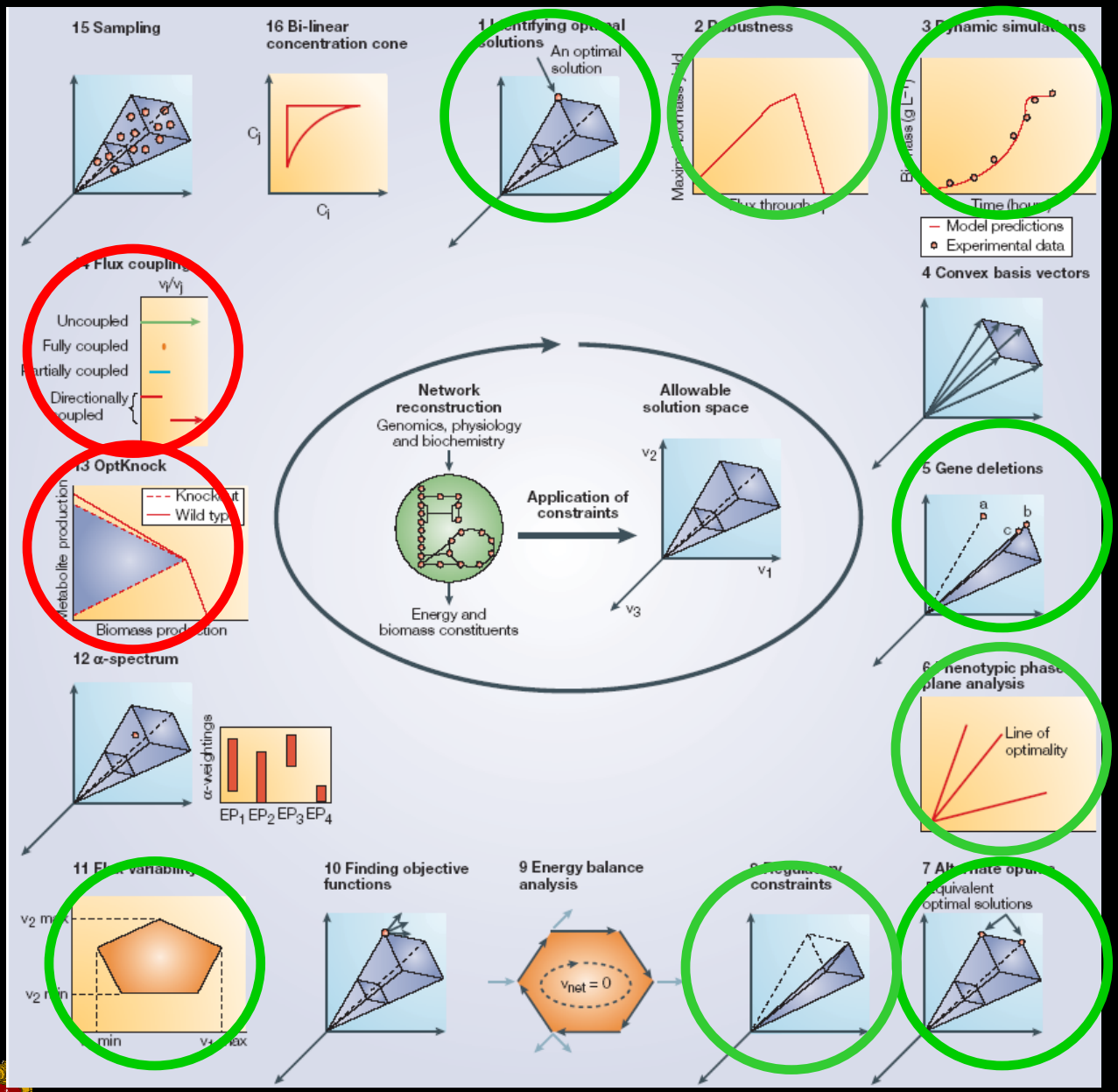
Constraint-Based Workshops

10. Flux Coupling (again) & Metabolic Engineering
February 28th, 2008

Constraint-Based Methods



Optimal Solutions
1. FBA
2. Flux Variability

Flux Dependencies
1. Robustness
2. Phase Planes
3. Flux Coupling

All Allowable Solutions
1. Extreme Pathways
2. Elementary Modes
3. Sampling

Altering Phenotypes
1. Genetic Mutations
2. Strain Design

Application of Additional Constraints
1. Regulation
2. Energy Balance

Price, Reed, and Palsson
Nat. Reviews Microbiol.
2004

Flux Coupling

- Used to see how pairs of fluxes affect one another.
- Done by calculating the minimum and maximum ratio between two fluxes
- Transformation needed to make it a linear problem

$$\begin{aligned}
&\text{maximize (or minimize)} && v_1/v_2 && \\
&\text{subject to} && \sum_{j=1}^{M} S_{ij} v_j = 0, && \forall\, i \in \mathrm{N} \\
& && v_j^{uptake} \le v_j^{uptake_max}, && \forall\, j \in \mathrm{M}_{\mathrm{transport}} \\
& && v_j \ge 0, && \forall\, j \in \mathrm{M}
\end{aligned}$$

$$\begin{aligned}
&\text{maximize or (minimize)} && \hat{v}_1 && (\mathrm{P}') \\
&\text{subject to} && \hat{v}_2 = 1 && \\
& && \sum_{j=1}^{M} S_{ij} \hat{v}_j = 0, && \forall\, i \in \mathrm{N} \\
& && \hat{v}_j^{uptake} \le v_j^{uptake_max} \cdot t, && \forall\, j \in \mathrm{M}_{\mathrm{transport}} \\
& && \hat{v}_j \ge 0, && \forall\, j \in \mathrm{M} \\
& && t \ge 0 &&
\end{aligned}$$

Burgard, AP, et al. Genome Research. 14(2):301-12 (2004).

Types of Coupling:

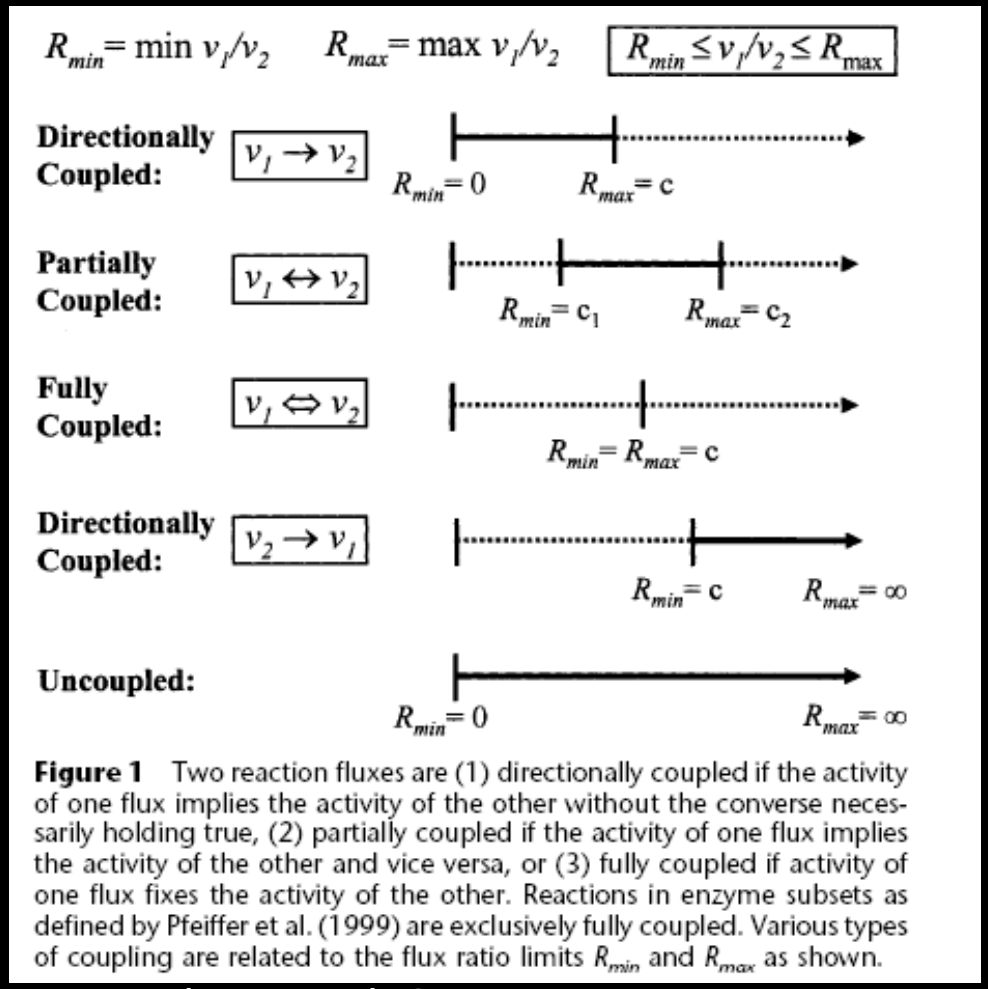
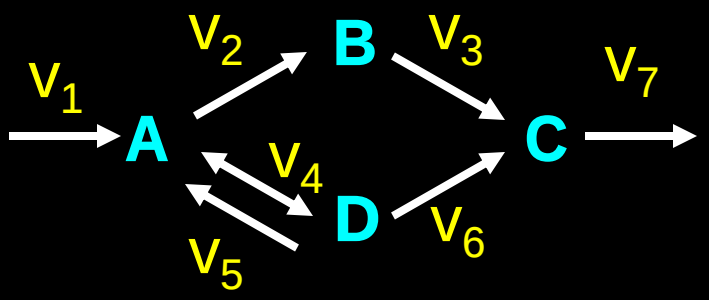


