Summer 6-4-2012

Understanding In-vivo Kinetics and Transport Through Stimulus Response Experiments Penicillium chrysogenum as Host Strain

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*Penicillium chrysogenum* as host strain

5th June 2012

Metabolic Engineering IX
Biarritz, France

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Background information/Motivation

- **Aim:** Demonstrate the application of stimulus-response studies to identify possible bottlenecks in a product pathway.
- **Model system:** PenicillinG production in a high producing industrial *P. chrysogenum* strain.

- **Previous studies:**
  - Flux control lies in the penicillin pathway: *but where?* (Jens Nielsen’s group)

- **Focus of this study:**
  - Application of different perturbations/quantitative metabolomics/kinetic modeling
  - Use models for bottleneck analysis and estimation of changes in *in-vivo* enzyme levels
The penicillin G pathway

ACVS → ACV → IPNS → IPN → 6APA

α-AAA, Cysteine, Valine

de novo synthesis, central carbon metabolism

AT → PACC0A → PenG

PCL

PAA → PenG

Cytosol
Possible bottlenecks

α-AAA, Cysteine, Valine

de novo synthesis
central carbon metabolism
Possible bottlenecks

ACVS → ACV → IPNS → IPN

α-AAC, Cysteine, Valine

de novo synthesis, central carbon metabolism

Peroxisome

AT → PACUA → PCL

PenG → PenG

Cytosol

6APA → 6APA
Possible bottlenecks

α-Amino acids (α-AAA), Cysteine, Valine

de novo synthesis

Peroxisome

AT

PACoA

PenG

PCL

PAA

PenG

Cytosol

6APA

IPN

ACVS → ACV → IPNS → IPN

6APA
Possible bottlenecks

\[ \begin{align*}
&\text{ACVS} \rightarrow \text{ACV} \rightarrow \text{IPNS} \rightarrow \text{IPN} \\
&\alpha\text{-AAA} \\
&\text{Cysteine} \\
&\text{Valine} \\
&\text{de novo synthesis} \\
&\text{central carbon metabolism} \\
&\text{6APA} \\
&\text{IPN} \\
&\text{AT} \\
&\text{PACoA} \\
&\text{PCL} \\
&\text{PAA} \\
&\text{PenG} \\
&\text{PenG} \\
&\text{6APA}
\end{align*} \]
Possible bottlenecks

\[ \text{ACVS} \rightarrow \text{ACV} \rightarrow \text{IPNS} \rightarrow \text{IPN} \rightarrow 6\text{APA} \]

- $\alpha$-AAA
- Cysteine
- Valine

\[ \text{de novo synthesis} \]
\[ \text{central carbon metabolism} \]

\[ \text{AT} \rightarrow \text{PACoA} \rightarrow \text{PCL} \rightarrow \text{PenG} \]

\[ \text{PAA} \rightarrow \text{PenG} \]
Possible losses of intermediates

ACVS → ACV → IPNS → IPN → 6APA

α-AAA
Cysteine
Valine

de novo synthesis
central carbon
metabolism
Approach

Local perturbation of the pathway at different time scales

Perturbations
- PAA transport mechanism
- PAA perturbation (sec)
- PenG transport mechanism
- PenG perturbation (min)
- In-vivo pathway kinetics
- PAA perturbation (hours)

Modeling & Simulation
- Formulate hypothesis
- Validate hypothesis by Modeling & simulation
- Obtain kinetic parameters
- Use

Application of model
- Identify bottlenecks/
- Estimate change in enzyme levels
Possible bottleneck no. 1: PAA uptake

- ACVS → ACV → IPNS → IPN
- 6APA → IPN
- AT
- PACoA
- PCL
- PenG
- α-AAA
- Cysteine
- Valine
- de novo synthesis
- central carbon metabolism
- PAA
PAA uptake in pulse experiment (seconds scale)

- Duplicate PAA pulse experiments to steady state chemostat:
  0 → 10 mM PAA

Rapid uptake of PAA!!

Douma R.D., Deshmukh A.T. et al., 2012
Is PAA uptake limiting?

