Title

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Journal

STAR Protocols, 4(2)

ISSN

2666-1667

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Publication Date

2023-06-01

DOI

10.1016/j.xpro.2023.102190

STAR Protocols

Heterologous Production of Polycyclopropanated Fatty Acids and their Methyl Esters in Streptomyces

--Manuscript Draft--

Manuscript Number:	STAR-PROTOCOLS-D-22-00897R3
Full Title:	Heterologous Production of Polycyclopropanated Fatty Acids and their Methyl Esters in Streptomyces
Article Type:	Protocol
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Corresponding Author Secondary Information:	
Corresponding Author's Institution:	
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Abstract:	Polycyclopropanated (POP) compounds show promise as fuels as their energy density can be greater than jet and rocket fuels in current use, but realizing their full potential requires significant development. This protocol guides the production of Polycyclopropanated fatty acids (POP-FAs) in Streptomyces; POP production in another host remains to be demonstrated. This method can serve as a baseline for further development of POP as well as other Polyketide products. For complete details on the use and execution of this protocol, please refer to Cruz-Morales et al. (2022)
Additional Information:	
Question	**Response**
Original Code<p>Does this manuscript report original code?	No
Standardized datasets<p>A list of datatypes considered standardized under Cell Press policy is available here. Does this manuscript report new standardized datasets?	No

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Cover Letter

This protocol paper outlines a platform for production of polycyclopropanated (POP) fatty acids in heterologous *Streptomyces* hosts. Using this platform, researchers can expand on current achievements in developing POP fuel compounds, as described in Cruz-Morales et al. 2022*, the publication with which this protocol is associated. More broadly, this method can be applied to the development of other polyketide products in *Streptomyces*.

*full citation:
Cruz-Morales, P., Yin, K., Landera, A., Cort, J.R., Young, R.P., Kyle, J.E., Bertrand, R., Iavarone, A.T., Acharya, S., Cowan, A., et al. (2022). Biosynthesis of polycyclopropanated high energy biofuels. Joule. 10.1016/j.joule.2022.05.011.

Main figures must be provided as high-resolution individual JPG files. ***Please upload the image under "Designing an Expression Plasmid" as a main Figure, and include a title in the main text.***

Thank you for this suggestion. We have uploaded this image as Figure 1, and adjusted all other figure numberings accordingly. We have made the following addition to step 2:

> 2. Log in and use DIVA to design plasmid(s). For detailed instructions, refer to the "DNA construction guide" found in the top right corner (see **Figure 1**).

and the following addition to the Figure Legend:

> **Figure 1: Navigation of DIVA home page.** For more guidelines on using DIVA to design plasmids, see the DNA Construction Guide located in the top right corner as indicated.

Please incorporate the following changes. Please ***be sure these meet the length limits****.*

- ***Summary:*** *Polycyclopropanated (POP) compounds show promise as fuels as their energy density can be greater than jet and rocket fuels in current use, but realizing their full potential requires significant development. Here, we present a protocol for producing Polycyclopropanated fatty acids (POP-FAs). We describe steps for plasmid design and its conjugation into Streptomyces. We then detail extraction, purification and esterification of POP-FAs. This protocol can serve as a baseline for further development of POP as well as other Polyketide products.*

For complete details on the use and execution of this protocol, please refer to Cruz-Morales et al. (2022).[1]

Thank you for the suggestion. We have reverted some additions to the summary in order to meet the world limit.

> Polycyclopropanated (POP) compounds show promise as fuels as their energy density can be greater than jet and rocket fuels in current use, but realizing their full potential requires significant development. This protocol guides the production of Polycyclopropanated fatty acids (POP-FAs) in *Streptomyces* ~~as well as the extraction, purification and esterification of these POP-FAs~~; POP production in another host remains to be demonstrated. This method can serve as a baseline for further development of POP as well as other Polyketide products.

For complete details on the use and execution of this protocol, please refer to Cruz-Morales et al. (2022).[1]

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Heterologous Production of Polycyclopropanated Fatty Acids and their Methyl Esters in *Streptomyces*

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Summary

Polycyclopropanated (POP) compounds show promise as fuels as their energy density can be greater than jet and rocket fuels in current use, but realizing their full potential requires significant development. This protocol guides the production of Polycyclopropanated fatty acids (POP-FAs) in *Streptomyces*; POP production in another host remains to be demonstrated. This method can serve as a baseline for further development of POP as well as other Polyketide products.

For complete details on the use and execution of this protocol, please refer to Cruz-Morales et al. (2022).[1]

Before you begin

Selecting POP Biosynthetic Gene Clusters (BGCs) for Expression

Timing: 3 hours

We used the CORASON pipeline[2] to mine genomes for biosynthetic gene clusters (BGCs) predicted to produce POP-FAs. This process is detailed in Cruz-Morales et al. (2022)[1], and the source code for CORASON can be found at: https://github.com/WeMakeMolecules/myCORASON

The package runs on unix (e.g. ubuntu)

1. Install the following dependencies:

```
sudo apt install blast+
sudo apt install iqtree
sudo apt install mafft
sudo apt install cpanminus
cpanm SVG
```

2. download and install the corason3 package:

```
wget
https://github.com/WeMakeMolecules/myCORASON/raw/master/CO
RASON3.tar.gz
tar -xvf CORASON3.tar.gz
```

3. make the nw_distance file executable:

```
cd CORASON3/bin
chmod +x nw_distance
```

Note: Corason3 requires a database where the entries are genomes from the GenBank and BGCs from the MiBig repository annotated using RAST. For the analysis reported in Cruz-Morales et al. (2022)[1] the database includes 9678 entries. Detailed instructions to create a database for Corason3 and run the pipeline are available at: https://github.com/WeMakeMolecules/myCORASON

4. In our database, the corason3 pipeline can be executed with the following command:

```
perl corason3.pl -q MCD9195254.query -r 9067 -e
0.000000001 -s 200 -f 10 -d full -x FORMATDB
```

Note: This command instructs the pipeline to run the analysis using the protein sequence of the SAM-dependent methyltransferase Jaw2 (Accession number: MCD9195254) as a query (-q) with an e-value cutoff (-e) of less than 1E-9 and score cutoff (-s) of 200 or more and the genome of *Streptomyces albireticuli* NRRL B-1670 (accession number: JAJQQS010000000) as reference (-r). In our database this genome is indexed under entry 9067. Our database is available upon request.

The blast e-value represents the number of expected hits found by chance within a database of sequences. An e-value cutoff equal to zero or close to zero (such as 1E-9) guarantees that the search delivers only significant hits.

The blast bit score represents the sequence similarity normalized with the alignment score independently of the length of the query and the size of the database. For a protein query the size of Jaw2, a bit score cut-off of 200 filters out hits that may comply with the e-value cut-off, but which alignment do not cover most of the query sequence.

This analysis takes 187 minutes using 20 cores in an HP Z4 workstation with an intel Xeon W-2295x3 GHZX36 processor and 250 GB in RAM.

This results in a set of orthologs for POP1-4, indicating that the *loci* identified are related to POP-FA production. The following table shows 11 sets of orthologs that were predicted to be involved in FA biosynthesis from a database containing 7,762 genomes[1]. A table of the resulting iterative polyketide synthase (iPKS), methyltransferase (MT), ketoreductase (KR) and thioesterase (TE) genes and their accession numbers is provided here for the reader's convenience. Any combination of four homologs, one from each column (iPKS, MT, KR, and TE) can be tested for heterologous production of POP-FAs in *Streptomyces*.

Host Genome	Accession No.			
	iPKS	MT	KR	TE
Streptomyces sp. V2	WP_165854398	WP_109360941	WP_109360940	WP_109360937
S. niveiscabiei NRRL B-24457	WP_055723442	WP_055723443	WP_055723444	WP_055723446
S. acidiscabies NCPPB 4445	WP_199832115	WP_075737731	WP_050375598	WP_075737730
Streptomyces sp. TLI 146	WP_101386433	WP_101386432	WP_101386431	WP_101386430
Actinobacteria bacterium OK074	KPI10148	KPI10147	KPI10146	KPI10145
S. caatingaensis CMAA 1322	WP_049715139	WP_206743342	WP_049715141	WP_049717980
S. albireticuli NRRL B-1670	MCD9195255	MCD9195254	MCD9195253	MCD9195252
S. eurocidicus ATCC 27428	MBF6056017	MBF6056016	MBF6056015	MBF6056012
S. luteoverticillatus CGMCC 15060	WP_126917589	WP_126917590	WP_126917591	WP_126917592
Streptomyces sp. MUSC 14	WP_079177048	WP_071376315	WP_071376314	WP_071376313
S. cellostaticus DSM 40189	KUM98616	KUM98615	WP_066991096	KUM98614

Designing an Expression Plasmid

Timing: 1-2 hours

Once a set of orthologous genes have been selected, design a plasmid to express these genes in a suitable heterologous host. To design our expression plasmid we used DIVA[3], a web tool that can generate recommended PCR primer sequences suitable for various plasmid assembly strategies including Gibson and Golden Gate. DIVA is particularly useful for combinatorial design of plasmids (for example, expressing different combinations of genes from homologous BGCs). While we will include DIVA in our protocol, any equivalent webtool or software, such as SnapGene® or Benchling (www.benchling.com), may be used for plasmid design.

