

# **iBiology.org Teaching Tools**

## **Chaitan Khosla's Lecture Part 2:**

### **Dissecting Polyketide Assembly Lines**

Teaching Tools were prepared by Becky Anderson and Ben Barsi-Rhyne.

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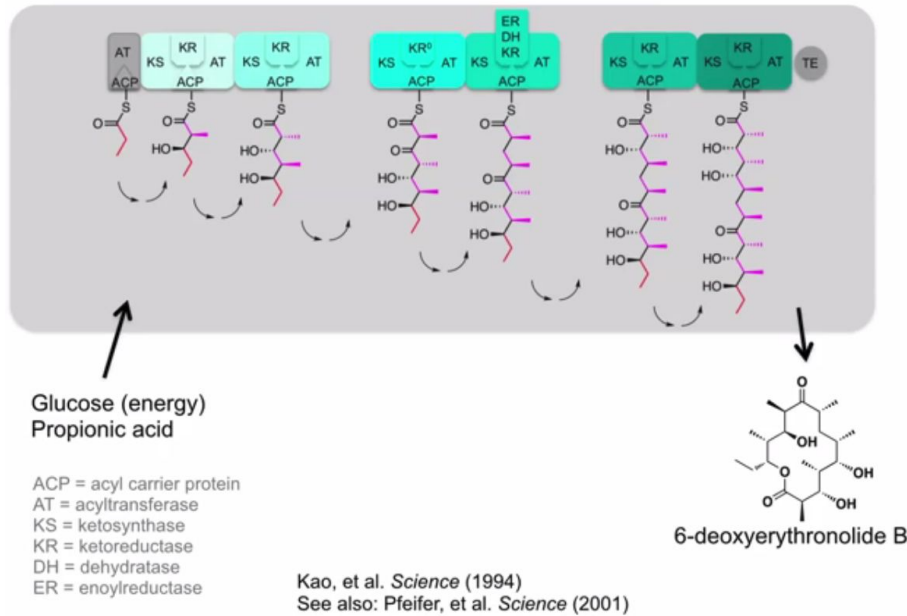
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#### **1. Keywords and Terms**

Kinetics, stereocenter, trans-complementation, rate-limiting step, substrate

#### **2. Lecture Notes**

## In vivo Refactoring



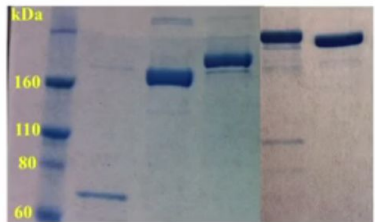
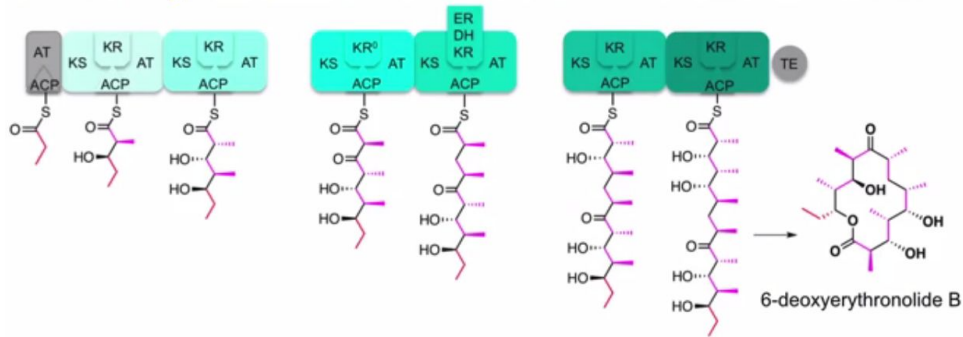
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### 1:25 min

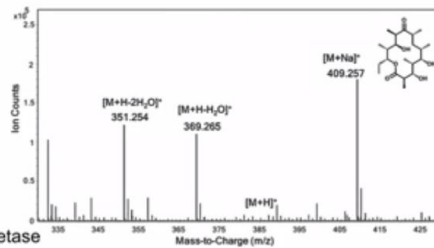
The DEBS pathway can be placed into *E. coli* through well-established genetic techniques. This allows:

- Production of 6-deoxyerythronolide B with glucose and propionic acid as precursors.
- Easy manipulation of the DNA that encodes the proteins to probe the function of each protein.

