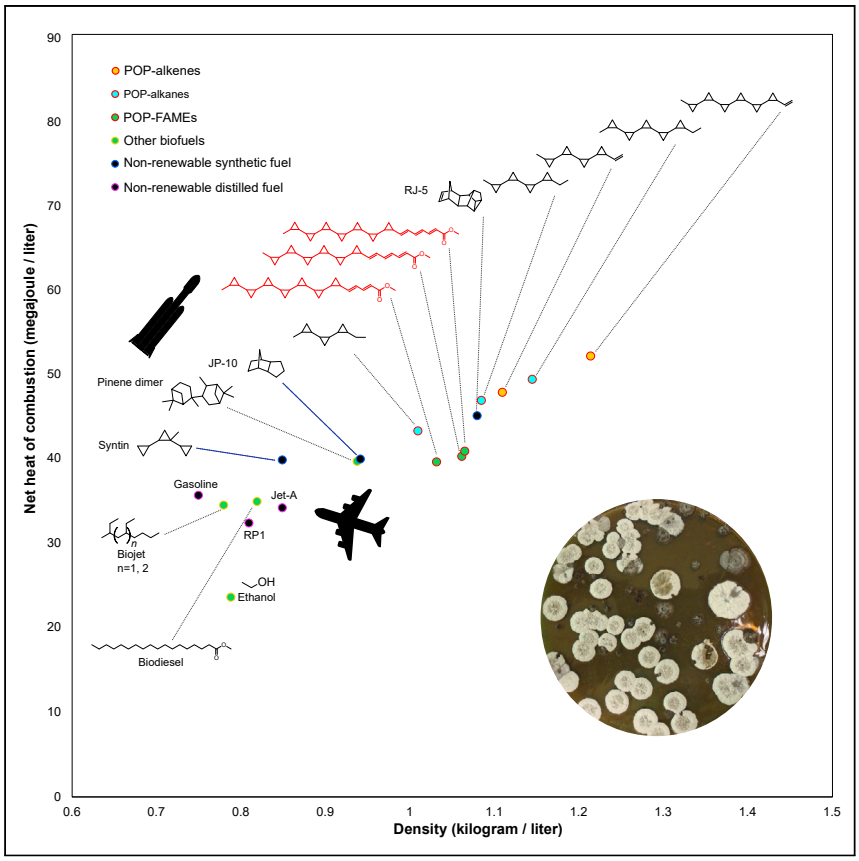

We produced new energy-dense biofuels that can replace rocket and jet fuels. The biosynthesis of the fuel precursor, a fatty acid, is directed by an iterative polyketide synthase (iPKS) that was discovered using phylogenomic methods. Production of the fatty acids was achieved after introduction of the iPKS in a *Streptomyces* host. The fuels were then obtained by esterification of the fatty acids. The energy density of the molecules was calculated and resulted higher than those of current fuels used in the aerospace sector.

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Highlights

Rocketry, aviation, and shipping require hard-to-replace, energy-dense fossil fuels

Cylopropanated fuels are energy dense, but their synthesis is challenging

We produced polycyclopropanated (POP) fuels using a bacterial host

The POP biofuels can have higher energy density than current aerospace fuels

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Biosynthesis of polycyclopropanated high energy biofuels

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SUMMARY

Cyclopropane-functionalized hydrocarbons are excellent fuels due their high energy density. However, the organic synthesis of these molecules is challenging. In this work, we produced polycyclopropanated fatty acids in bacteria. These molecules can be converted into renewable fuels for energy-demanding applications such as shipping, long-haul transport, aviation, and rocketry. We explored the chemical diversity encoded in thousands of bacterial genomes to identify and repurpose naturally occurring cyclopropanated molecules. We identified a set of candidate iterative polyketide synthases (iPKSs) predicted to produce polycyclopropanated fatty acids (POP-FAs), expressed them in *Streptomyces coelicolor*, and produced POP-FAs. We determined the structure of the molecules and increased their production 22-fold. Finally, we produced polycyclopropanated fatty acid methyl esters (POP-FAMEs). Our POP fuel candidates can have net heating values of more than 50 MJ/L. Our research shows that the POP-FAMEs and other POPs have the energetic properties for energy-demanding applications for which sustainable alternatives are scarce.

INTRODUCTION

Freeing the global economy from its dependence on petroleum is necessary to slow the pace of climate change. Advances in agriculture and biotechnology have enabled large-scale production of ethanol and biodiesel, both of which are viable replacements for some fossil fuels. The biofuels available today, in combination with increasing electric vehicle adoption and a decarbonization of the electric grid, are central to reducing petroleum demand and greenhouse gas (GHG) emissions in light-duty transportation. By 2030, light-duty EVs will represent a projected 8%–14% of the global light vehicle fleet and 17%–33% of new vehicle sales.[1] Despite these advances, replacing fossil fuels in applications that are more difficult to electrify such as long-haul terrestrial, maritime, and aerial cargo transport; heavy-duty machinery; transatlantic aviation; and rocketry is still challenging. These applications require high power and energy-to-weight ratios and, as a result, rely on energy-dense fuels.

The military and commercial aerospace sectors rely on petroleum distillates rich in cyclic and branched alkanes,[2] such as Jet-A (aviation), RP1 (rocketry), and synthetic polymers with high energy density such as hydroxyl-terminated polybutadiene

Context & scale

Freeing the global economy from its dependance on petroleum is key to slow down the pace of climate change. Energy-demanding applications like rocketry, aviation, and shipping are fueled with petroleum-derived hydrocarbons that are difficult to replace. These fuels are rich in cyclic molecules with strained bond angles allowing them to store more energy than non-cyclic molecules. The highest amount of energy can be stored in cyclopropanes, but these molecules are hard to produce via organic synthesis.

We produced polycyclopropanated fatty acid methyl ester (POP-FAME) fuels in bacteria. The POP-FAMEs can have energy densities of more than 50 MJ/L, which is larger than the energy of the most widely used rocket and aviation fuels. Although the next step is to scale up their production until the process is commercially viable, the availability of a biobased production method opens the possibility to replace fossil fuels in a very constrained sector.

(HTPB, rocketry). Marine transport of cargo relies on bunker fuel, which comprise heavier fractions (residual) produced through the distillation of petroleum and emits substantial NO_x, SO_x, and particulate matter to the atmosphere when combusted.[3]

Shipping is responsible for 1 billion tons of CO_2 and GHGs per year, accounting for around 3% of global emissions,[4] whereas aviation accounted for 2% with 915 million tons of CO_2 in 2019.[5,6] As climate impacts of light-duty transportation and electricity decline, these difficult-to-decarbonize sectors will increase as a share of global emissions in the next three decades. No reliable data are available for rocketry, a largely unregulated sector whose impact is considered insignificant. However, the current growth of the sector, including an increase in space tourism and other private-sector activities, puts it on a trajectory of inevitable collision with the objectives of climate change policy.[7] Given this outlook, new sustainable alternatives for high energy-demanding sectors are urgently needed.