1. Register for a public DIVA account at https://public-diva.jbei.org/register

   a. Select between commercial or non-commercial account type, and input your first and last name, email address, and a user ID.
   b. Click 'Register', wait ~24 hours to receive an email from Public DIVA with your initial login credentials. Upon first logging in, you will be prompted to change your password.
2. Log in and use DIVA to design plasmid(s). For detailed instructions, refer to the "DNA construction guide" found in the top right corner (see **Figure 1**).

Note: We designed our POP3.4 expression plasmid to be assembled using an NEBuilder® HiFi DNA Assembly Master Mix (New England Biolabs, Cat#E2621L). For considerations regarding assembly approaches, see the Limitations section. DIVA and SnapGene® support both Gibson Assembly[4] and Golden Gate[5] design strategies.

We designed an integrative vector carrying the POP pathway that can be introduced into our *Streptomyces* host by conjugative transfer. Essential components for our conjugated integrative plasmid include:

3. An origin of replication, which maintains the plasmid in cloning and conjugative strains of *E. coli*. We used ColE1 to obtain a high number of plasmid copies and increase the transformation efficiency.
4. An integrase, which expresses a bacteriophage enzyme that integrates the plasmid into the host chromosome, we selected the phage derived ΦC31 integrase[6].
5. An *attP* site, a specific DNA binding site onto which the cognate integrase attaches. We used the ΦC31 *attP* site.

Note: ΦC31 integrase sites are stable in *Streptomyces coelicolor*; however, they may cause chromosomal rearrangements and off-target effects in other *Streptomyces* hosts such as *S. albus* J1074[7]. It has been recently shown that the conserved ΦC31 integration site is found in most *Streptomyces* in the *pirA* gene, and *pirA* function is disrupted upon integration. *pirA* has been involved in redox balance and in major shifts in Acyl-coA pools. In the case of POP-FAs this effect may be favorable[1], however this may be an important consideration for other products[8].

6. An origin of transfer, a recognition site for the conjugative transfer of this plasmid from *E. coli* to *Streptomyces*. We used the RP4 origin of transfer.
7. A unique *Streptomyces* promoter positioned upstream of each gene to be expressed. We used constitutive promoters sourced from the genome of *S. albus* J1074 to minimize homology with DNA sequences native to *S. coelicolor*[9].
8. An antibiotic resistance gene, to select for successful transformations, conjugations and integrations.

Note: We recommend choosing either apramycin or spectinomycin as a resistance marker for conjugative expression plasmids. This allows for compatibility with the conjugating *E. coli* strain (ET12567/pUZ8002) which already uses kanamycin and chloramphenicol selection markers; furthermore, some other commonly used antibiotics are not effective markers in *Streptomyces*.

For a streamlined design of POP-BGC expression plasmids in *Streptomyces*, POP3.4 (which contains all of the listed components) may be used as a template in which the pop1-4 genes are replaced with

four genes of choice, e.g., homologs selected from the previous section. Annotated sequence information for POP3.4 can be found in The Joint BioEnergy Institute's Inventory of Composable Elements (ICE) as entry number JPUB_020600, or using the link: https://public-registry.jbei.org/entry/20600.

To access this entry, an account will need to be registered at https://public-registry.jbei.org/register. This is a separate account from the public DIVA account created in Step 1:

9. Input your first name, last name, email address, and institution.
10. You will receive an email with your login ID and password. You may then change the password by going to your profile page.

Readers may also request a sample of this plasmid by contacting Jay Keasling at keasling@berkeley.edu.

A more detailed discussion of expression plasmid design, including alternatives such as replicative plasmids, can be found in Chapters 9, 10, and 11 of *Practical Streptomyces Genetics*[10].

Preparation of Expression Plasmid

Timing: 3-4 days

Once an expression plasmid has been designed, begin plasmid construction by assembling its vector and gene parts. Whether expressing the native sequences of *Streptomyces* genes or codon optimizing for a *Streptomyces* production host, consider that the high complexity and G+C content of such DNA may increase the difficulty of PCR amplification, sequence verification, or other plasmid preparation steps.

11. Obtain the native strain or genomic DNA containing your selected genes from a repository such as NRRL or ATCC.

Note: If obtaining a *Streptomyces* or other gram-positive strain, extract genomic DNA using Qiagen DNeasy Powersoil Pro, following kit instructions (QIAGEN, Cat#47016).

Alternatives: Some native strains will not be accessible for purchase or ordering from a repository. In such cases, DNA synthesis of your selected genes is a viable option; vendors such as Genscript are able to produce high quality high G+C DNA clones within reasonable cost & time.

CRITICAL: When using genomic DNA as a PCR template, design silent mutations to replace any native TTA codons (e.g., replace with TTG codons). When ordering gene synthesis, we similarly recommend codon-optimizing these genes for expression in *Streptomyces*, which includes avoiding TTA codons. TTA codons are rare in native *Streptomyces* genomes and function to regulate protein expression[11], which is not compatible with heterologous production host platforms.

12. Order the DNA oligo synthesis of PCR primers (e.g., listed in the Oligo Synthesis section of a DIVA output file, or generated from SnapGene®, Benchling or equivalent tools).
13. Set up and run PCR reactions to amplify each plasmid fragment to be assembled.
    a. Most standard polymerase enzymes, buffers, and protocols are suitable. However, to improve the success rate of PCR reactions involving high G+C content, we recommend

using PrimeSTAR GXL Premix (TakaraBio, Cat#R051A) with the following protocol and/or up to adding up to 10 % DMSO (v/v) to the PCR mix:

PCR reaction master mix

Reagent	Amount
DNA Template	1-200 ng
Primer 1	10-15 pmol (final conc. 0.2- 0.3 µM)
Primer 2	10-15 pmol (final conc. 0.2- 0.3 µM)
PrimeSTAR GXL 2x Premix	25 µL
DMSO (optional)	up to 5 µL
ddH_2O	to 50 µL

PCR cycling conditions

Steps	Temperature	Time	Cycles
Denaturation	98 °C	10 sec	30 cycles
Annealing	55-60 °C*	15 sec	
Extension	68 °C	1 min/kb	
Hold	4 °C	forever	

*The PrimeSTAR protocol recommends an annealing temperature of 60 °C if the Tm value of the primers is more than 55 °C, and an annealing temperature of 55 °C if the Tm value is 55 °C or less.

14. Add 10 µL of Thermo Scientific™ DNA Loading Dye 6x (or an appropriate volume of a comparable loading dye) to 50 µL of each completed PCR reaction.
15. Separate DNA samples on a 1% agarose gel.
16. Identify the relevant SYBR™ Safe (Thermo Fisher, Cat#S33102) stained DNA bands using a blue light transilluminator and excise the bands using a fresh scalpel or razor blade.

Alternatives: Ethidium bromide stain and/or UV transilluminator may be used but take caution that UV exposure damages DNA samples over time and pose an additional safety risk to researchers.

17. Use QiaQuick Gel Extraction Kit (QIAGEN, Cat#28706) or an equivalent kit to purify the desired amplified fragment.
18. Assemble the plasmid(s) using NEBuilder® HiFi DNA Assembly Master Mix (New England Biolabs, Cat#E2621L), and following kit instructions.

Alternatives: If the plasmid was designed using Golden Gate Assembly approach, use NEBridge® Golden Gate Assembly Kit (New England Biolabs, Cat#E1602) or an equivalent kit, following kit instructions.

19. Transform the completed assembly product into DH5α—or some equivalent strain—competent *E. coli* cells.
    a. Remove a purchased or aliquoted tube of competent cells from -80 °C storage and thaw on ice. 50-200 µL of competent cells is sufficient for each transformation.
    b. Add 1-5 µL of the assembly reaction DNA to the cells via pipette. Mix by gently tapping 3-5 times. Do not pipette vigorously or vortex to mix.
    c. Incubate the cell and DNA mixture on ice for 30 minutes.

d. Heat-shock the cells at 42 °C for 30-60 seconds. The exact timing will depend on the strain; follow supplier instructions for best results.
e. Place tube back on ice for 1-2 minutes.
f. Add 450-950 µL of LB or equivalent recovery medium.
g. Spread transformed cells onto LB agar (see Key resources table; Materials and equipment) plates with the antibiotic that selects for the expression plasmid (e.g., 50 µg/mL of apramycin, or 100 µg/mL of spectinomycin).

