Engineering Thermophiles to Produce Drop-in Fuels from Syngas

U.S. Department of Energy (DOE), Bioenergy Technologies Office (BETO)
2017 Project Peer Review

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Biochemical Conversion

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Goal Statement:

- *Goal:* To develop microbial biocatalysts to convert syngas into monoterpenes.
- *Outcome:* The project will engineer novel metabolic capabilities into thermophilic bacteria that use hydrogen for metabolic energy and CO_2 and CO as carbon sources to produce monoterpenes useful as solvents and fuels.
- *Relevance:* Syngas feedstocks are relatively inexpensive and diverse, thermophiles have several unique advantages, and end products have value as fuel and solvent applications among others.

The diversity of feedstocks available to syngas processes (including waste) together with the continuous fermentation potential of thermophiles makes the economics of producing monoterpenes in this bioprocess highly competitive and flexible in dynamic fuel and solvent markets.

Quad Chart Overview

Project Timeline

Start: October 1, 2015
End: September 30, 2017
(67% Complete)

Technical Barriers

1) Transform carboxydotrophic thermophile
2) Identify thermostable monoterpene synthase
3) Circumvent monoterpene toxicity

Technical Target: **Conversion R&D**

Project Budget

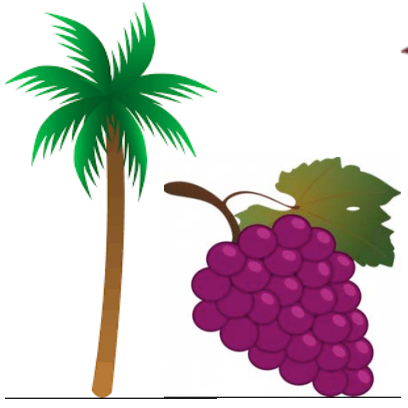
	Costs FY 12 –14	FY 15 Costs	FY 16 Costs	Total Funding (FY 17- End)
DOE Funded	0	0	$815,138	$784,184
Project Cost Share	0	0	$208,310	$191,522

Partner Spending

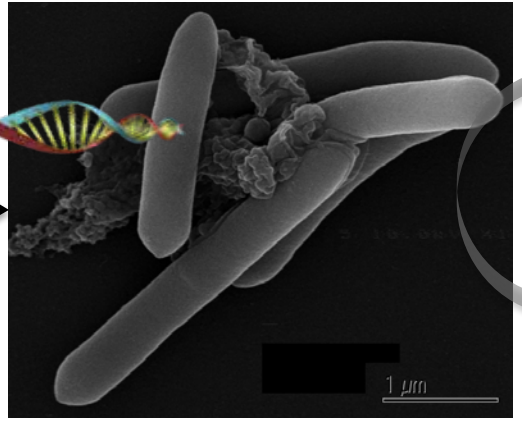
Kiverdi (69%)
NREL (31%)

Project Overview

Genes from monoterpene producing plant species



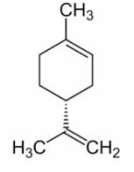
Genetically engineered thermophiles



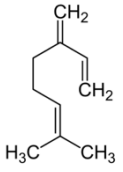
Monoterpene production

Syngas (H_2, CO, CO_2)

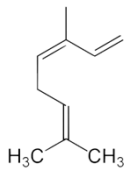
- Municipal waste
- Agricultural waste
- Industrial waste



Limonene



Myrcene



Ocimene

- Identified and characterized multiple terpene synthase enzymes
- Developed analytical methods for monoterpene products
- Identified new thermophile chassis strain and verified CO consumption
- Developed vectors for thermophilic carboxydotrophic bacteria (Go/NoGo)

Project Relevance

Inexpensive and flexible feedstocks

Coupled Syngas and Bioprocess

Limonene 

Myrcene 

Ocimene 

Applications for Monoterpenes

Industrial Solvents	Fuels
• Diluent • Oil sand solvent • Refining agent for crude petroleum • Asphalt grading • Industrial cleaner (e.g., marine vessels, concrete cleaners, parts washer)	• JP-10 jet fuel substitute (dimer) • Bio-gasoline • Gasoline or diesel additive • P-menthane (component of JP-4 / Jet B)

