Beyond transcriptomics. Genome-scale analysis of the adaptation of *Saccharomyces cerevisiae* to the winemaking environment
Some of what we know from transcriptomics (during AF).

Some limitations of transcriptomics.

The HIP-HOP strategy.

Our approach to HIP-HOP analysis of wine fermentation.

Results for the first step of alcoholic fermentation.

Directed evolution. A complementary approach.

The ubiquitin-proteasome pathway.
Transcription analysis of primary wine fermentation

L.F. Bisson group (2001)
• Switch to respiration in response to nitrogen starvation
• Expression of stress genes

B. Blondin group (2003)
• Response to anaerobiosis
• Response to nitrogen depletion (TOR mediated)
• Weak regulation of carbohydrate metabolism
• General stress response
• Osmotic stress response is transitory
• Nitrogen recycling (vacuolar and autophagic activities)

H.J.J. van Vuuren group (2008)
• Osmotic stress
• Attenuation of glucose repression
• Repression of genes related to cell growth and proliferation
• Nitrogen starvation
• Ethanol stress
Other transcriptomic approaches

Transcription analysis of particular winemaking conditions
• Rehydration
• Low temperature
• Second fermentation of sparkling wines

Comparative transcriptomics
• Strains showing different fermentation phenotypes
• Different nitrogen availability

Transcription analysis of the response to stress factors
• Ethanol stress
• Osmotic stress
• Low temperature
Some limitations of transcriptomic approaches

• Genes relevant for many biological processes are not subject to transcriptional regulation in response to environmental conditions that influence these processes (Birrell et al. 2002; PNAS).

• Not all genes showing a transcriptional change in response to a given culture condition are required for fitness under these conditions (Tai et al., 2007; Microbiology SGM).
Genome wide non-transcriptomic approaches (wine)

Proteomics

• Complementary information
• Usually no direct correlation with transcription data

Comparative genomics by hybridization
(aCGH or low coverage sequencing)

• Strains showing different fermentation phenotypes
• Wine vs. non-wine strains

Whole genome sequencing (new assembly)

• Horizontal transfer
• ¿New mobile elements?
HaploInsufficiency Profiling/HOmozygous Profiling
HIP/HOP

Construction of YKO *S. cerevisiae* collections

- **Uptag**
  - KanMX4
  - Deletion cassette
  - Barcoded deletion mutant

- **Downtag**
  - KanMX4
  - Original genomic sequence

**BY4743**
- Heterozygous strains
  - x6000

**BY4742**
- Homozygous strains
  - x4500

**BY4741**
- Heterozygous strains
  - x4500

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HaploInsuficiency Profiling/HOmozygous Profiling 
HIP/HOP

**HOP genes**

*Genes required for fitness or survival under the assayed conditions*  
(always non-essential genes)

**HIP genes**

*Genes whose products are the target of “toxicity” under the assayed conditions*  
(both essential and non-essential genes)
HaploInsufficiency Profiling/HOmozygous Profiling  
HIP/HOP

1. Pool tagged deletion strains

2. Grow deletion pool in condition of choice

3. Purify genomic DNA

4. PCR-amplify uptags and downtags
   - Uptag PCR
   - Downtag PCR

5. Hybridize PCR products to chip

6. Analyze data
   - Array intensity
     - Starting sample
     - Growth sample
   - Analyzed data
     - Growth rate
     - Deletion strain
Some considerations about HIP/HOP analysis of wine fermentation

• Environmental conditions experiment dramatic changes
• Low number of generations in similar conditions

Alternative approach
Continuous culture
Simulation of wine fermentation in continuous culture

