Metabolic Engineering of *Methylobacterium extorquens* AM1

S. J. Van Dien and M. E. Lidstrom

University of Washington

Seattle, WA

Use of Methanol as a Biofeedstock

- Methanol is inexpensive, soluble in water, clean
- Methanol is produced from natural gas, but can be produced from agricultural wastes
- Methylotrophic bacteria are amenable to genetic manipulation

Goal: to develop process strains for converting methanol to chemicals and materials biologically using methylotrophic bacteria and metabolic engineering

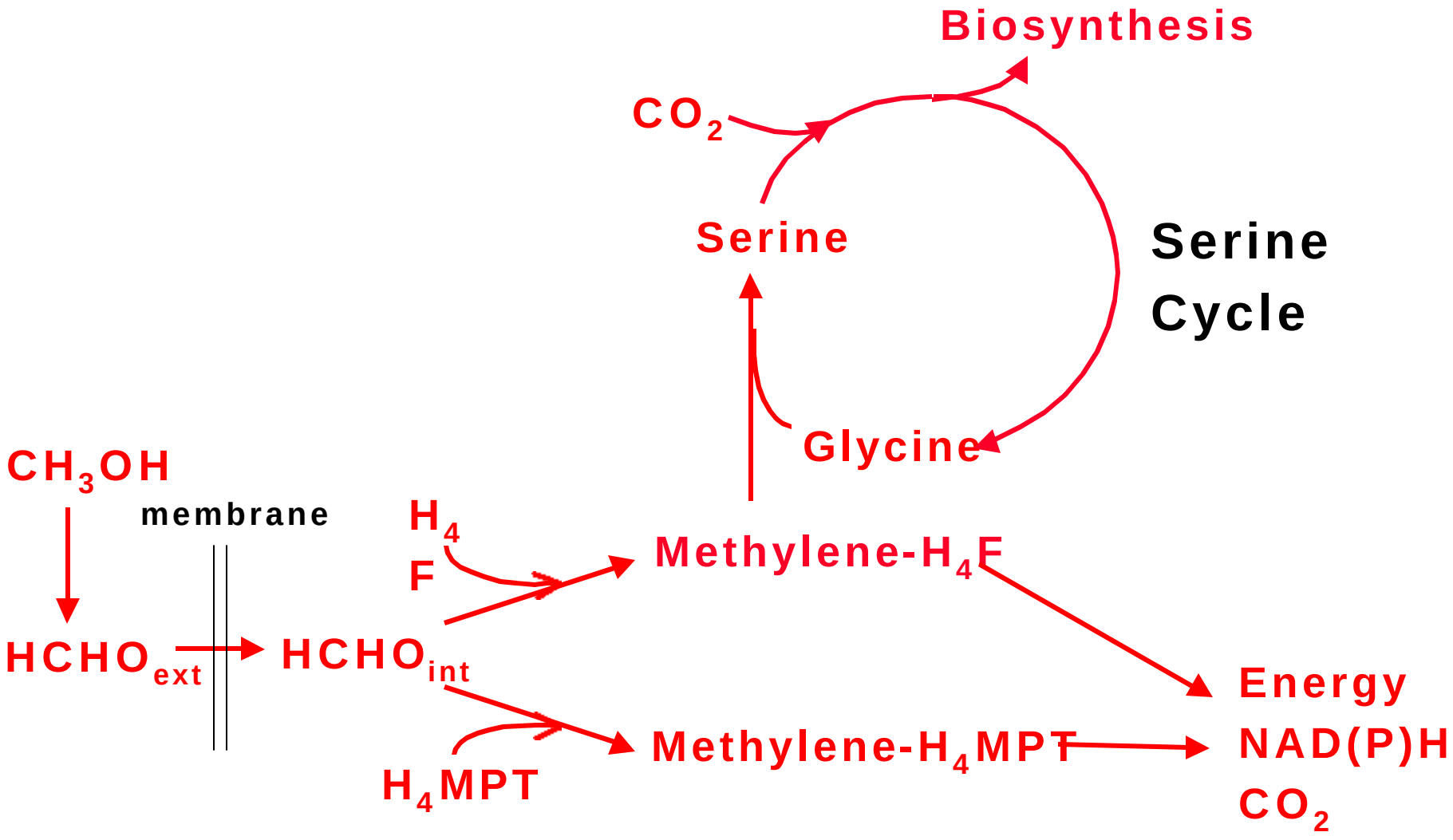
Potential Products

- Amino acids
- Industrial enzymes and cofactors
- Proteins for novel materials applications
- Polyhydroxyalkanoates (PHAs)
- Polysaccharides (viscosifiers)
- Carotenoids

