Metabolic Engineering of Solvent Tolerance in Anaerobic Bacteria (*Clostridium acetobutylicum*)

E. Terry Papoutsakis, H. Bonarius, Chris Tomas

Department of Chemical Engineering, Northwestern University, Evanston, IL

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Why?

- Solvent (and toxic chemical) tolerance in crucial for production of chemicals, bioremediation, whole-cell biocatalysis. But, ALSO, crucial basic knowledge: How do organisms adapt to “toxic” environments?
- Can we use ME (and genomic approaches) for targeted genetic changes to generate more tolerant strains for bioprocessing?
- Past efforts to produce tolerant strains have relied on selection under applied pressure and chemical mutagenesis: some good results, but not always consistent. Can we do better?

What constitutes solvent toxicity? Tolerance?

- The accepted dogma is that toxicity is due to the chaotropic effects of solvents on the cell membrane. Impaired membrane fluidity and functions (nutrient transport, energy metabolism, ion transport, transmembrane potential) inhibit cell metabolism, and result in cell death
- Thus, tolerance is associated with the ability of the membrane to withstand high levels of toxic chemicals without loss of "function": different membrane composition (and perhaps membrane proteins?)
- Some organisms tolerate solvents better than others (e.g., EtOH tolerance of some lactobacilli). WHY?

Is this model sufficient for solvent production tolerance?

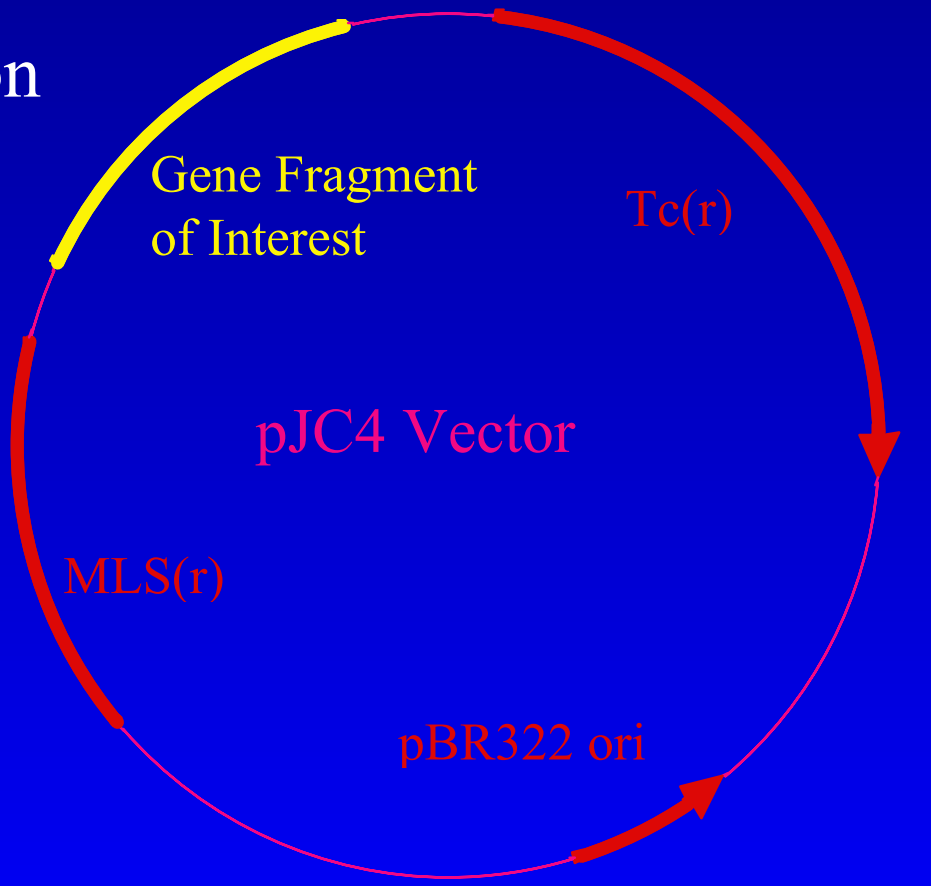
- Perhaps not: We have found that several well-defined genetic modifications impart solvent tolerance (by 40-70%) without strain selection (Butyrate kinase knockout, SolR knockout)
- What does that mean? We may need to re-examine the accepted dogma
- OBJECTIVE: Identify genes that may be also contributing to solvent tolerance and using genetic modifications (involving these genes) to generate solvent tolerant strains
- PROOF OF CONCEPT: Have examined such a set of genes and appears to work both in ATCC 824 and another Gram positive organism