Note: To ensure at least one plate results in well-spaced colonies for easier picking, we recommend spreading different volumes of transformation onto 2-4 plates, e.g., 10 µL, 100 µL and 200 µL.

h. Let the plates dry in a sterile environment, and incubate for 16-20 h at 37 °C.

20. Inoculate colonies from the plates into 1-10 mL LB and incubate for 16-20 h at 37 °C, shaking at 200 RPM.

Pause point: After incubation, prepare glycerol stocks by transferring 500 µL of bacterial culture to a sterile cryotube and add 500 µL of 50% glycerol. Store tubes at -80 °C. Any stocks that have been later determined to contain incorrectly assembled or mutated plasmids may be discarded.

Alternatives: Glycerol stocks may be prepared after sequence verification.

21. Extract plasmid DNA from the remaining liquid bacterial cultures using Qiaprep Spin Miniprep Kit (QIAGEN, Cat#27106X4) or an equivalent kit, following kit instructions.
22. Sequence the plasmid to verify that it has assembled correctly and does not contain any mutations.

Note: Any Sanger or Next Generation Sequencing service may be used for this step. Sample submission guidelines for Genewiz Sanger Sequencing (Plasmid, Premix) serve as an example:

a. Design sanger sequencing primers by selecting a 15-20 nucleotide sequence roughly 100bp upstream of the gene or region to be sequenced.
    i. Because Sanger sequencing results usually only reach up to ~800 bases long, design primers for every 700-800 bases until the desired sequencing coverage is achieved.
    ii. For improved coverage, consider designing primers for sequencing in the reverse direction as well.
b. Order and obtain the designed primers as DNA oligos from IDT or an equivalent vendor.
    i. Dilute sequencing primers to 5 µM with molecular biology grade water.
c. Using only one primer for each sequencing reaction, combine 5 µL of primer, 50-100 ng of plasmid, and water to a final volume of 15 µL, into 8-strip PCR tubes.
d. Deliver samples to Genewiz corresponding to the sequencing order

Streptomyces cultivation and spore preparation

Timing: 1-2 weeks

Cultivation and preparation of *Streptomyces* spores is detailed in Chapter 2 of *Practical Streptomyces Genetics*[10]. Our modified protocol is summarized below:

23. Using a sterile inoculation loop, L-spreader or glass beads, spread *Streptomyces* from liquid culture or a spore suspension stock onto Soy-Flour Mannitol (SFM) agar plates.

CRITICAL: To prevent contamination of strains, always work in a sterile environment (laminar flow hood, biosafety cabinet or flame) when handling *Streptomyces* bacteria, growth media, etc.

24. Incubate plates (agar-side up, lid-side down) at 30 °C for roughly 7 days to allow for spore formation.

CRITICAL: To permit air circulation and ensure *Streptomyces* grow and sporulate properly, use petri plates with vented lids, e.g., Disposable Petri Dishes (VWR®, Cat#25384-088).

Optional: To reduce desiccation of agar over time, wrap a thin strip of parafilm around the petri dish lid, forming a seal between the lid and plate.

25. When spores have formed, indicated by a fuzzy white hydrophobic top layer, transfer the plates to a sterile environment. See Troubleshooting 1; Troubleshooting 2.
    a. remove the upside-down plates from the bottom-facing lids, taking care not to allow any condensation that has collected on the lid to contaminate the spores.
    b. Place the open plates right-side up.
26. Add enough sterile water to cover the plate (approx. 9-10 mL).
27. Scrape the surface of the plated *Streptomyces* with a sterile cotton swab. See Methods Video S1.

Note: Spores being released by sufficiently forceful scraping should be indicated by an increase in opacity (or change in color) of the water. If the scraping is too forceful however, it will break the agar. Take caution and increase vigor gradually.

28. Using a sterile tweezer for handling, add a sterile cotton ball to the spore solution. This will serve as a filter against agar and mycelial fragments.
29. Press the tip of a sterile 10 mL pipette to the wet cotton ball and draw up the solution. Transfer the spore suspension to a sterile 15 mL falcon tube. See Methods Video S2.
30. Centrifuge for 5-10 minutes at 4000 x g to pellet the spores. Remove supernatant.
31. Add sterile 20% glycerol, transfer to a screw cap bottle and freeze at -20 °C.

Note: Regarding the selection of heterologous *Streptomyces* host strain(s), we recommend using *Streptomyces coelicolor* strain M1152 or related strains from Gomez-Escribano et al. (2011)[12], which have been engineered for heterologous production of secondary metabolites. The endogenous actinorhodin, prodiginine, CPK and CDA biosynthetic gene clusters have been deleted in these strains; this not only redirects key precursors such as malonyl-coA towards heterologous pathways, it also simplifies the metabolic background and expedites the analytical chemistry steps.

Preparation of POP-FA producer strain via plasmid conjugation

Timing: 2-3 weeks

To introduce our BGC into *Streptomyces*, we chose a conjugation approach for its simplicity; a standard protocol for conjugation is provided here. Conjugation from *E. coli*, as well as alternative methods—such as transformation, transfection or electroporation into mycelial cells or prepared protoplasts—are discussed in further detail in Chapter 10 of *Practical Streptomyces Genetics*[10].

32. Transform the expression plasmid into competent *E. coli* ET12567/pUZ8002[13] using standard electroporation methods.
    a. After 1 h of outgrowth at 37 °C, select for transformants by plating on LB agar containing kanamycin (50 µg/mL), chloramphenicol (25 µg/mL) and the antibiotic that selects for the expression plasmid (e.g., apramycin or spectinomycin). Incubate for 16-20 h at 37 °C.

Pause point: After 16-20 h incubation, plates with colony formation can be stored at 4 °C for up to 2 weeks.

33. Inoculate a colony of transformants into 10 mL LB with kanamycin, chloramphenicol, and the antibiotic that selects for the expression plasmid. Incubate for 16-20 h at 37 °C, 200 RPM.
34. Inoculate 100 µL of the incubated culture from Step 33 into 10 mL LB with kanamycin, chloramphenicol, and the antibiotic that selects for the expression plasmid. Incubate at 37 °C, 200 RPM until the culture reaches an OD600 of 0.4 (~4 h).

Pause point: To store transformed conjugation strains for future use, recover ~500 µL of the incubated culture from Step 33 and add an equal volume of sterile 50% glycerol. Store this glycerol stock at -80 °C.

35. Wash cells (centrifuge at 4000 x g for 10 minutes, remove supernatant, resuspend in fresh LB) twice to remove antibiotics. Resuspend in 0.1 mL LB.
36. Per conjugation, add 10 µL of *Streptomyces* spores to 500 µL 2x YT broth in sterile Eppendorf tubes.

Optional: Heat-shock spores at 50 °C for 1-10 minutes, then allow spores to cool down. For some *Streptomyces*, this may not improve the conjugation efficiency.

37. Mix 0.5 mL of *E. coli* resuspension with the 0.5 mL of *Streptomyces* spores prepared in Step 36. Centrifuge the mixture at 4000 x g for 5 minutes and remove supernatant, then resuspend in 50 µL of residual or added liquid.
38. Make a series of dilutions from 10^{-1} to 10^{-4}, each in a total of 100 µL of water; plate out 100 µL of each dilution on SFM agar + 10 mM $MgCl_2$ (without antibiotics).

CRITICAL: To allow *Streptomyces* sufficient time to receive and express the antibiotic resistance gene contained within the plasmid, incubate for 16-20 h at 30 °C before the next step.

39. After incubation, overlay the plate with 1 mL water containing 0.5 mg nalidixic acid and the antibiotic that selects for the expression plasmid, e.g., 1.25 mg apramycin.
    a. Continue incubation at 30 °C for about 1 week. The nalidixic acid is added after conjugation to select against the gram-negative ET12567/pUZ8002 cells. See Troubleshooting 3.

Note: When preparing an overlay solution containing a mix of nalidixic acid with other antibiotics, precipitation may be observed. To resolve this, slowly add NaOH until the precipitation clears.

Optional: To save time in waiting for the 1 mL overlay to dry out, we recommend "pre-drying" the agar plates, by removing the plate lid under a laminar flow hood, biosafety cabinet or otherwise sterile environment, for 1 h before plating.

40. Passage exconjugants to select for stable integration of the expression plasmid into the heterologous host chromosome.
    a. After 1 week of incubation from Step 39, *Streptomyces* spores will have formed. See Troubleshooting 1; Troubleshooting 2; Troubleshooting 4.
    b. Using a sterile cotton swab, pick colonies and thoroughly spread spores onto fresh and sterile SFM plates containing nalidixic acid and the antibiotic that selects for the expression plasmid.

Note: After conjugation, the *E. coli* strain ET12567/pUZ8002 is no longer necessary to maintain. Agar plates from this point should not include kanamycin or chloramphenicol.