Methylobacterium extorquens AM1

- **α-proteobacterium**
- **Grows on one-carbon compounds (methanol, methylamine)**
- **Also grows on multi-carbon compounds (succinate, pyruvate)**
- **Substantial toolkit for genetic analyses**
  - **110 genes identified**
  - **75 of those involved in methylotrophy**
  - **6x genome sequence complete**
  - **Cloning and expression vectors available**

Methylotrophic Metabolism



Issues for Metabolic Engineering

- Increase serine cycle flux by improving efficiency of formaldehyde handling
- Decrease by-product formation
  - Poly-**b**-hydroxybutyrate is 40 wt.% of cell during methanol growth
  - Carbohydrate is 12 wt.%, part of which is secreted
- Re-direct central metabolism toward essential precursors

Project Goals

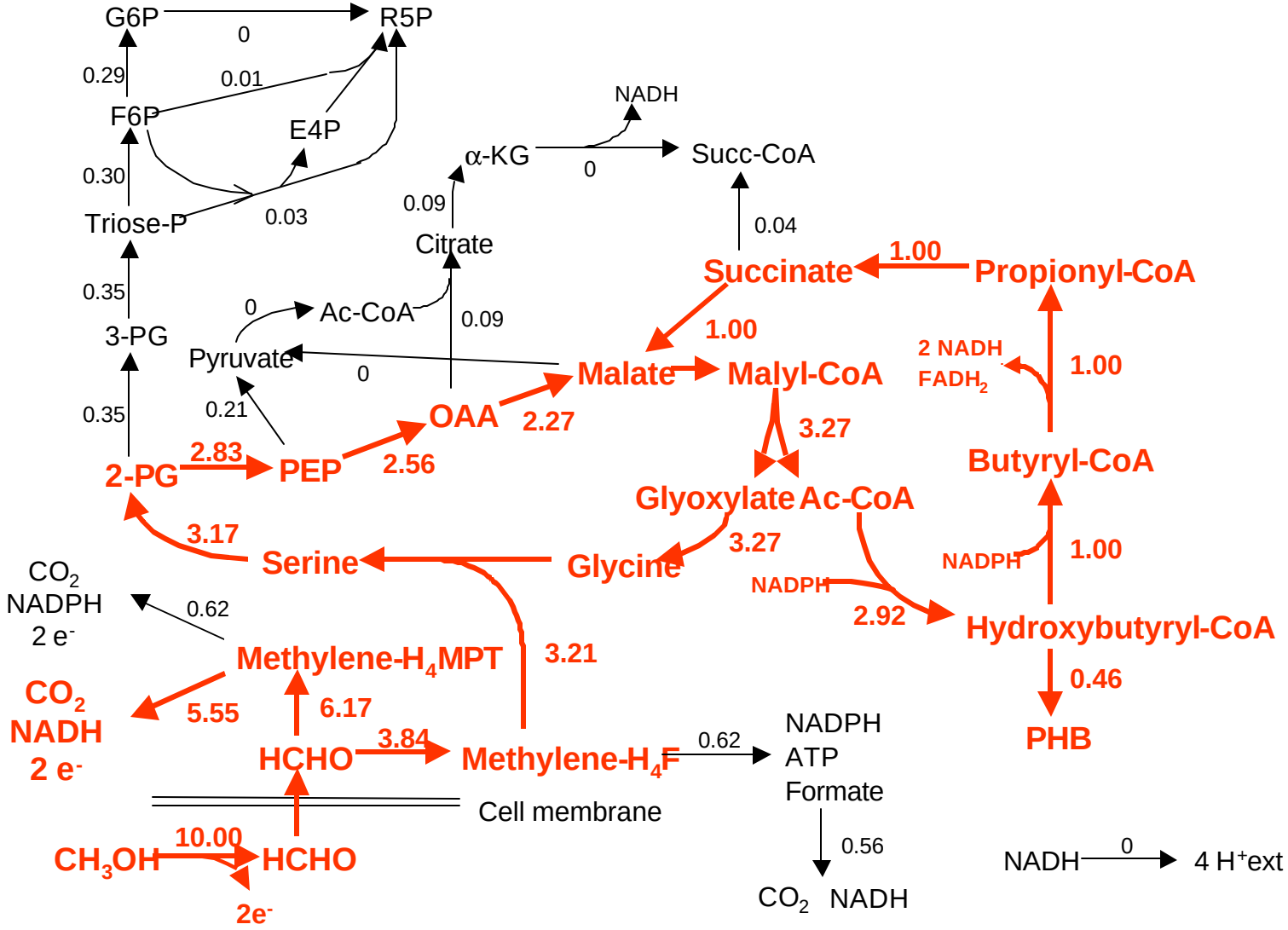
- Use flux balance analysis to develop a model of AM1 central metabolism
- Use model in conjunction with genome sequence and experiments to reconstruct growth on multicarbon compounds