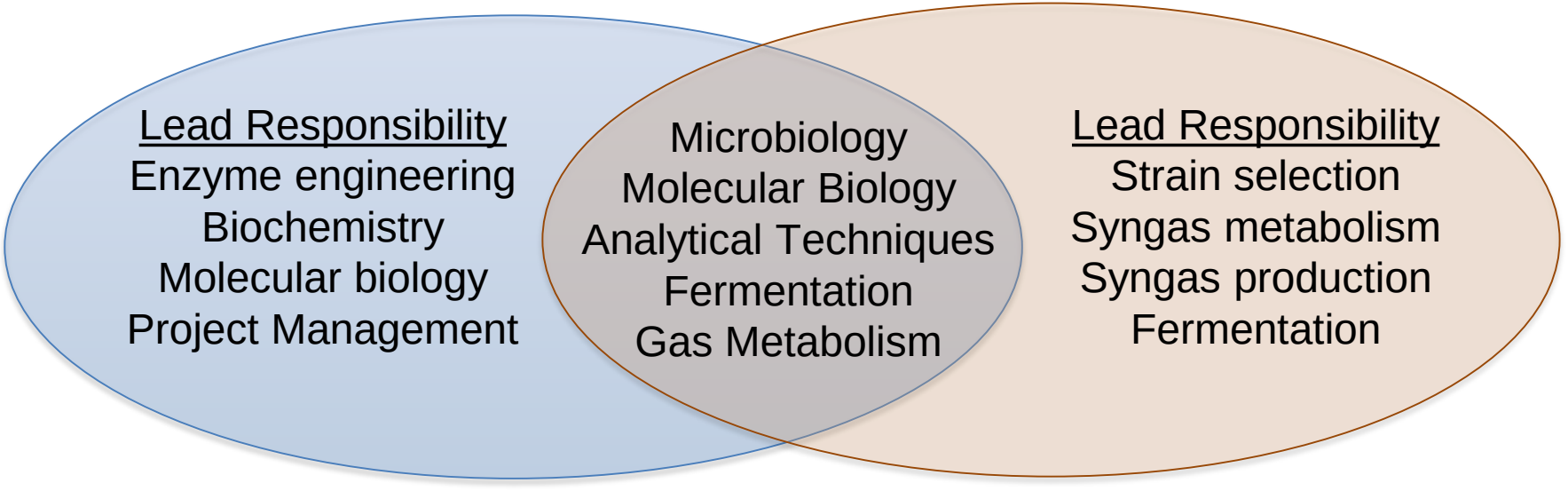
Additional Advantages for Bio-derived Monoterpenes:

- Non-carcinogenic and non-toxic hydrocarbon
- High energy density (37.8 MJ/L, limonene)
- Bio-degradable
- Multiple products with the same process and equipment
- Consumer and home product applications (cleaners and insecticides)

Project Management:

Kiverdi

NREL
NATIONAL RENEWABLE ENERGY LABORATORY



Highly cooperative team science approach. Frequent meetings and offline communication.

Technical Approach

Concurrent Development of Required Technological Components

Analytical methods
– Detection and quantitation of monoterpenes
– Detection of substrate molecules and side products

Microbiology
– Identification of new chassis strain (CO consuming thermophile)
– Development of routine manipulation of chassis strain(s)
- liquid culture, cryogenics, plating, antibiotic selection

Molecular biology
– Identification of candidate thermotolerant Terpen synthases
– Development of vectors to manipulate thermophilic chassis strain(s)
– Identified promoter to drive expression and thermo-tolerant selection method

Biochemistry
– Biochemical assays to test enzyme activity, solubility, and performance
– Biochemical methods to detect enzyme substrates and precursor enzymes

Converge all technical groundwork in year 2 to accomplish goals

Results: Quantitative Analytical Methods

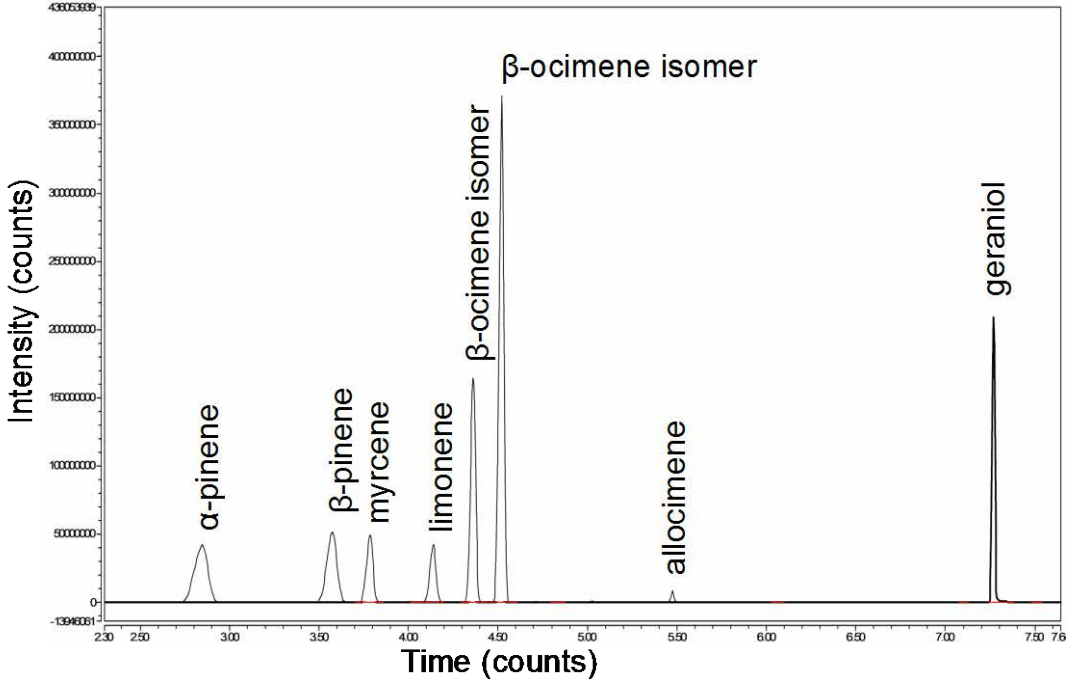
Quantitative analytical assays developed for:

- limonene
- a-pinene
- B-pinene
- Myrcene

Geraniol and ocimene isomers can be identified but not yet precisely quantified

Limit of detection for monoterpenes pushed down to 1ng, limit of quantitation is at 10 ng

Fully Resolved Analytical Detection of Monoterpenes



Identification of Candidate Genes

Literature and database searches identified set of candidate genes

Candidate genes were computationally compared, evaluated, and selected

Genes selected for cloning and evaluation

1) Mint limonene synthase
2) Mandarine orange limonene synthase*
3) Holly Oak pinene synthase*
4) Date Palm terpene synthase (ocimene)
5) Grape terpene synthase (new addition)

* Database annotations incorrect

Grape and holm oak terpene Synthase alignment

```
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            MAL +L+S  + ++S +P           ++ +G  A PVQC+V N+ S    + RR+A
Sbjct  1    MALKLLTSLPMYNFSRVPVSSKDPILLVTSRTRNGYLARPVQCMVANKVSTSPDILRRSA  60

Query  56   NYQAPIWDYDYVQSLRSDYTGETCIGRFDQLKREVKMMLGKVEKPLDQLELIDLLQRLGI  115
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Sbjct  61   NYQPSIWNHDYIESLRIEYVGETCTRQINVLKEQVRMMLHKVVNPLEQLELIEILQRLGL  120

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Sbjct  121  SYHFEEEIKRILDGVYNNDHGGDTWKAENLYATALKFRLLRQHGYSVSQEVFNSFKDERG  180

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Sbjct  181  SFKACLCEDTKGMLSLYEASFFLIEGENILEEARDFSTKHLEEYVKQNKEKNLATLVNHS  240

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Sbjct  241  LEFPLHWRMPRLEARWFINIYRHNQDVNPILLEFAELDFNIVQAAHQADLKQVSTWWKST  300

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             L E LSFARDR +ENF WTVG+IF+PQFGYCRRM TKV ALITTIDDVYDVYGTL+ELE
Sbjct  301  GLVENLSFARDRPVENFFWTVGLIFQPQFGYCRRMFTKVFALITTIDDVYDVYGTLDELE  360

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Sbjct  361  LFTDVVERWDINAMDQLPDYMKICFLTLHNSVNEMALDTMKEQRFHIIKYLKKAWVDLCR  420

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Sbjct  541  DRAASSPFSHIFIEIALNLARMAQCLYQHGDGHGLGNRETKDRILSLLIQPIPL  594
```
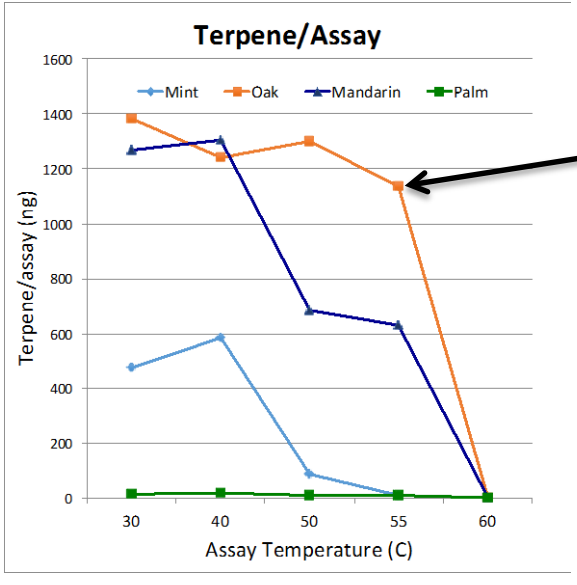

67% Identity, **81%** Similarity, 0% Gaps

Biochemical Evaluation of Enzymes

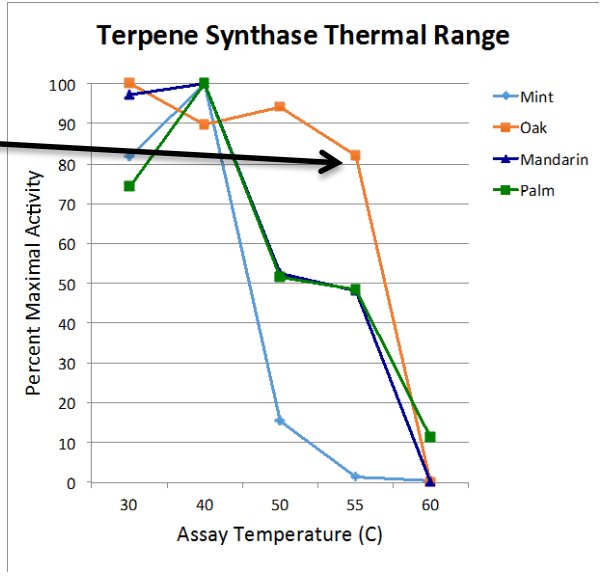
- Candidate genes were cloned into pET28 vectors (*E. coli* expression vectors).
- Epitope tags were added for detection of recombinant enzymes.
- Enzymes were expressed in *E. coli* and evaluated for a number of characteristics.