STRATEGY

- **Design expression cassettes, selection markers & gene inactivation protocols in order to be able to overexpress or knockout several genes at the same time**
- **Overexpression of several genes and use of 2-3 selection markers is now reasonably well developed. Gene chromosomal integration (inactivation) IS NOT**
- **IDENTIFY classes of genes that may play an important role in solvent tolerance or toxicity**
  - **We had thought that chaperonins and molecular pumps would be a sufficiently complete group: WELL, it is not…. WHAT THEN? More discovery? How? → DNA arrays (home made)**

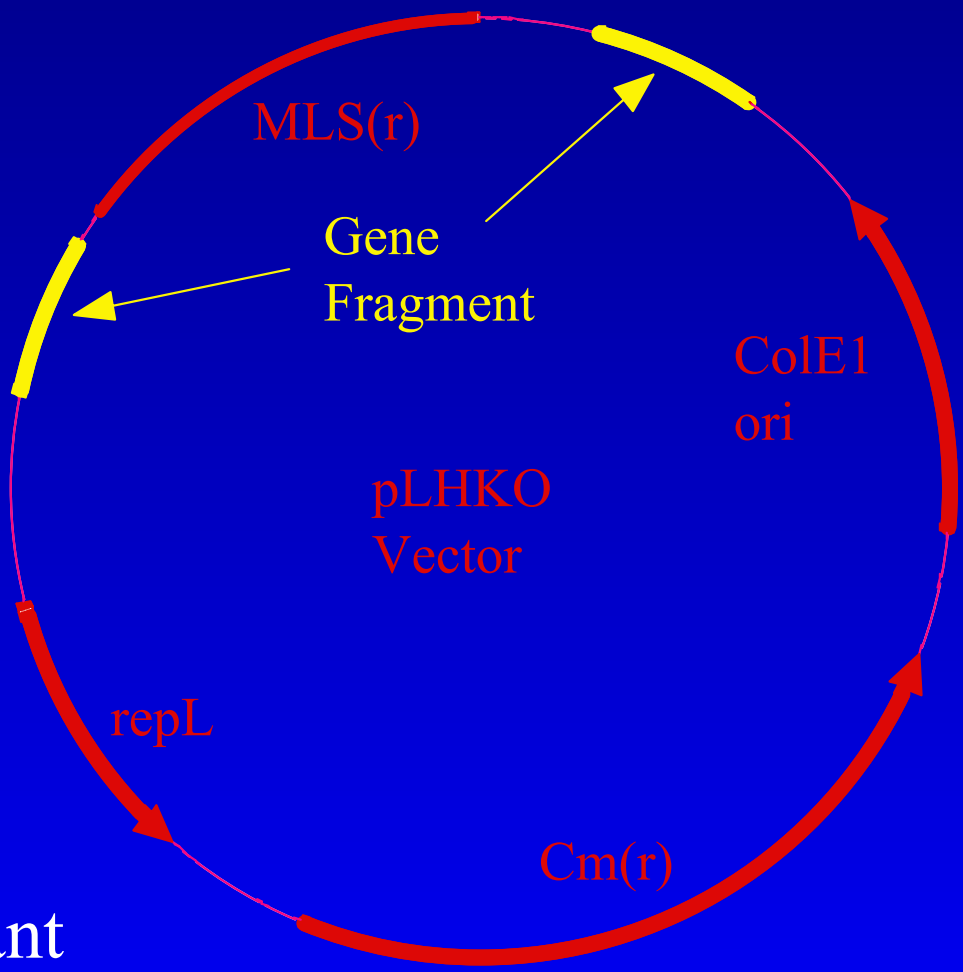
Non-Replicative Vector

- No *Clostridial* ori
- Requires high transformation efficiencies
- Very low recombination frequency
- Integration of entire plasmid
- Used to create *buk*, *pta*, *aad*, *sol*R mutants
- Not easily reproducible