- Use ^{13}C-tracing and GC-MS to improve accuracy of model predictions
- Test metabolic engineering effort using a model product

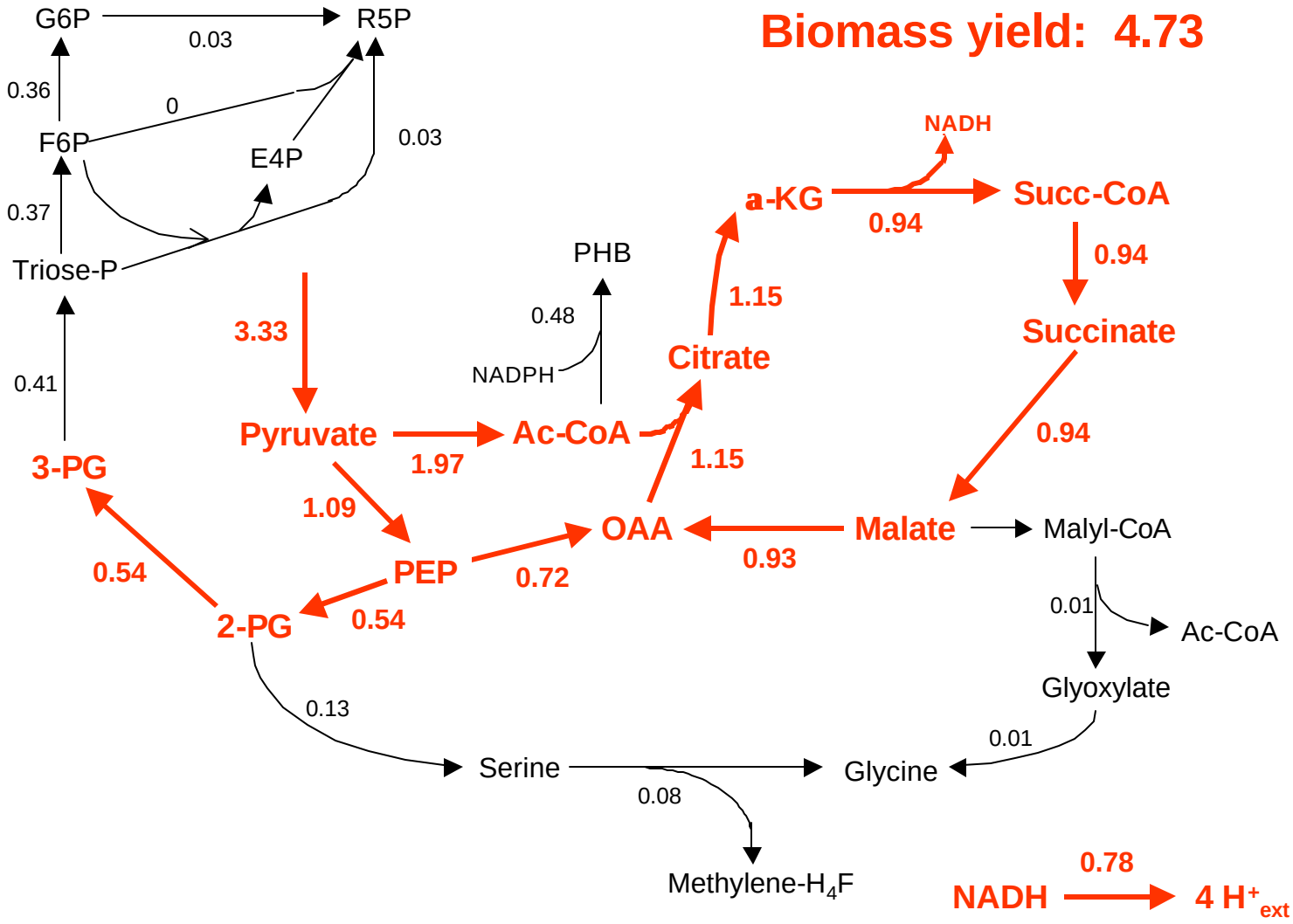
Development of FBA Model

- 68 reactions and 65 metabolites
- Growth on methanol, succinate, or pyruvate
- Experimental measurement of cell macromolecular composition
  - » Used *E. coli* biosynthetic reactions to calculate precursor requirements (EcoCyc, Pramanik and Keasling 1997)
- Calculated elementary modes (Schuster et al, 1999)
  - » Gives all extreme solutions, including optimal solution(s)
  - » Examined elementary modes to explore metabolic capabilities of the cell
  - » Choose elementary modes based on enzyme activity and mutant data