Amount of Active Enzyme



Holly Oak is leading candidate enzyme

Thermal Stability of Enzymes



Biochemical Evaluation of Enzymes

Characterization pipeline established for recombinant enzymes

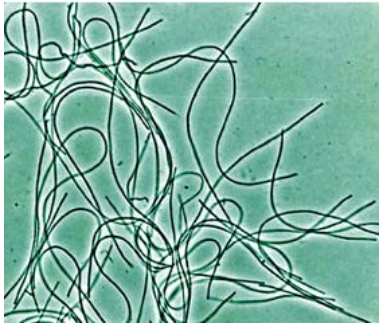
TS Construct	CBB	West.	Soluble	Activity (Insol.)	Activity (solub.)	Other Products	Product (ng)
Mint LS*							
Mint LS							
Date palm LS							*
							**
						e pinene	**1564**

* Ocimene quantitation is approximate

** One-off experiment has not been repeated yet

Chassis Strain Selection

Originally Proposed Strain*: Chlororflexus aurantiacus*



- Thermophilic green non-sulfur bacteria (40-50^{o}C)
- Photosynthetic (anaerobically), but capable of aerobic growth in the dark
- Grows autotrophically on H_2/CO_2, and heterotrophically (organic acids, sugars)
- Putative CO dehydrogenase identified
- High natural terpenoid pathway flux (photosynthetic pigments)

Early evaluation revealed *Chlororflexus aurantiacus* to be unsuitable host for project goals.

- **No/poor growth under dark aerobic conditions regardless of carbon source**
- **No evidence of any CO/Syngas uptake under any growth condition**

Early strain vetting revealed a need to identify suitable “chassis” strain for project goals

Chassis Strain Screening

12+ potential strains identified, 9 strains underwent extensive testing on criteria.

Strain evaluation criteria:

1) CO/Syngas consumption

2) Thermal range of growth

3) Available genome sequence

4) Genetic tractability

5) Metabolic properties
(e.g. terpene flux)

Microbe	CO uptake	Aerobic/ Anaerobic	Autotrophic growth on syngas components (with O_2 for aerobic strains)				Genetic tractability	Genome Sequence	High flux to terpenes	Growth Temp (^oC): Optimum (Min)
			CO	H_2/ CO_2	H_2/CO_2 /CO	H_2/CO				
Chloroflexus aurantiacus	-	Both	-	+	ND	ND	?	+	+	48 (40)
Rubrivivax gelatinosus CBS	+	Both	++ +	+++	+++	+++	+	+	+	35
Cupriavidus necator	-	Both	-	+++	ND	ND	+	+	-	30
Cupriavidus metallidurans	-	Both	-	+++	ND	ND	(+)	+	-	30
Thermomicrobium roseum	+	Aerobic	-	-	-	ND	?	+	+	70 (55)
Moorella thermoacetica	+	Anaerobic	ND	ND	+	ND	(+)	+	+*	55 (45)
Hydrogenobacter thermophilus	ND	Aerobic	ND	+	ND	ND	?	+	?	70
Carboxydothermus hydrogenoformans	(+)	Anaerobic	ND	ND	ND	ND	?	+	+*	65 (40)
Carboxydocella thermautotrophica	(+)	Anaerobic	(+)	ND	ND	ND	?	-	ND	58 (40)
Caldanaerobacter subterraneus sp. yonseiensis	+	Anaerobic	ND	ND	ND	+	?	+	+*	75 (50)
Hydrogenibacillus schlegelii	+	Aerobic	ND	ND	+	ND	?	+	-*	65 (42)

Identification of New Chassis Strains

Variables tested during growth optimization experiments:

1) Autotrophic vs. mixotrophic growth
2) Media composition
3) Uptake of Syngas components (CO, H_2, CO_2)
4) Culture: headspace ratio (optimized gas mixing
5) Temperature (minimal/maximal, optimal)

35+ growth experiments for top 6 candidate strains, each lasting 3-5 days

Three suitable candidate thermophilic strains identified as project chassis strains

Microbe	Aerobic/ Anaerobic	CO uptake	Growth and utilization of synthetic syngas	Genetic tractability	Genome Sequenced	High flux to terpenes	Optimum growth temp °C (Minimum)
Moorella thermoacetica	Anaerobic	+	+	(+)	+	+*	55 (45)
Thermomicrobium roseum	Aerobic	+	+	?	+	+	70 (55)
Caldanaerobacter subterraneus sp. tengcongensis	Anaerobic	+	+	(+)	+	+*	75 (50)

(+) reported in literature; * based on annotated pathways in genomes

Note: Two mesophilic backup strains also identified as contingency strains for this project.