In cyclic alkanes such as those used in aerospace applications, where the C–C bonds form angles other than 109.5°, the straining of the bonds causes an increase in the fuels' net heat of combustion. Angle strain has been used for production of tactical fuels with high energy density such as the bioderived pinene dimers and the synthetic JP-10 and RJ-5, all of them bearing strained ring systems.[8,9] The highest tension and heat of combustion possible is found in cyclopropanes (CPs), which have 60° bond angles. This property was exploited in Syntin, a synthetic fuel developed during the Soviet era for the 11D58M engine originally designed for kerosene/oxygen as fuel and oxidant, respectively. Syntin/oxygen was successfully used in the upper stages of the Soyuz and Proton rockets. Syntin allowed for a 3% increase in impulse equivalent to 200 kg in payload.[10–12] However, organic synthesis of Syntin and polycyclopropanated (POP) compounds in general is challenging, costly, and involves toxic and hazardous intermediates derived from petroleum,[13,14] making them a non-viable option for fuel production. Nevertheless, the performance of Syntin demonstrates that incorporation of multiple CP rings into hydrocarbon fuels can yield high energy density. In fact, recent work on catalytic cyclopropanation of bioderived terpenes has led to excellent candidates for high-performance jet fuel blend stocks or specialized rocket propellant.[15]

In this work, we designed POP molecules that can be used as high energy fuels. We explored the chemical diversity of microorganisms to identify and repurpose naturally occurring cyclopropanated molecules. To date, only two naturally occurring molecules are known to include multiple sequential CP rings (Figure 1), namely the antifungal FR-900848 also known as jawsamycin[16] produced by Streptomyces roseoverticillatus and the cholesteryl transfer protein inhibitor U-106305, produced by *Streptomyces* U-11136.[17] The biosynthetic pathway for jawsamycin has been elucidated[18,19] and involves a highly unusual iterative polyketide synthase (iPKS) that forms an olefin in each catalytic cycle, which in turn can be modified by an S-adenosyl-methionine (SAM)-dependent cyclopropanase (CP) to form a CP ring, leading to a POP acyl-acyl carrier protein (ACP) intermediate that is later tailored into the final product.

We identified a set of candidate iPKSs predicted to produce POP fatty acids (POP-FA) and obtained the products of one of them using a heterologous system. We determined the structure of the molecules and named them fuelimycins. We then engineered the heterologous system to increase fuelimycin production, obtained POP-FA methyl esters (POP-FAMEs), and calculated their enthalpy of combustion, energy density (as net heating values), and vapor pressure. We conclude that they are viable replacements for solid rocket propellants such as

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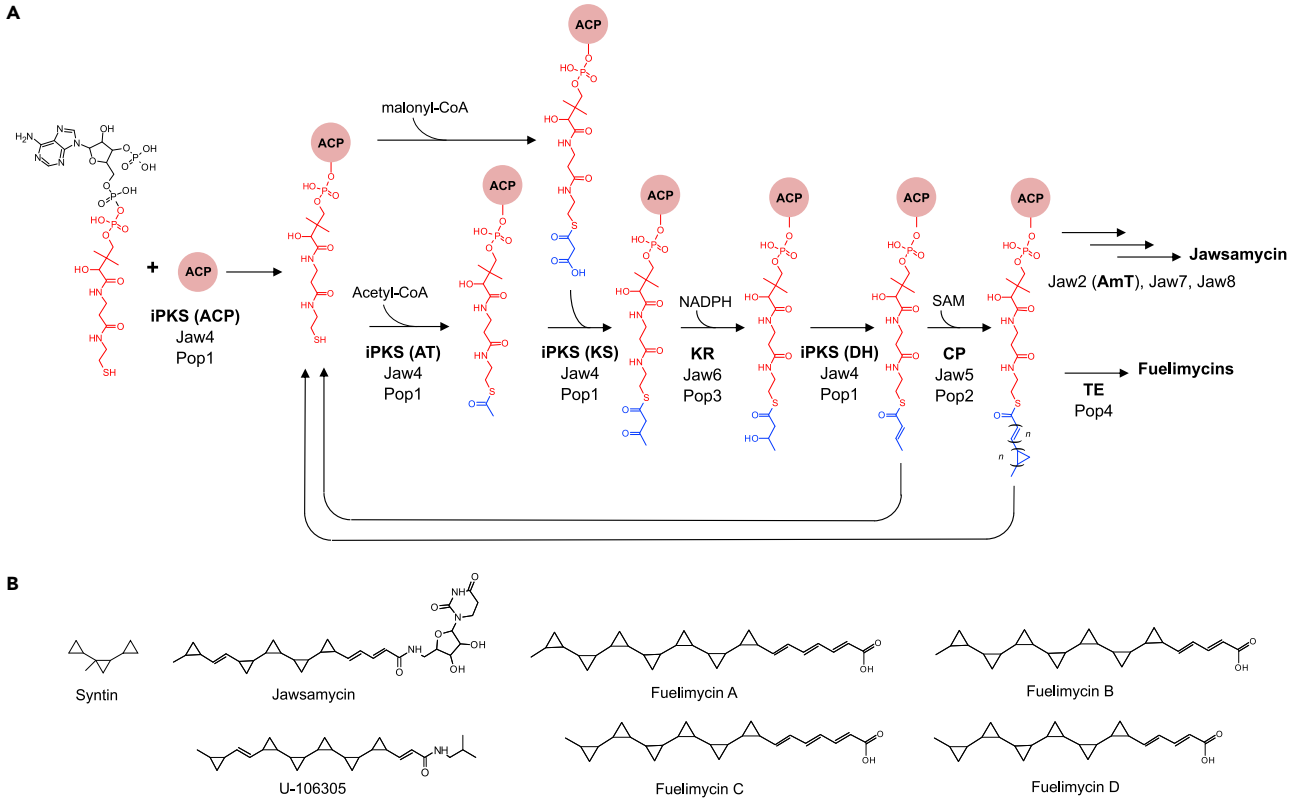


Figure 1. Biosynthesis of polycyclopropanated natural products by iterative PKS (iPKS)

(A) The biosynthetic mechanism for stepwise cyclopropanation via iPKS.

(B) Syntin, a synthetic rocket fuel, known polycyclopropanated natural products, and the fuelimycins are described in this work. ACP, acyl carrier protein; AT, acyl transferase; KS, ketosynthase; KR, ketoreductase; DH, dehydratase; CP, cyclopropanase; AmT, aminotransferase; TE, thioesterase; NADPH, nicotinamide adenine dinucleotide phosphate; SAM, S-adenosyl methionine.