## Assembly Line Reconstitution from Purified Proteins



+ propionyl-CoA + methylmalonate + malonyl-CoA synthetase  
+ NADPH + ATP + CoASH



Lowry, et al. *JACS* (2013)



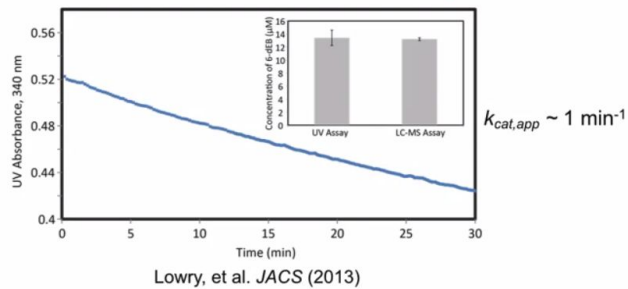
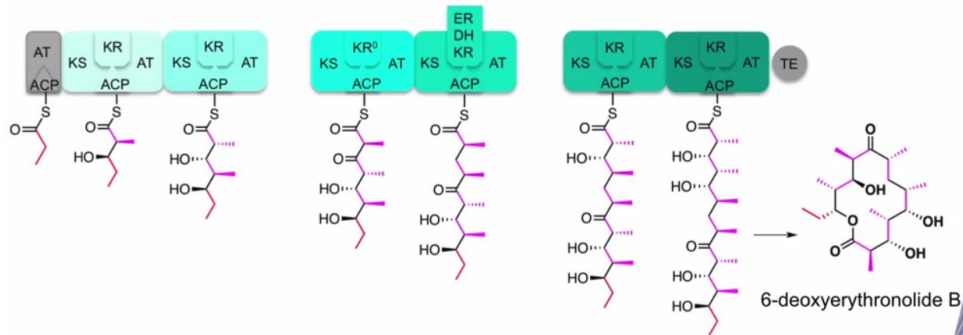
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### 3:53 min

The entire DEBS pathway can also be reconstituted from purified proteins.

- On the bottom left are the isolated proteins. The two bands on the right are whole proteins while the first three are a protein that was split into three parts.
- On the bottom right is the mass spectrum of the products of the reconstituted system showing that the desired product is being produced.

## Assembly Line Reconstitution from Purified Proteins



ACP = acyl carrier protein  
 AT = acyltransferase  
 KS = ketosynthase  
 KR = ketoreductase  
 DH = dehydratase  
 ER = enoylreductase

### 6:28 min

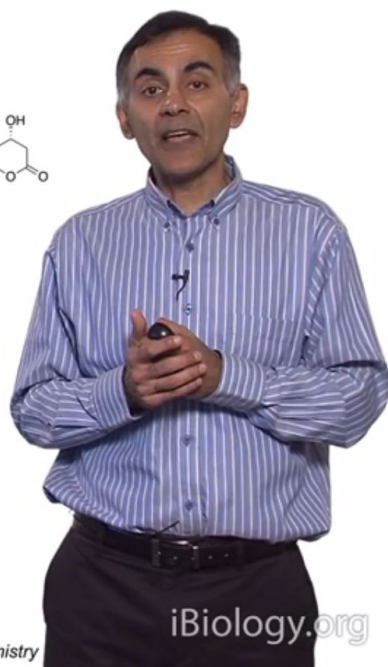
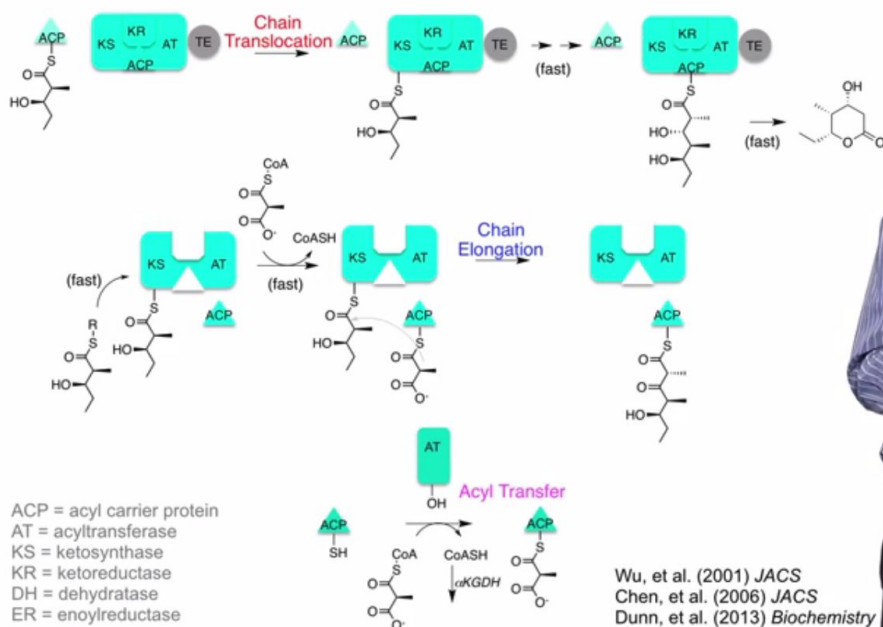
The graph shows the reconstituted system has a turnover rate of  $\sim 1$  molecule per minute.