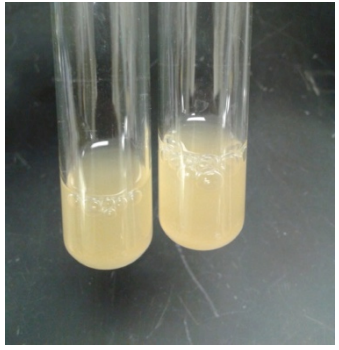
Genetic Tool Development: *Moorella thermoacetica*

Transformation protocol reported in literature was not reproducible in ATCC and DSM strains.

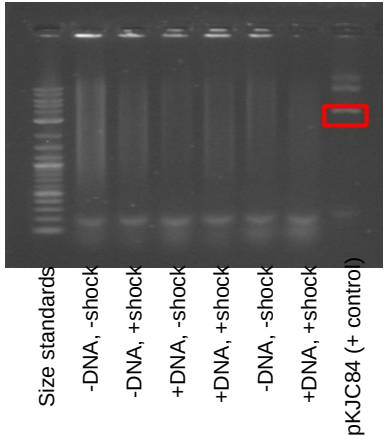
OD600	Plasmid (1.2 ug)	Wash	Electroporation	Recovery time	Thiamphenicol (liquid medium)	Growth in liquid?	Growth in liquid after dilution? (1:25)	# colonies* (Th 10)	Protocol
0.93	pKJC84	H2O	1200 V, sq. wave	30 min	ND	ND	ND	0	Lin et al
0.93	pKJC84	H2O	1200 V, sq. wave	30 min	15	—	ND	ND	Lin et al
0.93	pKJC84	EP buffer	1200 V, sq. wave	30 min	ND	ND	ND	0	NREL
0.93	pKJC84	EP buffer	1200 V, sq. wave	30 min	15	—	ND	ND	NREL
1	pKJC84	H2O	1200 V, sq. wave	1 hr	ND	ND	ND	0	Lin et al
1	pKJC84	H2O	1200 V, sq. wave	1 hr	10	++	ND	0	Lin et al
1	pKJC84	EP buffer	1200 V, sq. wave	1 hr	ND	ND	ND	0	NREL
1	pKJC84	EP buffer	1200 V, sq. wave	1 hr	10	++	ND	0	NREL
0.142	pKJC84	Sucrose	1500 V, exp.	19 hr	ND	ND	ND	0	Kita et al
0.142	pKJC84	Sucrose	1500 V, exp.	19 hr	10	ND	—	ND	Kita et al
0.142	pKJC84	Sucrose	none	19 hr	ND	ND	ND	0	Kita et al
0.142	pKJC84	Sucrose	none	19 hr	10	ND	—	ND	Kita et al
3	pKJC84	EP buffer	1200 V, sq. wave	0	10	++	ND	0	NREL
3	pKJC84	EP buffer	1200 V, sq. wave	0	35	++	—	0	NREL
3	pKJC84	EP buffer	1200 V, sq. wave	24 hr	10	ND	+	0	NREL
3	pKJC84	EP buffer	none	0	10	++	ND	0	NREL
3	pKJC84	EP buffer	none	0	35	—	ND	0	NREL
3	pKJC84	EP buffer	none	24 hr	10	ND	++	0	NREL
3	none	EP buffer	1200 V, sq. wave	0	10	++	ND	0	NREL
3	none	EP buffer	1200 V, sq. wave	0	35	+	ND	0	NREL
3	none	EP buffer	1200 V, sq. wave	24 hr	10	ND	+	0	NREL
3	none	EP buffer	none	0	10	++	ND	0	NREL
3	none	EP buffer	none	0	35	++	—	0	NREL
3	none	EP buffer	none	24 hr	10	ND	+	0	NREL

OD600	Plasmid (1.2 ug)	Wash	Electroporation	Heat shock (71°C)	Recovery time	Thiamphenicol (liquid medium)	Growth in liquid?	# colonies* (Th 10)	Protocol
0.177	pKJC84	Sucrose	1500 V, exp.	5 min	15 min	ND	ND	?	Kita et al plus Rest et al
0.177	pKJC84	Sucrose	1500 V, exp.	10 min	15 min	ND	ND	?	
0.177	pKJC84	Sucrose	1500 V, exp.	15 min	15 min	ND	ND	?	

Outgrowth observed in selective medium after transformation



No exogenous DNA detected in cells



Despite a number of attempts and strategies to optimize conditions, no Moorella transformants were verified.

Genetic Tool Development: *Thermicrobium roseum*

Development of electroporation protocols for transformation unsuccessful to date.

