

Development of tolerant and other complex phenotypes for biofuel production

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Complex phenotypes

- **Involve many genes, which are frequently or mostly not known in their entirety (class I)**
- **Even if we know the genes involved, we do not understand their dynamic behavior to allow us to alter or develop the phenotype (Class II)**

Examples?

- Cells that can tolerate harsh bio-processing conditions beyond what may currently exist in nature? **(Class I)**
- **Like ethanol toxicity: Can we create cells that can tolerate 30% ethanol? Why not? What would it take? What do we know? What is the problem? Biophysical?**

More examples...

- Plants with dramatically improved photosynthetic machinery that produce cellulosic biomass twice or thrice as fast as what is currently possible? (**Class II**)
- Cells that utilize quickly and effectively (**5-10x better than currently known**) cellulose or xylans to produce one major product (such as ethanol or butanol or other biofuels or commodity chemicals) (**Class II or I?**)

Which way to solve such problems?

- **An *ab initio* cellular design?**
 - How far can we go based on what we know?
 - What is holding us back? Synthetic biochemistry/biology or knowledge?
- **A hybrid first approach: some knowledge based, some empirical or semi-empirical?**
 - What is the evidence that it will work? What hypotheses must be tested to that effect?
- **Luck, and trial & error empiricism like mostly currently practiced?**

Our Research Goals

- **Identify genes and cellular programs in clostridia affected by solvent (e.g., butanol) and carboxylic acid (butyrate & acetate) stress in order to identify:**
 - Specific and general stress regulons
 - Genes which may impart solvent/acid tolerance

Applications: Bioprocessing for:

- Solvent-production: fermentations
- Biocatalysis
- Bioremediation

Production of solvents via fermentation provides a green alternative to petrochemicals

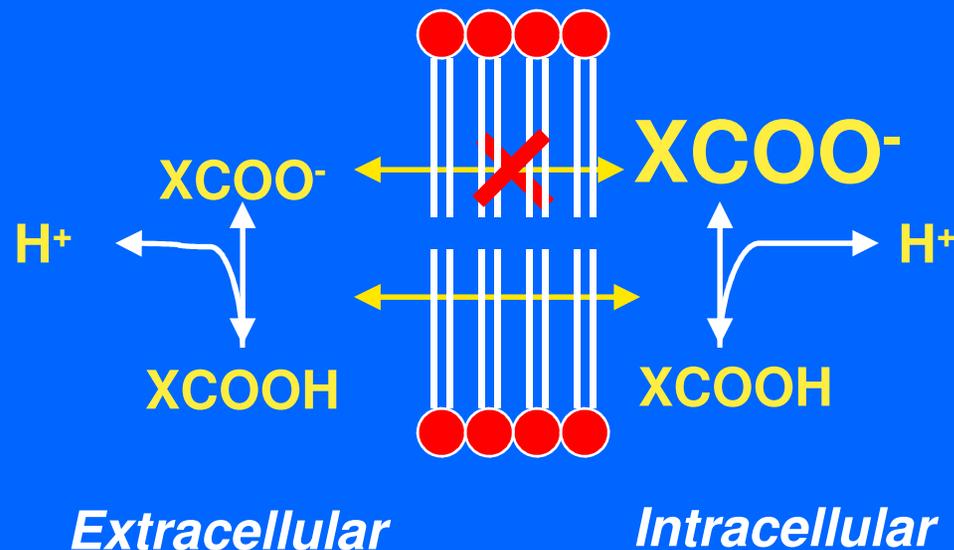
- 1910s-1950s: butanol produced by anaerobic fermentation: *C. acetobutylicum* (acetone, butanol, ethanol)
 - Fermentation yields 1.5% butanol (very low)
 - A limiting step in final titers: product inhibition (toxicity)
- Two phase fermentations
 - Exponential growth: production of butyrate, acetate
 - Stationary phase: uptake of butyrate and acetate, production of acetone, butanol, and ethanol
- TOXICITY: butanol (but also butyrate & acetate & interactions)