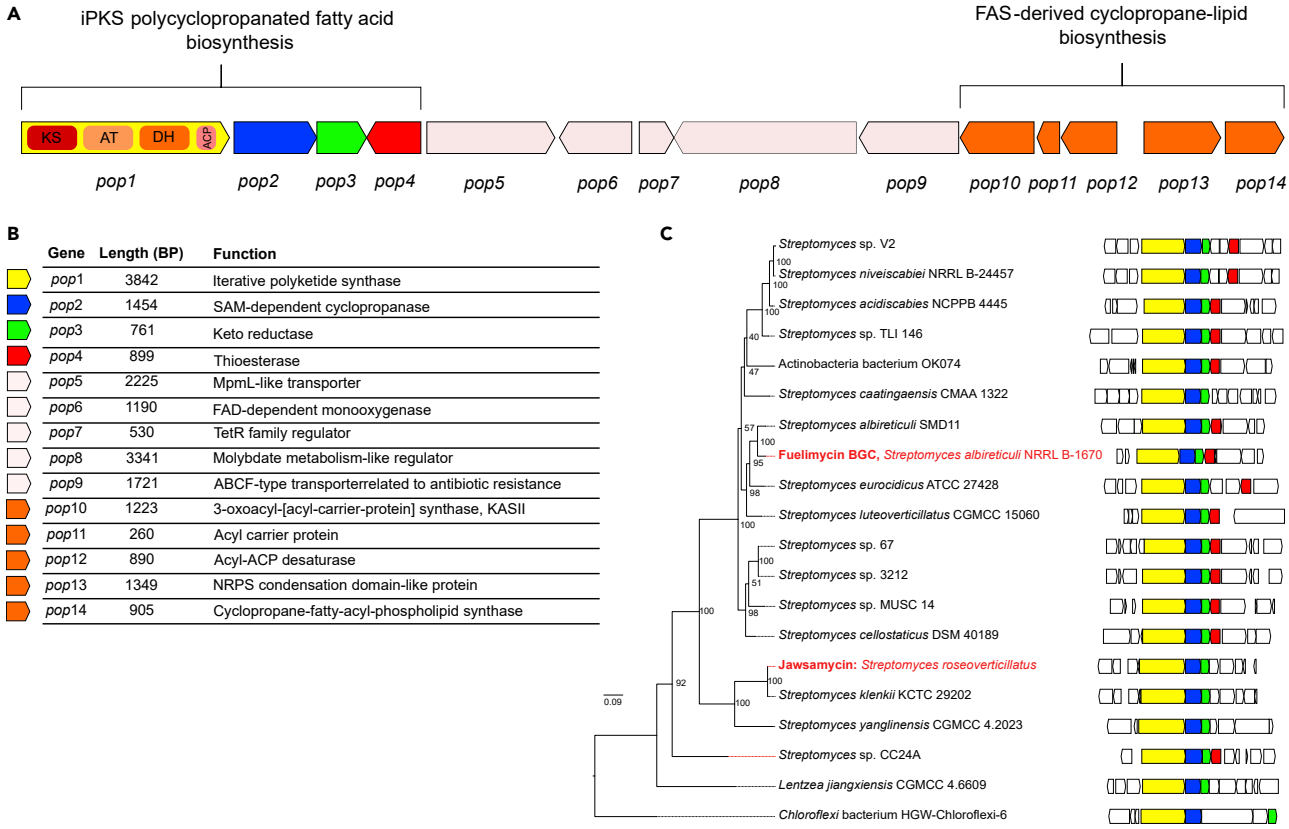
HTPB. Furthermore, our work shows the potential of the fuelimycins biosynthetic pathway for production of a variety of highly energy-dense biofuels.

RESULTS

Bioprospection of polycyclopropanating iPKSs

Release of an ACP-bound polyketide intermediate as a carboxylic acid enables its straight-forward functionalization into burnable molecules.[20] However, during jawsamycin biosynthesis (Figure 1), the release of the polyketide intermediate is concomitant with its condensation with an uridyl moiety by the aminotransferase Jaw2.[18,19] Therefore, to produce a POP fuel, our options were either to introduce a thioesterase (TE) from a known pathway to the jawsamycin pathway or to find and use a new polycyclopropanating iPKS (POP-iPKS) pathway that naturally includes a TE to produce a carboxylate. To identify candidate biosynthetic gene clusters (BGCs), we used the amino acid sequences of Jaw4 and Jaw5 as queries[18] (MiBiG: BGC0001002) for a phylogenomic search through a database of 7,762 *ad hoc* assembled bacterial genomes. A list of the genomes included in the database is available in Table S1, and a list of BGCS included in the database is available in Table S2. This search led to the identification of 19 new POP BGCs (Figure 2C), which we predicted would produce POP molecules.

The taxonomic distribution of the POP BGCs is almost exclusively confined to members of the genus *Streptomyces*, known for their outstanding natural product



	Gene	Length (BP)	Function
	pop1	3842	Iterative polyketide synthase
	pop2	1454	SAM-dependent cyclopropanase
	pop3	761	Keto reductase
	pop4	899	Thioesterase
	pop5	2225	MpmL-like transporter
	pop6	1190	FAD-dependent monooxygenase
	pop7	530	TetR family regulator
	pop8	3341	Molybdate metabolism-like regulator
	pop9	1721	ABCF-type transporterrelated to antibiotic resistance
	pop10	1223	3-oxoacyl-[acyl-carrier-protein] synthase, KASII
	pop11	260	Acyl carrier protein
	pop12	890	Acyl-ACP desaturase
	pop13	1349	NRPS condensation domain-like protein
	pop14	905	Cyclopropane-fatty-acyl-phospholipid synthase

Figure 2. Bioprospection of POP-iPKSs

(A) The genetic organization of the *pop* BGC found in *Streptomyces albireticuli* NRRL B-1670 that was characterized in this paper.

(B) Annotation of the *pop* BGC in *Streptomyces albireticuli* NRRL B-1670.

(C) Distribution, conservation, and diversity of the *pop* BGCs.

repertoire. The exceptions are a filamentous actinobacterium from the genus *Lentzea* and an unclassified member of the phylum Chloroflexi. Within *Streptomyces*, the taxonomic distribution of the BGCs is spread across the genus in multiple distinct clades, implying extensive lateral transfer of the BGC (Figure S1).

The POP BGC family has highly conserved features. **Pop1** is an iPKS including keto-synthase, acyltransferase, dehydratase, and ACP (KS-AT-DH-ACP) domains. Pop1 is closely related to other POP-iPKSs and is distantly related to other type I PKSs (Figure S2).

Pop2 is a standalone, SAM-dependent CP related to the HemN-like coproporphyrinogen III oxygenase family.[21] The closest orthologs unrelated to POP-iPKSs are also CPs involved in tailoring biosynthetic steps in other natural products (Figure S3), such as CC-1065, whose biosynthesis includes a cyclopropanation step catalyzed by a two-component system including a radical SAM enzyme named C10P (an ortholog of Pop2) and a methyltransferase named C10Q.[22] Interestingly, POP BGCs do not include C10Q orthologs, implying that Pop2 orthologs are sufficient for cyclopropanation in POP-iPKS systems, as we in fact later demonstrated. This observation implies that CPs have convergently been recruited multiple times in divergent natural product biosynthetic pathways.

Pop3 is a standalone ketoreductase (KR), an unusual feature in type I PKSs (iterative or multimodular) that typically have KR domains embedded within a multidomain peptide. POP-KRs belong to the same structural family as *bona fide* KRs but are more closely related to stand-alone KRs involved in aryl polyene and type II polyketide biosynthesis (Figure S4).

Pop4 is a stand-alone TE related to the Hot Dog protein family, which includes the acyl-coenzyme A (CoA) TE subfamily TesB23 and the acyl-ACP TEs from enediyne BGCs[24] (Figure S5). This is a distinctive feature as stand-alone TEs (often called type II TEs) are typically members of the alpha-beta hydrolase fold. Furthermore, the presence of genes coding for TEs in 70 % (14/20) of the POP BGCs implies that a free carboxylate intermediate or product is a common feature in POP-iPKSs systems.