<table>
<thead>
<tr>
<th>Culture time (h)</th>
<th>Corresponding batch phase</th>
<th>Chemostat steady state</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific growth rate (h⁻¹)</td>
<td>0.37 – 0.15</td>
<td>0.27</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Specific consumption / production rates (mmol g DW⁻¹ h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
</tr>
<tr>
<td>Fructose</td>
</tr>
<tr>
<td>Ethanol</td>
</tr>
<tr>
<td>Glycerol</td>
</tr>
<tr>
<td>Acetic acid</td>
</tr>
<tr>
<td>Succinic acid</td>
</tr>
<tr>
<td>Lactic acid</td>
</tr>
</tbody>
</table>
Simulation of first step of wine fermentation in continuous culture

• 10 generation times for homozygous competition (SM)
• 20 generation times for heterozygous competition (SM)
• Controls for 10 and 20 generation times in YPD

➢ 3 biological replicates for each of the above
HIP-HOP results for the first step of alcoholic fermentation

At least 150 heterozygous deleted strains showed deficient growth in synthetic must after 20 generations (>2-fold reduced fitness as compared to fitness in YPD)

At least 126 homozygous deleted strains showed deficient growth in synthetic must after 10 generations (>2-fold reduced fitness as compared to fitness in YPD)
Individual phenotypic characterization. Area under OD-time curve phenotypic index
Individual phenotypic characterization.
Growth rate phenotypic index
Individual phenotypic characterization. OD after arrest of alcoholic fermentation

Similar landscape as seen by comparing fermentation time-course
Relevant functions from HIP analysis

• Vacuolar functions, including autophagy
• Different functions in the “DNA-to-protein” pathway
  o mRNA processing and stability
  o Protein synthesis
  o Secretion (ER functions)

Relevant functions from HOP analysis

• Adenine and lysine biosynthesis
• Inositol biosynthesis
• Biosynthesis of phospholipids
Some additional genes to watch

From the HIP analysis

*SAM1* and *SAM2; URE2; DUR1,2; MAL12, OCA6; CDC19;* genes involved in Gap1p sorting; genes involved in chromatin remodeling and histone modification

From the HOP analysis

*NPR2, NPR3 and RTC1; CAR1 and CAN1; GPD1 y GPD2; UBR1; STB5; BCK1; BUL2; ADH3; AQR1;* genes coding for ribosomal proteins; genes involved in protein folding in the ER
Previous reports of HIP/HOP analysis of wine fermentation

Delneri et al. 2008

• Commercial grape must (100 g/L sugar) (among several other media)
• Chemostat
• Not supplemented with uridine. Aerobic.
• Single biological replicate, only HIP analysis
• No unstressed contrast
• Concluded all nutritional requirements were provided by must
Previous reports of HIP/HOP analysis of wine fermentation

Piggot et al. 2011

• Synthetic must (200 g/L sugar)
• Single biological replicate each (HIP and HOP analyses)
• Time-course
• YPD amplification of samples
• No unstressed contrast
• Autophagy and ubiquitin-proteasome functions required
• Proficient deleted strains also identified (ribosomal and peroxisomal functions)
• FUR4
Coincidences with previous studies

- 19 overlapping HIP genes
- 28 overlapping HOP genes
- 3 overlapping HIP genes
- Piggot (481 genes)
- Novo (150 genes)
- Delneri (210 genes)
- Piggot (300 genes)
- Novo (126 genes)
Apparent limitations of the HIP/HOP approach

- Limited to loss-of-function phenotypes
- Difficulty to estimate wine-related phenotypes in a BY4743 background

Complementary approaches

- Directed evolution of laboratory strains
- QTL mapping by high throughput methods
Directed evolution of laboratory strains

- **Haploid laboratory strain (BY4741)**
- Continuous culture in conditions emulating the first step of alcoholic fermentation
- Working volume 40-50 ml
- 150-250 generations (three biological replicates)
- Verification of the “evolved” phenotype
- Whole genome sequence analysis of the evolved strains
  - Alignment to consensus (medium coverage shotgun sequencing)
  - New assembly (high coverage shotgun sequencing)
  - aCGH
Phenotype of evolved strains. Batch culture