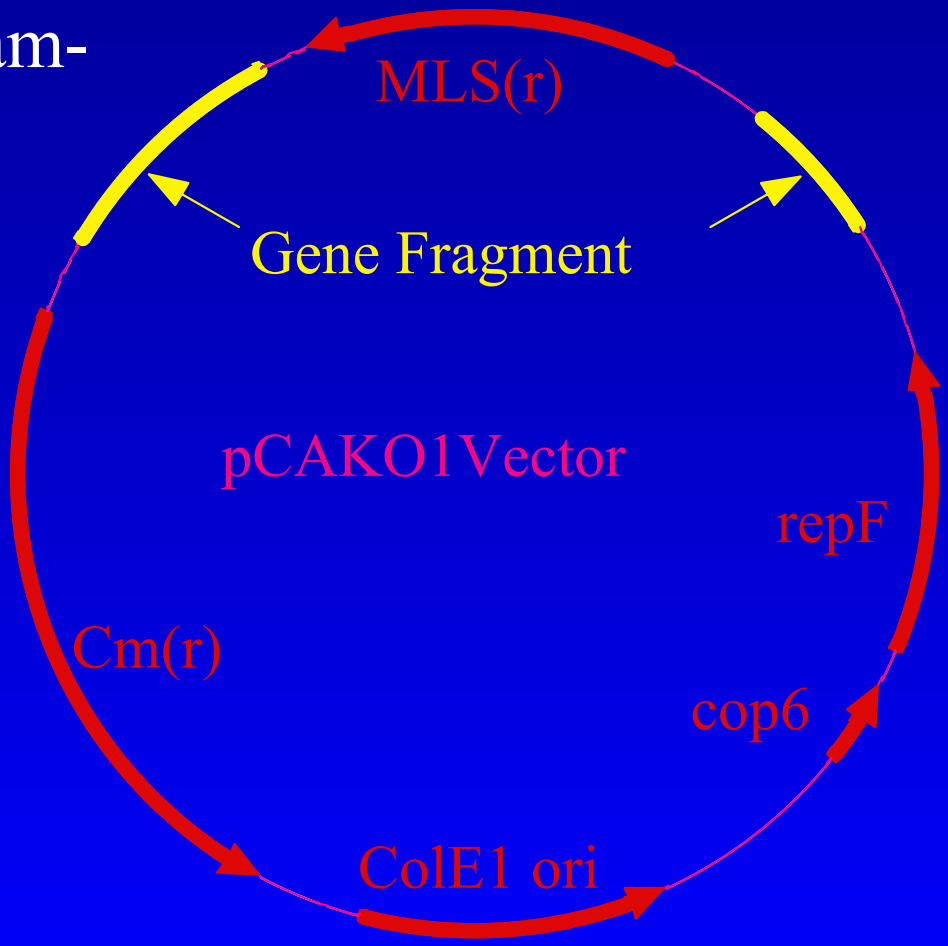
Replicative Vector

- Gram-positive origin, *rep*L
- Increased contact time with chromosomal DNA
- Capable of double crossover event
- Very stable replicon, making selection difficult
- Used to create *spo*OA mutant
- Tedious process, does not always work