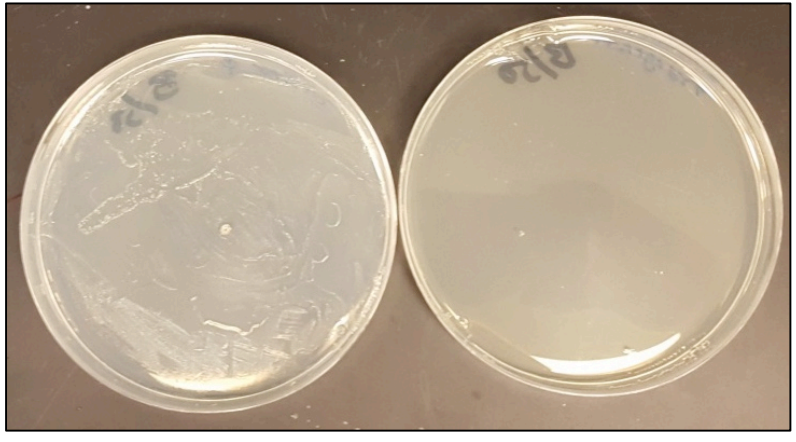
OD600	Plasmid (1 ug)	Wash	Electroporation	Recovery time	Thiamphenicol (liquid medium)	Growth in liquid?
0.435	pBBR1MCS	H2O	1000 V, exp	20 hrs	15	—
0.435	none	H2O	1000 V, exp	20 hrs	15	—
0.435	pBBR1MCS	H2O	none	20 hrs	15	—
0.435	none	H2O	none	20 hrs	15	—
0.435	pBBR1MCS	Sucrose	1000 V, exp	20 hrs	15	—
0.435	none	Sucrose	1000 V, exp	20 hrs	15	—
0.435	pBBR1MCS	Sucrose	none	20 hrs	15	—
0.435	none	Sucrose	none	20 hrs	15	—

Attempts using various transformation strategies failed to result in transformants to date.

Genetic Tool Development: *C.s. tengcongensis*

Anaerobic plating protocol developed

Antibiotic selection on plates established



- kanamycin + kanamycin

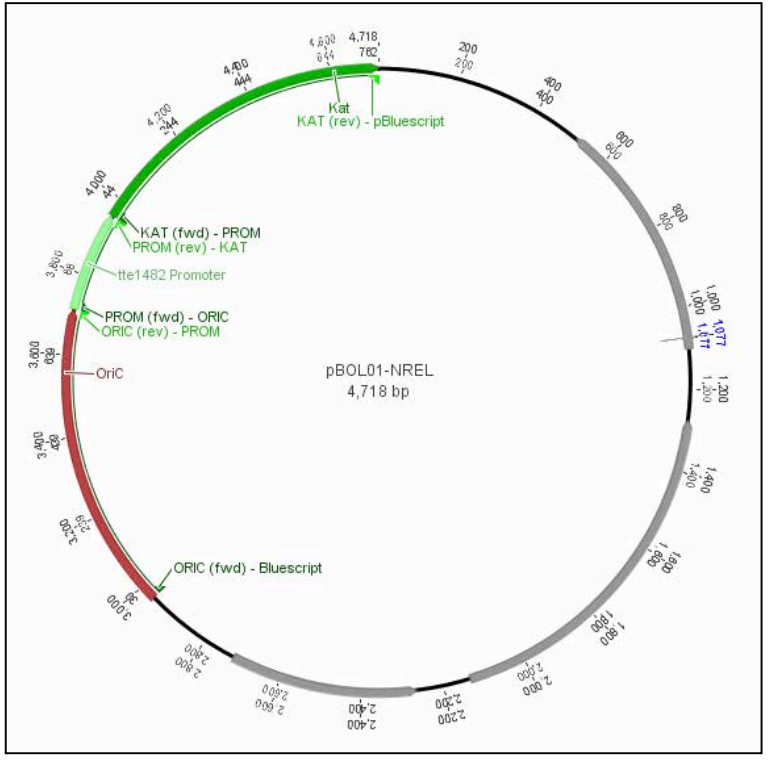
- *C.s. tengcongensis*: anaerobic thermophile that consumes CO
- Growth and transformation at 60^{o}C
- Incubation of plates in gasket-sealed anaerobic container
- Sensitivity to 400 μg/ml kanamycin (plate and liquid)

Thermophile Genetic Tools: *C.s. tengcongensis*

Shuttle Vector Development

- *tengcongensis* origin of replication
- *tengcongensis* promoter: phosphate acetyltransferase
- Thermostable resistance to kanamycin (kat):
  - from pMK18 (de Grado et al., 1998)
- Backbone: pBluescript II KS +
- Gibson assembly:
  - Designed PCR primers with Geneious software
  - PCR amplified origin and promoter from *tengcongensis* genomic DNA (Q5 polymerase)
  - PCR amplified Kat gene from pMK18
  - Assembled fragments (NEB Gibson Assembly kit)
- *E. coli* strain JM109 for propagation:
  - recA- and endA- to improve stability
  - Amp selection, blue-white screening

Novel Vector Constructed (pBOL01-NREL)