Figure 1 Two reaction fluxes are (1) directionally coupled if the activity of one flux implies the activity of the other without the converse necessarily holding true, (2) partially coupled if the activity of one flux implies the activity of the other and vice versa, or (3) fully coupled if activity of one flux fixes the activity of the other. Reactions in enzyme subsets as defined by Pfeiffer et al. (1999) are exclusively fully coupled. Various types of coupling are related to the flux ratio limits R_{min} and R_{max} as shown.

Burgard, AP, et al. Genome Research. 14(2):301-12 (2004).



- Fully Coupled:
  - V1 and V7
  - V2 and V3
- Directionally Coupled:
  - V2,V3 → V1,V7
  - V6 → V1,V4,V7
  - V5 → V4
- Uncoupled:
  - V5 w/ all other fluxes, except V4
  - V4 w/ all other fluxes, except V5 and V6
  - V6 w/ V2, V3 and V5

```
Sets
irr(j) /v1,v2,v3,v5,v6,v7/
rev(j) /v4/;
```

Separate Irreversible & Reversible Reactions

```
Variables
v_irr(irr) irreversible fluxes
v_for(rev) reversible fluxes forward direction
v_back(rev) reversible fluxes backward direction
Obj this is the value of the objective function for the FBA solutions;
```

In S·v=0 equation split reversible reactions (v_for and v_back fluxes)

```
Equations
massbalance(i) mass balance equations for each metabolite
calcobj calculates the dot product of the c vector the flux vector;
massbalance(i).. sum(irr,S(i,irr)*v_irr(irr)) + sum(rev,S(i,rev)*(v_for(rev)-v_back(rev)))=e=0;
calcobj.. Obj=e=sum(irr,c_irr(irr)*v_irr(irr))+sum(rev,c_for(rev)*v_for(rev)+c_back(rev)*v_back(rev));

Model FBA /massbalance, calcobj/;

v_irr.lo(irr)=0;
v_irr.up(irr)=Vmax;
v_for.lo(rev)=0;
v_for.up(rev)=Vmax;
v_back.lo(rev)=0;
v_back.up(rev)=Vmax;
```

All Fluxes Constrained Between 0 and Vmax

```
*********************************************************************
*This Section Finds All Blocked Reactions That Need to Be Removed*
*********************************************************************
set unblocked_irr(j)
    totalunblocked(j);
unblocked_irr(j)=no;
alias(dummyindex,irr);
loop(dummyindex, c_irr(dummyindex)
        solve FBA using lp maximi
        if(Obj.l=0,unblocked_irr(
        else unblocked_irr(dummyi
        c_irr(dummyindex)=0; );
```

Flux Variability Analysis to find actual Vmax and Vmin for each flux →
This will allow us to remove blocked reactions (including infeasible directions of reversible reactions)

```
*****************************************************************
***This Section Calculates the Minimum and Maximum Flux Ratios****
*****************************************************************
Parameters
d(j)
d_back(j)
S_mod(i,j);

S_mod(i,j)=S(i,j);
S_mod(i,unblocked_backonly)=-S(i,unblocked_backonly);
unblocked_irr(j)=unblocked_irr(j)+unblocked_backonly(j);

Variables
v_hat_irr(j) flux values through reaction in network
v_hat_for(j) flux values through reaction in network
v_hat_back(j) flux values through reaction in network
Obj_hat
t;

v_hat_irr.lo(j)=0;
v_hat_for.lo(j)=0;
v_hat_back.lo(j)=0;
v_hat_irr.up(j)=inf;
v_hat_for.up(j)=inf;
v_hat_back.up(j)=inf;
t.lo=0;

Equations
massbalance_hat(i) mass balance equations for each metabolite
calcobj_hat calculates the dot product of the c vector the flux vector
uptakelimit;

massbalance_hat(i).. sum(unblocked_irr,S_mod(i,unblocked_irr)*v_hat_irr(unblocked_irr)) + sum(unblocked_both,S_mod(i,
calcobj_hat.. Obj_hat=e=sum(unblocked_irr,d(unblocked_irr)*v_hat_irr(unblocked_irr))+sum(unblocked_both,d(unblocked_b
uptakelimit.. v_hat_irr('v1')=l=t*10;
Model FluxCoupling /massbalance_hat,calcobj_hat,uptakelimit/;
```

All Flux Ratios (v_hat) Constrained Between 0 and Inf

For the uptake flux, add additional constraint allowing its ratio to vary

GAMS File Results

- Two Text Files (row I, column J = Ratio for V_J/V_I:
  - "OneDirection" reports the max and min ratios in tables for the irreversible reactions compared to all other fluxes (irreversible and reversible fluxes).
  - "BothDirection" reports the max and min ratios in tables for the reversible reactions with each other (note that reversibility is redefined after FVA is performed)

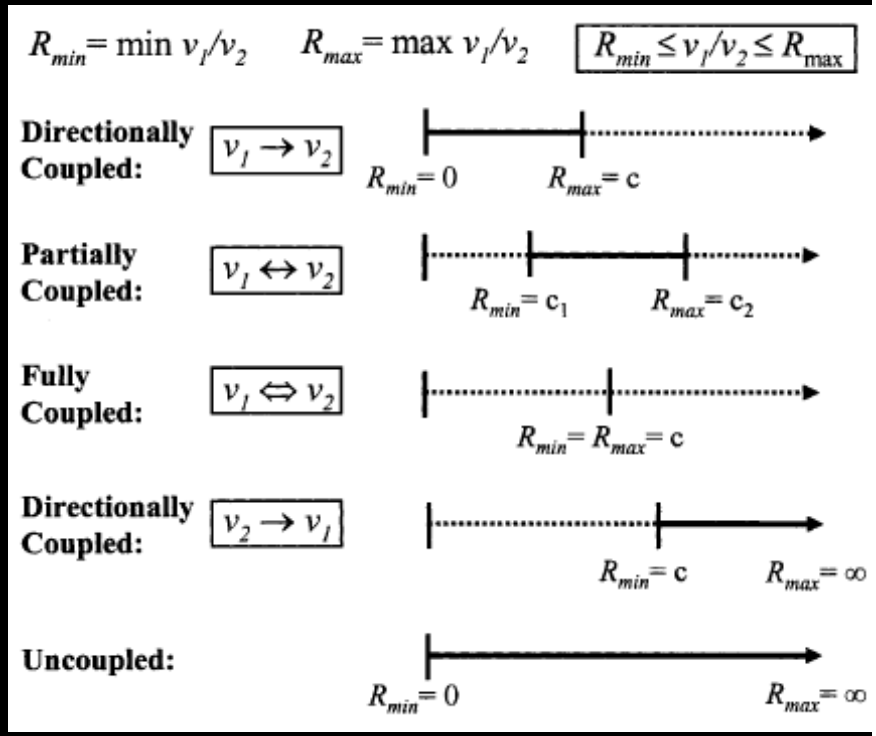
Results from the Example Network

FluxCouplingExample.txt - WordPad

File Edit View Insert Format Help

```
"MAXRATIO","v1","v2","v3","v4","v5","v6","v7"
"v1",1.0000,1.0000,1.0000,"+INF","+INF",1.0000,1.0000
"v2","+INF",1.0000,1.0000,"+INF","+INF","+INF","+INF"
"v3","+INF",1.0000,1.0000,"+INF","+INF","+INF","+INF"
"v4","+INF","+INF","+INF",1.0000,1.0000,1.0000,"+INF"
"v5","+INF","+INF","+INF","+INF",1.0000,"+INF","+INF"
"v6","+INF","+INF","+INF","+INF","+INF",1.0000,"+INF"
"v7",1.0000,1.0000,1.0000,"+INF","+INF",1.0000,1.0000

"MINRATIO","v1","v2","v3","v4","v5","v6","v7"
"v1",1.0000,0.0000,0.0000,0.0000,0.0000,0.0000,1.0000
"v2",1.0000,1.0000,1.0000,0.0000,0.0000,0.0000,1.0000
"v3",1.0000,1.0000,1.0000,0.0000,0.0000,0.0000,1.0000
"v4",0.0000,0.0000,0.0000,1.0000,0.0000,0.0000,0.0000
"v5",0.0000,0.0000,0.0000,1.0000,1.0000,0.0000,0.0000
"v6",1.0000,0.0000,0.0000,1.0000,0.0000,1.0000,1.0000
"v7",1.0000,0.0000,0.0000,0.0000,0.0000,0.0000,1.0000
```