THEORY:

Mechanisms of Solvent Toxicity

- **Inhibition of growth and glucose uptake**
- **Disruption of membrane integrity**
 - **Loss of membrane ΔpH and $\Delta\Psi$**
 - **Loss of ATP production efficiency**
 - **Cells adapt slowly by altering membrane fluidity**
 - **Adaptation may inhibit membrane function**

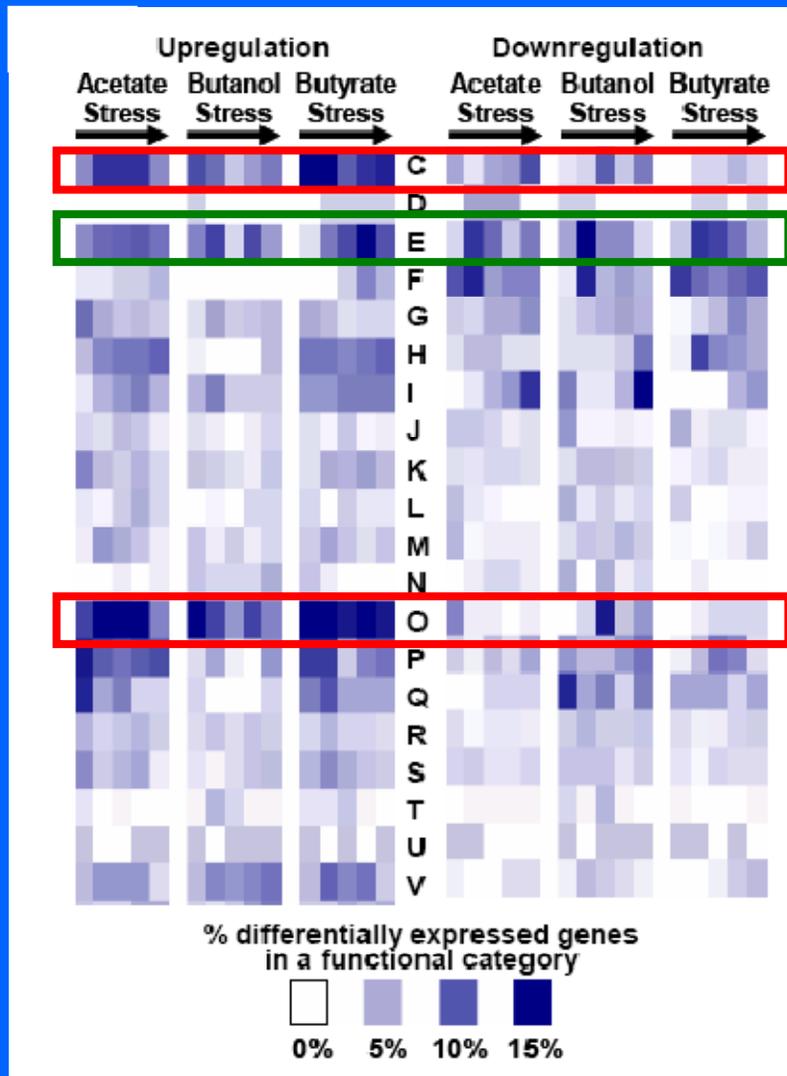
Uncoupling THEORY of Carboxylic Acid Toxicity: Undissociated acids cross cellular membrane and acidify the cytoplasm



Russell and Diez-Gonzalez, *Adv. Microb. Phys.*, 1998, 208

- Is it that simple?