Our bioinformatic analyses of the POP BGCs defined *pop1-4* as the minimal set of genes needed for production of POP-FA. Careful analysis of the annotation of the POP BGCs led to the identification of a BGC encoding unusual lipid and FA biosynthesis in *Streptomyces albireticuli* NRRL-B1670 (Figure 2B). In this strain, we found a group of genes (*pop*1-14) that encode for a POP-iPKS and a FA-derived mono-cyclopropanation system, which is often associated with cell membrane modifications, usually in response to stress.[25] This system is common in bacteria but not widely distributed in *Streptomyces* (Figure S6). Furthermore, we found an ortholog of MmpL (Pop5; Figure S7), a family of mycobacterial transporters associated with cyclopropanated cell membrane components.[26] Altogether, the annotations of these genes suggest a role in membrane modification featuring CP moieties. Based on these observations, we predicted that the *pop*1-4 orthologs from *S. albireticuli* NRRL B-1670 are involved in POP-FA production and selected them for characterization.

The POP BGC in *Streptomyces albireticuli* NRRL B-670 is silent

We obtained *S. albireticuli* NRRL B-1670 from the Northern Regional Research Laboratory (NRRL) strain collection. After cultivating it on agar plates, we obtained colonies with at least three phenotypes (A, B, and C; Figure S8), two of which (B and C) were not able to sporulate. These phenotypes were stable in different media after propagation. To confirm the taxonomic identity of the strain, we sequenced the genomes of the three different variants derived from the NRRL sample. Genome analysis confirmed that the three isolates were in fact *S. albireticuli* NRRL B-1670, but strains B and C are mutants that lost about 1.5 Mb from the arms of the chromosome (Figure S9) including the *pop* locus. Therefore, we focused on isolate A. Cultivation of isolate A followed by FA analysis yielded no cyclopropanated products (Table S3). We suspected that the putative FA product of the POP BGC is likely activated in a particular condition such as stress of the cell membrane or its incorporation into an unknown product that we could not easily predict (i.e., it is a cryptic BGC). This unknown, in addition to the genetic instability of *S. albireticuli*, NRRL B-1670 made heterologous expression a more attractive approach to produce POP molecules.

Heterologous production of POP-FAs

For heterologous expression of the POP BGC, we first selected *Escherichia coli* as a host. Expression of Jaw4-5-6 and Pop4 was confirmed by proteomics (Figure S10; supplemental information). However, we could not detect the products in the pellets or supernatants of the heterologous strain. We then opted for an *in vitro* strategy: we cloned *pop*1-4 into separate vectors with N-terminal 6-His-tag and His-SUMO tags. Then, we introduced them into *E. coli* hosts and assessed expression in various

conditions (supplemental information). Soluble expression of the enzymes was achieved using the His-Sumo tags (Figure S11; supplemental information). After removal of the tags, we conducted an *in vitro* pathway reconstruction assay that evaluated whether the POP enzymes can transform building blocks into polyketides. However, we were unable to detect products using liquid chromatography-mass spectrometry (LC-MS) with an electrospray ionization source in the negative and positive mode. We reasoned that this may be due to instability of the proteins or lack of proper folding.

We then moved back to an *in vivo* approach, this time using *Streptomyces* as a host. For expression in *Streptomyces*, we refactored the *pop*1-4 operon (Figure S12) and introduced it into the widely used *Streptomyces* hosts *S. albus* J1074, *S. lividans* TK24, and *S. coelicolor* M1152 via chromosomal integration. We assessed the expression of Pop1-4 using targeted proteomics and found that *S. coelicolor* M1152 (Strain POP3.1; Figure S12) was the best host as *pop*1-4 expressed in both tested media. Furthermore, the native metabolic profile of *S. coelicolor* M1152 is simpler after deletion of its most highly expressed BGCs.[27] We cultivated the POP3.1 strain and used LC-MS to analyze the spent media and cell pellet. We detected ions consistent with unsaturated POP-FAs (listed in Table S3) in the pellets of POP3.1 but not in the unmodified host (Figure S13).

Structural characterization of fuelimycins

To further confirm that POP3.1 produces POP-FAs, we exploited the fact that each CP ring in the products is derived from SAM.[19] We cultivated POP3.1 in media supplemented with methionine (methyl-^{13}C), which is in turn incorporated into SAM by the host. The labeled SAM is then used for cyclopropanation by Pop2 leading to a shift in the isotopic distribution of the products corresponding to the number of ^{13}C exo-methylene groups in the molecule. This experiment allowed us to identify ions consistent with the predicted mass shifts using high-resolution MS measurements. For example, such a mass shift was observed for the isotopic distribution of one of the products having a chain length of eighteen carbons, six CP rings, and two olefins (C18:CP6) (Figure S14). In further experiments, we used high-resolution MS to obtain evidence of the incorporation of ^{13}C exo-methylene moieties upon feeding of ^{13}C-labeled methionine in four of the most abundant POP-FA products (Figure 3).

For definitive confirmation of the presence of CP rings, we used one-dimensional (1D) ^{1}H and ^{13}C nuclear magnetic resonance (NMR) and two-dimensional (2D) correlated spectroscopy (COSY), total correlation spectroscopy (TOCSY), heteronuclear single quantum coherence spectroscopy (HSQC), HSQC-TOCSY, and heteronuclear multiple bond correlation (HMBC) NMR. The spectra of fuelimycin-rich fractions (Figure S15) from the cultures of POP-FAs producer strains supplemented with labeled methyl-^{13}C methionine and with regular media were dominated by intense signals from saturated FAs; however, signals consistent with CP exo-methylene protons (exo-CH_2), as well as some of their backbone methine protons, were evident in the ^{1}H 1D spectra due to their characteristically upfield-shifted resonances[28] (Figure S16). 1D ^{13}C NMR (DEPTQ-135) and 2D HSQC spectra confirmed the identification of the exo-CH_2 resonances (Figures S17 and S18). All exo-CH_2 resonances detectable in the unlabeled sample were also observed in the labeled sample and the enrichment afforded the detection of another 6 ^{1}H-^{13}C correlations as well (Figure S19). Table S4 lists the ^{1}H and ^{13}C chemical shifts for 17 CP exo-CH_2 correlations observed between the labeled and unlabeled samples.

