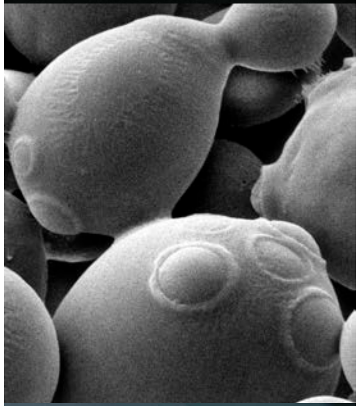
INTACT – EIB.10.008

INTegral engineering of ACetic acid Tolerance in yeast



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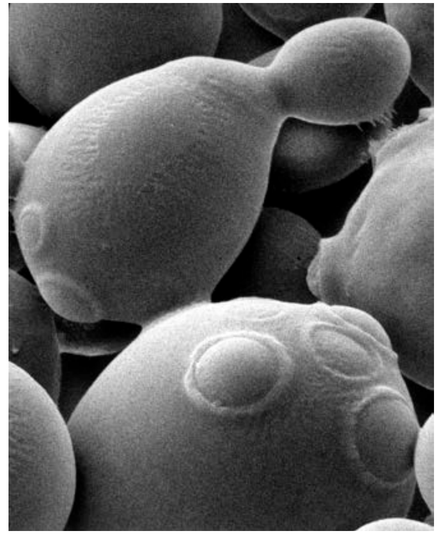
Delft, the Netherlands

Warsaw, February 26, 2014

... the Team:

Isabel Sa-Correia
Nuno Mira
Margarida Palma
Joana Guerreiro
& students

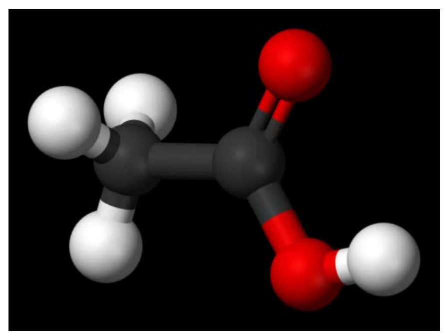


UAB

Joaquin Arino
Boris Rodriguez

JACOBS UNIVERSITY

Elke Nevoigt
Steve Swinnen
& students

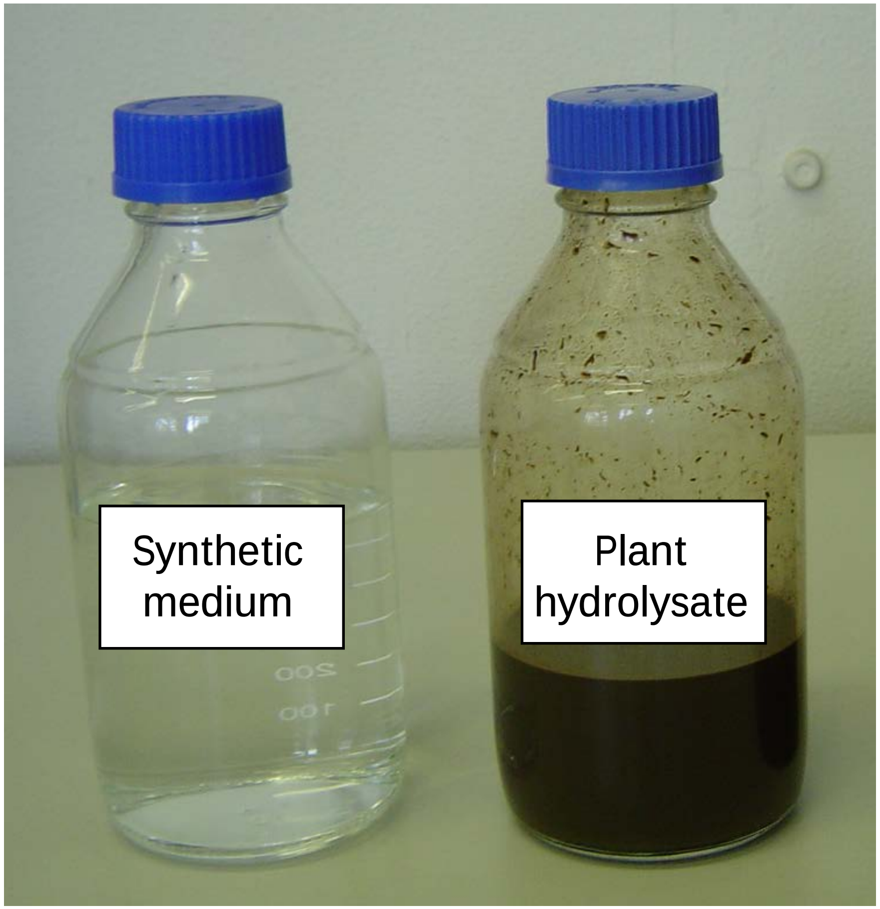


TUDelft

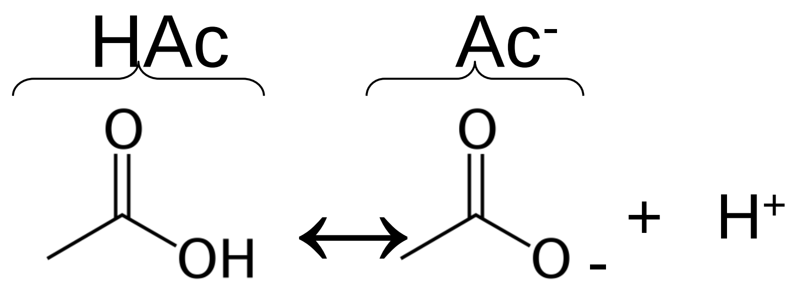
Ton van Maris
Dani Gonzalez Ramos
Erik de Hulster
Bianca e.d. Bianca (Bra)
& students