Ontological Analysis of multi-stress responses (based on Genome-scale μ -array analysis)

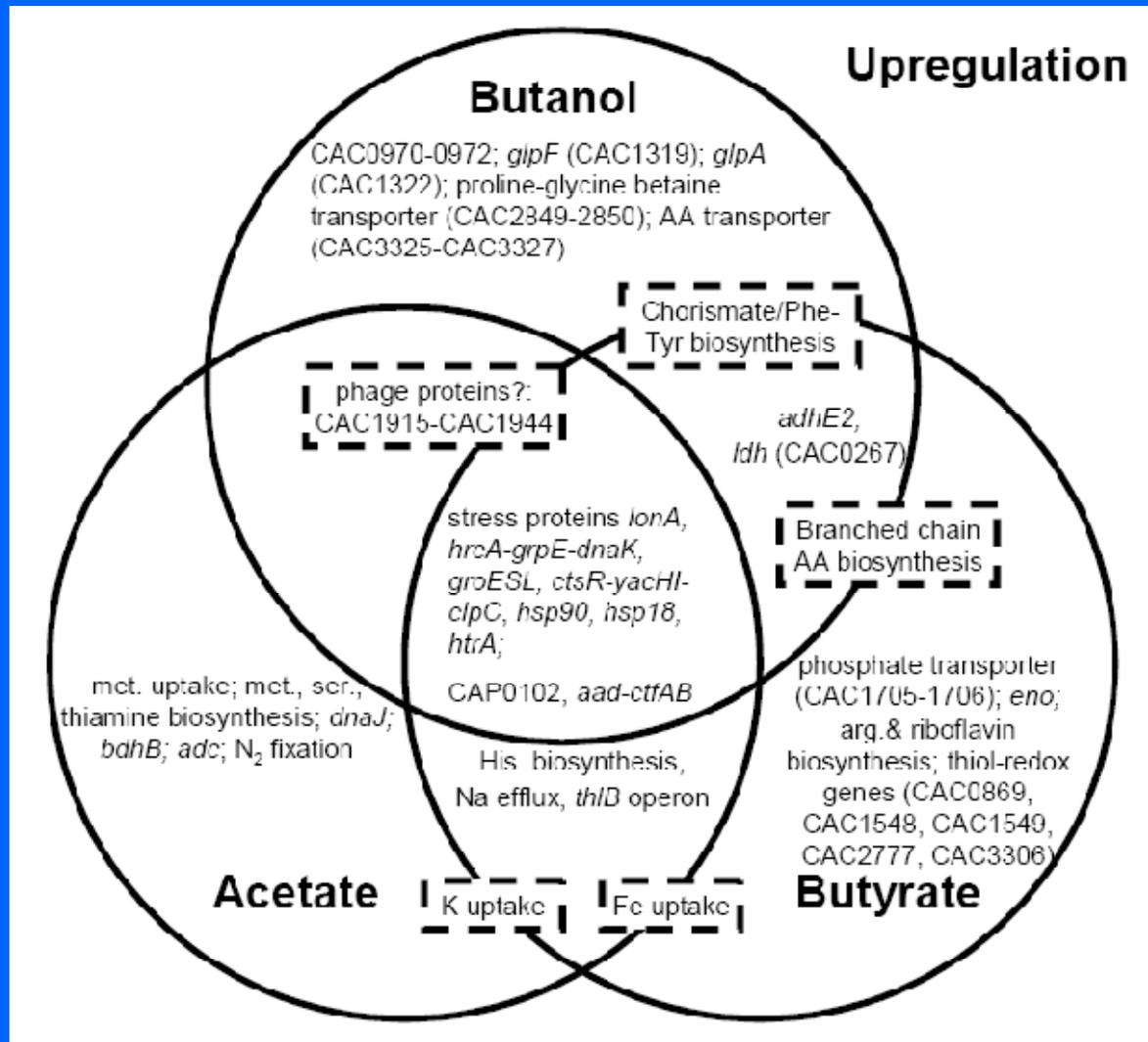


→ Energy Production & Metabolism

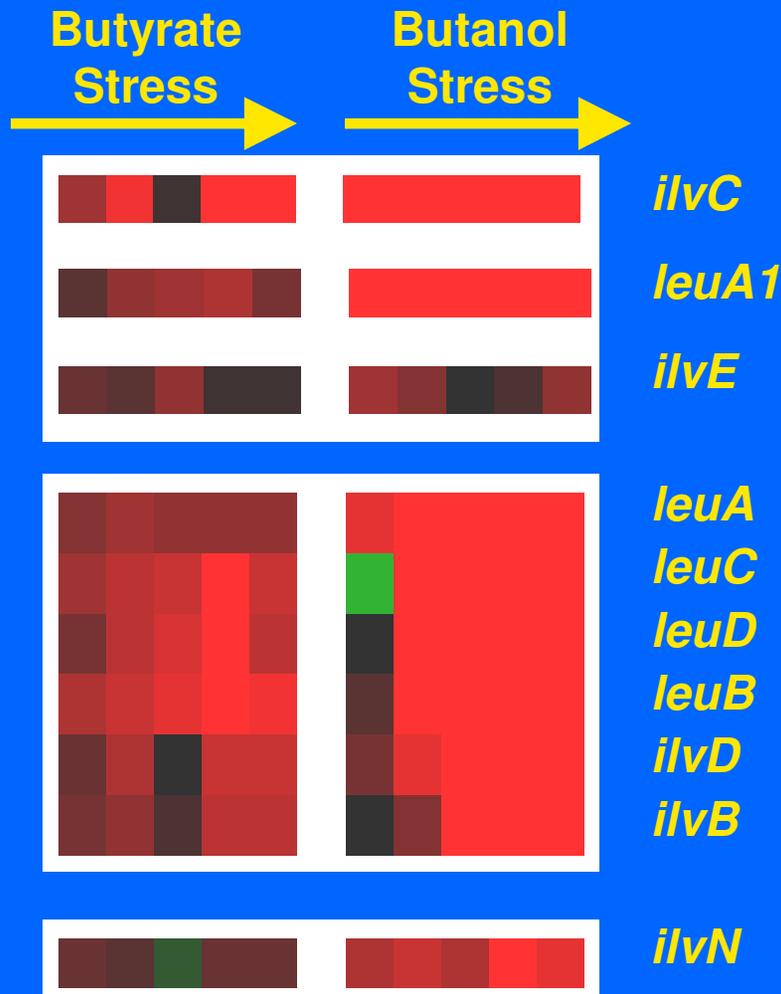
→ Amino acid transport & metabolism

→ Post-translational
modification, protein turnover,
and chaperones

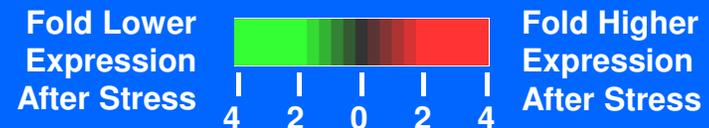