ROWS = denominators
COLS = numerators

Max V_3/V_2 = 1 & Min V_3/V_2 = 1 so they are Fully Coupled

Max V_1/V_5 = Inf & Min V_1/V_5 = 0 so they are Uncoupled (V6→ V1)

Max V_1/V_6 = Inf & Min V_1/V_6 = 1 so they are Directionally Coupled (V6→ V1)

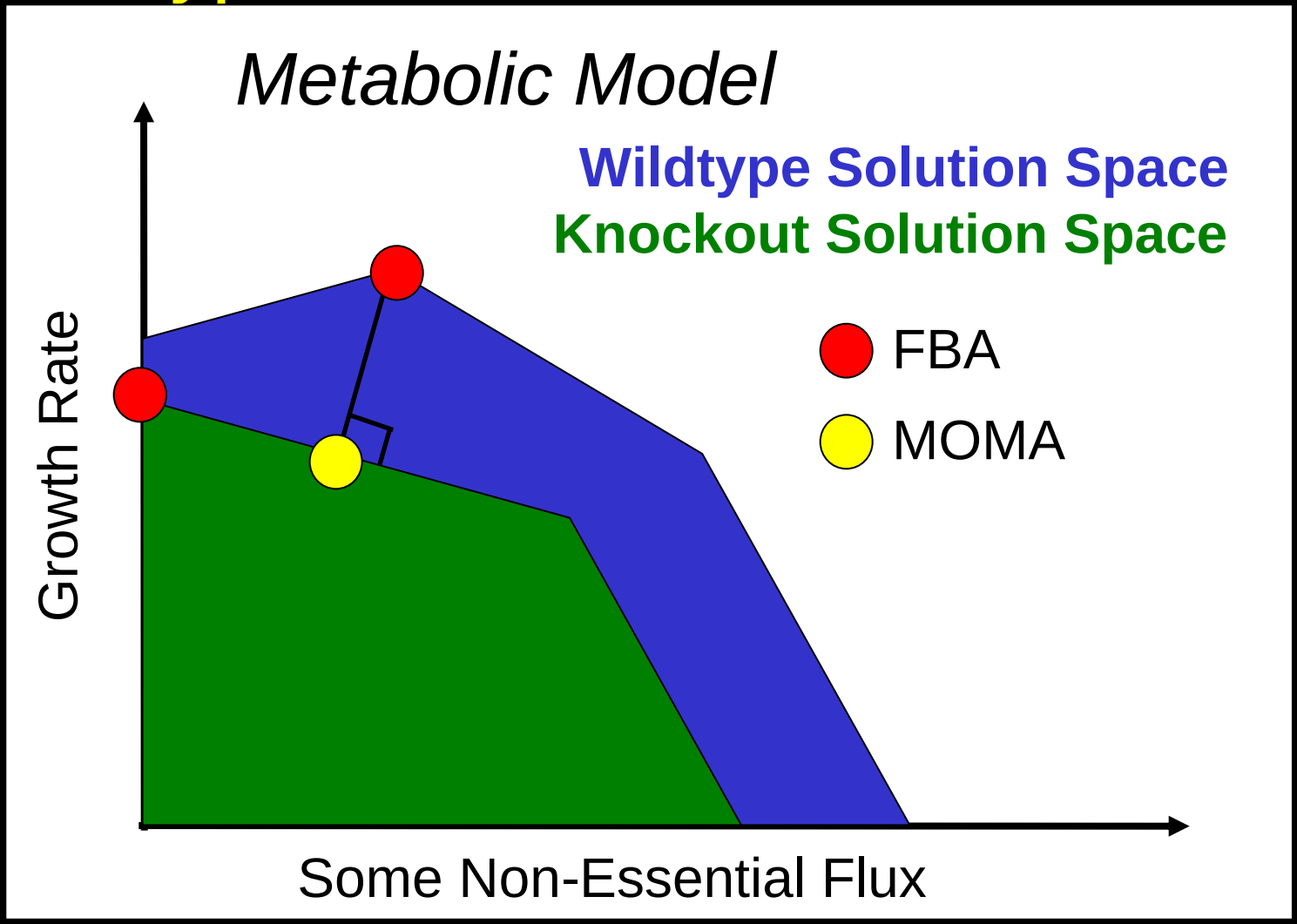
Flux Coupling Calculations

- What are all the fully coupled reaction pairs?
- What are all the uncoupled reaction pairs?
- What are all the directionally coupled reaction pairs ($v_j \rightarrow v_i$)?