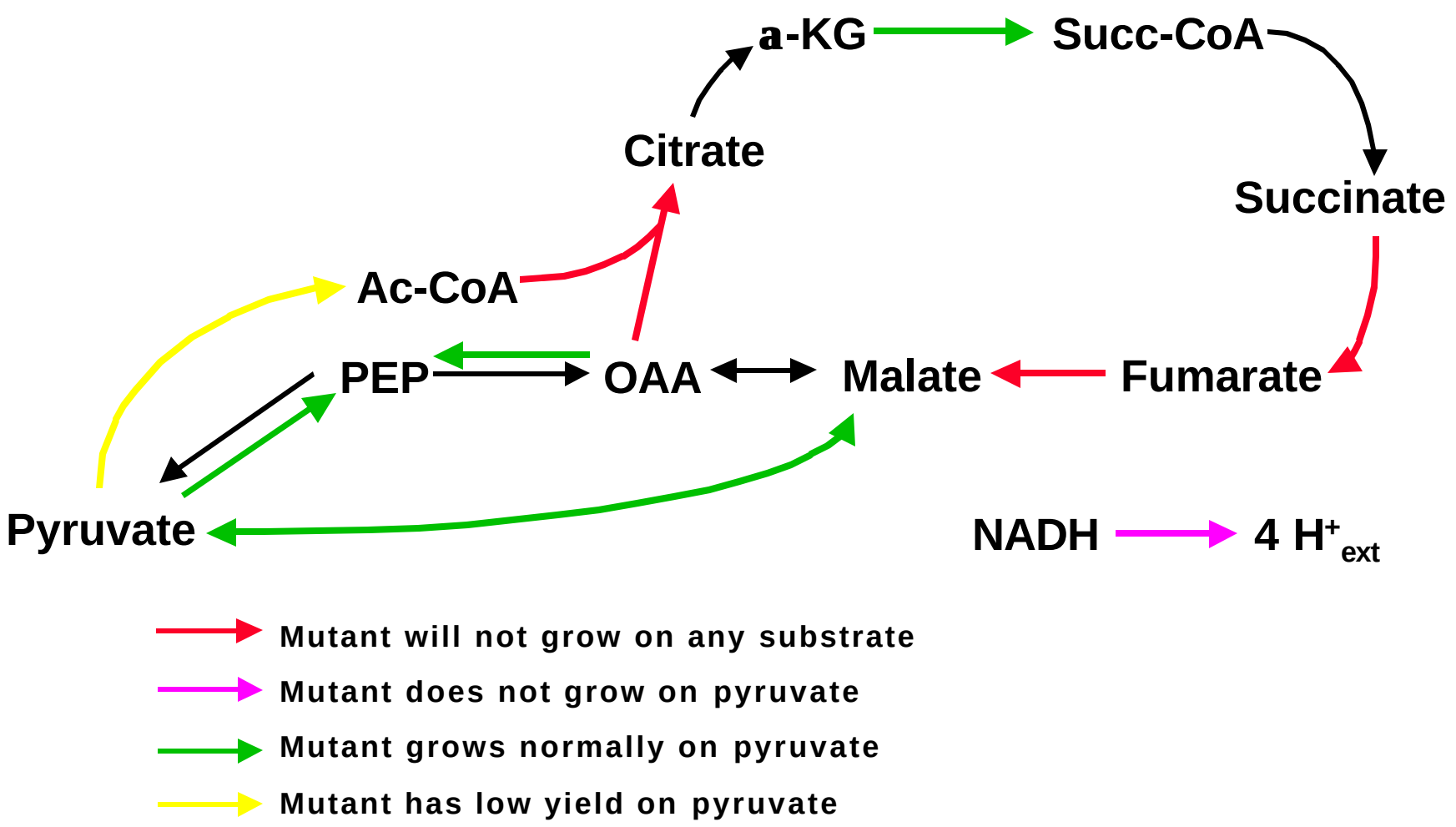
Simulation Results- Methanol Growth



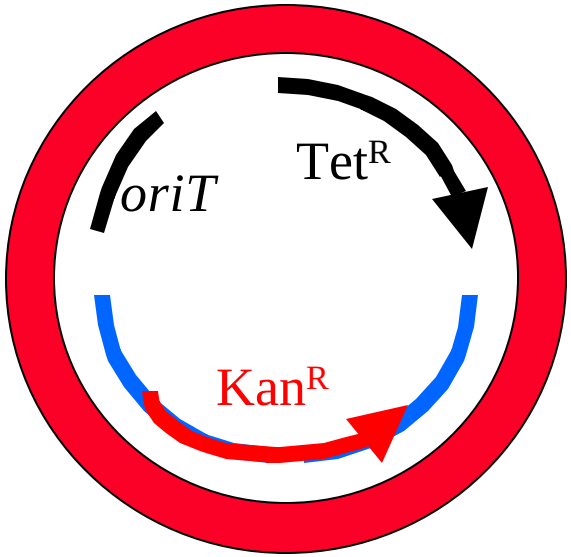
Simulation Results- Pyruvate Growth



Phenotypes of *in silico* Mutants



Construction of Insertional Mutants



Make suicide plasmid with pAYC61

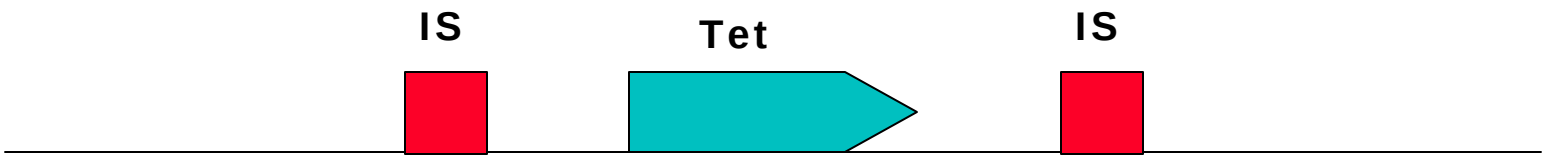
Select on MeOH or Succinate medium containing Kan