Common & Specific Stress Responses (based on Genome-scale μ -array analysis)



Differential Expression of Branched Chain Amino Acid Synthesis Genes



•Has this specific gene induction been seen in other microarray stress studies in other organisms?

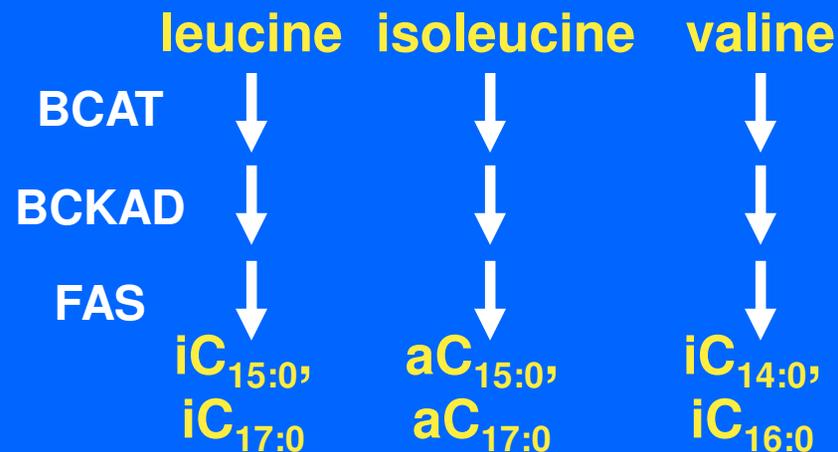


•Adaptation due to cold-shock of *B. subtilis* includes:

•decreased membrane fluidity

•downregulation of branched-chain amino acids (Kaan et al. *Microbiol.*, 2002)

•*Bacillus subtilis* can convert branched-chain amino acids into branched-chain fatty acids



•*C. acetobutylicum* increase membrane fluidity in response to butanol (Vollherst-Schneck et al. *J. Bacteriol.*, 1984)

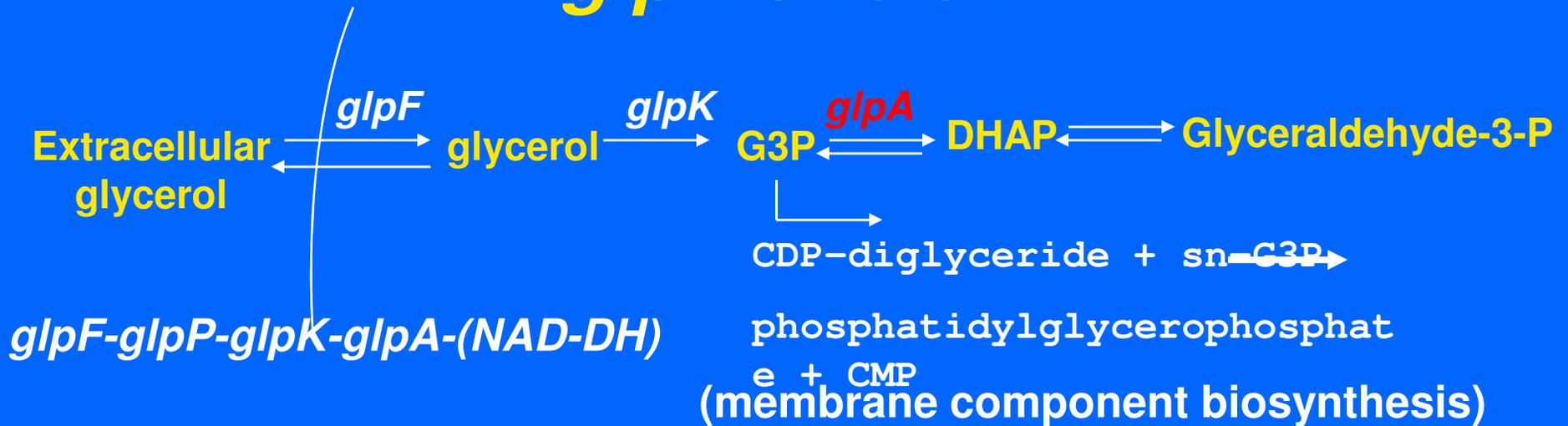
•Synthesis of branched-chain fatty acids in *C. acetobutylicum* may help cells adapt to metabolite stress

Genes Specifically Induced by Butanol Stress



- In *E. coli*, homolog *glpC* is associated with solvent tolerance (Shimizu, *AEM*, 2005), although cannot act alone...

glpA's role?