Note: The incubation period will vary with the strain of *Streptomyces* used, but 1 week was sufficient for *Streptomyces coelicolor* strain M1152.

    c. Incubate freshly streaked plates at 30 °C to cultivate spores for 1 week. Harvest resulting spores, and store at -20 °C. See Troubleshooting 5.
        i. Refer to the section titled '*Streptomyces* cultivation and spore preparation' for details.
41. Inoculate spores into liquid Tryptic Soy Broth (TSB) (or an equivalent media broth) with the antibiotic that selects for the integrated plasmid.
    a. Incubate at 30 °C and 200 RPM for 2-3 days to allow *Streptomyces* mycelia to grow.
42. Recover the mycelial pellet by centrifugation at 4000 x g for 10 minutes, or via vacuum filtration.
43. Extract genomic DNA using DNeasy Powersoil Pro Kit (QIAGEN, Cat#47016).
44. Using the extracted genomic DNA as a template, and re-using primers that were designed for assembly of the conjugative plasmid (or design new PCR primers), amplify a selected gene within the integrated plasmid using PCR.
    a. Any of the heterologous genes may be selected; we selected the iPKS gene for this confirmation step.
45. Add appropriate volumes of DNA Loading Dye to the completed PCR reaction.
46. Separate DNA samples on a 1% agarose gel.
47. Observe the stained DNA bands using a transilluminator; a successful PCR confirms integration of the plasmid into the *Streptomyces* host chromosome.
    a. For a negative control, extract and include the genomic DNA of the non-conjugated *Streptomyces* host in a separate PCR reaction.

Note: For publication-quality confirmation of plasmid integration, we recommend Next Generation Sequencing of the full host chromosome. However, PCR is sufficient for initial experiments.

Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and Virus Strains		
E. coli strain ET12567/pUZ8002	MacNeil et al., 1991	N/A
E. coli DH5α Competent Cells	Thermo Fisher Scientific	Cat#18265017
Streptomyces coelicolor M1152: *Δact Δred Δcdk Δcpa rpoB[C1298T]*	Gomez-Escribano et al., 2010	N/A
Chemicals, Peptides, and Recombinant Proteins		
LB broth (MILLER)	Sigma-Aldrich	Cat#1.10285
Methanol	Sigma-Aldrich	Cat#34860
Chloroform	Fisher Scientific	Cat#14-650-505
Trace Metals Mixture 1000X	Teknova	Cat#T1001
Critical Commercial Assays		
NEBuilder® HiFi DNA Assembly Master Mix	New England Biolabs	Cat#E2621L
DNeasy Powersoil Pro Kit (250)	QIAGEN	Cat#47016
PrimeSTAR GXL Premix	TakaraBio	Cat#R051A
SYBR™ Safe DNA Gel Stain	Thermo Fisher	Cat#S33102
QiaQuick Gel Extraction Kit	QIAGEN	Cat#28706
NEBridge® Golden Gate Assembly Kit	New England Biolabs	Cat#E1602
Qiaprep Spin Miniprep Kit (1000)	QIAGEN	Cat#27106X4
Recombinant DNA		
POP3.4 plasmid	Cruz-Morales et al., 2022	https://public-registry.jbei.org/entry/20600
Software and Algorithms		
CORASON	Navarro et al., 2019	https://github.com/WeMakeMolecules/myCORASON
DIVA (public)	Hillson et al., 2012	https://public-diva.jbei.org/
Other		
Disposable Petri Dishes	VWR®	Cat#25384-088
Black Plastic caps	Sigma Aldrich	Cat#Z106429

Materials and equipment

Note: Media recommendations and recipes are based on selection of *Streptomyces coelicolor* M1152 as the heterologous host. If a different heterologous host is being used, consider that the strain may have different optimal growth and production conditions and refer to Chapter 19 of *Practical Streptomyces Genetics*[10] for media alternatives.

To make agar plates, pour autoclaved and melted media onto sterile Petri plates and cool to 20-22°C in a sterile environment. Use LB media for cultivation of *E. coli*. Use Soy Flour Mannitol (SFM) media for cultivation, conjugation, and spore preparation of *Streptomyces*. 2x YT liquid media is used in the conjugation protocol. Use R5 or Tryptic Soy Broth (TSB) media for production of POP-FAs. R5 is recommended, but TSB pre-made powder can be purchased and is sufficient for initial experiments.

LB agar

Reagent	Final concentration	Amount
Agar	15 mg/mL	15 g
LB Broth (MILLER)	25 mg/mL	25 g
Water	n/a	to 1000 mL
Total	**n/a**	**1000 mL**

Omit agar if making liquid media. Sterilize in the autoclave using a liquid cycle at 121°C for 45 minutes. Store at 20-22°C and away from light for 3 to 6 months.

Soy Flour Mannitol

Reagent	Final concentration	Amount
Agar	20 mg/mL	20 g
Mannitol	20 mg/mL	20 g
Soy flour	20 mg/mL	20 g
Water	n/a	to 1000 mL
Total	**n/a**	**1000 mL**

Omit agar if making liquid media. A laboratory supplier is not necessary for soy flour; store bought soy flour is sufficient. For recommendations on properly dissolving the soy flour see Troubleshooting 1. Sterilize in the autoclave using a liquid cycle at 121°C for 45 minutes. Store at 20-22°C and away from light for 6 to 12 months.

2x YT

Reagent	Final concentration	Amount
Difco Bacto tryptone	16 mg/mL	16 g
Difco Bacto yeast extract	10 mg/mL	10 g
NaCl	5 mg/mL	5 g
Water	n/a	to 1000 mL
Total	**n/a**	**1000 mL**

Sterilize in autoclave using a liquid cycle at 121 °C for 45 minutes. Store at 20-22°C and away from light for 6 to 12 months.

Trace element solution (for R5)

Reagent	Final concentration	Amount
$ZnCl_2$	0.04 mg/mL	40 mg
$FeCl_3 \cdot 6H_2O$	0.2 mg/mL	200 mg
$CuCl_2 \cdot 2H_2O$	0.01 mg/mL	10 mg
$MnCl_2 \cdot 4H_2O$	0.01 mg/mL	10 mg
$Na_2B_4O_7 \cdot 10H_2O$	0.01 mg/mL	10 mg
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.01 mg/mL	10 mg
Water	n/a	to 1000 mL
Total	**n/a**	**1000 mL**

Alternatives: Instead of making Trace Elements Solution, 1 mL of Trace Metals Mixture 1000X (Teknova, Cat#T1001) may be used for every 1 L of R5. We recommend this option as a time-saver. Store at 20-22°C and away from light for 6 to 12 months.

R5

Reagent	Final concentration	Amount
Sucrose	103 mg/mL	103 g
K_2SO_4	0.25 mg/mL	0.25 g
$MgCl_2 \cdot 6H_2O$	10.12 mg/mL	10.12 g
Glucose	10 mg/mL	10 g
Difco Casamino acids	0.1 mg/mL	0.1 g
Trace Element Solution	n/a	2 mL
Difco Yeast Extract	5 mg/mL	5 g
TES Buffer	5.73 mg/mL	5.73 g
Distilled water	n/a	to 1000 mL

Alternatives: When optimizing media conditions, consider omitting sucrose.
Sterilize in autoclave using a liquid cycle at 121°C for 45 minutes. Once cooled, add filter-sterilized (only NaOH does not need to be sterilized) ingredients in the order listed:

Reagent	Final concentration	Amount
KH_2PO_4 (0.5%)	0.005%	10 mL
$CaCl_2 \cdot 2H2O$ (5M)	20 mM	4 mL
L-Proline (20%)	0.3%	15 mL
NaOH (1M)	7 mM	7 mL
Total	**n/a**	**~1000 mL**

Store at 20-22°C and away from light for 6 to 12 months.
CRITICAL: NaOH is corrosive; use caution and wear protective gloves when handling.

Tryptic Soy Broth (TSB)

Reagent	Final concentration	Amount
Tryptone (tryptic digest of casein)	17 mg/mL	17 g
Soytone (porcine digest of soy)	3 mg/mL	3 g
NaCl	5 mg/mL	5 g
KH_2PO_4	2.5 mg/mL	2.5 g
Glucose	2.5 mg/mL	2.5 g
Distilled water	n/a	to 1000 mL
Total	**n/a**	**1000 mL**

Store at 20-22°C and away from light for 6 to 12 months.

Step-by-step method details

Production of POP-FAs in *Streptomyces*

Timing: 6 days

Once integration of the expression plasmid has been confirmed in the *Streptomyces* producer strain, culture this strain and allow it to grow and produce POP-FAs. The following protocol was optimized for *Streptomyces coelicolor* strain M1152 expressing our POP3.4 plasmid; parameters such as incubation time or media may vary depending on the selected heterologous host.

1. Inoculate spores into 50 mL of R5 or TSB media with antibiotics in 250 mL Erlenmeyer flasks.
    a. We recommend baffled flasks for increased oxygenation and break-up of mycelial clumps for easier extraction.
2. Incubate for 6 days, at 30 °C, 180 RPM.