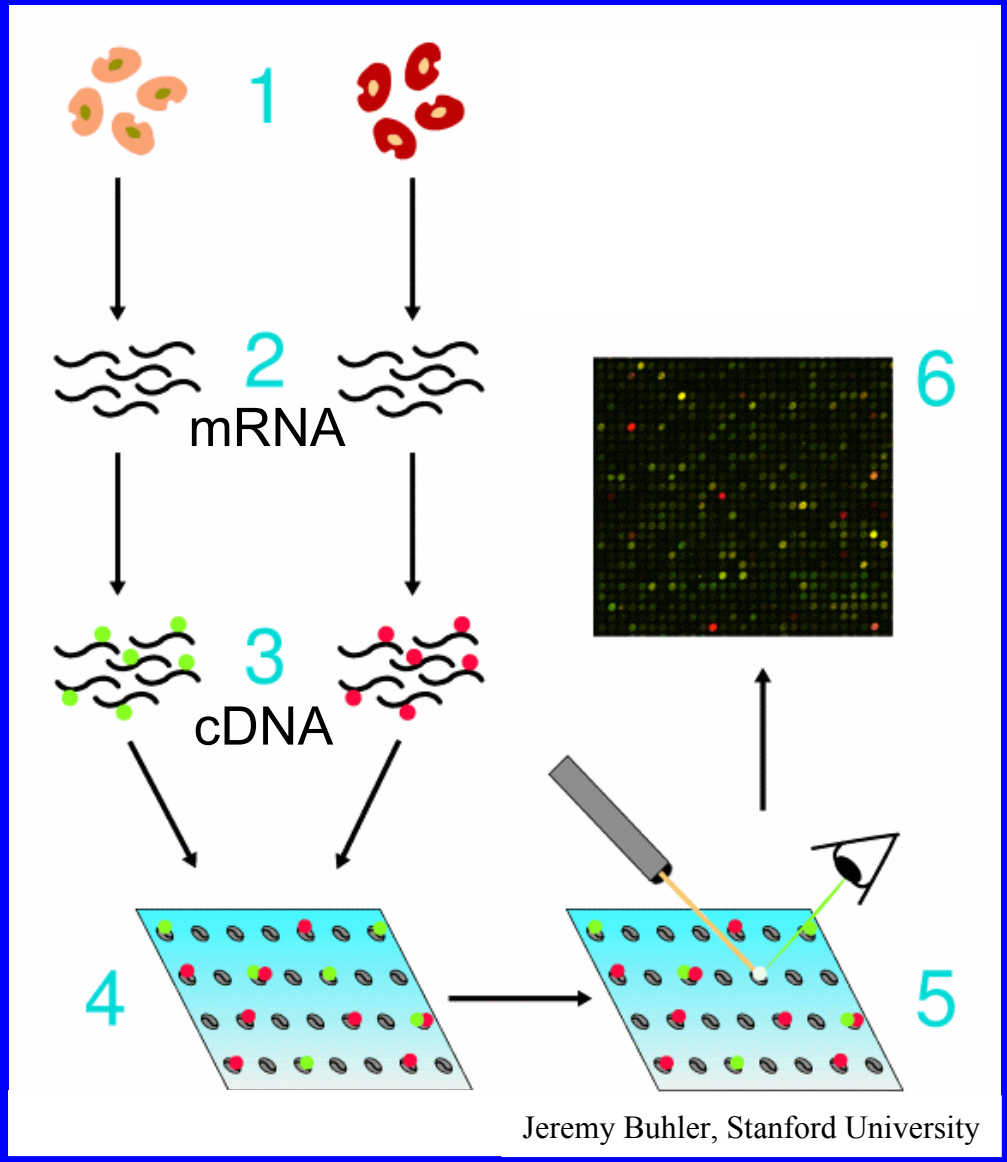


Temperature Sensitive Replicative Vector

- Temperature sensitive, Gram-positive origin, *rep*F
- Increased contact time with chromosomal DNA
- Capable of double crossover event
- Easier selection of mutant strains



DNA microarrays give comparative expression data



Expression analysis of *C. acetobutylicum*

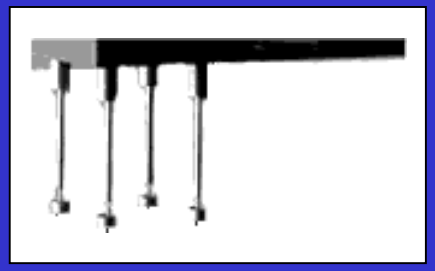
- PCR genes from *C. acetobutylicum* genome

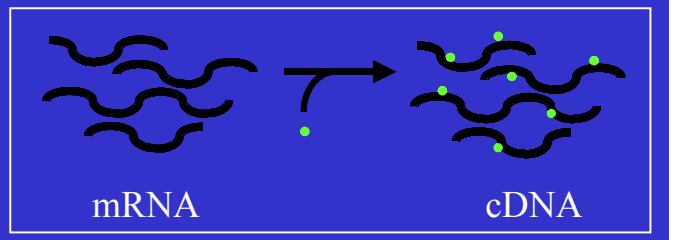
- Spot genes on aminosilane-coated glass slides with pin-and-ring arrayer