The inset of this graph shows that there is stoichiometric mapping of equivalents of 6-deoxyerythrothronolide to propionyl-CoA equivalents by NADPH assay.



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## Analysis of the Core Reactions

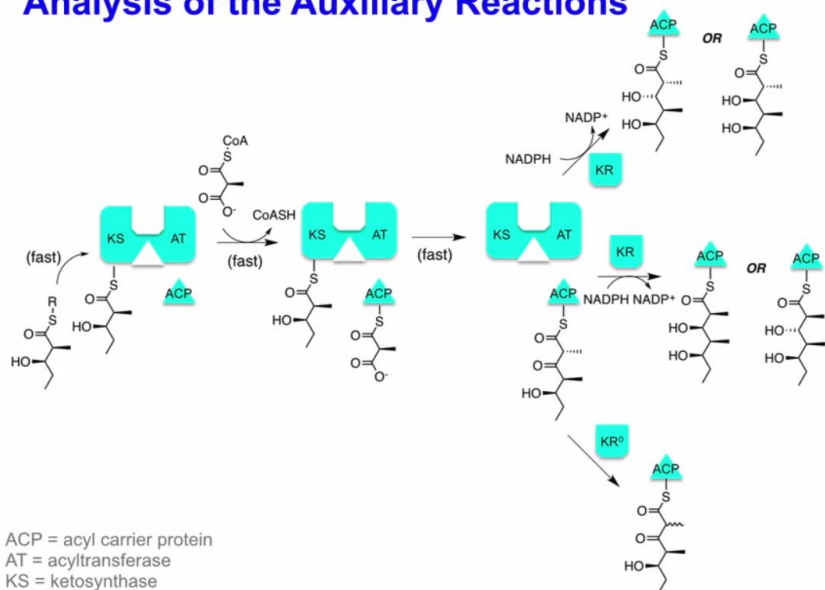


**10:28 min**

To tease apart the core reactions kinetic isolation was used. This involves taking a component of the system and making it the slowest step in the reaction by manipulating the reaction mixture.

This general scheme was used for chain translocation, chain elongation, and acyl transfer steps in the assembly line.

## Analysis of the Auxiliary Reactions



ACP = acyl carrier protein  
 AT = acyltransferase  
 KS = ketosynthase  
 KR = ketoreductase  
 DH = dehydratase  
 ER = enoylreductase

Chen, et al. (2006) *Chem. Biol.*  
 Castonguay, et al. (2007) *JACS*  
 Valenzano, et al. (2010) *JACS*

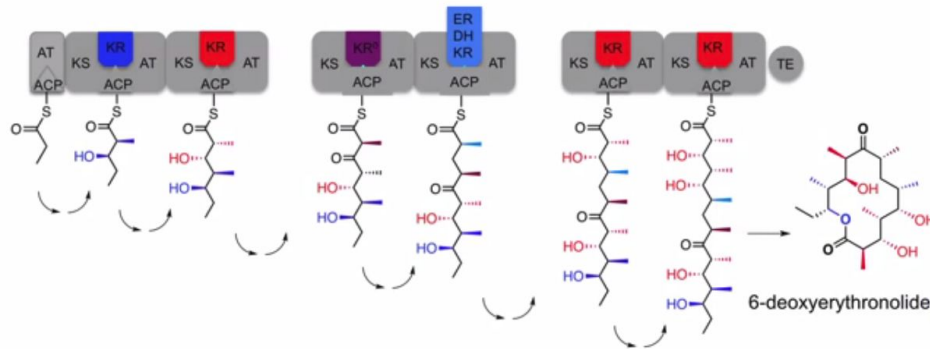


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**10:49 min**

Kinetic isolation can also be used for auxiliary reactions such as chain modification.