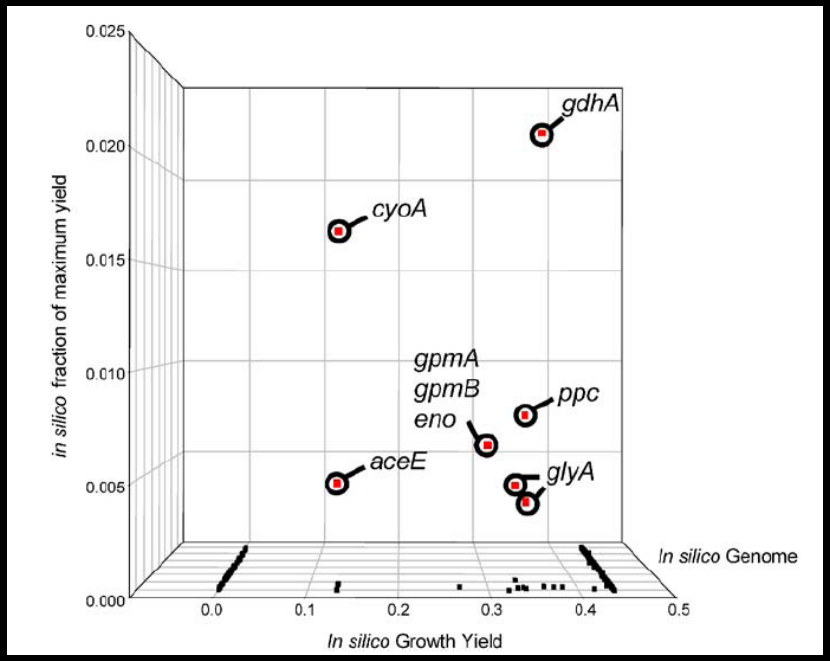
Metabolic Engineering

1. Knockout Prediction Tools (FBA, MOMA, ROOM)
2. OptKnock

MOMA: Minimize Distance Between Wildtype & Mutant Flux Distributions



MOMA for Increasing Lycopene Production



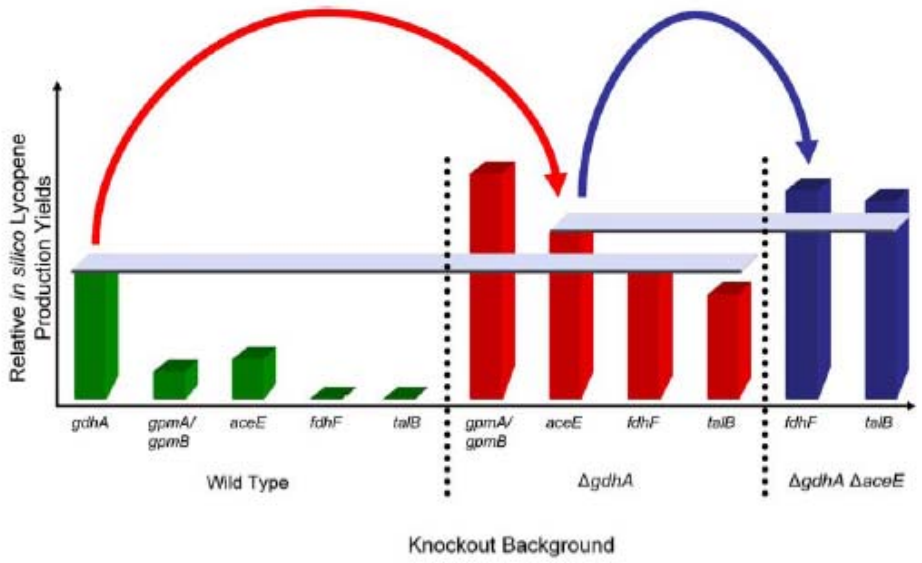


Table 1
Experimental results of single and multiple gene knockouts

Knockout construct	Growth rate	Percent increase in lycopene content (PPM)
None	0.67	0% (4700 PPM)
Single knockouts		
gdha	0.55	13% (±4)
gpma	0.44	−8% (±3)
gpmb	0.55	7% (±2)
acee	0.52	9% (±4)
fdhf	0.57	4% (±3)
Double knockouts		
gdhA, aceE	0.52	13% (±4)
gdhA, gpmA	0.37	12% (±3)
gdhA, gpmB	0.49	18% (±3)
gdhA, talB	0.46	3% (±4)
Triple knockouts		
gdhA, aceE, talB	0.44	19% (±4)
gdhA, aceE, fdhF	0.38	37% (±3) (6600 PPM)

ng

Alper, Jin, Moxley, & Stephanopoulos. Metabolic Engineering. 7:155-64 (2005)

Improving Valine Production in *E. coli*

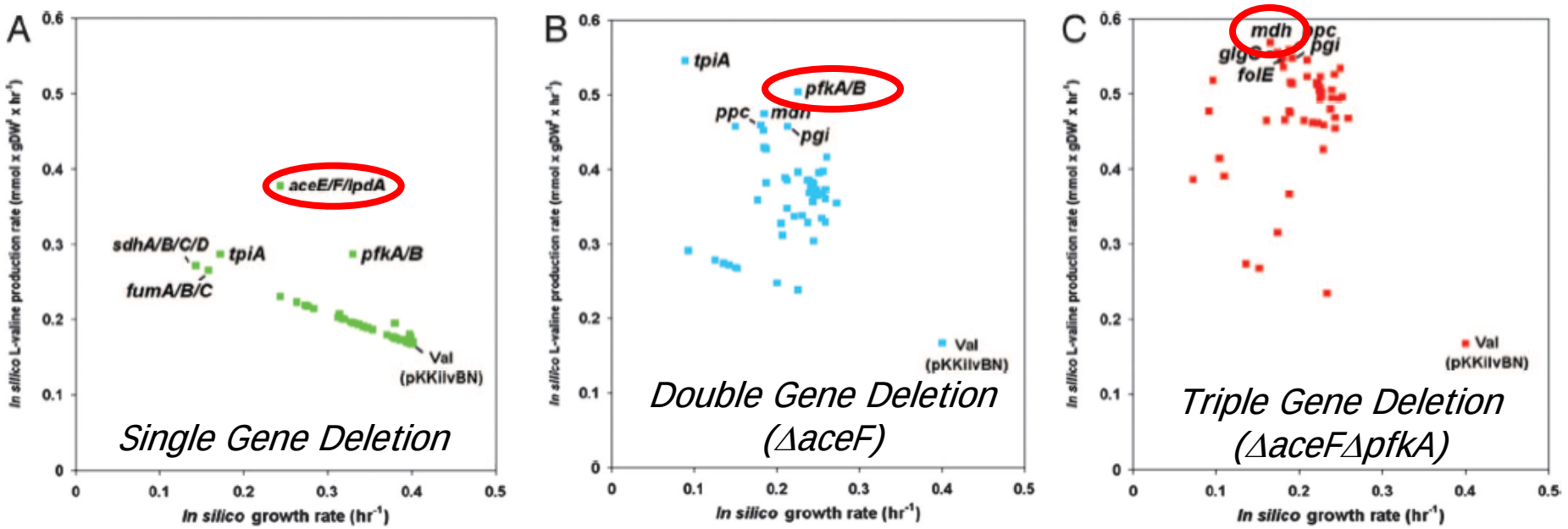


Fig. 3. Results of *in silico* gene knockout simulations by using the genome-scale metabolic model of *E. coli* MBEL979. The results of single (*A*), double (*B*), and triple (*C*) gene knockout simulations with respect to L-valine production and growth rates are shown. Only the five best candidates with respect to the L-valine production rate are shown for each stage of knockout simulation. Slashes indicate isoenzymes or subunits of the enzyme complex. The L-valine production and growth rates of the control Val strain harboring pKKilvBN are also indicated for comparison.

Park, J.H. Lee, K.H., Kim, T.Y., and Lee, S.Y. *PNAS*, 104(19):7797-7802 (2007).

Model calculations led to an improved strain design for valine production (~2 fold increase in valine yields)

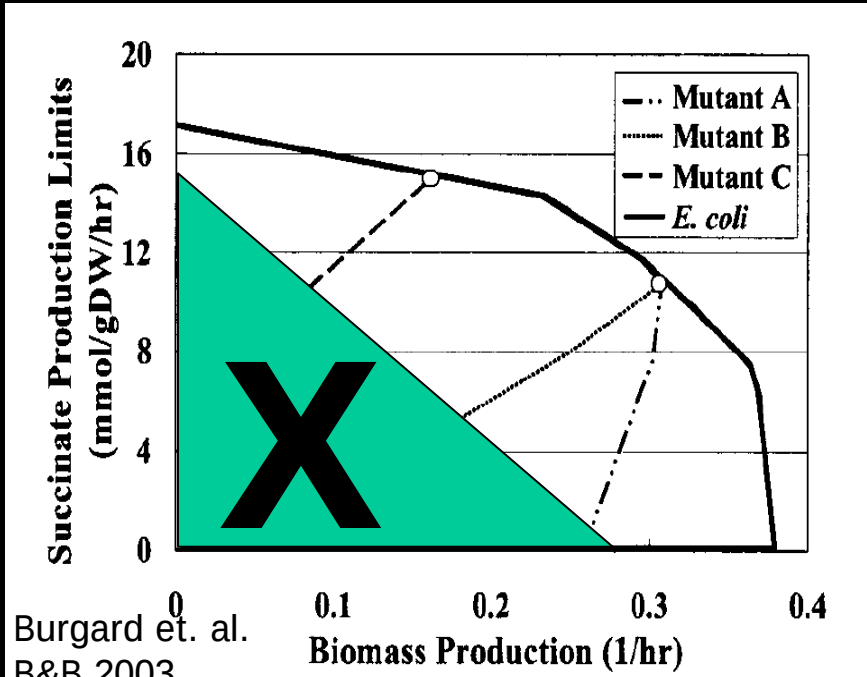
OptKnock:

- *Finds reactions, that if removed, couple of biomass production and metabolite production (ie. higher growth requires higher metabolite production levels)*

- *REFERENCES:*
  - *Burgard, Pharkya, Maranas. Biotechnology & Bioengineering. 84(6): 647-657 (2003)*
  - *Pharkya, Burgard, Maranas. Biotechnology & Bioengineering. 84(7): 887-899 (2003)*
  - *Pharkya, Burgard, Maranas. Genome Research. 14(11): 2367-76(2004)*
  - *Fong, et al. Biotechnology & Bioengineering. 91(5): 643-648*

Computational Design of Mutant Strains

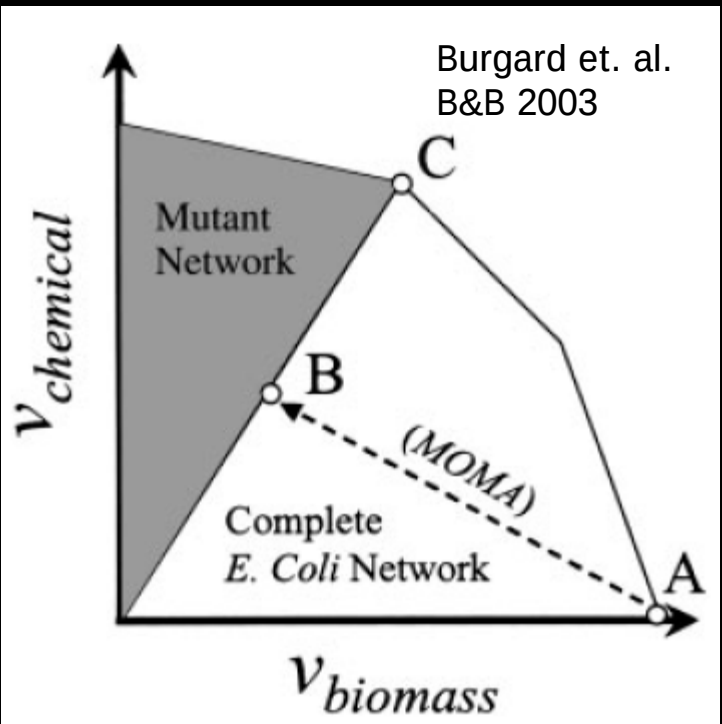
OptKnock: Find gene deletions needed such that maximizing biomass is coupled with maximizing metabolic engineering objective