PAA uptake is not limiting

Douma R.D., Deshmukh A.T. et al., 2012
PAA uptake mechanism

- Uptake by passive diffusion of the undissociated form of PAA
- Permeability coefficient: $5 \times 10^{-6}$ m/s

$$K_{eq} = \frac{C_{PAA,in}}{C_{PAA,ex}} = \frac{1 + 10^{pH_{in}-pK}}{1 + 10^{pH_{ex}-pK}} = 5$$

$pH_{in} = 7.2$
$pH_{ex} = 6.5$

Experimental PAA ratio $<< K_{eq}$

PAA catabolism?

PAA export?

Douma R.D., Deshmukh A.T. et al., 2012
PAA pulse increases respiration

Online data

**Offgas oxygen**

**Dissolved oxygen**

Increased respiration
PAA catabolism?
NO !! Closed PAA balance

Douma R.D., Deshmukh A.T. et al., 2012
PAA active export?

*S. cerevisae* studies - Hazelwood et al. 2006
Transcript studies - Harris et al. 2009

- Export of PAA: possibly by an ABC transporter

Uptake: passive diffusion
Export: active, using ATP

Douma R.D., Deshmukh A.T. et al., 2012
Active export leads to futile cycling of PAA

- PAA cycling rate 100 times higher than PenG production rate
- Significant ATP loss (2 mol ATP/molPAA exported)

Metabolic engineering target → eliminate active PAA export

Douma R.D., Deshmukh A.T. et al., 2012
Possible bottleneck no. 2: PenG export

- ACVS → ACV → IPNS → IPN
- IPN → 6APA → 6APA
- AT → PACoA → PCL
- PCL → PenG
- PAA → PenG

**α-AAA, Cysteine, Valine**
- de novo synthesis
- central carbon metabolism
Determine PenG (ex/in) ratio in ramp experiment

- Apply PenG ramp (2 → 10 mM) to PenG producing steady state chemostat.

PenG is taken up by the cell !! → Reversible transport

Douma R.D., Deshmukh A.T. et al., 2012
Export mechanism of PenG anion?

\[
K_{eq, penG} = \frac{C_{ex}}{C_{in}} = e^{-\frac{1}{R} \cdot F \cdot \Psi_{in}} = 23 \sim 158
\]

Measured ratio corresponds with reversible facilitated transport

Douma R.D., Deshmukh A.T. et al., 2012
PenG anion export: kinetic model

- Reversible facilitated transport

\[ q_{sec, PenG} = q_{sec, PenG}^{max} \left( 1 - \frac{C_{PenG, ex}}{C_{PenG, in} \cdot K_{eq, penG}} \right) \]

- Maximum transport capacity:
  \[ q_{sec, PenG}^{max} = 0.83 \pm 0.20 \text{ mmol/Cmol/h} \]
  \[ K_{eq, penG} = 73 \pm 17 \]

- PenG exporter capacity is same order of magnitude as PenG secretion rate (0.56 mmol/Cmol/h)

**Metabolic engineering target**

**Increase capacity of PenG export**

Douma R.D., Deshmukh A.T. et al., 2012
Possible losses of IPN and 6APA

\[ \text{ACVS} \rightarrow \text{ACV} \rightarrow \text{IPNS} \rightarrow \text{IPN} \rightarrow \text{6APA} \]

\[ \text{IPN} \rightarrow \text{AT} \rightarrow \text{PACoA} \rightarrow \text{PCL} \rightarrow \text{PAA} \rightarrow \text{PenG} \]

\[ \alpha\text{-AAA, Cysteine, Valine} \]

\[ \text{de novo synthesis, central carbon metabolism} \]
Chemostat without PAA

In the absence of PAA:
- IPN and 6APA are expected to accumulate in the cell and secreted
Chemostat without PAA

- No steady state! Levels of pathway intermediates change over time.
- Changing enzyme levels?
  
  Accumulation of IPN and 6APA in the cell and significant secretion
Transport mechanisms of IPN and 6APA

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Ratio (Ex/In)</th>
<th>$K_{eq}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPN⁻</td>
<td>0.012 ± 0.003</td>
<td>0.2</td>
</tr>
<tr>
<td>6APA⁻</td>
<td>1.73 ± 0.06</td>
<td>73 ± 17</td>
</tr>
</tbody>
</table>

IPN⁻ : proton symport
6APA⁻ : uniport

Metabolic engineering target ➔ eliminate the transporters
Integral kinetic model of PenG biosynthesis pathway

