Synthetic Biological Systems

1. Metabolic Engineering with Synthetic Biology
What is Metabolic Engineering?

“Metabolic engineering is the practice of optimizing genetic and regulatory processes within cells to increase the cells' production of a certain substance”

Wikipedia

01/04/2011

Dr Tom Ellis
Metabolic engineering for biosynthesis

An attractive alternative to chemical synthesis
- Generate fuels from renewable resources
- Convert biomass into chemicals – both bulk and speciality
- Produce therapeutic compounds that are a chemical challenge
- Tap into nature’s huge diversity

A 21st century goldmine
- Major push by US Department of Energy – “12 value-added compounds”
- Huge investments from BP, Chevron, Bill Gates
- Green alternative to the petrochemical industry
In this lecture we’ll learn about:

1. The complexity of metabolism in a cell and the major challenges in metabolic engineering
2. What synthetic biology offers for metabolic engineering
3. The data and models associated with metabolic engineering
4. A major project to engineer the synthesis of a crucial anti-malarial in both *E.coli* and yeast cells
5. Engineering bacteria to produce biopetrol from sunlight and CO2
6. Engineering bacteria to turn plant waste into diesel biofuels
7. Novel synthetic biology methods to optimise metabolic pathways
8. The future for synthetic biology and metabolic engineering
Cellular metabolism is a complex network

- Cellular metabolism is a complex inter-dependent network
- The metabolome is all the small molecules of a cell
- Metabolic networks are defined by pathways
- Flux is the rate of turnover of molecules through a pathway
- Flux is regulated by the enzymes in a pathway
Metabolic engineering is like managing traffic

- Carbons and other atoms are the people
- Metabolites are their location
- Enzymes are the roads and railways they travel on
Synthetic biology for metabolic engineering

Synthetic Biology offers:

1. Predictable, designed genetic engineering
2. Regulation of gene expression
3. Insertion of new genes and new functions
4. Scalability – the addition or modification of many genes

This gives metabolic engineering:

1. Engineering by design
2. Control of enzyme levels, and so control of flux
3. Synthesis of new products
4. Assembly of whole new pathways
Basic metabolic engineering: change gene expression

• Increase the expression of enzymes involved in synthesis
  add extra copies of enzyme gene into the cell
  over-express gene using strong, regulated promoter

• Remove (knock-out) enzymes that takeaway from synthesis
  use homologous recombination to delete host genes

• Careful balancing act – toxic precursors, growth rates
  accumulation of products inhibits pathway production
  many intermediates are toxic at high levels
  cell global metabolism is always effected
    - resources must be taken from somewhere
    - slower growth is less yield
Further metabolic engineering: change the genes

- Modify the enzymes
  - remove regulatory sub-units, change cell localisation

- Add enzymes from across nature – heterologous pathways
  - 400,000 known proteins and millions unknown

- Design new enzymes from scratch
  - *de novo* design of active sites

- Directed evolution
  - mutate the enzymes, select improvements, repeat
Measuring the results of metabolic engineering

- GC Mass Spec
  - Always on CSI
    Gas liquid chromatography plus mass spectrometry
- Growth rate
- Radio-labelled atoms
- Enzyme assays

- Gene expression data
  mRNA levels: cDNA array chips / sequencing
  protein levels: proteomics / MALDI-TOF
Predicting metabolic engineering

How to avoid bottlenecks, toxins, and negative feedback?

• Flux-balance analysis (FBA)

Mathematical analysis of the metabolic network under perturbations

Model built using GC-MS data under different conditions

Most metabolism across the cell relies on the levels of a few chemicals: ATP/ADP, NAD+/NADH, NADP+/NADPH, Acyl-CoAs, TCA cycle

Next Gen: FBA with gene expression = Metabolomics + Systems Biology
Kegg Pathway has thousands of annotated pathways

Example: Carotenoids – e.g. Lycopene, Carotene
Example: Caffeine synthesis

MetaCyc

References:


Bioinformatics for Metabolic Engineering

- **BRENDA** – gives hard information on enzymes. For metabolic engineering, it links to MetaCyc and Kegg.