Burgard et. al. B&B 2003

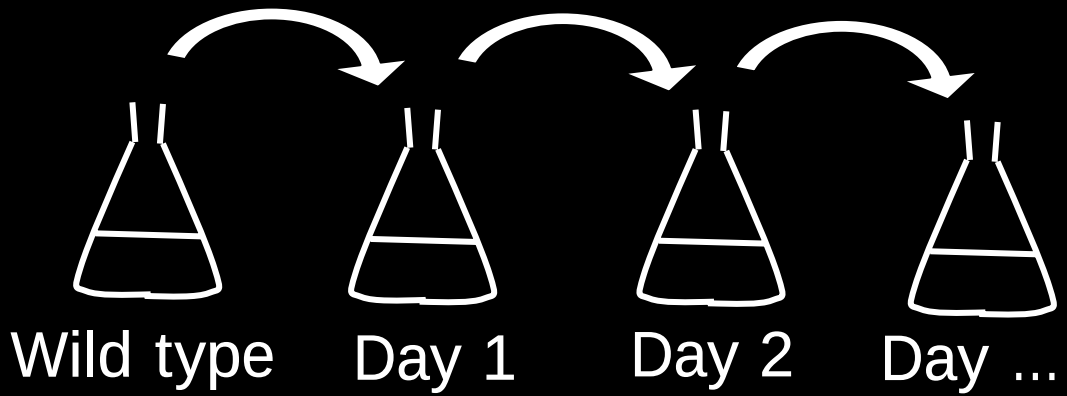
Strain Designs for:

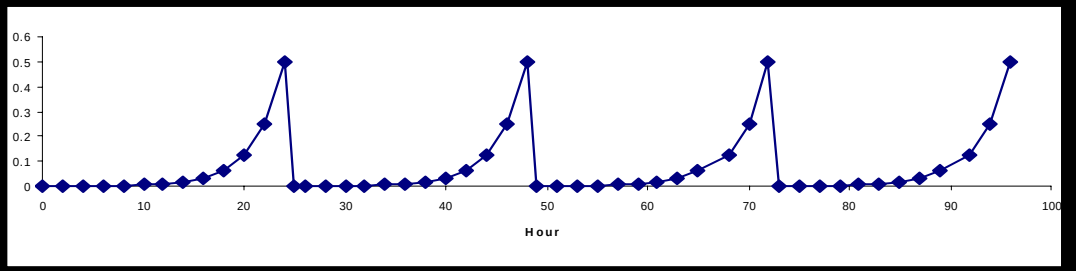
Lactate Production
Succinate Production
1,3 Propanediol Production
Chorismate Production
Alanine Production
Serine Production
Aspartate Production
Glutamate Production



Methods – adaptive evolution

- Cultures grown in 250ml minimal medium supplemented with 2g/L carbon source
- Serial passage during exponential growth
- Stable growth rate achieved at end of evolution
- Cells frozen throughout evolution for phenotype testing





Phenotype testing

Phenotype testing

OptKnock Problem Statement

$$
\begin{aligned}
&\underset{y_j}{\text{maximize}} && v_{chemical} && \text{(OptKnock)}\\
&\text{subject to} && \underset{v_j}{\text{maximize}} \; v_{biomass} && \text{(Primal)}\\
& && \text{subject to} \; \sum_{j=1}^{M} S_{ij} v_j = 0,\\
& && \quad v_{pts} + v_{glk} = v_{glc\_uptake}\\
& && \quad v_{atp} \geq v_{atp\_main}\\
& && \quad v_{biomass} \geq v_{biomass}^{target}\\
& && \quad v_j^{\min} \cdot y_j \leq v_j \leq v_j^{\max} \cdot y_j, \quad \forall j \in \mathcal{M}\\
& && y_j = \{0,1\}, \qquad \forall j \in \mathcal{M}\\
& && \sum_{j \in M} (1 - y_j) \leq K
\end{aligned}
$$

Cells have to grow

If a reaction (j) is removed, set y_j=0 so that v_j has to equal 0.

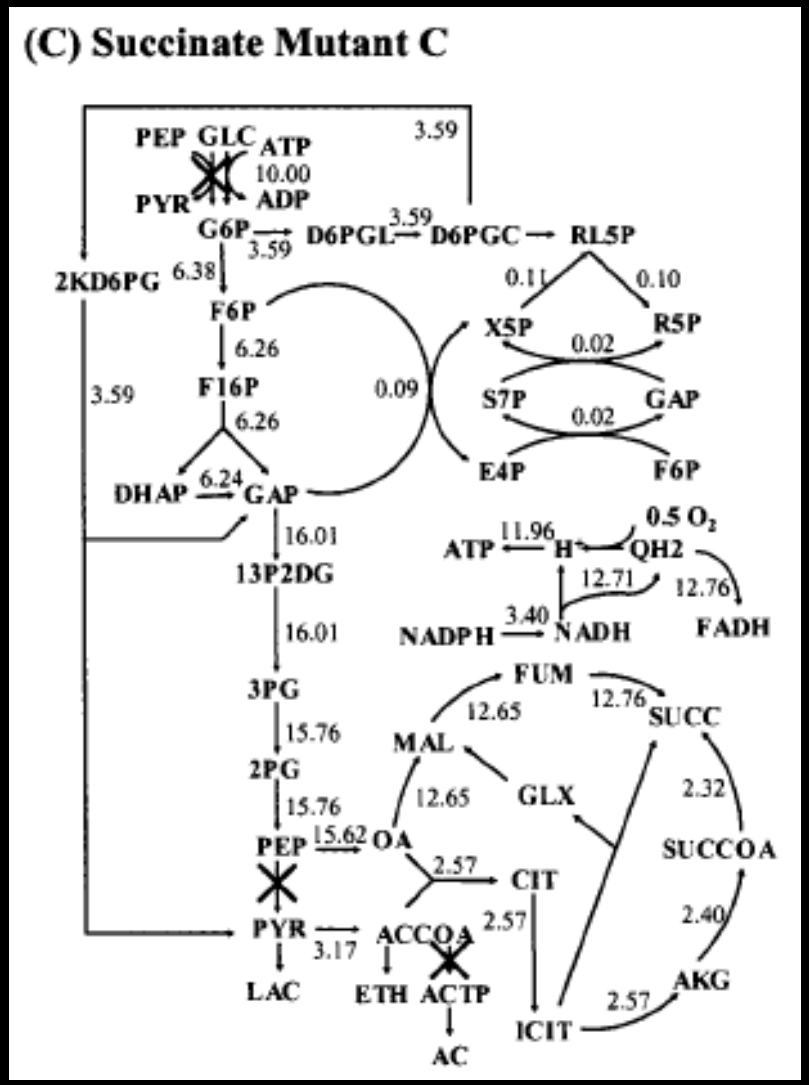
Specify the maximum number of reactions reactions you want to delete, this is K.