PenicillinG pathway model including transport

\[ \text{ACVS} \rightarrow \text{ACV} \rightarrow \text{IPNS} \rightarrow \text{IPN} \rightarrow 6\text{APA} \]

\[ \text{α-AAA} \rightarrow \text{Cysteine} \rightarrow \text{Valine} \]

\[ \text{de novo synthesis central carbon metabolism} \]

\[ \text{PCL} \rightarrow \text{PenG} \]

\[ \text{PAA} \rightarrow \text{PenG} \]
Obtain *in-vivo* kinetics of pathway enzymes

- PAA step experiment

![Diagram showing in-vivo kinetics experiment](image)
Obtain *in-vivo* kinetics from PAA step experiment

- Kinetic parameters obtained from dynamic state: first hour after PAA step

![Diagram showing PAA concentration over time with phases I and II, and kinetic parameters from dynamic state with enzyme levels assumed constant.]

- Rapid sampling in seconds or minutes scale.
Immediate formation of PA- CoA (< 5 sec)
PCL expressed in absence of PAA
PAA rapidly enters the peroxisome
PAA step experiment

- Intracellular IPN and 6APA pools deplete rapidly

- Immediate formation of PenG << 10 sec and export of PenG

All enzymes and transporters are expressed even in absence of PAA !!
Modeling & Simulations

- Dynamic model based on mechanistic, Michaelis-Menten type rate equations

Example: Equation of PenG production from IPN and PA-CoA

\[ v_{PenG,IPN} = \frac{k_{IAT}(X_{AT})}{1 + \frac{K_{m,IPN,PAACoA}}{C_{IPN}} + \frac{K_{m,PAACoA}}{C_{PAACoA}}} \]

No. of kinetic functions: 16
No. of parameters : 20
No. of data points : 308

Balance (Constant volume)

\[ \frac{dc}{dt} = S_c \cdot q \cdot c_x + \frac{\Phi_{in}}{V} \cdot c_{feed} - \frac{\Phi_{out}}{V} \cdot c \]

No. of balances : 12
S : Stoichiometric matrix

\[ \frac{dx}{dt} = S_x \cdot v - \mu \cdot x \]
PAA step during 1\textsuperscript{st} hour

- Model fit to the experimental observations
PAA step until 165 hours

- Model fit to the experimental observations
Model Application: Identification of bottlenecks

Pathway fluxes during the first hour after the PAA step

- In abundance of IPN and 6APA:
  - High initial PenG secretion rate.
  - IAT has limited flux capacity.
Model Application: Identification of bottlenecks

- Flux control coefficients: 100h after PAA step

<table>
<thead>
<tr>
<th></th>
<th>Control coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACVS</td>
<td>0.816</td>
</tr>
<tr>
<td>IPNS</td>
<td>0</td>
</tr>
<tr>
<td>IAH</td>
<td>0</td>
</tr>
<tr>
<td>IAT</td>
<td>0.070</td>
</tr>
<tr>
<td>AAT</td>
<td>0.004</td>
</tr>
<tr>
<td>PCL</td>
<td>0.070</td>
</tr>
<tr>
<td>PAA diffusion</td>
<td>0.050</td>
</tr>
<tr>
<td>PAA export</td>
<td>0</td>
</tr>
<tr>
<td>PenG export</td>
<td>0</td>
</tr>
</tbody>
</table>

Metabolic engineering target ➔ increase capacity of ACVS
Model Application: Estimate changes in enzyme levels

- Observed changes of pathway intermediates in SS chemostat are caused by changes in enzyme levels

ACVS level

IPNS level

AT level

- Enzymes also induced in the absence of PAA
- Partial degeneration of ACVS
Conclusions

- Stimulus response experiments with different time scales combined with quantitative metabolomics helps to:
  - unravel transport mechanisms of precursors, products
  - generate dynamic data for construction of kinetic models

- Kinetic models/thermodynamics help to:
  - validate the different hypotheses of transport kinetics
  - estimate in vivo changes in enzyme levels

- Results for penicillin biosynthesis pathway of high producing strain:
  - not limited by transport of PAA
  - possibly limited by PenG export
  - pathway enzymes are also induced in absence of PAA and (partly) degenerate (ACVS) over time
  - pathway flux mainly controlled by ACVS
Acknowledgements

Rapid sampling team
Rutger Douma
Lodewijk de Jonge
Elaheh Jamalzadeh
Luisa da Cruz
Katelijne Bekers
Camilo Suarez-Mendez
Christiaan van der Hoek
Hugo Cueto Rojas
Sushil Gaykawad