Alternatives: If doing large-scale production, after 48 h of incubation, inoculate this 50 mL culture into 450 additional mL of R5 or TSB with antibiotics, in 2 L baffled flasks, and incubate for another 4 days at 30 °C, 180 RPM.

Note: Culturing times will vary between *Streptomyces* strains.

3. Cultures are now ready to be harvested for POP-FA extraction.

Extraction of POP-FAs

Timing: 1-4 days

Because POP-FAs are produced intracellularly and not secreted, the mycelia must be harvested from liquid culture and lysed to extract the POP-FA products. The following protocol outlines a freeze-thaw and sonication step for cell lysis, and a methanol chloroform extraction for the recovery of POP-FAs.

4. Harvest mycelial cells from the culture broth.
    a. For 50 mL scale extractions, transfer liquid culture into 50 mL Falcon tubes via pipette or pouring. For 500 mL scale extractions, transfer the liquid culture into 1 L Polypropylene centrifuge bottles.
        i. Scrape the sides of the culture flasks to collect any visible mycelial growth there as well.
    b. Centrifuge at 4000 x g for 10 minutes to pellet mycelial cells, and discard the supernatant media.

Alternatives: For large, 500 mL scale extractions, consider recovering mycelia via vacuum filtration with a Buchner funnel and filter paper, rather than centrifugation of large sample volumes. When using vacuum filtration, continue to transfer the pellet to 1 L centrifuge bottles for downstream steps.

Pause point: Freeze the pellet at -20 °C for up to 2-4 weeks. Thaw to continue. While this step may be skipped, we recommend a freeze-thaw cycle to aid in the lysis of mycelial cells.

5. Resuspend mycelial pellet in water.

a. Use roughly 10 mL for small-scale extractions, or 100 mL for large-scale extractions.

6. Lyse the mycelial cells.
    a. Use a Qsonica Q500 Sonicator with a 2 mm microtip, and keep a sample tube on ice.
    b. Adjust the height of the sample tube until the microtip is at least ~1 cm submerged into the sample.

CRITICAL: To prevent hearing damage, wear ear protection and/or use a soundproof cabinet while using a probe sonicator. Shut doors and sonicate in a room separate from people who are not wearing ear protection.

CRITICAL: To prevent melting plastic or shattering glass tubes, avoid contact between the sonicator microtip and the sample tube.

c. We recommend the following sonication settings: 20 minutes (total) sonication **Time** (10 minutes of active sonication), 40% **Amplitude**, and a **Pulse** setting of 30 seconds On, 30 seconds Off.

CRITICAL: After sonication, ensure acidification of POP products by slowly adding HCl until the sample pH has been adjusted to ~4. Transfer to conical glass tubes.

CRITICAL: Wear gloves and personal protective equipment and work in a fume hood when handling acids.

Alternatives: For large-scale extractions, transfer to a glass bottle.

7. Add methanol and chloroform in a 1:1:1 ratio with the sample. For small-scale extractions, this means roughly 10 mL of methanol and 10 mL of chloroform for a total mixture volume of 30 mL.

Optional: We found best results with first adding the methanol, gently mixing, then adding the chloroform last.

CRITICAL: Chloroform reacts with most plastics; use only glass labware (tubes, pipettes, flasks, etc.) when handling chloroform.

CRITICAL: Wear gloves and personal protective equipment and work in a fume hood when handling toxic chemicals such as methanol and chloroform.

8. Mix vigorously and then allow fractions to settle and separate for 15-30 minutes; repeat this 2 times. See Troubleshooting 6.

CRITICAL: For the first 1-2 minutes of mixing, pause intermittently to open the tube cap and release any pressure buildup.

a. Recover the chloroform (bottom, transparent) fraction into a new glass tube using a glass Pasteur pipette.

CRITICAL: The top fraction contains methanol and water (transparent), while a middle layer (cloudy) contains lipids from the lysed cells; see **Figure 6.** Avoid the two upper fractions when recovering the chloroform fraction.

Pause point: The recovered fraction may be stored for 16-20 h at 20-22°C (or 1-2 weeks at 4 °C) to allow any residual methanol, water, or lipids to collect at the top of the sample and be removed via glass pipette. Because POP-FA compounds may precipitate at 4 °C, bring the samples back to 20-22°C before proceeding.

Alternatives: For large-scale extractions, agitate for 16-20 h using a magnetic stirrer. The following day, pour the mixture into a glass separatory funnel with the stopcock closed. Allow the chloroform and methanol fractions to separate. Open the stopcock and allow the chloroform fraction to flow into a glass tube, flask or beaker. Close the stopcock to prevent the lipid layer or methanol fraction from flowing through.

9. Evaporate the chloroform fraction using a rotary evaporator.
    a. In our preparations we used a Buchi Rotavapor R-124. Any equivalent Rotary Evaporator may be used; follow standard or manufacturer's protocols.

CRITICAL: To preserve sample quality, do not heat the sample beyond 40 °C during this step.

10. Add 0.5 mL (5 mL for large-scale) of methanol at 20-22°C. Fully resuspend the sample in methanol, using gentle vortexing if the small-scale sample is in a centrifuge-grade glass tube, or swirling if the large-scale sample is in a rounded glass flask.
11. Transfer the resuspension to appropriately sized centrifuge tubes (1.7 mL Eppendorf or 15-50 mL Falcon).

Pause point: The resuspension may be stored at 4 °C for up to several weeks. Because POP-FA compounds may precipitate at 4 °C, bring the samples back to 20-22°C before proceeding.

12. To remove any remaining solid and insoluble particles, centrifuge this solution for 10 minutes at 10000 x g or maximum speed and recover the supernatant.
13. The sample is now ready for LC-MS analysis; transfer to GC vials.

LC-MS analysis of POP-FAs and POP-FAMEs

Timing: 1 hour per sample

After POP-FA molecules have been extracted from mycelial cells (or after they have been esterified to POP-FAMEs), analyze the samples using an LC-MS. Based on the resulting molecular weights, the chain length and the number of cyclopropanes present in the extracted molecules can be determined.

14. Run the samples using a LC-MS with a 100 mm Viva C4 column (Restek, catalog number 9512511. 100 mm long, 1.0 mm inner diameter, 5 µM particle size) and the following conditions:
    a. Injection volume = 10 µL
    b. Flow rate = 200 µL/minute
    c. column temperature = 45 °C
    d. Mobile phase solvent A = water with 0.1% formic acid

e. Mobile phase solvent B = acetonitrile with 0.1% formic acid

Use the following HPLC gradient elution program as shown in Cruz-Morales et al. (2022)[1], supplementary methods table 1 (reprinted with permission):

Time (minutes)	*Solvent A (%)*	*Solvent B (%)*
0	*55*	*45*
2	*55*	*45*
35	*50*	*50*
35.5	*5*	*95*
40	*5*	*95*
40.5	*55*	*45*
60	*55*	*45*

Supplementary Table S3 from Cruz-Morales et al. (2022)[1] lists a range of theoretical m/z ratios for both hypothetical and detected POP-FA molecules in negative ion mode. A shortened version of this table is replicated with permission here for quick reference. To use the table, follow the C[X]:CP[Y] molecule naming scheme, where X = chain length as measured by the number of carbons, and Y = the number of cyclopropanes present. Observed LC gradient retention times were also recorded and should be used as an additional reference when following our LC-MS run parameters.

Molecule Name	Chemical Formula	Theoretical $[M-H]^-$	Retention Time
C14:CP4	$C_{18}H_{24}O_2$	271.170354	not detected*
C14:CP5	$C_{19}H_{26}O_2$	285.186004	17.6
C14:CP6	$C_{20}H_{28}O_2$	299.201654	not detected*
C16:CP4	$C_{20}H_{26}O_2$	297.186004	not detected*
C16:CP5	$C_{21}H_{28}O_2$	311.201654	17.8
C16:CP6	$C_{22}H_{30}O_2$	325.217304	18.3
C16:CP7	$C_{23}H_{32}O_2$	339.232954	not detected*
C18:CP4	$C_{22}H_{28}O_2$	323.201654	not detected*
C18:CP5	$C_{23}H_{30}O_2$	337.217304	18.1
C18:CP6	$C_{24}H_{32}O_2$	351.232954	18.4
C18:CP7	$C_{25}H_{34}O_2$	365.248604	18.9
C18:CP8	$C_{26}H_{36}O_2$	379.264254	not detected*
C20:CP5	$C_{25}H_{32}O_2$	363.232954	not detected*
C20:CP6	$C_{26}H_{34}O_2$	377.248604	18.8
C20:CP7	$C_{27}H_{36}O_2$	391.264254	19.2
C20:CP8	$C_{28}H_{38}O_2$	405.279904	19.5

C20:CP9	$C_{29}H_{40}O_2$	419.295554	not detected*
C22:CP6	$C_{28}H_{36}O_2$	403.264254	not detected*
C22:CP7	$C_{29}H_{38}O_2$	417.279904	19.3
C22:CP8	$C_{30}H_{40}O_2$	431.295554	19.67
C22:CP9	$C_{31}H_{42}O_2$	445.311204	not detected*
C24:CP7	$C_{31}H_{40}O_2$	443.295554	not detected*
C24:CP8	$C_{32}H_{42}O_2$	457.311204	18.27
C24:CP9	$C_{33}H_{44}O_2$	473.342504	not detected*

*These theoretical molecules were not detected in the study by Cruz-Morales et al. (2022)[1]. However, these molecules may be detected in other experiments, particularly if new homologous pathways are tested.