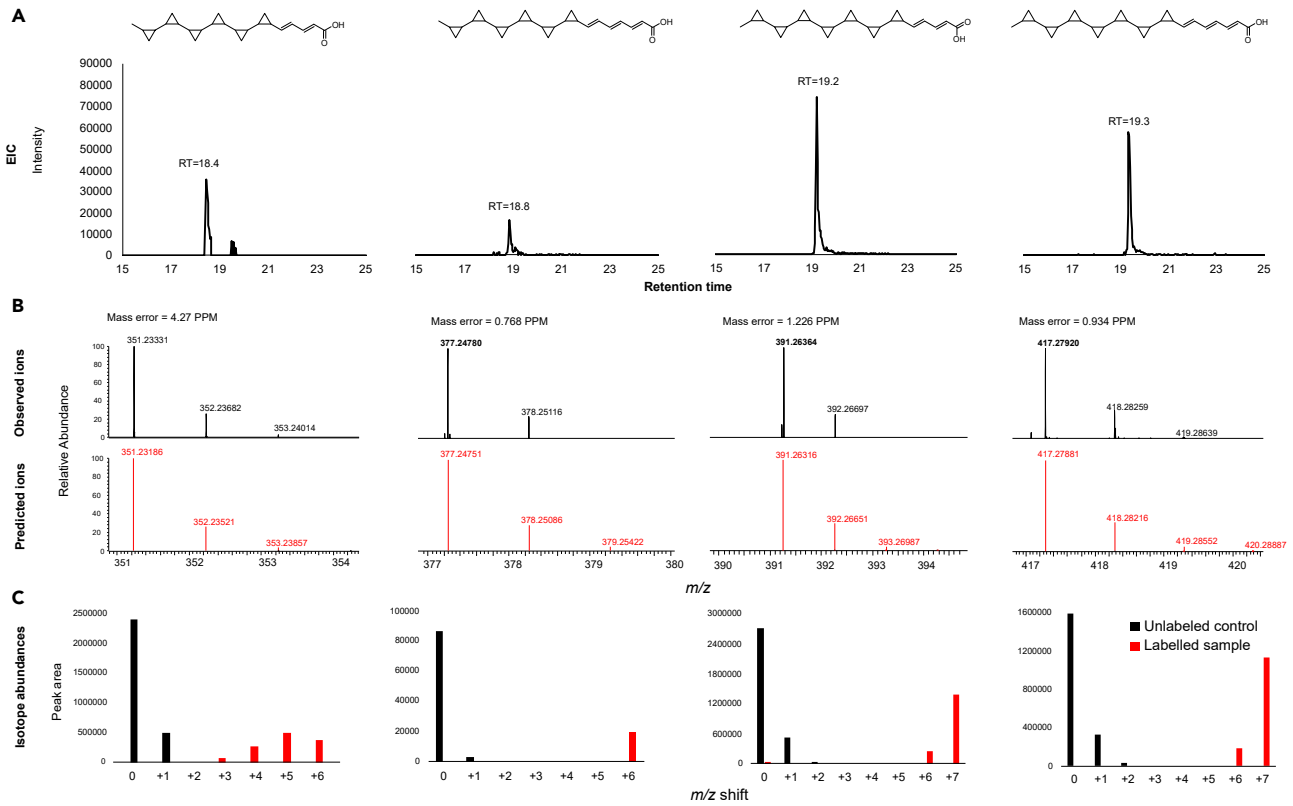


Figure 3. Structural analysis of the fuelimycins

Mass spectrometry data (negative ion mode) collected for fuelimycin D, C, B, and A (left to right).

(A) Extracted ion chromatograms.

(B) Observed and predicted ions.

(C) Isotopic analysis showing mass shifts corresponding with incorporation of ^{13}C exo-methylene groups.

Unfortunately, the intense saturated FA signals prevented full structural elucidation of individual POP-FAs. Nevertheless, there were clear correlations between CP resonances (methine and exo-methylene) with those of olefins (see HMBC; Figure S20). Additionally, a mapping from sequentially located exo-CH_2 units with one adjacent to an alkene was also possible (see Figure S21 for an example). The CP-containing chains in the predicted fuelimycins have many similarities with the same regions in jawsamycin.[18,29] Comparison of reported $^{1}H/^{13}C$ chemical shifts with those observed in this study are quite consistent for the environments within the POP chain region[18,29] (ca. shifts from ^{1}H = 0.05–0.58 ppm, ^{13}C = 7.6–14.4 ppm). Additionally, CP exo-CH_2 resonances outside this region (ca. shifts from ^{1}H = 0.60–1.38, ppm, ^{13}C = 14.8–16.3 ppm) were also noted in the fuelimycin samples, suggesting that other types of CP substitutions were also present in the mixture. Interestingly, at least one exo-CH_2 (^{1}H = −0.333, 0.565 ppm, ^{13}C = 10.8 ppm) was consistent with a CP moiety within a saturated carbon chain[28]. Although its signal was observed in both samples, we did not consider it a product of the POP BGC because it was not enriched in ^{13}C.

Finally, we used the fragmentation patterns from liquid chromatography-tandem MS experiments[30] (LC-MS/MS) to determine the positions of the olefinic and exo-methylene groups in the structure of the most abundant POP-FAs, namely, fuelimycins A, B, C, and D (Figures S22–S25). Overall, our experiments demonstrated that we achieved heterologous production of POP-FAs. We then proceeded to improve their production.

Improving fuelimycins production

Rare codon usage

The first strategy to improve fuelimycin production was to optimize the expression of *pop*1-4. A simple improvement strategy became apparent after inspection of the natural DNA sequence of the *pop*1-4 genes in search of TTA codons (UUA-Leu). These codons are an infrequent variant coding for leucine, and its cognate tRNA (encoded in the *bldA* gene) is known to be expressed during late growth stages of *Streptomyces*[31]; as a result, expression of heterologous genes carrying these codons is inefficient. We found two TTA codons in *pop1* and one in *pop2*. Therefore, we introduced an extra copy of *bldA* under control of a constitutive promoter in POP3.1 to obtain strain POP3.1-bldA. In parallel, we edited the sequences of *pop*1 and *pop*2 to obtain TTA-free variants of them (strain POP3.2). Both POP3.1-bldA and POP3.2 produced more fuelimycins than POP3.1 (Figure 4A). We selected POP3.2 for further experiments as it showed the largest increase in production. To further improve the production of fuelimycins, we constructed a series of strains in which we introduced modifications that were previously described by others as beneficial for polyketide production and/or for natural products production in general. A list of these strains and the plasmids used for the construction of the strains is available in Table S5 and a list of *Streptomyces* strains constructed for this project is available in Table S6.

Control of oxidative stress

PirA is a pirin involved in control of oxidative stress, and its mutation causes a *relaxed* phenotype[32] involving major physiological changes including shifts of acyl-CoA pools.[33] Remarkably, *pirA* (SCO3798 in *S. coelicolor*) is conserved across *Streptomyces* and contains the *att*B site for the φC31 phage integrase. This site is often regarded as neutral and is widely used for integration of heterologous genes. We disrupted *pirA* by integrating a φC31 phage integrase-containing plasmid carrying an mCherry reporter.[34] As a control, we introduced an extra copy of *pirA* under control of a constitutive promoter by integration in the R4 *att*B site in POP3.2. We found that disruption of *pirA* leads to a small decrease in POP-FA production (Figure 4C) and its overexpression leads to repression of the production of POP-FA. Given the small effect of the disruption of *pirA*, we decided to use the φC31 *att*B site for integration of other alleles that may increase production of POP-FAs.

Precursor supply

FadD1 (SCO6196 in *S. coelicolor*) is a long-chain acyl-CoA ligase that has been functionally associated with mobilization of carbon from triacylglycerols (TAGs) into polyketides. Its overexpression in *S. avermitilis* has led to spectacular yields of avermectins, a native product of this species.[35] We placed an extra copy of *fadD1* under the control of a constitutive promoter and integrated it into the φC31 *att*B site of POP3.2. We did not observe any increase in the production of POP-FAs (Figure 4C). We speculate that this may be linked to the expression onset of the *pop* BGC in POP3.2 as it is under the control of constitutive promoters, which drive expression before TAGs have accumulated.