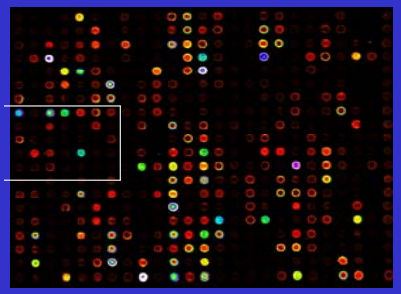
- Isolate mRNA and label cDNA from control and experimental condition with Cy3-dUTP or Cy5-dUTP

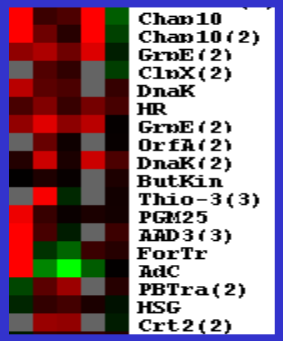
- Hybridize and scan at 550 and 650 nm

- Analyze expression and cluster genes



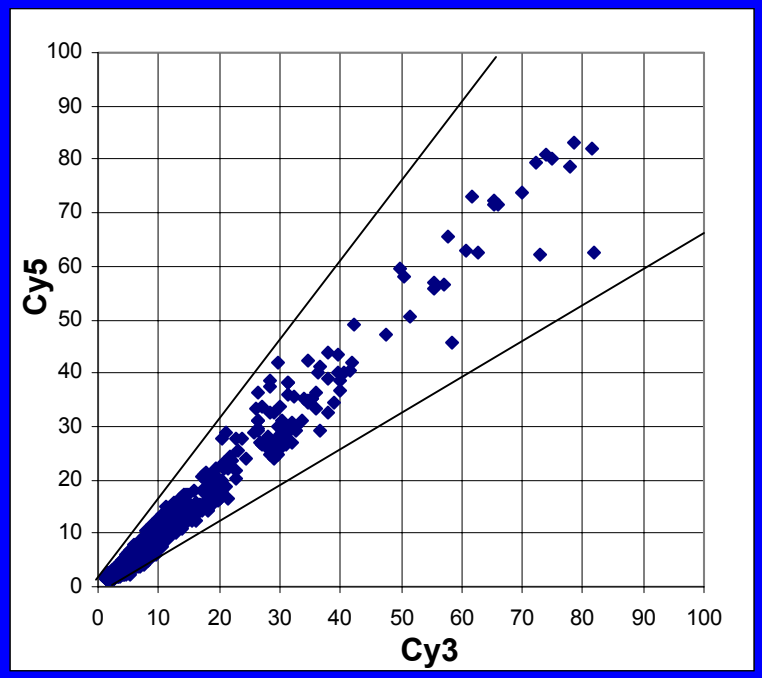




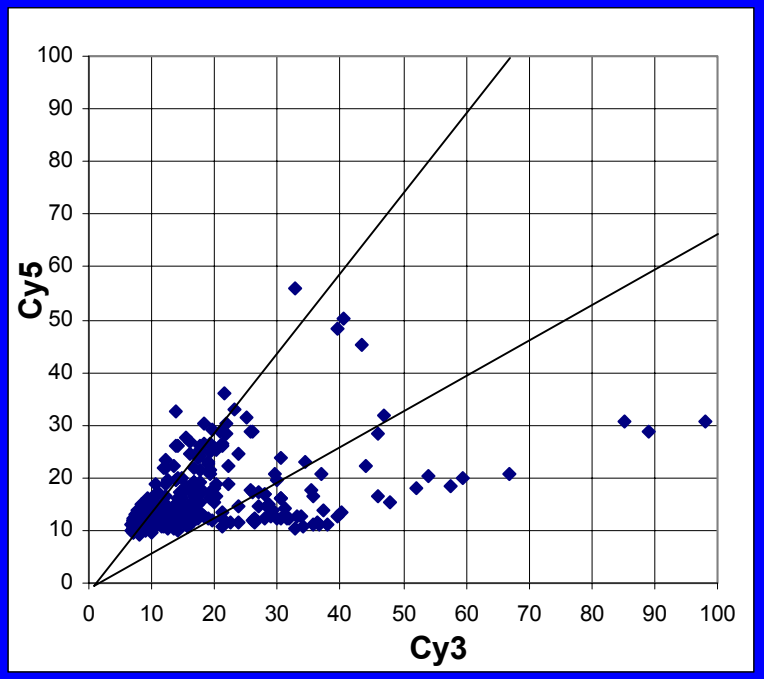


Comparative transcript levels for 100 genes (3x) on one slide