## Stereospecificity



ACP = acyl carrier protein  
 AT = acyltransferase  
 KS = ketosynthase  
 KR = ketoreductase  
 DH = dehydratase  
 ER = enoylreductase

Castonguay, et al. (2007) *JACS*  
 Castonguay, et al. (2008) *JACS*  
 Valenzano, et al. (2009) *JACS*  
 Valenzano, et al. (2010) *JACS*  
 You, et al. (2013) *JACS*  
 Garg, et al. (2013) *JACS*



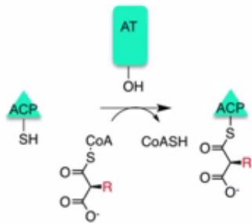
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### 12:41 min

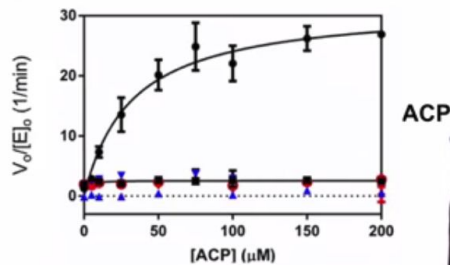
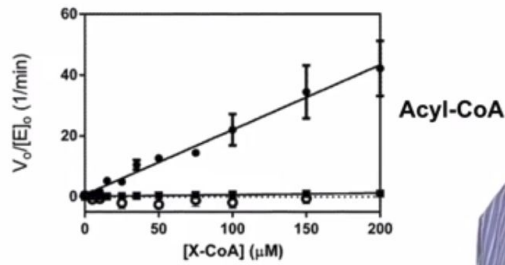
The DEBS assembly line is remarkably good at setting stereocenters. The product has 10 stereocenters meaning there 210 possible chiral forms of which only 1 is formed.

The structure on right has its stereocenters colored to match the colors of the enzymes in the assembly line diagram.

## Extender Unit Specificity



ACP = acyl carrier protein  
 AT = acyltransferase  
 KS = ketosynthase  
 KR = ketoreductase  
 DH = dehydratase  
 ER = enoylreductase



Dunn, et al. (2013) *Biochemistry*



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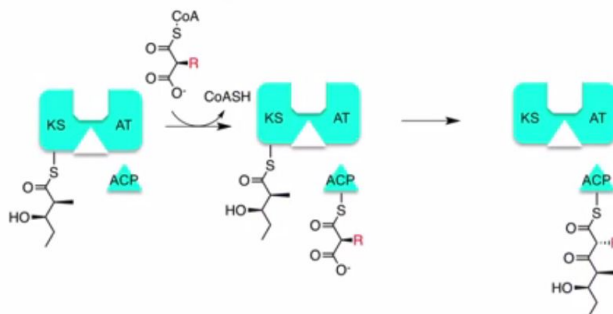
**16:46 min**

The reaction scheme on the left shows the addition of an extender unit.

The R group is normally a methyl, but when it is changed to a hydrogen or an ethyl, shown in the top right graph, the rate of product formation is drastically reduced.

Another way to generate specificity is with protein-protein interactions. The bottom right graph shows the rate of product formation when an acyltransferase is paired with different acyl carrier proteins. Rate of product formation is drastically reduced when a mismatched ACP is used.

## Extender Unit Specificity of Downstream Enzymes



Module	R	Relative rate
Module 3	Me	100%
Module 3	H	27%
Module 6	Me	100%
Module 6	H	70%

ACP = acyl carrier protein  
 AT = acyltransferase  
 KS = ketosynthase  
 KR = ketoreductase  
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 ER = enoylreductase

Chen, et al. (2006) JACS

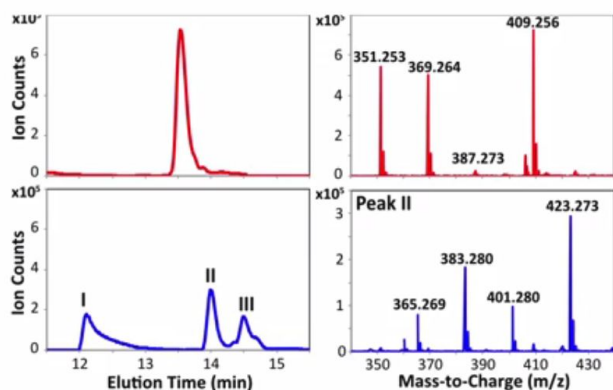
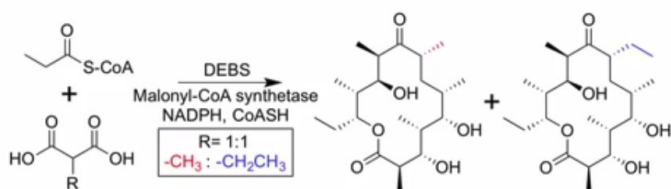


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### 21:21 min

Specificity for a methyl-malonyl extender unit is higher in earlier modules. Module 6 is much less specific than module 3 and can incorporate a hydrogen-malonyl more easily.