Desired feedstocks for Industrial Biotechnology

Acetic acid

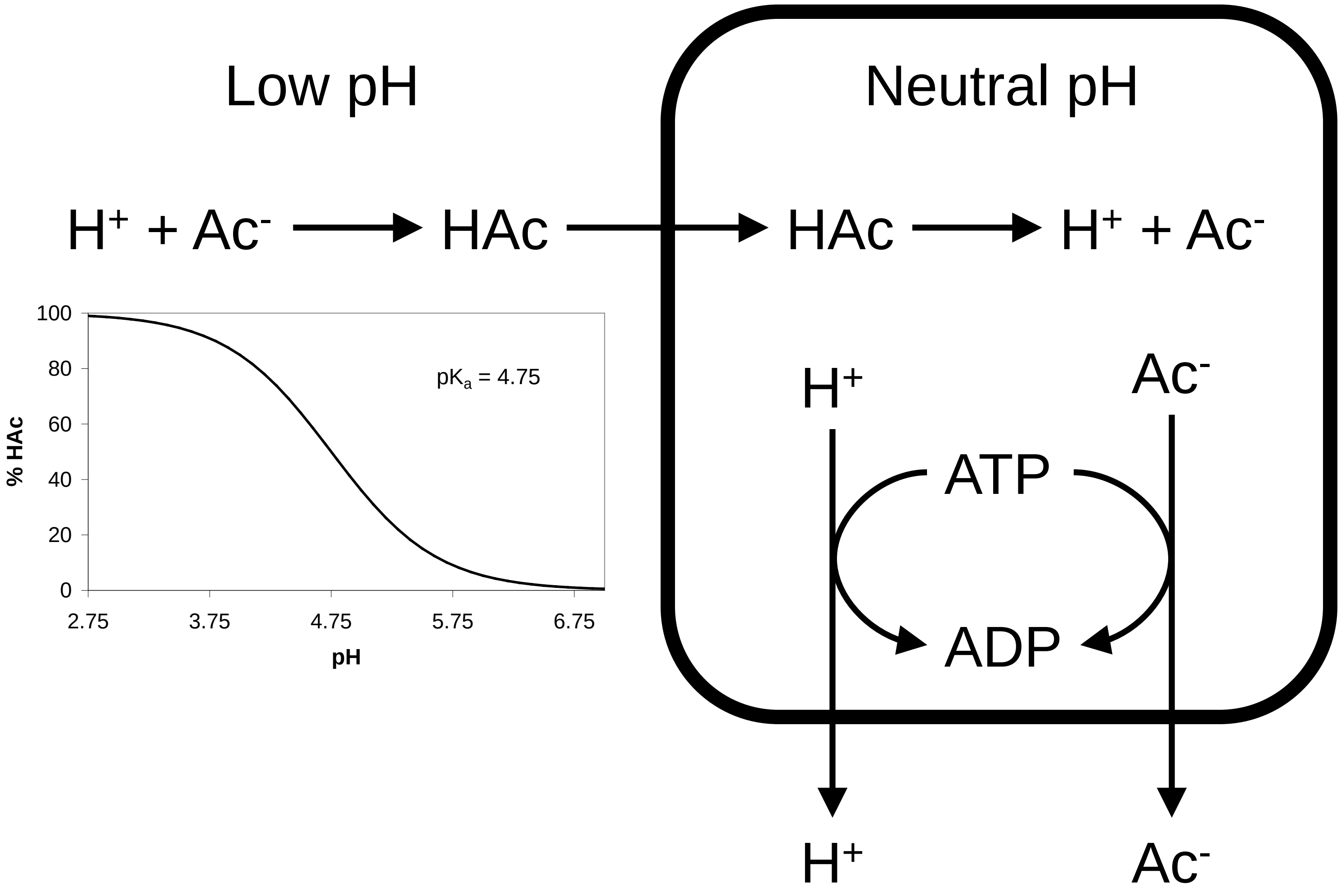


Structural component of lignocellulosic biomass



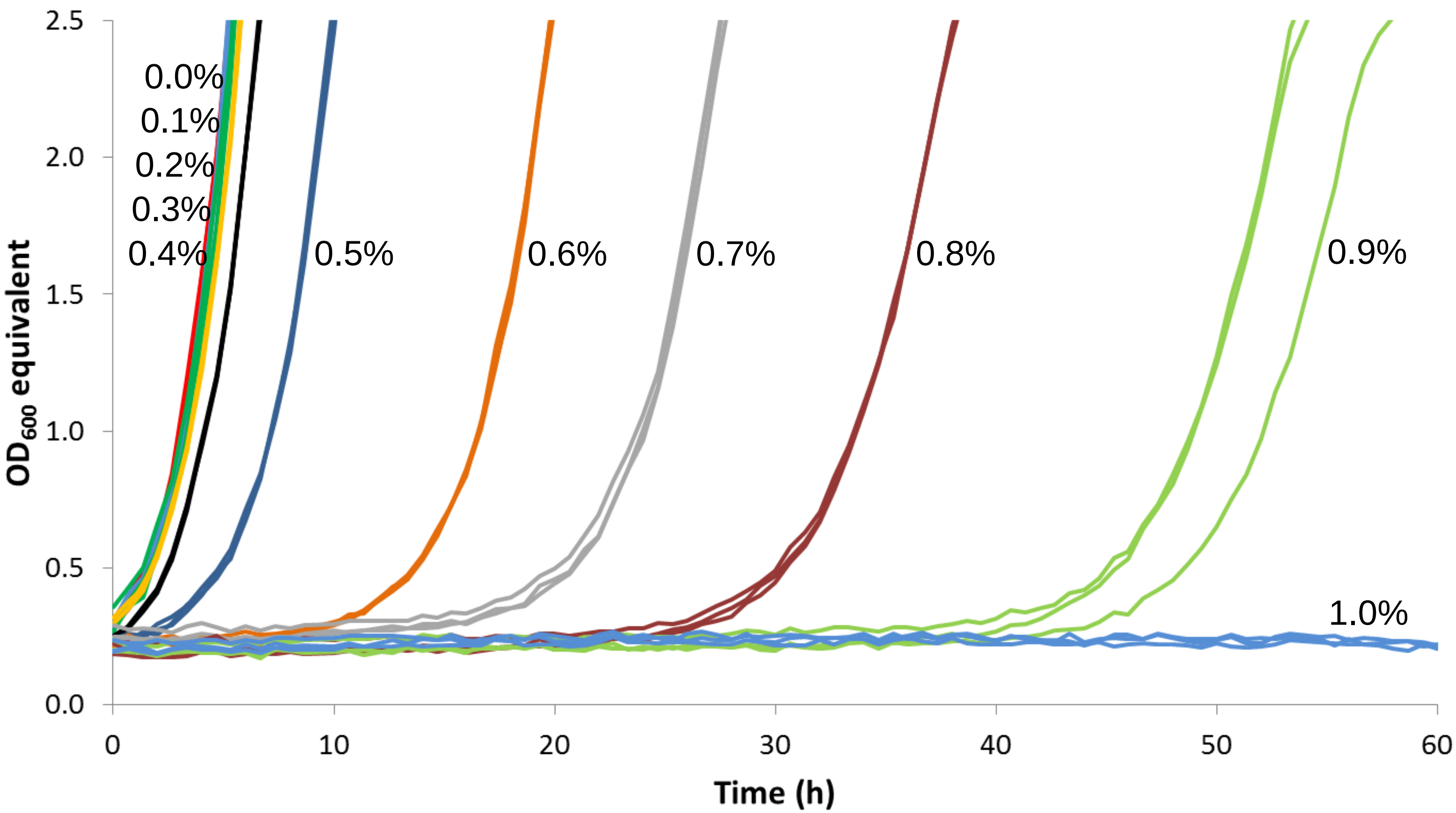
Weak organic acid, pKa = 4.75

Main mode of toxicity