POP-FAME molecules should instead be analyzed in positive ion mode:

Molecule Name	Chemical Formula	Theoretical $[M+H]^+$	Retention Time
C22:CP7-FAME	$C_{30}H_{40}O_2$	433.31011	19.57
C20:CP7-FAME	$C_{28}H_{38}O_2$	407.29446	19.44

Preparative HPLC purification of POP-FAs

Timing: 2-3 days

If analyzing samples with NMR, LC-MS/MS, or esterifying into POP-FAMES, preparative HPLC is necessary to further purify POP-FA after extraction.

15. Transfer 1 mL of sample via pipette into 2 mL amber vials (GC vials) for use in HPLC.
16. We used a Zorbax SB-C18 column (9.4x50mm, 5 microns particle size; Agilent PN: 846975-202). With the following conditions:
    a. Injection volume = 900 µL.
    b. flow rate: 1 mL/minute.
    c. mobile phase solvent A = water + 0.1% formic acid.
    d. mobile phase solvent B = acetonitrile + 0.1% formic acid.
    e. The gradient elution program used was the same as for LC-MS.
    f. Collect the resulting fractions every 4 minutes until the end of the run.
        i. Note: using these parameters, we usually found our desired products in fractions 9-12. Once the fractions containing desired products have been identified via LC-MS, these fractions can be combined for downstream steps.
17. Cover the fraction tubes lightly with foil (poke some holes in the foil) and dry for 16-20 h under a fume hood.
18. Freeze-dry the fractions in a lyophilizer.
    a. First, freeze the samples at -80 °C for 1 h in 50 mL falcon tubes with holes in the tube cap (holes can be drilled, or a heated metal tweezer could be used to poke the holes, etc.).
    b. Manually turn on the refrigeration function for the lyophilizer at least 20 minutes before adding frozen samples.
    c. Add frozen samples, close the valve and turn on the vacuum.
    d. After 1-2 days, or once lyophilization is complete (white-ish particles may be observed in the falcon tubes, but no frozen pellets should remain), open the valve, turn off refrigeration and vacuum functions, and remove the sample tubes.

Alternatives: These methanolic fractions may instead be dried using rotary evaporation; do not heat samples beyond 40 °C.

19. Resuspend the samples in 500-1000 µL methanol. Centrifuge at 6000 RPM for 5 minutes and transfer the supernatant to GC vials.

Preparation of POP-FAMEs

Timing: 1-2 days

After POP-FAs have been extracted, they can be esterified into POP-FAMEs for improved fuel properties. We used a mild HCl protocol for its simplicity, but will present an aggressive Boron Trifluoride protocol as an alternative. Both protocols begin with a POP-FA sample extracted from a large-scale production run (500 mL culture) and processed using the preparative HPLC protocol.

20. After preparative HPLC protocol, resuspend lyophilized sample in 100 µL of methanol and transfer to a small (e.g., 5 mL) screw-capped glass vial.
21. Add 0.75 mL methanol, followed by 0.15 mL of 8.0% HCl solution. The final HCl concentration should be 1.2% (w/v) or 0.39 M.
22. Stir and then incubate at 42 °C for 22 h.
23. Cool the sample to 20-22°C.
24. Add 1 mL of H_2O and extract three times with hexanes.
    a. To extract, add hexane, vigorously mix the sample, and recover the hexane (upper) fraction.
25. Combine the extracted organic layers. Dry the sample using a Nitrogen Evaporator or an equivalent apparatus, or under a fume hood.
    a. Expected result: a white residue around the vial edges.

Alternative protocol using Boron Trifluoride:

20. After preparative HPLC protocol, resuspend lyophilized sample in 500 µL of methanol and aliquot 100 µL into small (e.g., 5 mL) screw-capped glass vials.
21. Dry samples under a fume hood.
    a. Expect around 1-3 mg of dried product.
22. To the dried residue, add 1 mL of a 14% (w/w) solution of Boron Trifluoride in Methanol (Sigma-Aldrich catalog number B1252).
23. Seal the vial using a plastic cap, and heat to 100 °C for 1 h.
    a. Use a bakelite or phenolic cap, e.g., Black plastic caps (Sigma Aldrich, Cat#Z106429).
24. After 1 h, cool the solution to 20-22°C and evaporate the solvent under a fume hood.
25. Add 1 mL of H_2O and extract three times with hexanes.
    a. To extract, add hexane, vigorously mix the sample, and recover the hexane (upper) fraction.
26. Combine the extracted organic layers. Dry the sample using a Nitrogen Evaporator or an equivalent apparatus, or under a fume hood.
    a. Expected result: a white to yellow residue around the vial edges.

Expected outcomes

Our protocol describes a platform for producing POP-FAs using a *Streptomyces* heterologous host. Following this protocol, you can expect to introduce POP pathway genes into *Streptomyces* and extract the resulting POP-FA products. After a preparative HPLC step, you can then esterify these POP-FAs to POP-FAMEs. Finally, with our described LC-MS analytical guidelines, you can detect these POP-FA and/or POP-FAME products with their characteristic m/z and retention times.

Limitations

When following this protocol to construct plasmids, the high complexity and G+C content of DNA to be expressed in *Streptomyces* (whether native to *Streptomyces* or codon-optimized) can reduce the success rate of Gibson Assembly[14]. Golden Gate Assembly may be considered as an alternative; however, the recognition sequences of Type IIs restriction enzymes commonly used for Golden Gate (e.g., *BsaI* and *BsmBI*) are themselves high in G+C content, and these enzymes will often digest your genes unless all undesired & internal recognition sites are removed prior to assembly. To reduce the frequency of undesired recognition sites, consider alternative Type IIs enzymes such as *SapI* or *PaqCI,* which recognize longer or less G+C rich sequences. If using DIVA to design primers for Golden Gate Assembly with an alternative restriction enzyme such as *PaqCI*, note the following inputs under "J5 parameters": Golden Gate Overhang BPs (for *PaqCI* this is 4); Golden Gate Recognition Sequence (for *PaqCI* this is CACCTGC); Golden Gate Term Extra Seq (for *PaqCI* this is CACCTGCNNNN where N = any nucleotide). Additionally, USER® Cloning (New England Biolabs, Cat# M5505S) may be considered as an alternative to both Gibson Assembly and Golden Gate Assembly, but was not explored in this project.

Troubleshooting

Problem 1:

When propagating *Streptomyces* or making spore preparations, the selected strain grows poorly or not at all on agar plates (**Figure 2**) (related to Before you begin Step 25 and Before you begin Step 40).

Potential solution:

- It is critical to use petri plates with vented lids; if plates without vents are used, the cells may fail to grow and sporulate properly.
- Different growth and sporulation rates may be observed when propagating *Streptomyces* on different media plates. Many *Streptomyces* species sporulate well on Soy Flour Mannitol (SFM) agar.
- If a strain that is known to sporulate on SFM, such as *S. coelicolor*, fails to do so, it may be due to suboptimal preparation of media. In order to break down the soy flour for better nutrient availability for *Streptomyces*, we recommend boiling and stirring (a hot plate/stirrer may be used) the SFM media preparation for ~5 minutes before sterilizing in the autoclave.

Alternatively, the media may be autoclaved twice. See **Figure 3** for a comparison of SFM plate preparations.

Problem 2:

When propagating *Streptomyces* on agar plates, an uneven distribution of spores is observed (**Figure 4**) (related to Before you begin Step 25 and Before you begin Step 40). For the spore preparation step, such plates result in lower yields as a fraction of the plate does not contain spores.

Potential solution:

Using a sterile cotton swab, gently streak across the entire plate surface to redistribute spores and incubate at 30 °C for 1 week or until spores have uniformly developed.

Problem 3:

When preparing an antibiotic overlay for use in *Streptomyces* conjugations, the formation of precipitate is observed (**Figure 5**) (related to Before you begin Step 39).

Potential solution:

Precipitation can occur when mixing nalidixic acid with other antibiotics, such as spectinomycin or apramycin. To resolve this, slowly increase the pH with incremental additions of NaOH, and shake the solution to mix between additions.