## Extender Unit Specificity of the Assembly Line



Lowry, et al. *JACS* (2013)

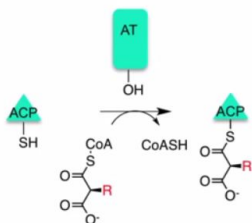


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### 23:04 min

The addition of methyl and ethyl malonyl-CoA into the reaction at equal concentrations results in a variety of products. The mass spectrum of methyl and ethyl products are shown on the bottom right. The location of addition of ethyl group is currently unpredictable.

## Trans-Complementation with Highly Active Acyltransferases



AT	Acyl-CoA	$k_{cat}/K_M$ ( $\mu\text{M}^{-1} \text{min}^{-1}$ )
DEBS	methylmalonyl-CoA	1
DSZS (also low specificity for ACP substrates)	malonyl-CoA	230

ACP = acyl carrier protein  
 AT = acyltransferase  
 KS = ketosynthase  
 KR = ketoreductase  
 DH = dehydratase  
 ER = enoylreductase

Dunn, et al. *Biochemistry* (2011)

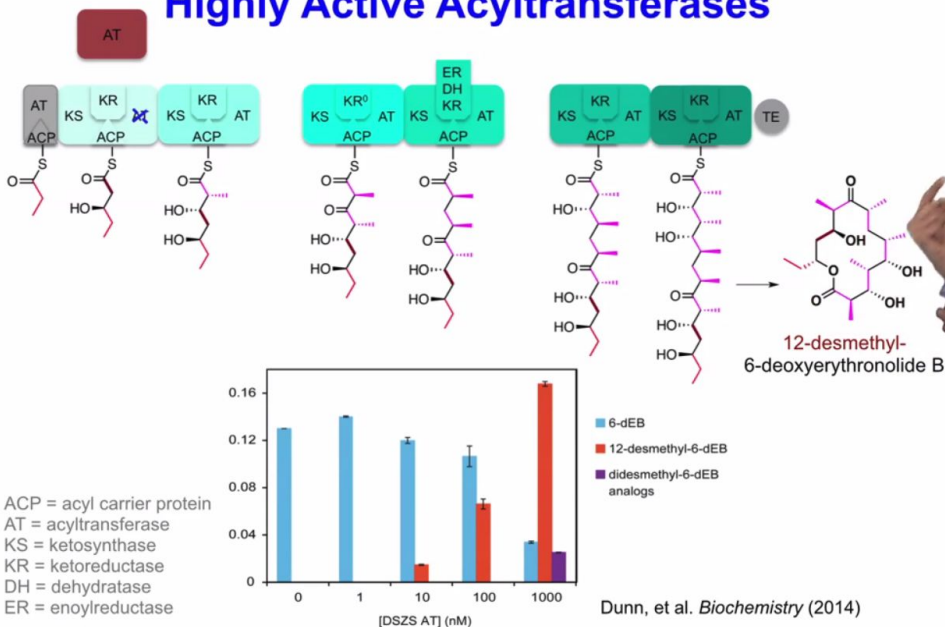


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### 25:17 min

It is possible to use another acyltransferase (AT) to alter the structure of the product, but this requires a standalone AT with a high rate and low specificity for its ACP.

## Trans-Complementation with Highly Active Acyltransferases



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25:59 min

Trans-complementation requires knocking out the native AT and replacing it with an exogenous AT. In the example shown here the red AT favors malonyl instead of methyl-malonyl resulting in a single change to the macrocycle.

### 3. Review Questions

1. What is an advantage of having the DEBS assembly line in *E. coli*?
2. How were reactions of interest kinetically isolated?
3. How many stereocenters does each enzyme control?
4. What R group is preferred in the assembly line and how was this shown?
5. What happened when methyl and ethyl-malonyl-CoA were mixed?
6. What are the requirements for trans-complementation of acyltransferase?

#### 4. Answers to Review Questions

1. *E. coli* DNA can be easily manipulated allowing you to probe protein function.
2. They were able to reconstitute particular parts of the assembly line so that the reaction of interest was the rate-limiting step.
3. 2
4. Methyl is preferred. This was shown by adding or removing a carbon to the substrate and observing the rate of production.
5. Ethyl was incorporated into the product, but its location was unpredictable.
6. The AT must be a stand-alone protein that doesn't have specificity for its own module and must have a significantly higher rate than the native AT.