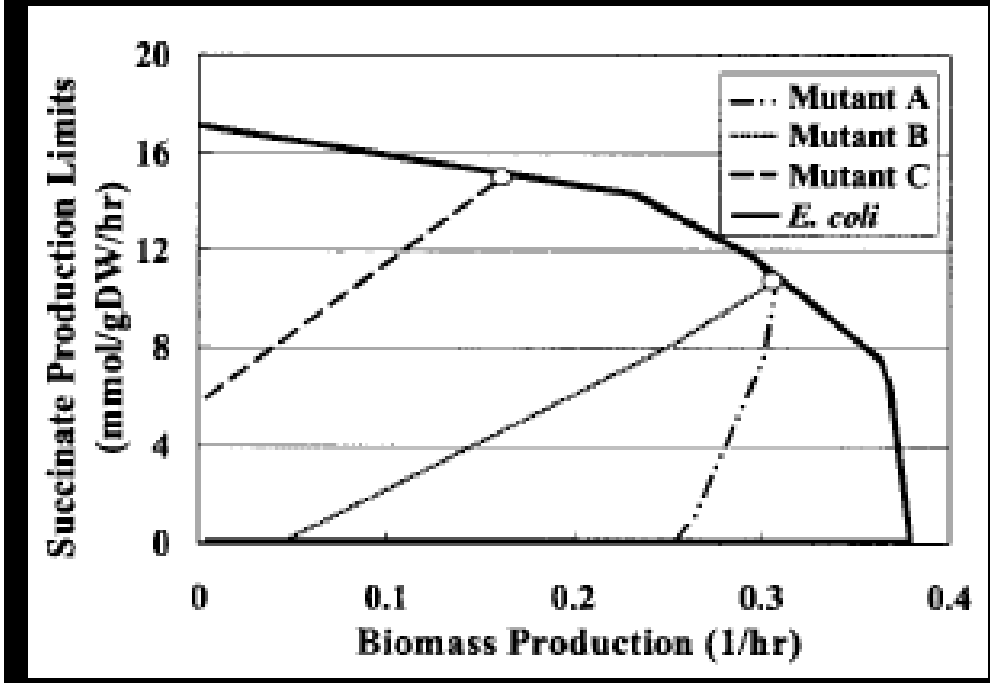
To solve this problem, you transform it by using the dual constraints for the primal problem, in addition to the primal constraints

Succinate				OptKnock		MOMA
ID		Knockouts	Enzyme	Biomass (1/hr)	Succinate (mmol/hr)	Succinate (mmol/hr)
Wild		''Complete network''		0.38	0.12	0
A	1	COA + PYR → ACCOA + FOR	Pyruvate formate lyase	0.31	10.70	1.65
	2	NADH + PYR ↔ LAC + NAD	Lactate dehydrogenase			
B	1	COA + PYR → ACCOA + FOR	Pyruvate formate lyase	0.31	10.70	4.79
	2	NADH + PYR ↔ LAC + NAD	Lactate dehydrogenase			
	3	ACCOA + 2 NADH ↔ COA + ETH + 2 NAD	Acetaldehyde dehydrogenase			
C	1	ADP + PEP → ATP + PYR	Pyruvate kinase	0.16	15.15	6.21
	2	ACTP + ADP ↔ AC + ATP or	Acetate kinase			
		ACCOA + Pi ↔ ACTP + COA	Phosphotransacetylase			
	3	GLC + PEP → G6P + PYR	Phosphotransferase system			

Lactate				OptKnock		MOMA
ID		Knockouts	Enzyme	Biomass (1/hr)	Lactate (mmol/hr)	Lactate (mmol/hr)
Wild		''Complete network''		0.38	0	0
A	1	ACTP + ADP ↔ AC + ATP or	Acetate kinase	0.28	10.46	5.58
		ACCOA + Pi ↔ ACTP + COA	Phosphotransacetylase			
	2	ACCOA + 2 NADH ↔ COA + ETH + 2 NAD	Acetaldehyde dehydrogenase			
B	1	ACTP + ADP ↔ AC + ATP or	Acetate kinase	0.13	18.00	0.19
		ACCOA + Pi ↔ ACTP + COA	Phosphotransacetylase			
	2	ATP + F6P → ADP + F16P or	Phosphofructokinase			
		F16P ↔ GAP + DHAP	Fructose-1,6-biphosphatate aldolase			
C	1	ACTP + ADP ↔ AC + ATP or	Acetate kinase	0.12	18.13	10.53
		ACCOA + Pi ↔ ACTP + COA	Phosphotransacetylase			
	2	ATP + F6P → ADP + F16P or	Phosphofructokinase			
		F16P ↔ GAP + DHAP	Fructose-1,6-biphosphatate aldolase			
	3	ACCOA + 2 NADH ↔ COA + ETH + 2 NAD	Acetaldehyde dehydrogenase			
	4	GLC + ATP → G6P + PEP	Glucokinase			