MetK (SCO1476 in *S. coelicolor*) is a SAM synthetase, and its overexpression has been used to successfully increase titers of other natural products.[36] We placed an extra copy of metK under the control of a constitutive promoter and integrated it into the φC31 *att*B site of POP3.2. The resulting strain produced greater amounts of fuelimycins in comparison with POP3.2 (Figure 4C).

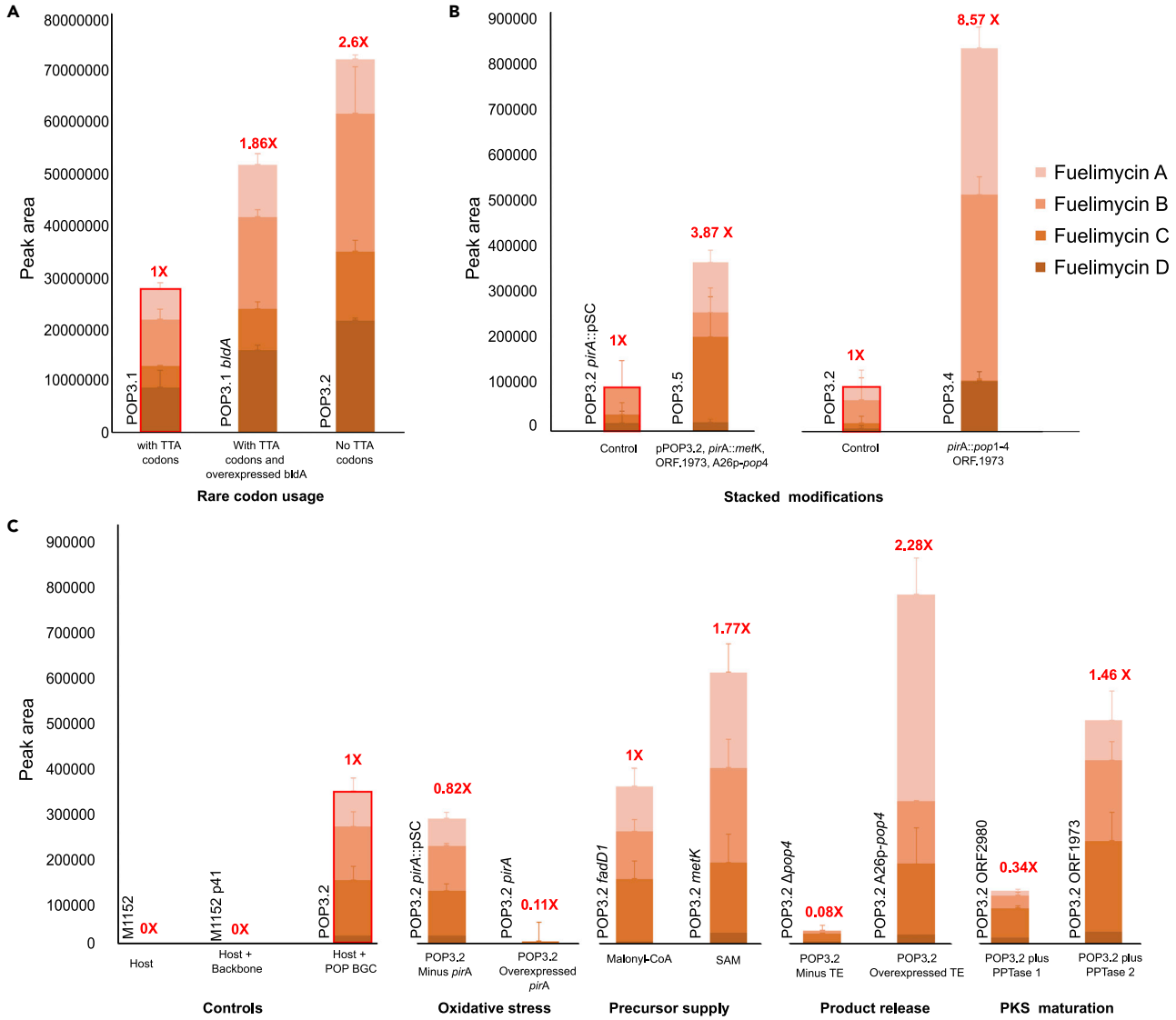


Figure 4. Effect of selected strain or pathway modifications in fuelimycins production

(A) Effect of buffering or editing the rare TTA codon.

(B) Effect of stacked modifications.

(C) Individual modifications tested on strain POP3.2.

Each panel shows independent experiments. The error bars represent the standard deviation from three biological replicates.

PKS maturation

Phosphopantetheinylation is an essential posttranslational modification of PKSs, FA synthases (FASs), and non-ribosomal peptide synthetases (NRPSs). It consists of the addition of a phosphopantetheinyl group from CoA to the ACP domain at a conserved serine residue (Figure 1A). This reaction is catalyzed by phosphopantetheinyl transferase (PPTase), which exists in different subfamilies, the AcpS-type, specialized in FAS (AcpS-type), and the SFP-type, specialized in NRPSs and PKSs.[37] Given that *S. coelicolor* has two SFP-type PPTases (SCO5883 and SCO6673), it is often assumed that they are sufficient for heterologous production of polyketides. However, it has been recently shown that their range is limited.[37] To assess if the *S. coelicolor* PPTases are sufficient to process the Pop1 iPKS, we reproduced the analysis by Kim et al.[37] but focused on the differences in PTTase family

conservation between strains with POP-FA BGCs and *S. coelicolor* (Figure S26). We identified two PPTases, ORF.1973, and ORF.2980 from *S. albireticuli* NRRL B-1670 that are divergent from the *S. coelicolor* PPTases. We cloned them, placed them under the control of a constitutive promoter, and integrated them in the φC31 *attB* site of POP3.2. We found that the expression of ORF.1973 increased the production of POP-FAs (Figure 4C).

Product release

The last step in the production of POP-FAs is the release of the ACP-bound product. We predicted that this reaction will be performed by Pop4, a TesB-like TE of the hot dog family.[23] We reasoned that increasing the concentration of this enzyme relative to Pop1-3 may alter the length of the product or increase the processivity of the pathway. To test this idea, we cloned Pop4 under the control of a synthetic constitutive promoter A26, which is among the strongest promoters reported in *Streptomyces*.[38] A POP3.2 strain carrying this extra copy of *pop4* integrated into the φC31 site produced greater amounts of fuelimycin A, whereas a version of POP3.2 without *pop4* produced only a small amount of POP-FAs (Figure 4C). We speculate that this residual activity may be driven by one of the two hot dog TEs in the genome of *S. coelicolor*. This result confirms that Pop4 is a new POP FA-ACP TE.

Finally, after testing a selected set of pathway and host modifications, we assembled two strains where these features were stacked: Strain POP3.4 included the *pop*1-4 and ORF.1973 genes integrated in a single event at the φC31 site, causing disruption of *pirA*. Strain POP3.5 included constitutively expressed copies of *metK*, ORF.1973, and *pop4*, integrated into the φC31 site, causing disruption of *pirA* and *pop*1-4 integrated into the VWB site. Strain POP3.4 produced 8.57 times more product than POP3.2 (Figure 4B). Since POP3.2 was 2.6 times more productive than POP3.1, our efforts led to an overall 22-fold increase in production from our initial POP3.1 strain.