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**New BREND A release online since January, 5th 2011**

**New publications on BRENDA**

<table>
<thead>
<tr>
<th>Nomenclature</th>
<th>Reaction &amp; Specificity</th>
<th>Functional Parameters</th>
<th>Organism-related information</th>
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<tbody>
<tr>
<td>Enzyme Names</td>
<td>Pathway</td>
<td>Km Value</td>
<td>Organism</td>
</tr>
<tr>
<td>EC Number</td>
<td>Catalysed Reaction</td>
<td>kcat/Km Value</td>
<td>Source Tissue</td>
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<td>Reaction Type</td>
<td>Ki Value</td>
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<td>pI Value</td>
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<td>Turnover Number</td>
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<td>Natural Product</td>
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<td>Localization</td>
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<td>Inhibitors</td>
<td>Temperature Optimum</td>
<td>Specific Activity</td>
<td>Organism-related information</td>
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<td>Temperature Range</td>
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<td>Enzyme Related Compound</td>
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<td>Localization</td>
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<tr>
<td>Activating Compounds</td>
<td>PIs</td>
<td>PIp</td>
<td>Organism-related information</td>
</tr>
<tr>
<td>Ligands</td>
<td>PIp</td>
<td>PIp</td>
<td>Source Tissue</td>
</tr>
<tr>
<td>Ligand Views</td>
<td>PIp</td>
<td>PIp</td>
<td>Localization</td>
</tr>
<tr>
<td>Biochemical Reactions Aligned</td>
<td>PIp</td>
<td>PIp</td>
<td>Organism-related information</td>
</tr>
</tbody>
</table>

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**EC 3.5.1.11 - penicillin amidase**

<table>
<thead>
<tr>
<th>Activating Compound</th>
<th>ORGANISM</th>
<th>COMMENTARY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naorectum</td>
<td><em>Escherichia coli</em></td>
<td>the enzyme activity in a 25% <em>B. megaterium</em> is 1.98fold than activity in water. The enzyme activity is decreased in higher concentration of <em>B. megaterium</em></td>
</tr>
</tbody>
</table>

**Km VALUE (mM)**

- 2.5 - 2-benzonitrile-3-trifluoroacetic acid methyl ester
- 1.6 - 2-nitro-5-phenylacetylamide benzoic acid
- 0.915 - 2-nitro-5-(chloroacetyl)imidazo-benzoic acid
- 0.00063 - 2-penicillamine dicarbonyl acid
- 0.0004 - 1-penicillamine dicarbonyl acid

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**Additional information**

- no inhibition with sulfhydryl reagents
- no product inhibition by hydroxine

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**Contact Information**

- Webmaster: Maurice Scheer

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**01/04/2011**

**Dr Tom Ellis**
Examples of metabolic engineering by synthetic biology

1. Yeast production of artemisinic acid for anti-malarials  
   Jay Keasling Group

2. Bacterial production of iso-butanol biopetrols  
   James Liao Group

3. Bacterial production of fatty acids biodiesels  
   Jay Keasling Group
Artemisinin – a valuable therapeutic compound

• Artemisinin is a natural plant product from the ‘sweet wormwood’ plant (A.annua)
• It is highly effective against multi drug-resistant Plasmodium falciparum malaria
• Releases free-radicals in blood cells to kill off the malarial parasite
• P.falciparum malaria is a major problem, particularly in Africa

A.annua grows naturally only in China and Vietnam
Harvest yields of artemisinin from A.annua are very low

High need but low supply and high cost

Difficult and expensive to do complete chemical synthesis
Synthesis from Artemisinic Acid pre-cursor is cheap and easy
Artemisinic acid – designing its biosynthesis

Can the difficult synthesis of artemisinin be done by microbes?

*A. annua* converts sugars into artemisinin

→ Import *A. annua* metabolic pathways into *E. coli* or Yeast

Arteminin from artemisinic acid can be done easily by chemistry

Artemisinic acid comes from amorphadiene

Armophadiene is made from isoprenoids

Isoprenoids are synthesised through the mevalonate pathway, already common to yeast and *E. coli*

**Engineer native pathways and add *A. annua* enzymes to make artemisinic acid**
Re-engineering microbes to make amorphadiene

Yeast and *E.coli* mevalonate pathway enzymes were engineered into operon units.

Each operon controlled by a promoter and on a plasmid.

Heterologous pathways created alongside existing isoprenoid pathway.

*A.annua* genes added to make product.

Done in *E.coli* and Yeast.
Semi-synthesis of artemisinin by engineered yeast

- Blue gene = directly up-regulated and dual-copy

- Purple gene = indirectly up-regulated by upc2-1 – a global yeast biosynthesis regulator transcription factor

- Red line = strain has repression built-in

- Green = *A. annua* enzymes codon-optimized for yeast

DK Ro, EM Paradise et al., Nature 2006
Efficiency of translation is affected by the codon used.
Coding sequence of a protein has an effect on how much is produced.
Tuning the enzymes of the isoprenoid pathway

Key pathway to artemisinin – isoprenoids

How to up-regulate without creating bottlenecks and toxins?