Screen colonies on Tet plates

Single crossovers (Tet^{R}) - **contain both mutant and wild type gene**

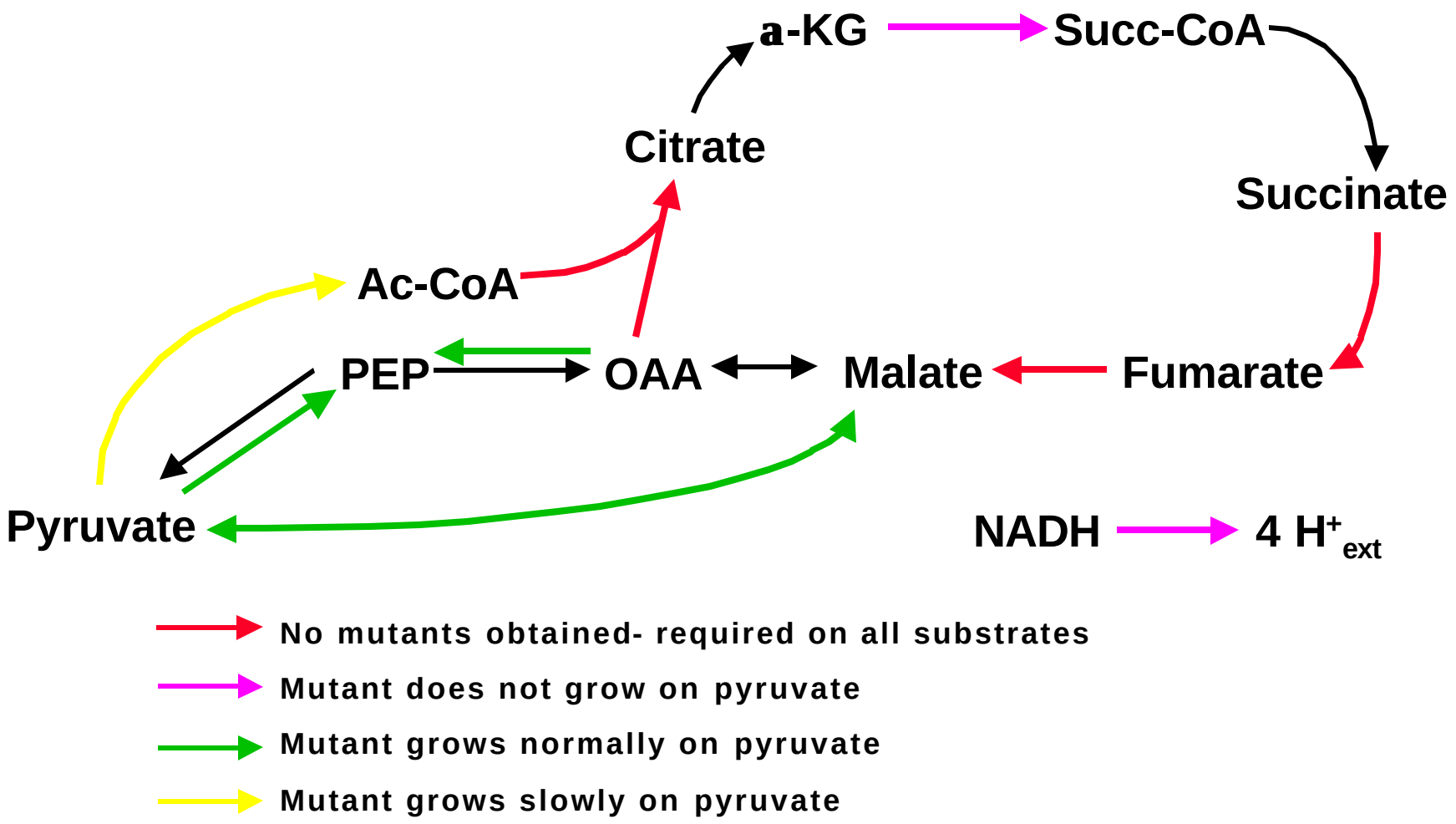
Double crossovers (Tet^{S}) - **contain only mutant gene, and have mutant phenotype**

Mini-Tn*phoA* Transposon Mutagenesis



- The mini Tn*phoA* transposon inserts into the genome creating random insertion mutations.
- These mutations are selected for by growth on methanol with tetracycline resistance.
- Once identified, the mutants are screened for slow or no growth on pyruvate and succinate