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Joseph J. Heijnen
Peter J. T. Verheijen

Co-operation
DSM
Rémon Boer
Wouter van Winden
Roel Bovenberg
Questions

Thank you for Your Attention

Poster No. 136 & 205
Figure 1: Schematic representation of the reaction pathway for synthesis of metabolites during production of PenG
Acyl transferase has three different activities

Acyl Transferase (AT)

- PA-CoA
- IPN
- IAT
- IAH
- 6APA
- AAT
- PenG
Increase in respiration +PAA: SS conditions

Table 2. Predicted and Experimentally Determined Changes in the Biomass Specific Rates of Glucose Consumption (\(q_S\)), Oxygen Consumption (\(q_O\)), and Carbon Dioxide Production (\(q_C\)) as a Result of the Presence of PAA in the Feed Medium of a Glucose Limited Chemostat Culture of \(P.\) chrysogenum DS17690

<table>
<thead>
<tr>
<th></th>
<th>PenG Biosynthesis</th>
<th>PAA Cycling</th>
<th>Total</th>
<th>Experimental Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\Delta q_S)</td>
<td>0.9</td>
<td>1.8</td>
<td>2.7</td>
<td>3.0 ± 0.7</td>
</tr>
<tr>
<td>(\Delta q_O)</td>
<td>2.1</td>
<td>11</td>
<td>13.1</td>
<td>14.4 ± 2.6</td>
</tr>
<tr>
<td>(\Delta q_C)</td>
<td>2.6</td>
<td>11</td>
<td>13.6</td>
<td>14.7 ± 2.6</td>
</tr>
</tbody>
</table>

All rates are expressed in mmol Cmol\(^{-1}\) h\(^{-1}\). Experimental data were obtained from Ref. 32.
Penicillin pathway modeled scheme
PenG transport mechanism

\[ T + PenG_{in} \xleftrightarrow{\text{ex}} (TPenG)_{in} \]

\[ (TPenG)_{in} \xleftrightarrow{\text{ex}} (TPenG)_{ex} \]

\[ (TPenG)_{ex} \xleftrightarrow{k_{sec}} \xrightarrow{k_{up}} T + PenG_{ex} \]

\[ K_1 = \frac{C_T \cdot C_{PenG,in}}{C_{TPenG,in}} \]

\[ K_2 = \frac{C_{TPenG,ex}}{C_{TPenG,in}} \]

\[ q_{sec, PenG} = k_{sec} \cdot C_{TPenG,ex} - k_{up} \cdot C_T \cdot C_{PenG,ex} \]
PAA step

\[ C_{T_o} = C_T + C_{TPenG,in} + C_{TPenG,ex} \]

\[ q_{sec,PenG} = \frac{k_{sec} \cdot C_{T_o} \cdot \frac{K_2}{K_1}}{1 + \frac{C_{PenG,in}}{K_1} + \frac{K_2}{K_1} \cdot C_{PenG,in}} \cdot \left( \frac{C_{PenG,in}}{K_1} \cdot \frac{C_{PenG,ex}}{K_2 \cdot k_{sec} \cdot k_{up}} \right) \]

When \( C_{PenG,ex} = 0 \)

\[ \left( q_{sec,PenG} \right)_{PenG,ex=0} = \frac{k_{sec} \cdot C_{T_o} \cdot \frac{K_2}{K_1} \cdot C_{PenG,in}}{1 + \frac{C_{PenG,in}}{K_1} + \frac{K_2}{K_1} \cdot C_{PenG,in}} \]

At equilibrium \( q_{sec,PenG} = q_{up,PenG} = 0 \)

\[ \frac{C_{PenG,ex}}{C_{PenG,in}} = \frac{K_2}{K_1} \cdot \frac{k_{sec}}{k_{up}} = \left( K_{eq,PenG} \right)_{fac} \]

When, \( K_2 \gg K_1 \)

\[ q_{sec,PenG} = q_{sec,PenG}^{\text{max}} \cdot \left( 1 - \frac{C_{PenG,ex}}{C_{PenG,in} \cdot \left( K_{eq,PenG} \right)_{fac}} \right) \]
Acyl transferase (AT)
PAA step during 1\textsuperscript{st} hour

Model fit to the experimental observations