Need to tune expression of each enzyme
  • Separate promoters for each?
  • Directed evolution on each enzyme?

‘Tunable Intergenic Regions’ (TIGRs)

Modular RNA system to allow genes under the control of the same promoter to have different expression levels.

Modular = shuffling = directed evolution

Works in E.coli and yeast

BF Pfelger et al., Nature Biotech 2006
Beyond artemisinic acid – the value of isoprenoids

Gates Foundation, Sanofi-Aventis - Africa

Full synthesis to Artemisinin
Improved synthesis: every ATP counts

Other plant products – Taxol

Industry - Amyris (biodiesels, aviation fuels and more)
Increased synthesis of mevalonate with scaffolds

Mevalonate synthesis in *E.coli*

Create a 3-enzyme mevalonate pathway:
1 x *E.coli*, 2 x codon-optimized Yeast

Fuse each enzyme to a binding domain
Co-express a modular yeast scaffold protein

Scaffold protein binds all 3 enzymes next to each other

1. Increases pathway flux
2. Reduces toxic intermediate build-up
3. Prevents negative feedback inhibition from metabolite accumulation

Dueber et al., Nature Biotech 2009
Mevalonate and glucaric acid synthesis with scaffolds

Dueber et al., Nature Biotech 2009
Petrol from *E. coli* – engineering iso-butanol production

Iso-butanol can be a direct replacement for petrol
Naturally synthesized by some strains of *Clostridium* – one of the oldest biotechnologies
Not produced naturally by any non-fermenting cells growing on glucose

- Use keto-acid pathways (amino acids synthesis)
- Overexpress entire pathway operons on plasmids
- Delete (knock-out) by-product forming enzymes
- Add a variety of different decarboxylases and dehydrogenases and look for product yields: *E. coli*, Yeast, *Clostridium*, *L. lactis*, *B. subtilis*

S Atsumi et al., Nature 2008

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**Table 1: Alcohol production with KDC and ADH in *E. coli***

<table>
<thead>
<tr>
<th>Product (g/L)</th>
<th>KDC/pc1A50</th>
<th>KDC/pK4A16</th>
<th>Pak/Pc1A40</th>
<th>Thd/pkA47</th>
<th>Pak (C. acetobutylicum)/pK4A8</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Propanol</td>
<td>520</td>
<td>290</td>
<td>125</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Isobutanol</td>
<td>3,126</td>
<td>762</td>
<td>50</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2-Butanol</td>
<td>492</td>
<td>95</td>
<td>92</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2-Methyl-1-butanol</td>
<td>766</td>
<td>602</td>
<td>59</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3-Methyl-1-butanol</td>
<td>1,495</td>
<td>1,099</td>
<td>92</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2-Phenylethanol</td>
<td>324</td>
<td>489</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

The strain was ATCC80 with various his genes and S. cerevisiae ADH2 expressed from plasmids. Culture was grown in M9 medium with 0.2 M glucose plus 0.1 mM IPTG at 30 °C for 40 h. These products were identified by GC-MS and quantified by GC-FID (see Methods). ND, not detectable.

**Table 2: Alcohol production with the supply of 2-keto acids***

<table>
<thead>
<tr>
<th>Product (g/L)</th>
<th>2-Keto isovalerate</th>
<th>2-Keto-3-methyl-valerate</th>
<th>2-Keto-4-methyl-valerate</th>
<th>2-Keto-3-methyl-pentanoate</th>
<th>Phenylpyruvate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Propanol</td>
<td>2.131</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Isobutanol</td>
<td>98</td>
<td>10,416</td>
<td>ND</td>
<td>ND</td>
<td>64</td>
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<tr>
<td>2-Butanol</td>
<td>492</td>
<td>3,126</td>
<td>ND</td>
<td>ND</td>
<td>23</td>
</tr>
<tr>
<td>2-Methyl-1-butanol</td>
<td>1,315</td>
<td>ND</td>
<td>ND</td>
<td>5,284</td>
<td>ND</td>
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<tr>
<td>3-Methyl-1-butanol</td>
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<td>ND</td>
<td>52</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2-Phenylethanol</td>
<td>26</td>
<td>309</td>
<td>66</td>
<td>3,766</td>
<td>105</td>
</tr>
</tbody>
</table>