Problem 4:

After conjugation and antibiotic overlay steps are performed, no *Streptomyces* growth is observed (related to Before you begin Step 40). Before conjugation, the selected *Streptomyces* strain can grow on the same media in the absence of antibiotics, and the plasmid components pertaining to conjugation (origin of transfer, integrase, antibiotic resistance gene) have been confirmed by literature, sequencing, and/or control experiments to be present and compatible with the selected *Streptomyces* strain.

Potential solution:

- Increase the amount of *Streptomyces* spores and/or *Echericia coli* conjugating parental cells.
- Skip the heat shock stage as some *Streptomyces* strains become unviable after heat shock.
- Allow for an increased incubation time between the conjugation and antibiotic overlay steps.

Problem 5:

After conjugation and antibiotic overlay steps are performed, the resulting *Streptomyces* colonies fail to propagate when transferred to new agar plates (related to Before you begin Step 40).

Potential solution:

- Check that both the media as well as the antibiotic concentrations are consistent between conjugation, antibiotic overlay, and propagation.
- Increase selective pressure by decreasing incubation time between the conjugation and antibiotic overlay steps

Problem 6:

If the middle lipid layer is thick and/or indistinguishable from a cloudy bottom fraction, this is an indication of incomplete separation. See **Figure 6** for an example of the problem before and after its solution (related to Step 8).

Potential solution:

- Gently swirl or tap the sample tube to facilitate the separation of chloroform and lipid layers.
- Storage of samples for 16-20 h at 4 °C may also aid in separation.
- Gently centrifuge the sample tubes if further separation is needed.

CRITICAL: To avoid shattering glass tubes, use snugly fitting centrifuge rotor adaptors and check the manufacturer's specifications for maximum centrifugation force.

Resource availability

Lead contact

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

Materials availability

Materials generated in this study, such as the POP3.4 plasmid, are available from the lead contact upon completing a Materials Transfer Agreement.

Data and code availability

The source code for CORASON can be found at https://github.com/WeMakeMolecules/myCORASON; a version of record has been archived at Zenodo (DOI: 10.5281/zenodo.7643073). No unique datasets were generated in this study.

Acknowledgments

The authors acknowledge J.L. ''Clem'' Fortman for facilitating our work and Leonard Katz for valuable scientific discussions. This work was funded by the DOE Joint BioEnergy Institute (http://www.jbei.org) supported by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research and by the Co-Optimization of Fuels & Engines (Co-Optima) project sponsored by the U.S. Department of Energy (DOE) Office of Energy Efficiency and Renewable Energy (EERE), Bioenergy Technologies, and Vehicle Technologies Offices, under contract DEAC02-05CH11231 between DOE and Lawrence Berkeley National Laboratory. Part of this research was performed at Sandia National Laboratories, which is a multi-mission laboratory managed and operated by National Technology and Engineering Solutions of Sandia, LLC., a wholly owned subsidiary of Honeywell International, Inc., for the U.S. Department of Energy's National Nuclear Security Administration under contract DE-NA0003525. The portion of this research conducted at Pacific Northwest National Laboratory (PNNL) was supported by Co-Optima, a collaborative project of

multiple National Laboratories initiated to simultaneously accelerate the introduction of affordable, scalable, and sustainable biofuels and high-efficiency, low-emission vehicle engines. PNNL is operated by Battelle Memorial Institute for the Department of Energy under contract no. DE-AC05-76RL01830. NMR data collection was performed on a project award (https://doi.org/10.46936/cpcy.proj.2021.51813/60000334) from the Environmental Molecular Sciences Laboratory, a DOE Office of Science User Facility sponsored by the Biological and Environmental Research program and located at PNNL. A.A.N. was supported by a National Science Foundation Graduate Research Fellowship, fellow ID 2018253421. P.C.-M. was partially funded by the SENER-CONACYT Sustainable Energy Fund, Mexico with grant 282203. C.M.W. was funded by grants from the Novo Nordisk Foundation (NNF20CC0035580, NNF16OC0021746).

Author contributions

J.D.K. conceived the project and obtained funding. P.C.-M. designed and coordinated the research. P.C.-M. and K.Y., performed molecular biology experiments and fermentations. P.C.-M. performed POP-FA extractions, preparative HPLC, and LC-MS analyses. P.C.-M., prepared and analyzed POP-FAMES. P.C.-M. and K.Y. performed structural analysis of fuelimycins. K.Y. wrote the original draft. C.M.W. tested the protocol steps. All authors revised the manuscript and approved it.

Declaration of interests

P.C.-M, K.Y., 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, Zero Acre Farms, and ResVita Bio.

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Figure legends

Figure 1: Navigation of DIVA home page. For more guidelines on using DIVA to design plasmids, see the DNA Construction Guide located in the top right corner as indicated.

Figure 2: Complete or partial failure to sporulate. In this example, *Streptomyces coelicolor* was grown on ISP2 agar. On the left, spores developed but in small amounts. On the right, *Streptomyces* mycelial growth can be observed but there is a complete lack of spore formation.

Figure 3: Differences in Soy Flour Mannitol plate preparation methods. When the SFM agar is not boiled before autoclaving, the soy flour remains clumped and visually distinct in the resulting plates (left). By boiling and stirring the SFM agar for 5 minutes before autoclaving, these soy flour clumps have dissolved and broken down, and the resulting nutrients are more available for consumption by *Streptomyces* (right).

Figure 4: Uneven spore distribution. Two examples (left and middle) are shown where the distribution of spores (white) are uneven. After gentle spreading with a sterile cotton swab, the plate is uniformly covered with spores (right).

Figure 5: Precipitation in antibiotic overlay. When preparing an antibiotic overlay solution containing nalidixic acid and apramycin/spectinomycin/etc., formation of a white precipitate may be observed (left). After precipitation has been cleared, the solution will return to transparency (right).

Figure 6: Troubleshooting methanol chloroform extractions. Due to incomplete separation of fractions, the lipid layer is thick and the chloroform layer is cloudy (left). After gentle tapping of the sample tube for several minutes, the lipid layer has condensed and the chloroform fraction is transparent (right).

Methods Video S1: Scraping matured *Streptomyces* surface to release spores, related to Before you begin Step 27 and Before you begin Step 40.

Methods Video S2: Filtering out mycelia with cotton and drawing up released spores, related to Before you begin Step 29 and Before you begin Step 40.

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Figure 1

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Figure 2

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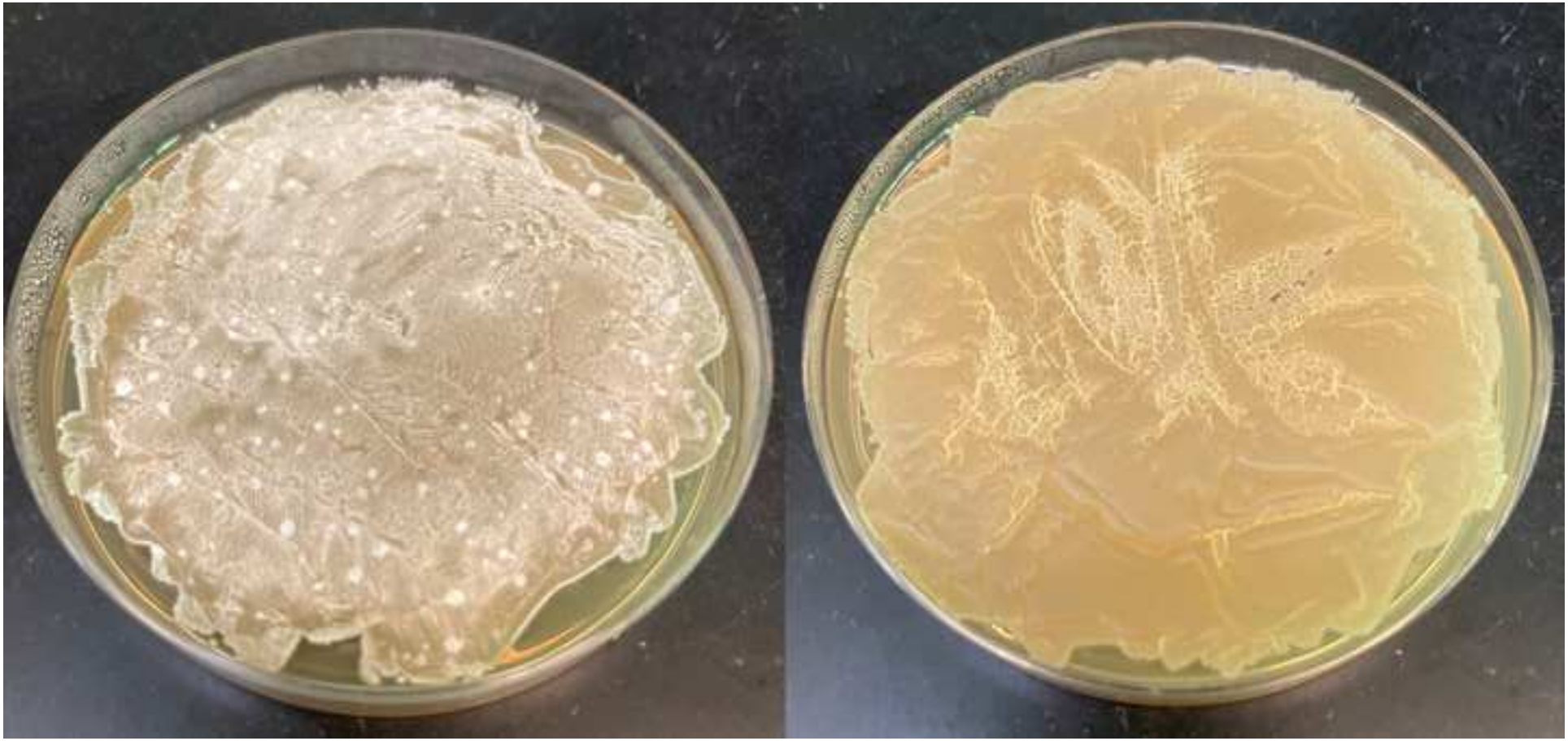