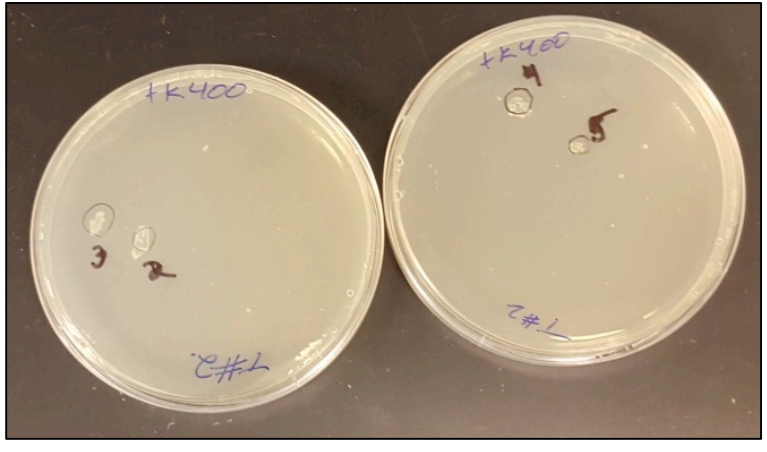


Thermophile Genetic Tools: *C.s. tengcongensis*

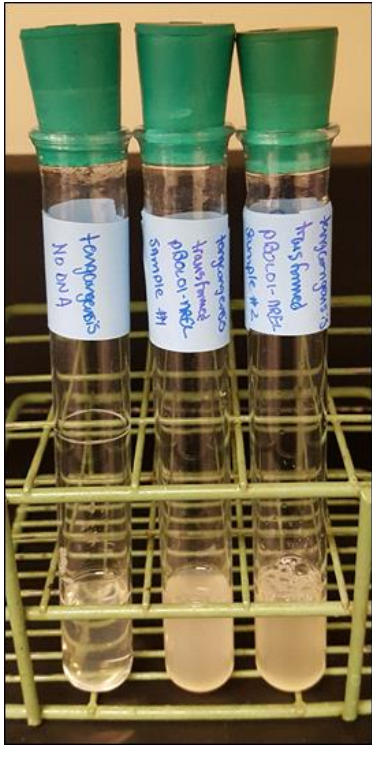
Transformation method: **Natural competence**

Grow liquid culture to OD (600 nm) 0.8-1.2 (in TTE + starch)
Dilute and incubate culture +/- plasmid, 8 hours at 60°C (unshaken)
(1) Apply to plates +/- kanamycin and incubate at 60°C for 66 hours
(2) Inoculate liquid +/- kanamycin, incubate at 60°C

Transformants: growth on selective plates and liquid (Kan)



Four identified colonies on selective plates transferred to liquid selection and propagated



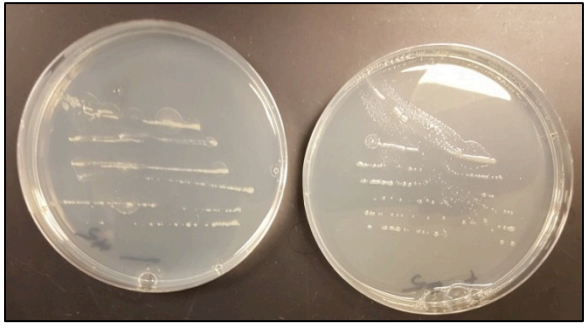
Vector: - + +

Thermophile Genetic Tools: *C.s. tengcongensis*

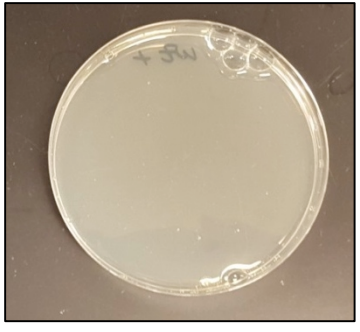
Transformed kanamycin resistance is stable

4 transformants retain antibiotic resistance when re-streaked or grown in selective liquid (400 μg/ml Kan)

No-DNA controls remain sensitive to Kan and fail to grow (mock transformations)



Transformant - kanamycin	Transformant + kanamycin



No-DNA control (WT) + kanamycin

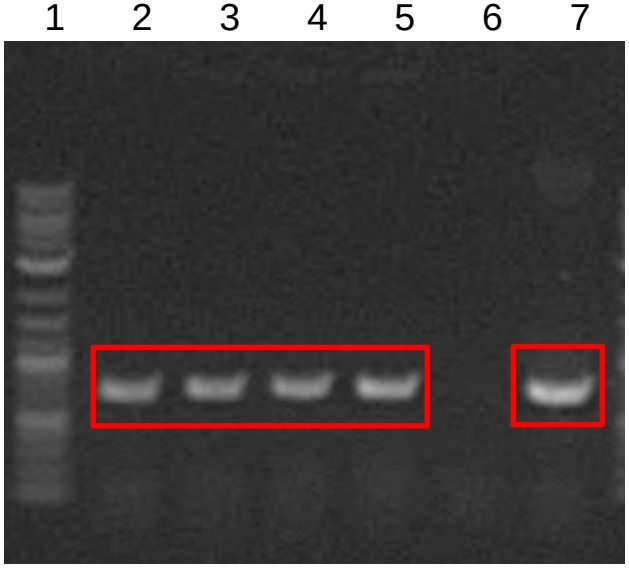
Transformants + kanamycin	WT - kan	WT + kan