Strains and culture conditions are the same as described in Table 1. A total of 8 g L⁻¹ of 2-keto acids was added, except for 2-ketoisovalerate, where 1 g L⁻¹ was added because of its toxicity. ND, not detectable.
Petrol from *E.coli* keto-acids – systems biology analysis

- Network analysis follow-up study
- How does gene expression change when iso-butanol production is added?
- Do expression array studies and build a network map to identify master controllers

MP Brynildsen and JC Liao, Mol Sys Biol 2009
Petrol from CO₂ – extending iso-butanol production

**Synechococcus elongatus** – cyanobacteria
- Overexpress related Rubisco enzymes
- Add keto-acid genes from *E.coli*, *B.subtilis*, *L.lactis*
- Chromosomal integration required

S Atsumi et al., Nature Biotech 2009
Fatty-acid biodiesel production in plant-digesting *E.coli*

1. Remove feedback inhibition from native fatty acid synthesis
   - Overexpress and free thioesterase
   - Overexpress 1st step of degradation

2. Prevent fatty acid degradation
   - Delete 2\textsuperscript{nd} step of pathway from the cell genome (*fadE*)

3. Convert fatty acids into fatty alcohols
   - Express codon-optimized mouse FAR enzyme

4. Assemble new pathway to synthesize ethanol
   - Express *Zymomonas pdc* and *adhB*
Fatty-acid biodiesel production in plant-digesting *E.coli*

5. Combine ethanol and fatty acids and alcohols to get biodiesels, wax esters
   - Express *Acinetobacter* AT gene

6. Use hemicellulose as a food source
   - Fuse codon-optimized cellulases from *Clostridium* and *Bacteroides* to cell surface proteins

Fatty products secrete from the cells

Swapping thioesterase (TES) from different organisms gives different fatty acid product lengths

All done by adding plasmids with genes with strong, regulated promoters

EJ Steen and Y Kang et al., Nature 2010
Fatty-acid biodiesel production in plant-digesting *E.coli*

BBC4 “The Cell” episode 3, 2009
Summary of synthetic biology used so far

• Up-regulate enzymes and pathways by cloning techniques
  - strong promoters, plasmids, codon-optimisation

• Add foreign enzymes from all across nature
  - create heterologous pathways, test out different versions of enzymes

• Co-ordinated expression of many enzymes
  - express genes from same regulated promoter
  - tune relative levels in an operon with TIGRs / IRES / regulation units

• Knock-out competing pathways and enzymes by modifying genomic DNA
• Use mutation/shuffling and selection to evolve increase yields
• Co-localize pathway enzymes on a scaffold to increase flux to product
• Combine FBA and systems biology to determine global regulators
The future for metabolic engineering

- The minimal cell – “tailor-made” cell chassis (JC Venter)
- Industrial metabolic engineering – e.g. LS9, Amyris
- Yeast – cytochromes, post-translation protein modifications
- Algae – biofuel from sunlight, hydrogen, even olive oil

Automation of flux balance in pathways by shuffle/evolution
Self-regulation built in to pathways
Predictive network models linking genome to metabolic flux
Consortia of engineered microbes growing with different roles
What you should now know and read up on!

You could get exam questions on...

1. The complexity and diversity of metabolism and how these are a challenge to engineering
2. The synthetic biology tools that can be used to overcome these challenges in metabolic engineering
3. The use of modeling, bioinformatics and systems biology in metabolic engineering
4. Examples of high-value products that could be made by metabolic engineering
5. How microbes have been engineered to help in the synthesis of artemisinin
6. Production of biofuels by engineered microbes (x2)

What you would synthesise, why and how?

01/04/2011  Dr Tom Ellis
Reading – useful reviews and perspectives

Toward engineering synthetic microbial metabolism – GH McArthur and SS Fong

Synthetic Biology for Synthetic Chemistry – JD Keasling
ACS Chemical Biology, Vol. 3, No. 1. (1 January 2008), pp. 64-76.

Engineering for biofuels: exploiting innate microbial capacity or importing biosynthetic potential? – H Alper and G Stephanopoulos

Chemical synthesis using synthetic biology. – JM Carothers et al.

Synthetic Metabolism: Engineering Biology at the Protein and Pathway Scales – CH Martin et al

Advances in flux balance analysis – KJ Kauffman et al.