Phenotypes of Selected Mutant Strains



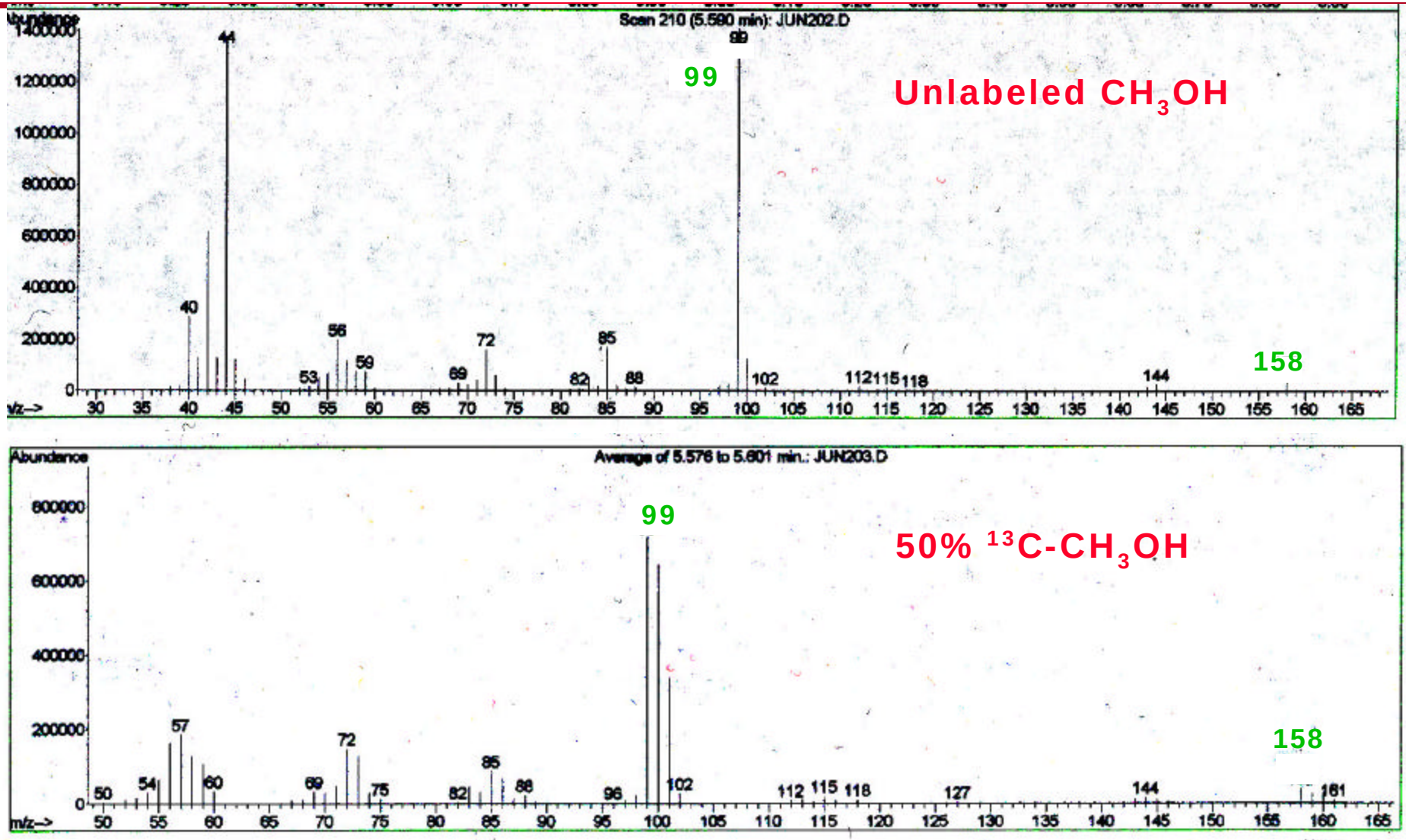
Summary of Results

- Growth yields are within range of measured values
- Cells are NADH-limited during methanol growth, and ATP-limited when grown on non-C1 substrates
- Model correctly predicts most mutant phenotypes tested
- Used model in conjunction with mutant analysis to begin metabolic reconstruction of non-methylotrophic growth

Isotopomer Analysis by GC-MS

- Goal: use ^{13}C-label analysis to gain more information about internal fluxes
- Method (Christensen and Nielsen, 1999)
  - » Grow cells with given ratios of labeled/unlabeled substrate
  - » Isolate total protein, hydrolyze, and analyze derivatized amino acids by GC-MS
  - » Amino acids can be directly linked to their precursors
  - » Write balance equations on each possible isotopomer of each metabolite

GC-MS Results- Alanine Spectra



Acknowledgments

We thank the NSF/EPA Metabolic Engineering Program for financial support

NSF grant # BES9819957
EPA grant # G8H20574

Laboratory and technical assistance:

Melinda Hough (Tn5 mutagenesis)
Yoko Okubo (directed insertional mutants)
Tim Strovas and Martin Sadilek (GC-MS)