POP-FAMES are viable biofuels for high energy applications

Finally, to convert the fuelimycins into fuels, we further purified them (Figure S15) using preparative high-performance liquid chromatography (HPLC) and then esterified them to obtain POP-FAMEs. We used LC-MS to confirm the production of at least two POP-FAMEs derived from fuelimycin A and B (Figure S27). We then used the CBS-QB3 *ab initio* quantum mechanics method[39] and the SAFT-γ-Mie equation of state to calculate thermo-physical properties of the molecules.[40–42] These calculations allowed us to predict the net heating values and vapor pressures of several POP fuels including alkanes, alkenes, and POP-FAMEs, these values are available in Table S7. Our analysis shows that the POP-FAMEs have high energy density (Figure 5) and will likely be very stable solids or dense liquids at room temperature (Table S7) making them promising candidates with energy densities similar or superior to those of other rocket propellants such as RP1, HTPB, JP10, or RJ-5. Furthermore, our analysis of potential fuelimycin-derived alkanes, alkenes, and other MEs showed that these biofuels have potential use in a broad range of energy-demanding applications.

DISCUSSION

CP -functionalized molecules are excellent fuels. However, their organic synthesis is challenging.[13–15] Our research shows that the bioproduction of novel POP fuels with high energy density is possible. We discovered the biosynthetic pathway for an unusual group of POP-FAs, the fuelimycins, and prototyped a microbial system for POP fuel production using a refactored BGC in *Streptomyces*. Our theoretical

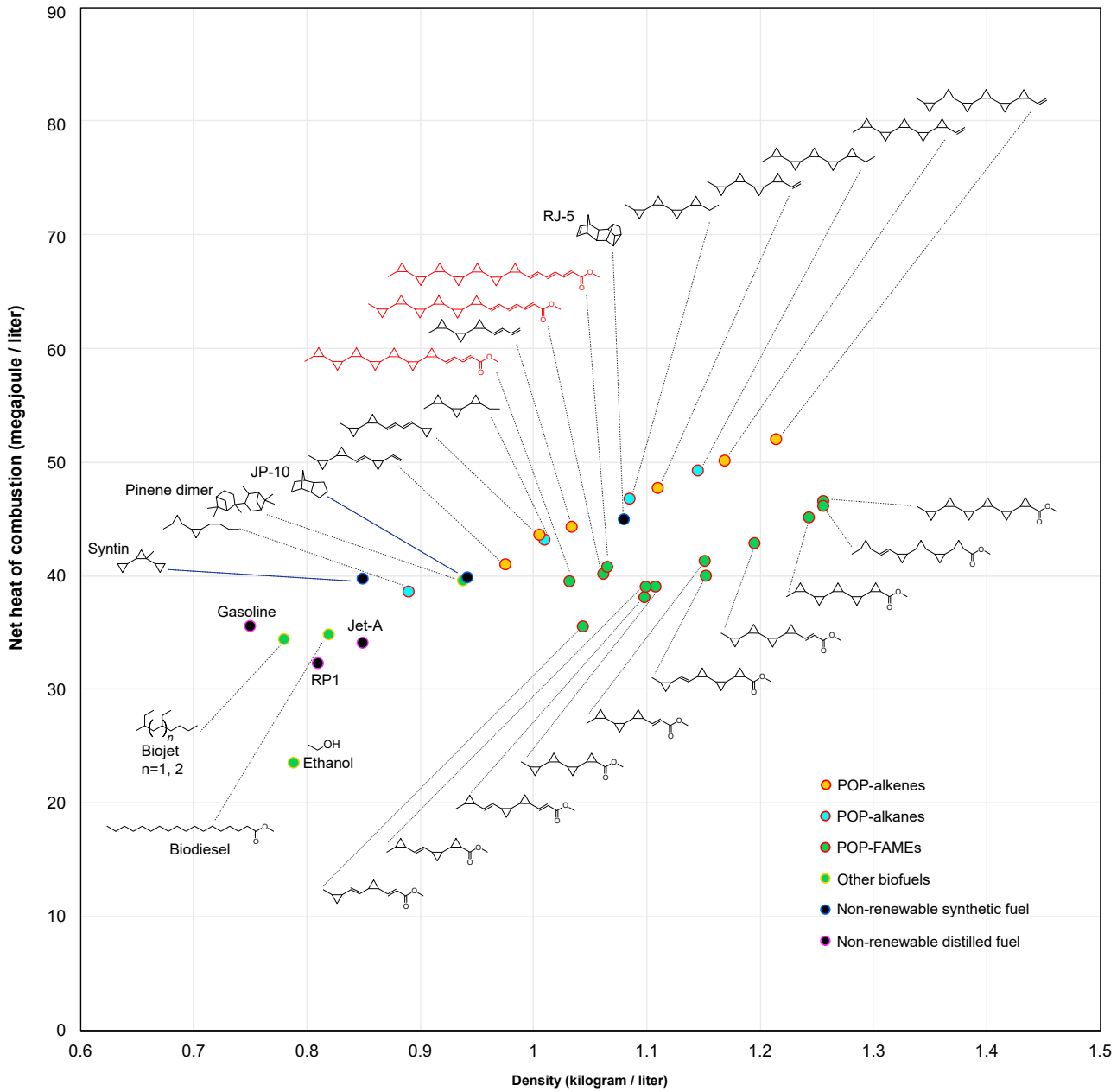


Figure 5. Energy density of POP-FAMES and renewable and nonrenewable fuels

The energy densities, calculated as net heating values, and structures of different POP fuel candidates are shown. The energy density of the fuelimycin-derived POP-FAMEs (highlighted in red) is close to that of the synthetic nonrenewable fuels JP-10 and RJ5, which are tactic rocket fuels.

calculations suggest that the POP-FAMEs derived from fuelimycins A, B, and C are energetically superior to previously described high energy-bioderived fuels,[9] with densities and volumetric heating values comparable with those of RJ-5, a petroleum-derived tactical fuel used in rockets. Furthermore, our analysis suggests that the properties of other potential fuelimycin-derived MEs, alkenes, and alkanes are suitable for long-haul transport, aviation, or shipping (Figure 5; Table S7). To obtain such fuels, engineering of the fuelimycins BGC and of the host organism is needed to produce larger amounts of these molecules and to obtain saturated products and/or products with lower molecular weight.

The path to commercial viability requires production of sufficiently large volumes of fuel to enable further properties testing and optimization to dramatically increase the titer, rate, and yield for POP fuel production. Melting point, boiling point, flash point, yield sooting index, and net heat of combustion are all important properties for aviation fuels. In diesel applications, cetane number is also important. Bench-scale experiments produce thousands of times less fuel than the minimum volumes typically needed to test these properties, and machine learning models struggle to extrapolate them to molecules that are very different from what is in their training sets.[43] However, new methods are being developed to enable small-volume (<1 mL) fuel testing.[44] The potential payoffs of early testing and further production scale-up could be substantial.