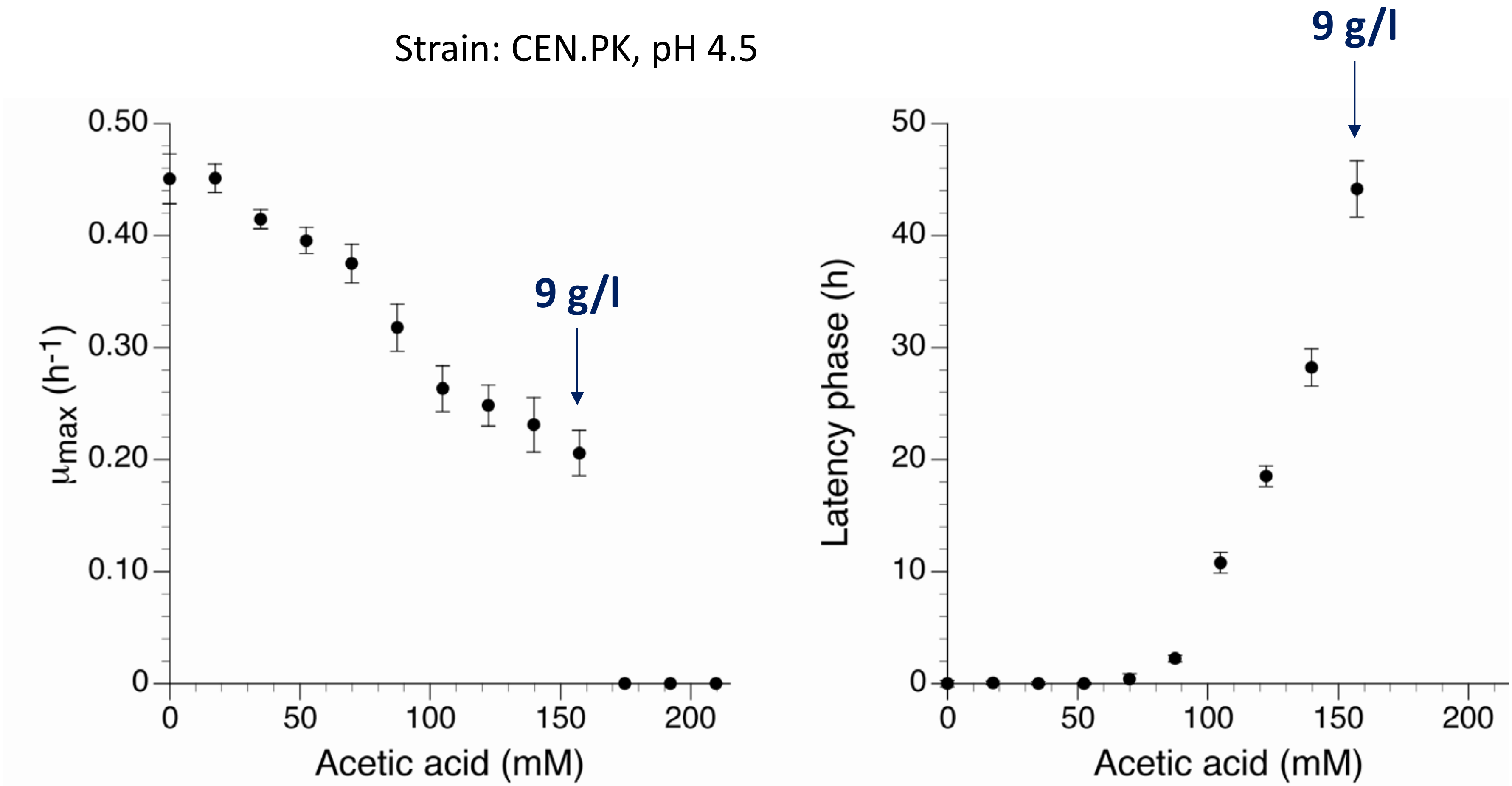
Growth of lab strain (CEN.PK) at various concentrations

pH 4.5 defined media



Exposure of exponentially growing cells to acetic acid decreases specific growth rate (μ_{max}) and lag (latency) phase