Growth rate in Synthetic must

<table>
<thead>
<tr>
<th>Strain</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHS BY 4741</td>
<td>0.1683</td>
</tr>
<tr>
<td>AV 8</td>
<td>0.2254</td>
</tr>
<tr>
<td>BV 19</td>
<td>0.2569</td>
</tr>
<tr>
<td>E18</td>
<td>0.2760</td>
</tr>
<tr>
<td>AV 16</td>
<td>0.2779</td>
</tr>
</tbody>
</table>

Fermentation kinetics in Synthetic must

Adaptation to first steps does not involve improved overall fermentation performance, rather the opposite.
### Phenotype of evolved strains. Continuous culture

<table>
<thead>
<tr>
<th>Cell count</th>
<th>OD 600</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>D=0.20 h⁻¹</strong></td>
<td></td>
</tr>
<tr>
<td>BY4741</td>
<td>4.5 x 10⁷ /ml</td>
</tr>
<tr>
<td>Av16</td>
<td>8.7 x 10⁷ /ml</td>
</tr>
<tr>
<td><strong>D=0.25 h⁻¹</strong></td>
<td></td>
</tr>
<tr>
<td>BY4741</td>
<td>0.28</td>
</tr>
<tr>
<td>Av16</td>
<td>0.74</td>
</tr>
</tbody>
</table>
Sequencing of evolved strains

- Illumina Solexa platform
- 100 cycles per run
- Average 40x coverage
- Alignment to S288C consensus genome
- SNP analysis almost complete
- Additional runs (up to 100x coverage) required for further genome sequence analysis (new assembly)
Summary of mutations already identified

- 50% mutations (coding or non-coding regions)
- SNPs in non-coding regions
- Nonsense mutations
- Missense mutations

Mutations requiring confirmation

- Small deletions
- Changes in copy-number
- Chromosomal rearrangements

<table>
<thead>
<tr>
<th>Strain</th>
<th>SNPs</th>
<th>50%</th>
</tr>
</thead>
<tbody>
<tr>
<td>E18</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>BV19</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>AV8</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>AV16*</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>
Ubiquitin-proteasome pathway mutants

<table>
<thead>
<tr>
<th>Gene</th>
<th>Strain</th>
<th>Mutation</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSP5</td>
<td>E18</td>
<td>Asn&gt;Lys</td>
<td>E3 Ubiquitin ligase</td>
</tr>
<tr>
<td></td>
<td>BV19</td>
<td>Glu&gt;Asp</td>
<td>E3 Ubiquitin ligase</td>
</tr>
<tr>
<td></td>
<td>AV16</td>
<td>Asn&gt;Thr</td>
<td>E3 Ubiquitin ligase</td>
</tr>
<tr>
<td>CDC4</td>
<td>E18</td>
<td>Ser&gt;Leu (50%)</td>
<td>Part of a complex with ubiquitin ligase activity on a CDK inhibitor</td>
</tr>
<tr>
<td>BRE5</td>
<td>E18</td>
<td>Glu&gt;STOP</td>
<td>Ubiquitin protease cofactor</td>
</tr>
<tr>
<td>UBC6</td>
<td>BV19</td>
<td>Small deletion*</td>
<td>Ubiquitin-conjugating enzyme</td>
</tr>
<tr>
<td>BUL1</td>
<td>AV8</td>
<td>Asp&gt;His</td>
<td>Ubiquitin-binding component of the Rsp5p E3-ubiquitin ligase complex</td>
</tr>
</tbody>
</table>
Upcoming

• Complete sequence analysis of evolved strains
• High throughput QTL analysis of wine/laboratory yeast crosses
• Analysis of the adaptation to further fermentation steps
• Analysis of the adaptation to isolated wine related stress factors or growth conditions
  o Ethanol
  o SO₂
  o Extreme temperatures
  o Osmotic stress
  o Microoxygenation
  o Acetaldehyde
  o ...

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www.icvv.es/winehiphop