- **What is the common role of glycerol-3-phosphate dehydrogenase in solvent tolerance?**
 - First step in P-lipid biosynthesis as the need for an altered membrane composition develops (H. Goldfine et al.)
 - Yeast: *glp* genes play role in osmotolerance (Brisson, *BioEssays*, 2001)

Universally Upregulated Genes by Acetate, Butyrate, and Butanol

- Stress proteins

- Chaperones: *dnaKJ*, *groESL*, *clpC*, *hsp90*, *hsp18*
- Protease: *lonA*
- Benefits of *groESL* overexpression in enhancing solvent tolerance has been established by our group^{1,2}

- ***Establishing protein stability and functionality seems to be key in responding to toxic stress***

- ***THUS, stress response can (sometimes) predict stress tolerant phenotypes***

1. Tomas, CT, NE Welker, ET Papoutsakis. 2003. *Appl. Environ. Microbiol.* 69: 4951-4965.

2. Tomas, CT, J Beamish, ET Papoutsakis. 2004. *J. Bacteriol.* 186: 2006-2018.

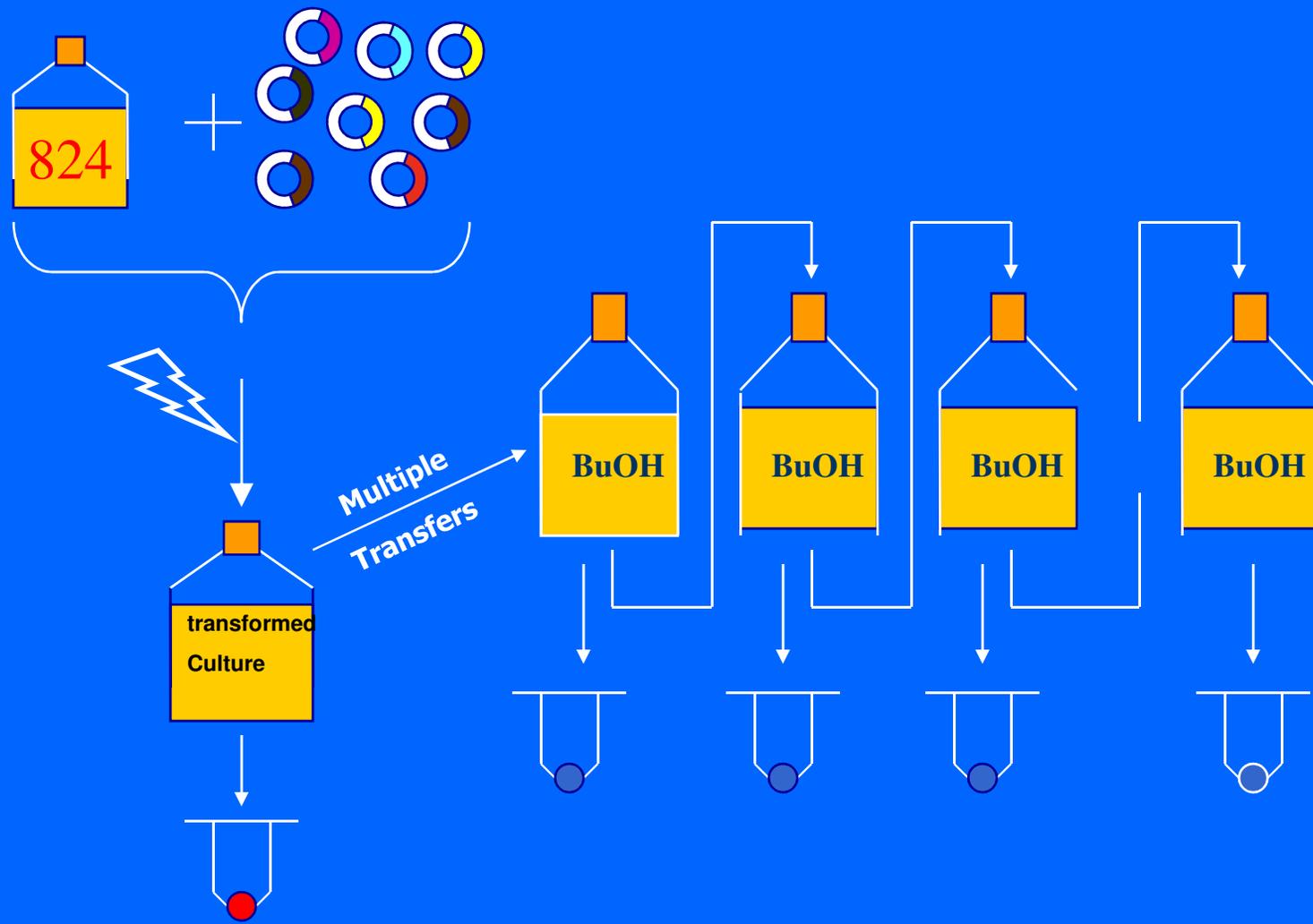
A lot of new information...