As Baral et al.[45] demonstrated in their analysis of advanced bio-jet fuels, fuels with greater energy densities can improve aircraft efficiency because the weight of fuel required for a given flight distance is reduced, thus allowing planes to carry more cargo and/or use less fuel. To be competitive with conventional fuels, the required titer, rate, and yield will vary depending on the target market and feedstock costs. To reach minimum selling prices around $3 USD per gallon of Jet-A equivalent (approximately $1 USD/L Jet-A equivalent), highly reduced aviation fuels generated aerobically from plant-derived sugars must be produced at yields approaching 90% of stoichiometric theoretical yield.[46] For fuelmyicin A, this target yield would be approximately 0.29 g of product per gram of sugar, based on a previously reported method.[47] Achieving this yield, along with dramatic improvements in the titer and rate, will require further effort to optimize production. Beyond optimizing the conversion of glucose to POP fuels, engineering hosts that are capable of catabolizing plant-derived sugars and even lignin-derived aromatics can further reduce costs and the emissions footprint of producing these fuels.

Recent developments in sustainable energy generation and storage as well as biofuel production[20,48] opened the possibility of powering the transportation sector with a wide range of fuels. However, factors such as fuel cost, range, stability, cloud point, volumetric requirements, safety, and national security may limit their use in the aerospace and the long-haul terrestrial and maritime transport sectors. The fact that petroleum-derived hydrocarbon fuels have extensively and successfully been used for such applications is evidence of their excellent properties. Unfortunately, their fossil origin renders them unsustainable. Our work shows that production of hydrocarbon fuels with superior properties to current high energy fossil fuels is possible using biological production, and they should be excellent alternatives for modes of transportation for which renewable fuels are sorely needed.

EXPERIMENTAL PROCEDURES

Resource availability

Lead contact

Further information and requests for resources should be directed to the lead contact, Dr. Jay D Keasling (keasling@berkeley.edu)

Materials availability

Strains and plasmids used in this study are available upon request through the Joint BioEnergy Institute Strain Registry (https://public-registry.jbei.org/).

Data and code availability

The code used for retrieval, processing and analysis of the genomes used herein as well as the modified code for CORASON are freely available at https://github.com/pablo-genomes-to-vials-cruz.

The genome sequences of *Streptomyces albireticuli* NRRL B-1670, its wild-type strain (GenBank: JAJQQS000000000) and its variants B (GenBank: JAJQQR000000000) and C (GenBank: JAJQQQ000000000) have been deposited at DDBJ/ENA/GenBank as BioProject PRJNA774980: SUB10575957.

The targeted proteomic data and skyline files are available in the Panorama Public repository at this link, https://panoramaweb.org/polycyclopropanated-biofuels.url, and the data are available via ProteomeXchange with the dataset identifier PXD029122.

The shotgun proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD031402 and https://doi.org/10.6019/PXD031402.

The structures of fuelimycins A, B, C, and D have been deposited in PubChem: 162421478, 162421479, 162421480, 162421481 . The DNA sequences of the fuelimycins BGC from *Streptomyces albus* NRRL-B1670 is deposited at MiBig: BGC0002138.

Methods

To identify the POP BGCs, the genome sequences of 7,762 bacterial strains (Table S1) were obtained from the GenBank and analyzed with the CORASON pipeline,[49] whose code was modified to fit our database and improve phylogenetic analysis. All phylogenetic analyses were performed with IQ tree[50] further bioinformatic methods are available in the supplemental information (section 1). *Streptomyces coelicolor* M1152 was obtained from the John Innes Centre and *S. albireticuli* NRRL B-1670 from the NRRL collection. The pop1, 2, 3 and 4 genes were cloned from genomic DNA or synthesized depending on the construct (Table S5). Promoter sequences were obtained from *S. albus* or synthesized depending on the construct. A list of these promoters is available in Table S8. All plasmids used, except for pPOP 3.2, were assembled using the Golden Gate assembly method[51] and were introduced into their corresponding hosts (Table S5) using standard protocols. Confirmation of successful integrations was done by polymerase chain reaction (PCR). Detailed methods for the refactoring of the POP BGC are available in the supplemental information (section 6). The genomes of strains POP3.4 and *S. albireticuli* NRRL B-1670 (strain A) and its derivative strains (B and C) were sequenced using the Illumina MiSeq platform in the 2 x 150 format (supplemental information [section 2]).

For POP-FA production (supplemental information [section 8]) the *Streptomyces* strains were cultivated in R5 medium in 250-mL Erlenmeyer baffled flasks with 50 mL of culture volume. Strains POP3.2 and POP3.4 were cultivated in R5 medium in 2-L flasks with 500 mL of culture volume to assess POP-FA production, to obtain products for structural characterization and for POP-FAMEs preparation.

The POP-FA products were obtained from the mycelium (supplemental information [section 9]), which was lysed by sonication. The pH of the lysate was adjusted to 4 and extracted using 1:1:1 methanol:chloroform:water. The chloroform fraction was recovered, and the solvent evaporated, leaving a dense liquid. This product was then resuspended in methanol up to one hundredth of the original culture volume. Any solids in the sample were separated by centrifugation. These extracts were used without further processing for LC-MS analysis to determine production of POP-FAMES (supplemental information [section 10]). The extracts were further processed by preparative HPLC, and the eluted fractions were collected (supplemental

information [section 11]). The fractions enriched in POP-FAMEs were identified by LC-MS and used for NMR and LC-MS/MS analysis[29] (supplemental information [sections 12 and 13]) and for POP-FAME preparation (supplemental information [section 14]).

The POP-FAs in methanolic solution were esterified into POP-FAMEs using methanol as the methyl donor with 0.39 M HCl (incubated at 42°C for 22 h) or 14% weight/weight BF_3 in methanol (incubated at 100°C for 1 h). The products were extracted with hexanes and analyzed by LC-MS. The physicochemical properties and the enthalpies of combustion of the obtained POP-FAMES and several potential fuels were calculated using the SAFT-γ approach (supplemental information [section 15]).

Detailed descriptions of all the methods and materials used are available as supplemental information.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.joule.2022.05.011.

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AUTHOR CONTRIBUTIONS

P.C.-M., K.Y., R.B., A.C., and S.A. performed molecular biology experiments. P.C.-M., E.S., C.A.-B., and K.Y. performed fermentations. P.C.-M., C.A.-B., A.T.I., and Y.L. performed POP-FA extractions, preparative HPLC, and LC-MS analyses. S.K., P.C.-M., and A.T.I. prepared and analyzed POP-FAMES. P.C.-M., K.Y., J.R.C., R.P.Y., and J.E.K. performed structural analysis of fuelimycins. A.L. and A.G.

performed SAFT-γ calculations. Y.C., J.W.G., C.J.P., and P.C.-M. performed proteomic analyses. P.C.-M., A.A.N., and S.A. performed bioinformatic analyses; C.D.S. analyzed the viability of the POP fuels. J.D.K. conceived the project and obtained funding. P.C.-M. designed and coordinated the research. All authors participated in writing the manuscript.

DECLARATION OF INTERESTS

P.C.-M, K.Y., R.B., A.C., E.S., Y.L., and J.D.K. have filed a patent related to the subject matter of the contribution. J.D.K. has a financial interest in Amyris, Lygos, Demetrix, Maple Bio, Napigen, Apertor Pharma, Ansa Biotechnologies, Berkeley Yeast, and Zero Acre Farms.

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