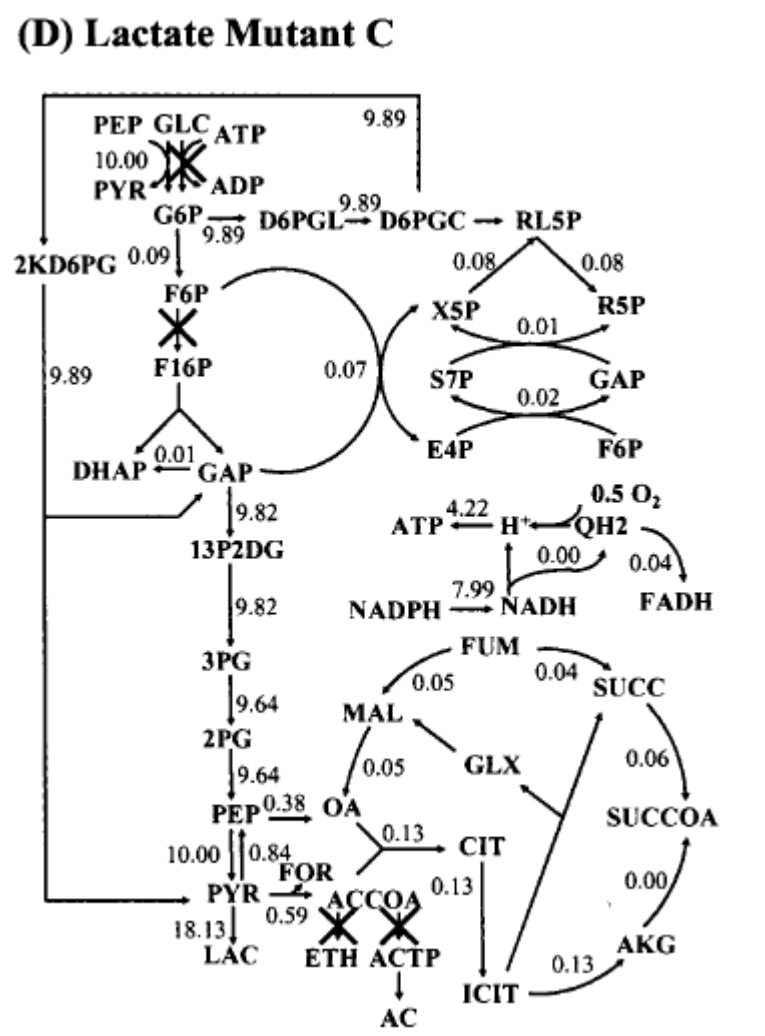
Succinate Production Strains

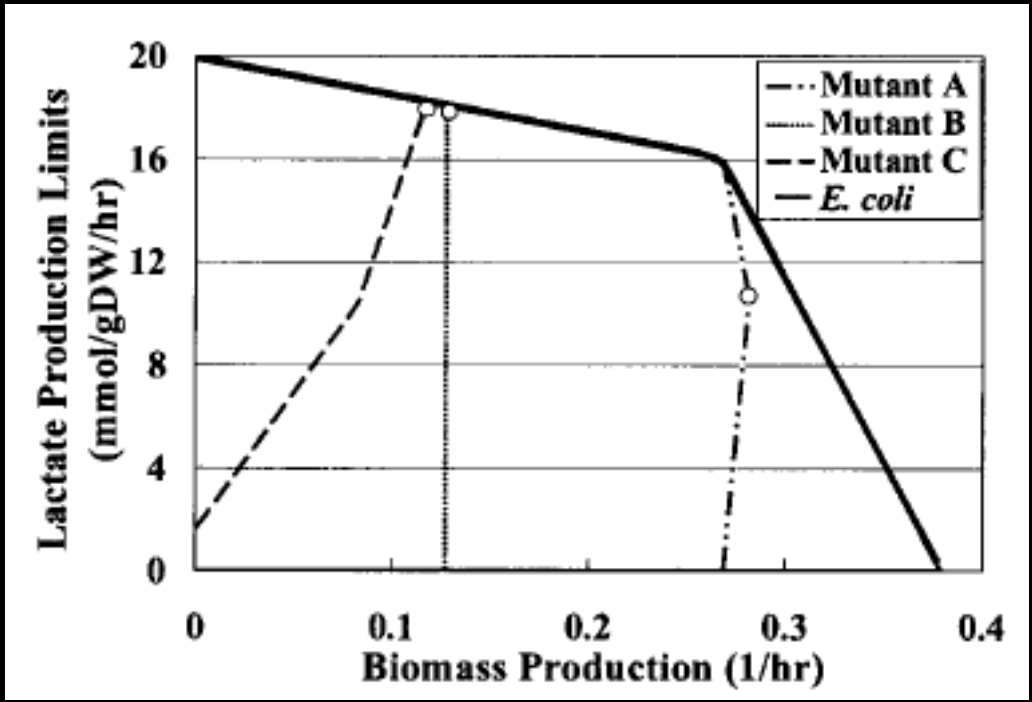




Glucose Uptake = 10
Oxygen Uptake = 0

Lactate Production Strains





Glucose Uptake = 10
Oxygen Uptake = 0

Calculating the Flux Envelop

- This is a combination of flux variability analysis (for just the production flux) and robustness analysis (varying growth rate)!

```
Sets
steps /step1*step15/
maxmin /maxprod,minprod/;

Parameter
c(j)    used to define the objective function for optimizatio
n_steps  number of steps that will be taken and is defined b
range_max maximum flux value through the flux to be varied
range_min minimum flux value through the flux to be varied
flux_value(steps)  stores the values for the varied flux
store_obj(steps,maxmin) stores the value of the objective fu
pick_flux(j) a vector of zeros except for the one flux which

*Determine the number of steps based on the number of elemen
n_steps=card(steps);
*Select the flux that you want to vary
pick_flux('Biomass')=1;
```

How many steps along the x-axis

Flux along the x-axis

```
*reset the objective function to maximize objective of interest
c(j)=0;
c('EX_lacD_e')=1;
loop(steps,
         flux_value(steps)=range_min+(ord(steps)-1)*(range_max-range_min)/(n_steps-1);
         loop(j, if(pick_flux(j)<>0,v.fx(j)=flux_value(steps);  ); );
         solve FBA using lp maximizing Obj;
         store_obj(steps,'maxprod')=Obj.l;
         solve FBA using lp minimizing Obj;
         store_obj(steps,'minprod')=Obj.l;
);
```

Flux along the y-axis

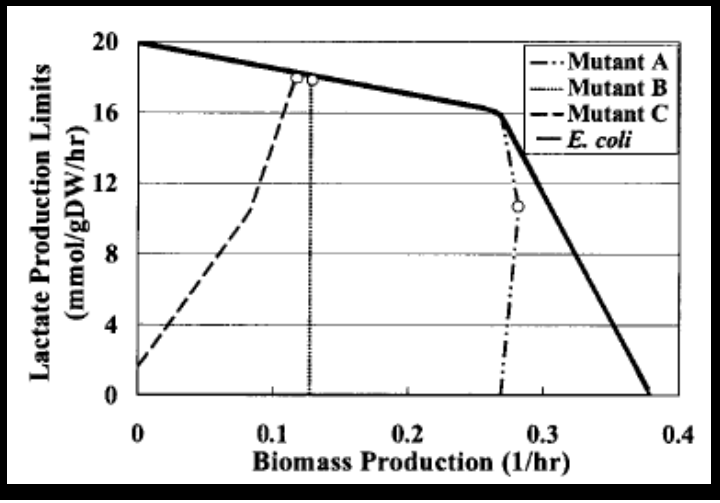
Max & Min the y-axis flux

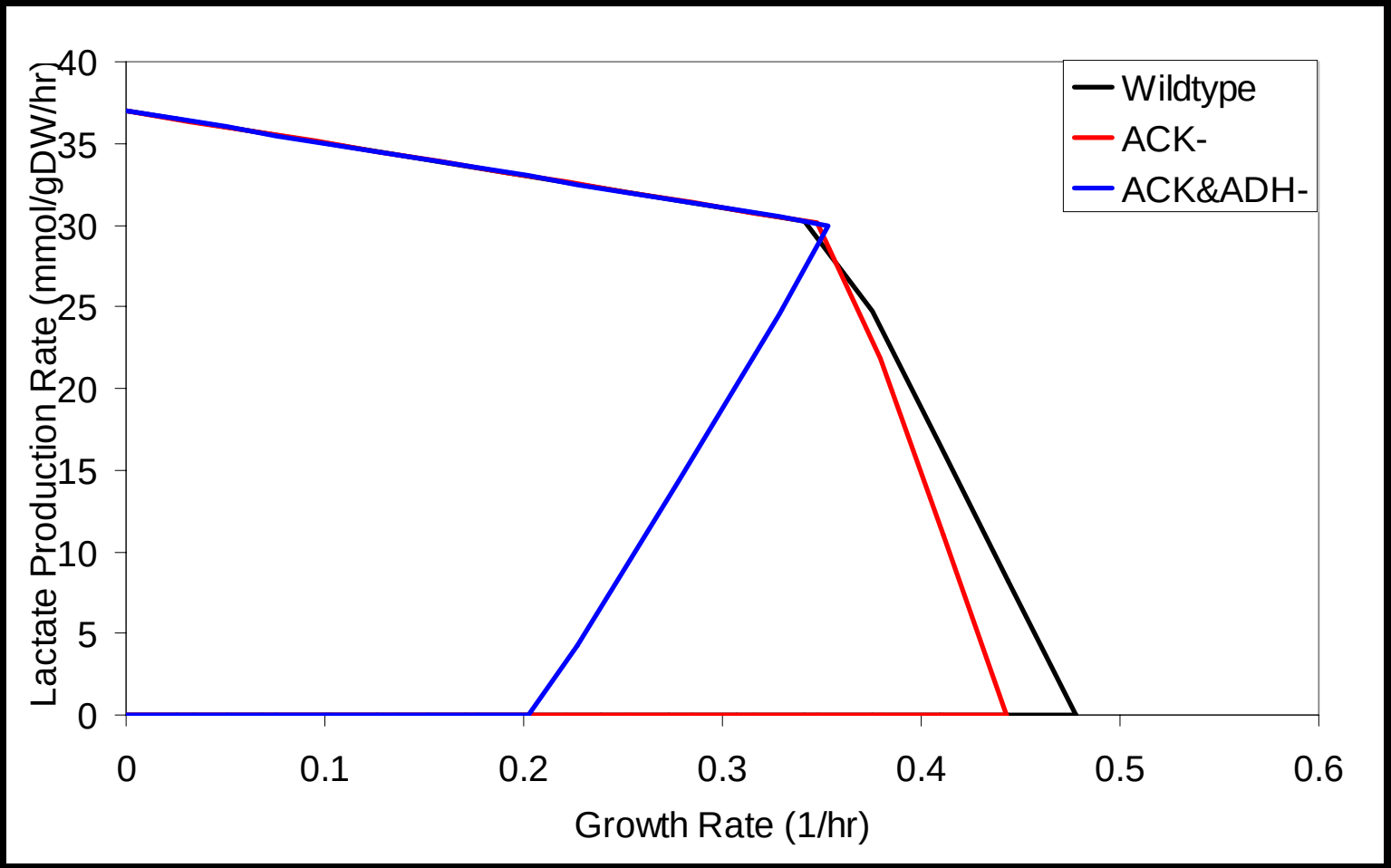
Calculations (Answers on Next Slide)

- Calculate and graph the flux envelops for lactate production under glucose ANAEROBIC conditions for:
  - Wildtype solution
  - Acetate Kinase mutant (ACKr reaction)
  - Acetate Kinase & Aldehdehyde Dehydrogenase double mutant (ACKr and ADHEr reactions)