- But no obvious high-throughput way to test and use such information for achieving strong tolerant phenotypes...
- Need then for such (empirical for now) tools, that can be more broadly used for developing other complex phenotypes
 - **Genomic or expression libraries have been used extensively to identify genes or loci (or their variants) imparting a selectable phenotype, incl. tolerant ones**
 - **What is NOT known is how well and completely that works for either SINGLE loci/genes (let alone for INTERACTING MULTIPLE LOCI) and if these genes/loci are related to stress regulons**

Library approach

- Several types of libraries
- Have applied them for identifying tolerance to **butanol** and **butyrate** and have tested some of the identified genes

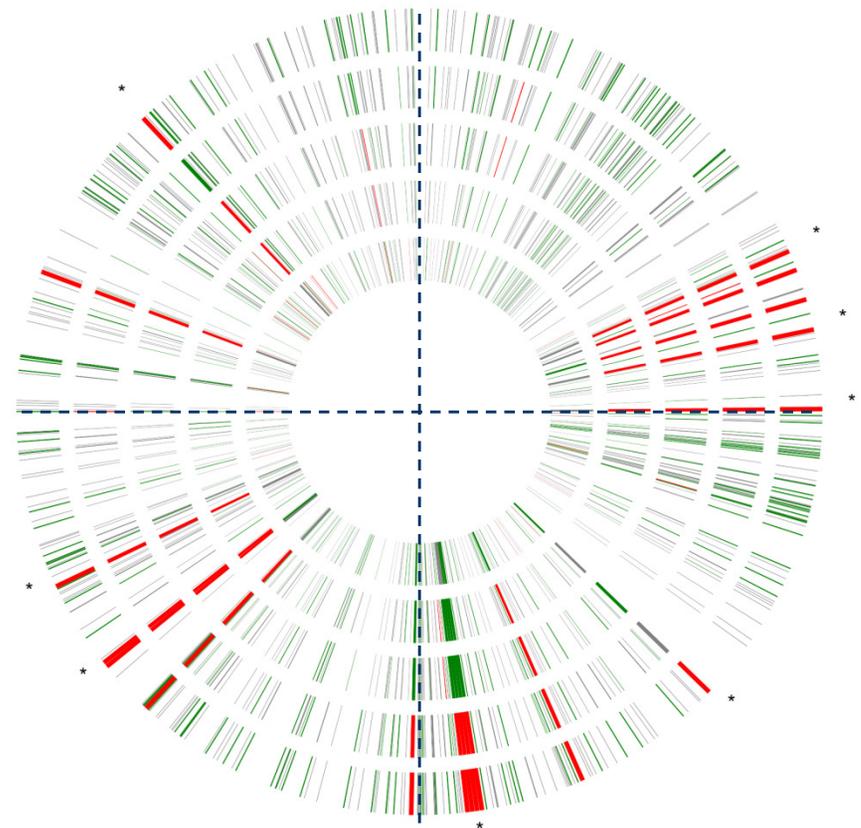
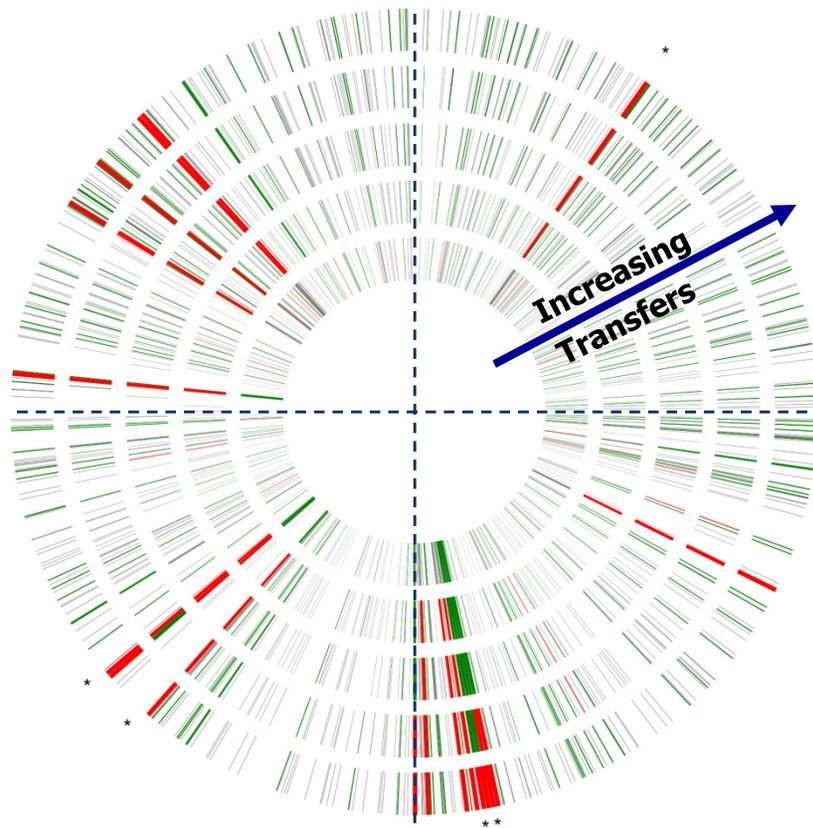
Library Selection