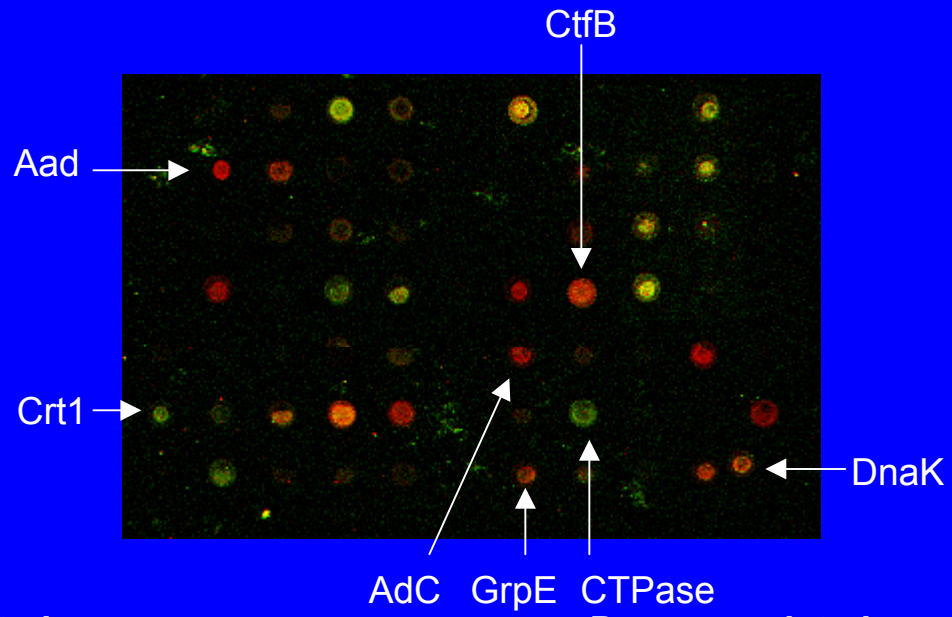
control vs control



butanol challenge vs control

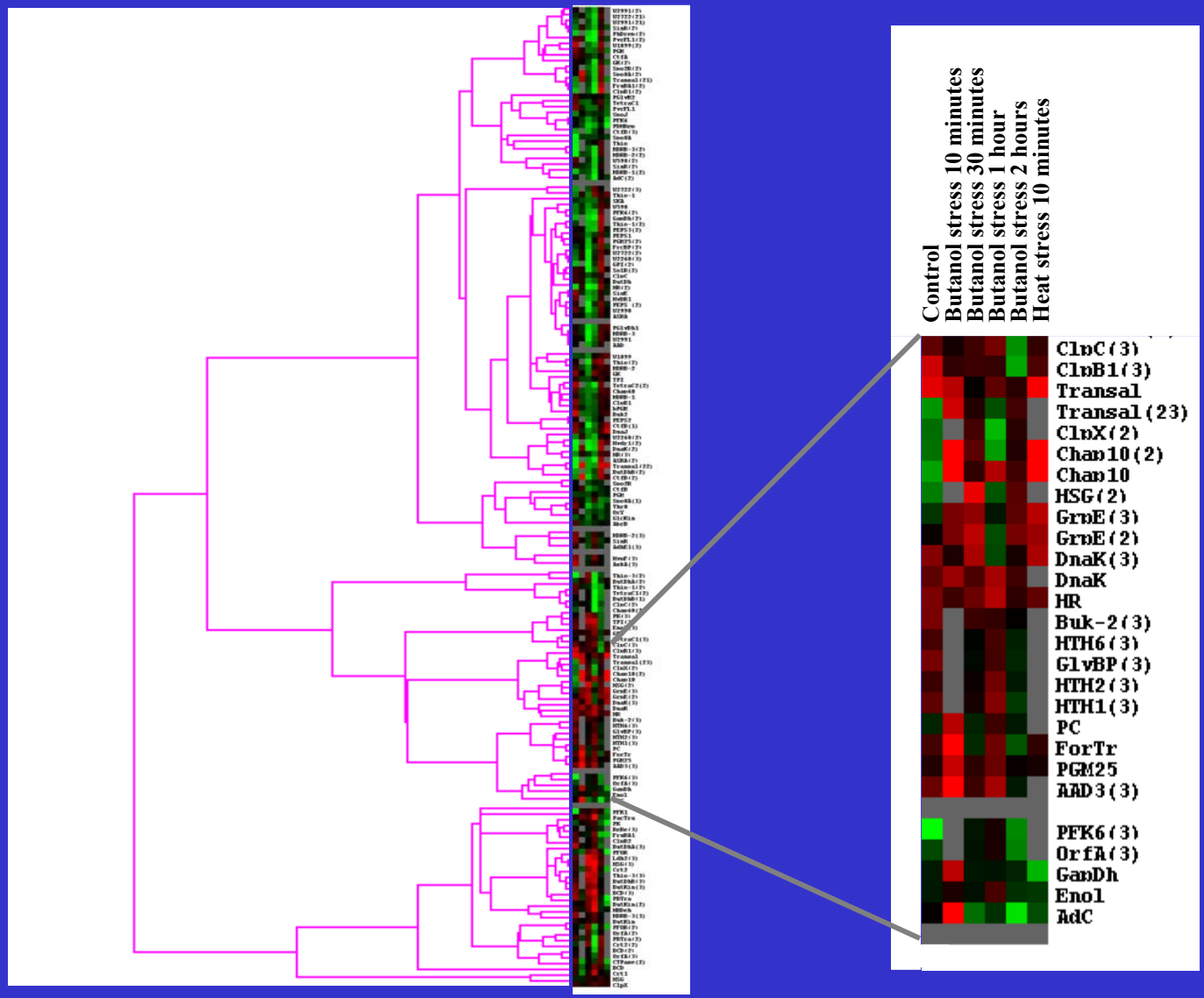


Butanol challenge (cy3) vs Control (cy5)



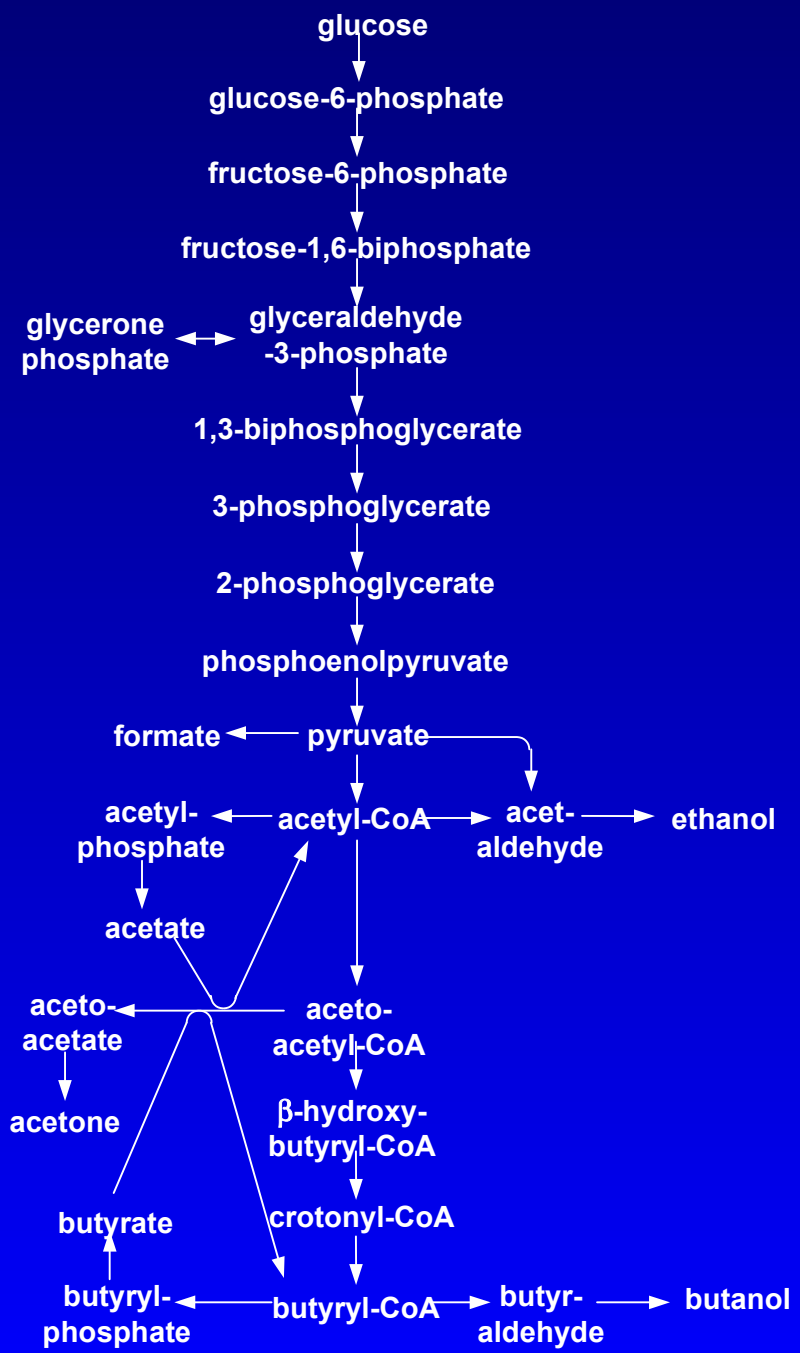
Up-regulated genes		Down-regulated genes	
Aldehyde alcohol dehydrog.	3.4	CTP synthetase	0.4
Formate acetyltransferase	3.3	Crotonase1	0.5
Acetoacetate decarboxylase	3.1	Buyrate kinase	0.5
Acetyl-CoA transferase B	2.5	Pyruvate-Fe Oxidoreductase	0.6
Chaperonin (10 kb)	2.4	Acyl-CoA dehydrogenase	0.6
Transaldolase	2.0	Hydroxybutyryl dehydrogenase	0.6
Sporulation kinase A	1.9	Phosphotransbutyrylase	0.7
Phosphoglucomutase	1.7	Crotonase2	0.7
Glyceraldehyde-P dehydr.	1.7		
Chaperone DnaK	1.5		
Stress protein GrpE	1.5		