Validation of Transformation: *tengcongensis*

Direct probe for exogenous DNA in host strain

PCR from transformed cells to probe for thermostable kanamycin resistance gene (KAT)

PCR product of KAT gene (761 bp)



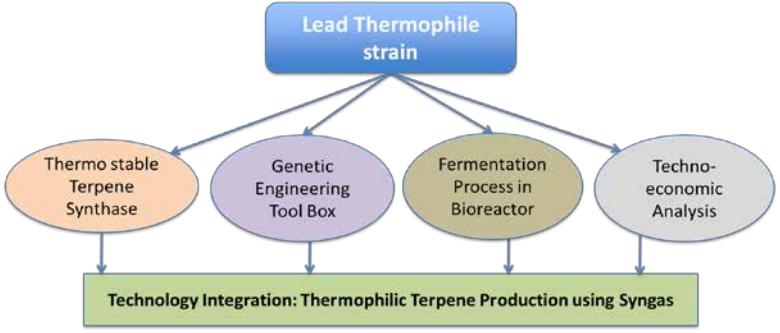
1. Size standards-2-log ladder
2. Transformant #2
3. Transformant #3
4. Transformant #4
5. Transformant #5
6. Cells with no plasmid
7. pMK18 DNA (+ control)

Unambiguous validation of exogenous DNA present in host cells

Current and Future Work

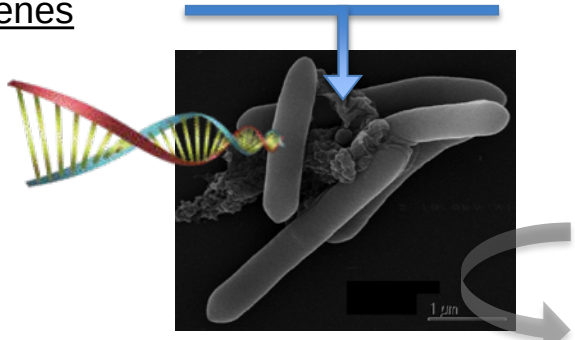
Year one accomplishments:

- New chassis strain identified
- Thermostable enzymes characterized
- Vector and transformation established
- Gas fermenttion established



Year two: Technology integration for monoterpene production from syngas.

Identified Thermostable Genes
- Myrcene synthase
- Limonene synthase
- Ocimene synthase
- Limonene synthase



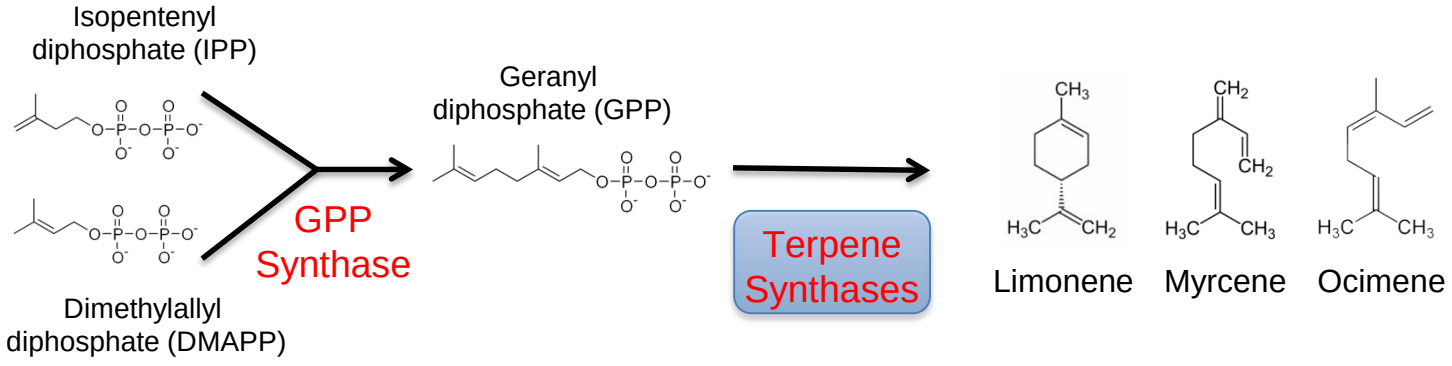
H_2, CO, CO_2 Syngas Fermentation

Monoterpene Production

Validated microbial and molecular tools
- Identified chassis strain
- Shuttle vector, transformation, selection
- Functional promoter

Current and Future Work

Metabolic Pathway Manipulation strategy



- Clone terpene synthases into *C.s. tencongensis*
- Probe for monoterpene production
- Probe for GPP pools
- Probe for GPP synthase activity
- Identify thermostable GPP synthase genes

Acknowledgements

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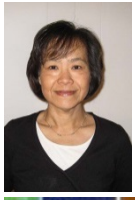
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