  **We will use physiological measurements for glucose anerobic uptake and ATP maintenance

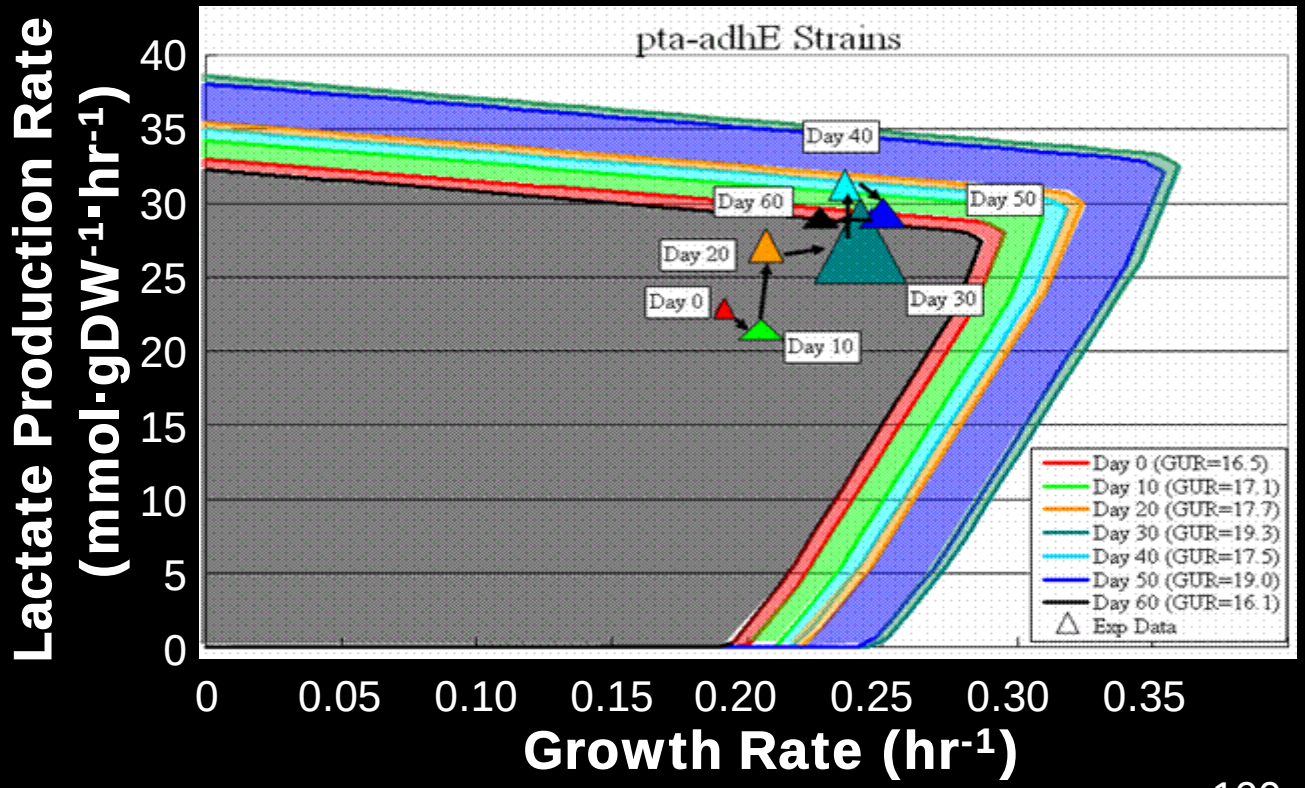
- Why might your graphs look different from those in the publication?



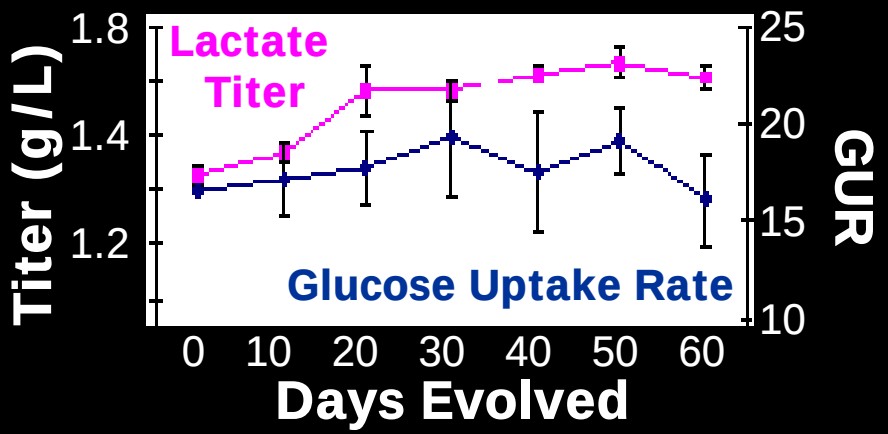


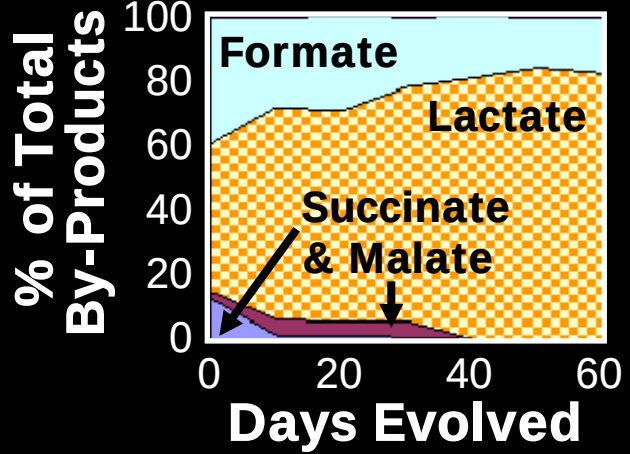
- Why might your graphs look different from those in the publication?
  - Different metabolic networks are used (their network overpredicts anaerobic growth rates)!
  - Different glucose uptake rates are used (they used a value of 10 rather than 18.5)!

Experimental Testing of a Lactate Strain

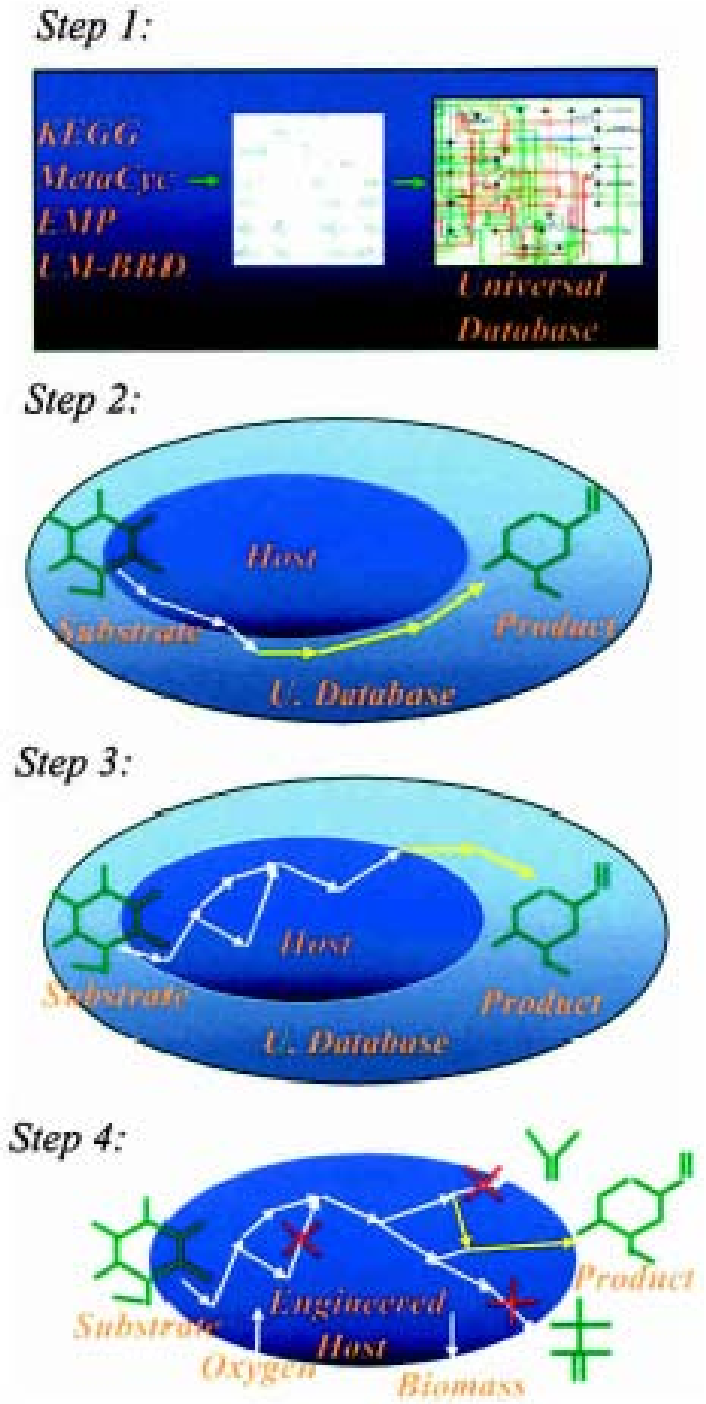


- Lactate secretion rate increased with increasing growth rate
- Lactate yield increased ~35% over 60 day evolution
- 2° by-product secretion decreased





S.S. Fong et al. B&B 2005



OptStrain:

1. Currate KEGG database to find balance reactions.
2. Identify reactions for database with highest production yields for product
3. Identify fewest number of reactions needed from database
4. Run OptKnock to find reaction deletions which couple growth and production rates

Pharkya, Burgard, & Maranas, Genome Research 2004