Hierarchical clustering ~ 100 genes over 6 stress experiments



Several classes of new genes identified as changing gene expression under solvent stress

- Molecular pumps
- Chaperonins (HSP)
- Several primary metabolism genes
- ATPases
- Sporulation genes
- Transcriptional regulators
- Carbohydrate metabolism genes
- FINDINGS DO NOT MAKE ALWAYS SENSE
  - E.g., why would butanol formation genes be upregulated and butyrate formation genes downregulated? One would expect the opposite


glucose
glucose-6-phosphate
fructose-6-phosphate
fructose-1,6-biphosphate
glycerone phosphate
glyceraldehyde -3-phosphate
1,3-biphosphoglycerate
3-phosphoglycerate
2-phosphoglycerate
phosphoenolpyruvate
formate
pyruvate
acetyl-phosphate
acetyl-CoA
acet-aldehyde
ethanol
acetate
aceto-acetate
aceto-acetyl-CoA
acetone
β-hydroxy-butyryl-CoA
butyrate
crotonyl-CoA
butyryl-phosphate
butyryl-CoA
butyr-aldehyde
butanol

Several classes of new genes identified as changing gene expression under solvent stress

- Will overexpression (or down regulation) of these genes impart solvent tolerance? We are about to find it out

- If these effects (toxicity and tolerance) result from the action of the products of several genes, one many need multigene ME strategies
  - AsRNA
  - Powerful expression cassettes & gene knockout technologies
  - Enabling promoters
  - Regulatory gene approaches

COMPLEXITY, METABOLIC ENGINEERING, AND A NEW ERA IN MICROBIAL PHYSIOLOGY?

- **High throughput technologies**
- **DNA microarrays: THE Transcriptional program**
- **Protein microarrays (tomorrow & forever…): THE Proteome**
- **The hybrid ones (protein/protein, DNA/protein, protein/cell interactions, …)(tomorrow & forever…)**

Calculations, Computations & Systems Theory

- Models, lots of them…

- **Regulatory & Genetic Networks**
- **Systems Biology and Organismal Biocomplexity**
- **Pathways & Fluxes, BUT ALSO "systems" interactions all levels**