#### 5. Discussion Questions

1. Why might a molecule like 6-deoxyerythronolide B need this type of assembly line instead of a standard enzymatic pathway?
2. Why do you think adding other R groups (other than hydrogen and ethyl) to the growing polyketide chain was not discussed?
3. What do you think is an interesting open question presented in this lecture and why?

#### 6. Answers to Discussion Questions

1. 1. One answer might be that complex molecules require complex protein processes to be created. A more interesting answer might be a discussion about what sets this system apart from a run of the mill enzymatic pathway.
2. 2. It is likely that other R groups that are larger or more charged than ethyl are not shown because they do not incorporate well into the growing polyketide chain. This might be significant because it would mean that new proteins would have to be identified or current proteins would have to be evolved in order to add interesting functional groups. The less interesting answer is of course that they have tested other R groups, but elected not to show it.

3. To me the most interesting question involves stereochemistry. Is this finely tuned machine that is capable of producing 1 chiral form of a possible 1024 something that slowly evolved over time? Are there versions of this pathway that have less fidelity than the one described? If one of these pathways can be found might it have any biomedical applications?

## 7. Questions for Discussion Paper

Walker, MC. et al. Expanding the fluorine chemistry of living systems using engineered polyketide synthase pathways.

Science. 2013 Sep 6; 341(6150): 1089–1094.

1. What was the overall goal of this research? Why would achieving this goal be useful?
2. How are the experiments shown in figures 2A and 2B similar? What is different about them?
3. Describe the experimental set-up depicted in Figure 3. What are the two main results from this experiment?
4. What is the purpose of making the different mutations shown in Figures 4C and D? How do these mutations affect the DEBS modules, and how is this useful?
5. What traits of *E. coli* are important for the *in vivo* chain extension reactions using fluoromalonyl-CoA?

## 8. Answers to Questions for Discussion Paper

1. The goal of this research was to develop a method of introducing fluorine into small molecules that could serve as building blocks for complex fluorinated products. Specifically, fluoromalonyl-CoA could act as an extender unit for polyketide biosynthesis, enabling the introduction of fluorine into structurally complex and biologically active compounds. This would be useful because many pharmaceutical compounds contain at least one fluorine atom. Organofluorines are also used in diverse fields including diagnostics and agriculture.

2. They are similar because they both result in the production of fluoromalonyl-CoA. However, they use different pathways to create this product. In 2A, a two-step reaction is used starting from fluoroacetate. This is important because fluoroacetate is produced naturally by certain bacteria. In 2B, CoA is directly ligated to fluoromalonate.
3. This experiment tests whether a fluorinated extender can be used in a chain elongation reaction. Fluoromalonyl-CoA was used as an extender to lengthen an acetyl-CoA starter. This reaction was catalyzed by a simple ketosynthase NphT7 and the ketoreductase PhaB. The two main results are that the ketosynthase could efficiently use fluoromalonyl-CoA as an extender to create acetofluoroacetyl-CoA, and that the ketoreductase was capable of reducing this fluorinated product. Since chain elongation and ketoreduction are key steps in the catalytic cycle of polyketide synthases, it was important to verify that both of these processes could occur with the fluorinated substrate.
4. These mutations were made in order to alter the specificity of the DEBS modules. Normally these modules have a high preference for methylmalonyl-CoA over fluoromalonyl-CoA. However, the mutations shown in these figures confer a preference for fluoromalonyl-CoA. This is useful because by combining these mutated and normal DEBS modules, fluorine could be incorporated in a site-specific manner. Importantly, in 4D they find that a two-step chain elongation reaction can occur even when fluorine is incorporated in the first step, indicating that fluorinated intermediates can be tolerated in downstream reactions.
5. The DEBS modules have a preference for methylmalonyl-CoA over fluoromalonyl-CoA, however *E. coli* contain almost no methylmalonyl-CoA. While they do have a sizable pool of malonyl-CoA, this is much less efficiently incorporated by the DEBS modules than fluoromalonyl-CoA is. Therefore, fluoromalonyl-CoA would face little competition as an extender unit. Another useful aspect of performing *in vivo* chain extension reactions is that *E. coli* continually generate ATP through normal metabolic processes, whereas ATP needed to be added to all of the *in vitro* reactions.