Assessment by high resolution HT (μ -array or deep sequencing analysis): stochasticity vs determinism

Biological Replicate 1

Biological Replicate 2



Genes color coded according to percentile rank in a given transfer: the top 5% are red, 5-33% are green, and 33-100% are gray

Several of the identified genes

- Impart solvent tolerance alone...
- But better as a group in a mixed population rather than in pure recombinant strains (extracellular molecules? Cell-to-cell communication?)
- Novel transcriptional regulators...

Other interesting findings

- Butyrate-tolerance studies lead consistently to enrichment of IR (non-coding) DNA of the rRNA locus. Regulon has been determined by μ -array analysis but mechanism not known yet: a srRNA or what?
- **Based on these identified DNAs, tolerance to butyrate imparts also tolerance to all tested carboxylic acids and leads to higher butanol formation (and tolerance?)**

To summarize, Library approach

- Useful for identifying many loci, but not all
 - Little overlap with BuOH stress regulon
 - Significantly, it missed chaperons (why?) and likely more tolerance genes...
 - Appears to identify more transcriptional regulators (solo players)
- Misses large, multigenic programs...
- It is not strictly deterministic, and depends on
 - Selection assay (select for growth only?)
 - Application mode of selection assay
 - Insert size and regulation of gene expression

Stress-regulon approach works best...

- When there is a dormant adaptive response such as in, e.g., clostridial adaptation to butanol and butyrate accumulation
- For identifying potential target programs and pathways

Current & future work in my lab: methods

- HT methods to capture and combine distant multi-locus effects and allogeneic traits (for tolerant and other phenotypes, ...)
- Coupled with “enhanced”, “temporarily-induced” recombination, ...
- to develop strains that quickly and simultaneously directly and efficiently ferment cellulose and xylans without enzymatic pre-treatment

...and exploration of the relationship between differentiation (sporulation) and tolerance?

- Mature or even immature spores are extremely resistance to chemicals, radiation, everything,...
- Can we use “Differentiation Engineering” to freeze “differentiation” stage at cellular type which is tolerant and a good producer?