Figure 3

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Figure 4

Figure 5

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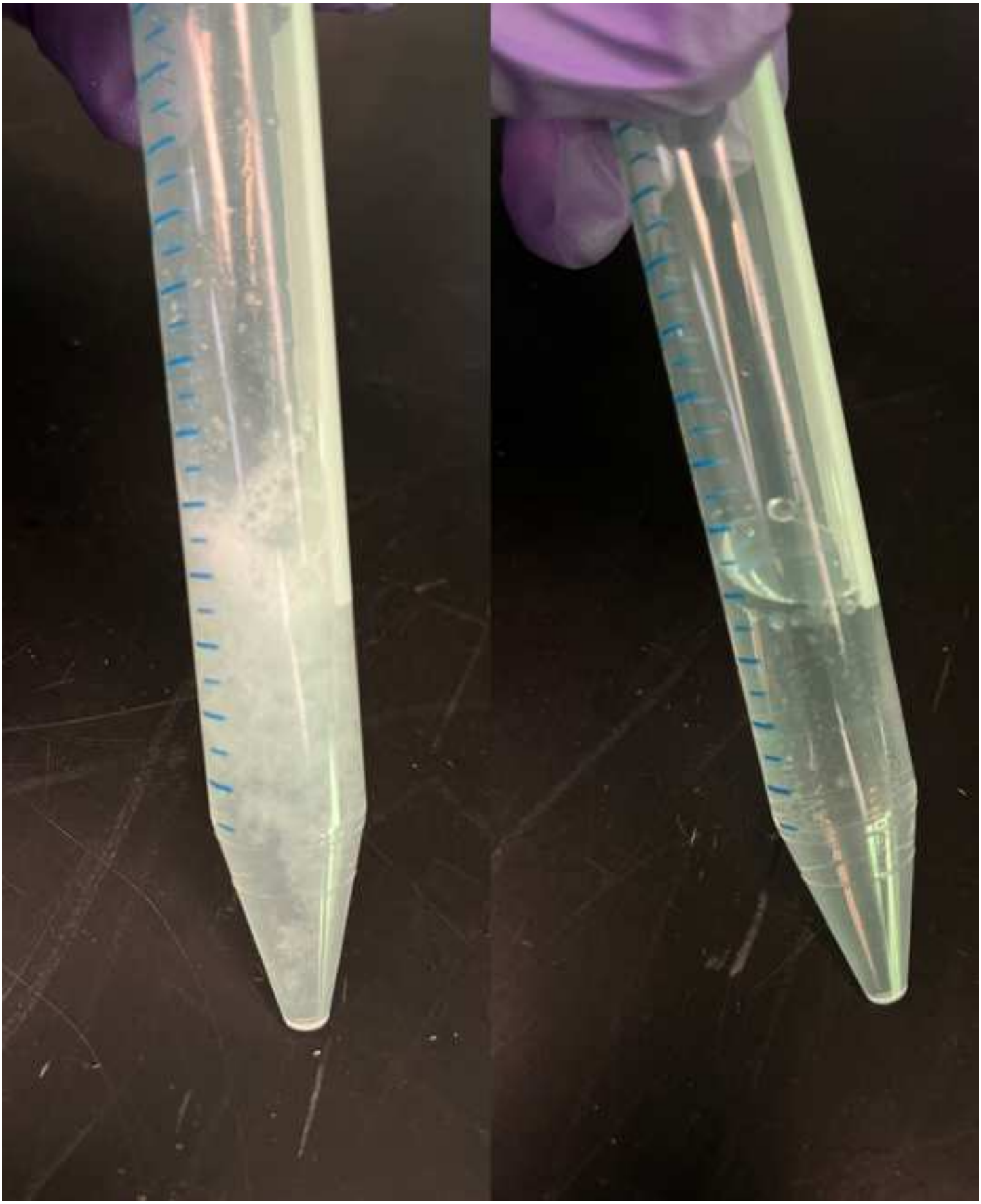

Figure 6

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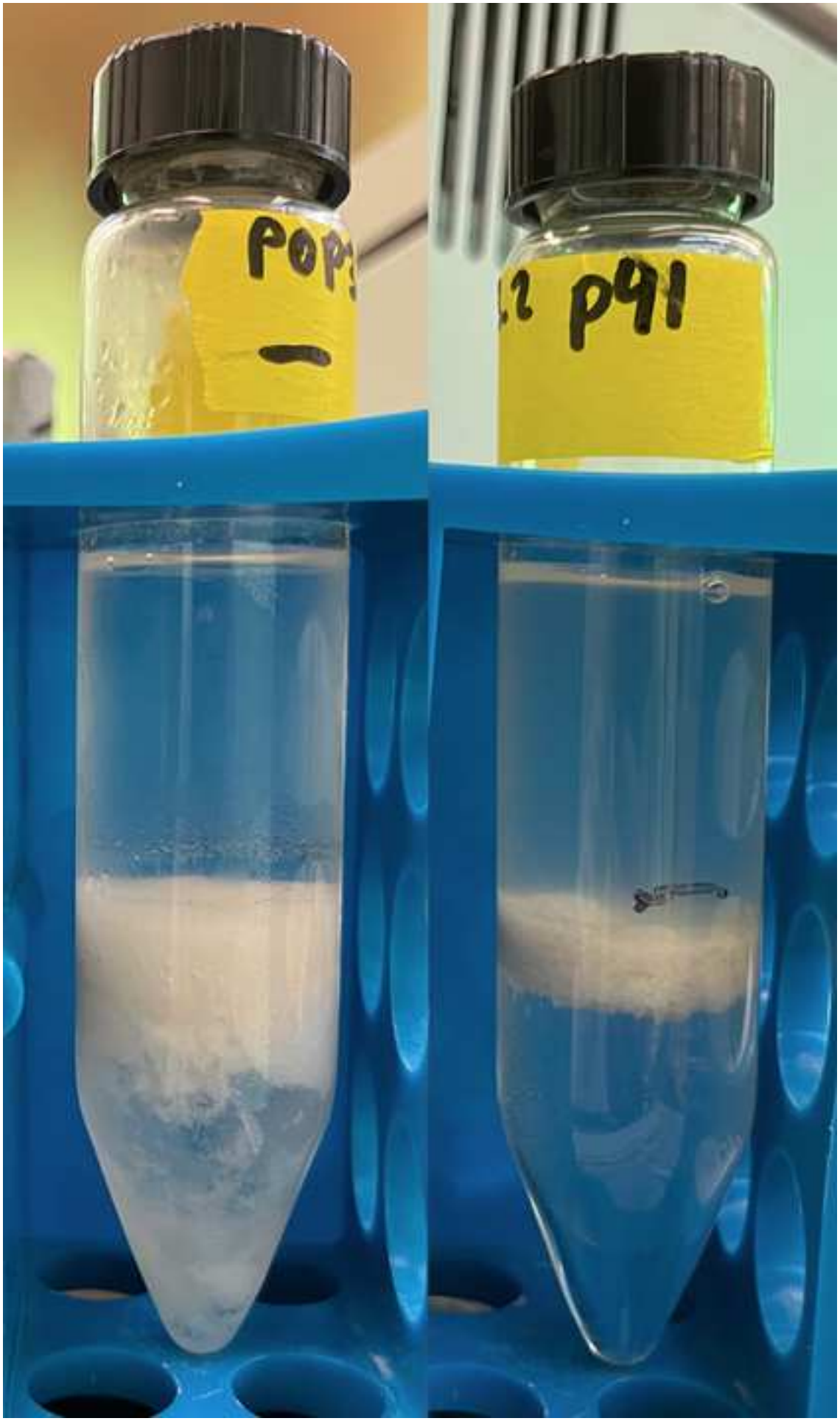

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Figure 360 and Methods Videos

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