Strain: CEN.PK, pH 4.5



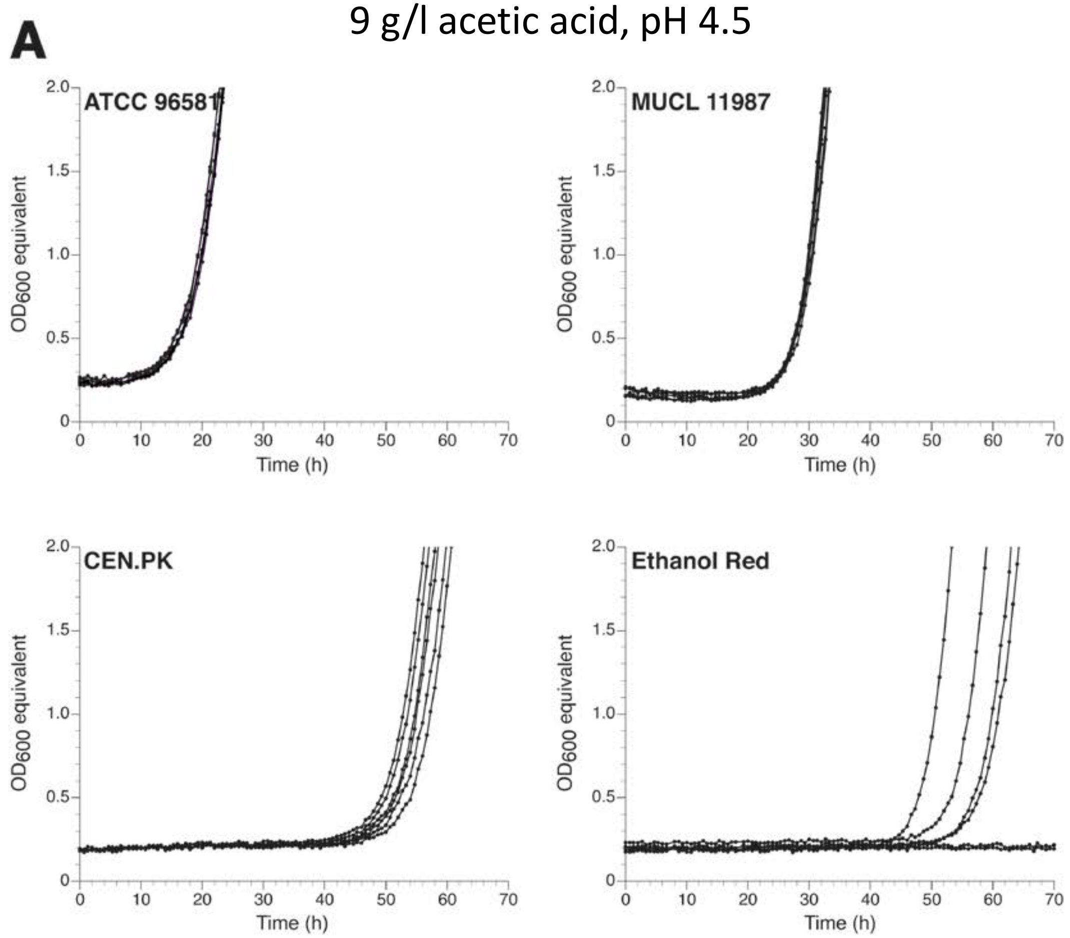
Swinnen *et al.*, submitted

Consortium Aim

- Understand and rationally improve acetic acid tolerance of *S. cerevisiae*, through integrating:
  - Identification of tolerant natural isolates
  - Genetic mapping and comparative genomics
  - Transcription factor engineering
  - Evolutionary engineering
  - Physiological analysis including ion homeostasis
  - (Reverse) metabolic engineering

S. cerevisiae strains strongly differ in acetic acid tolerance (particularly lag phase)

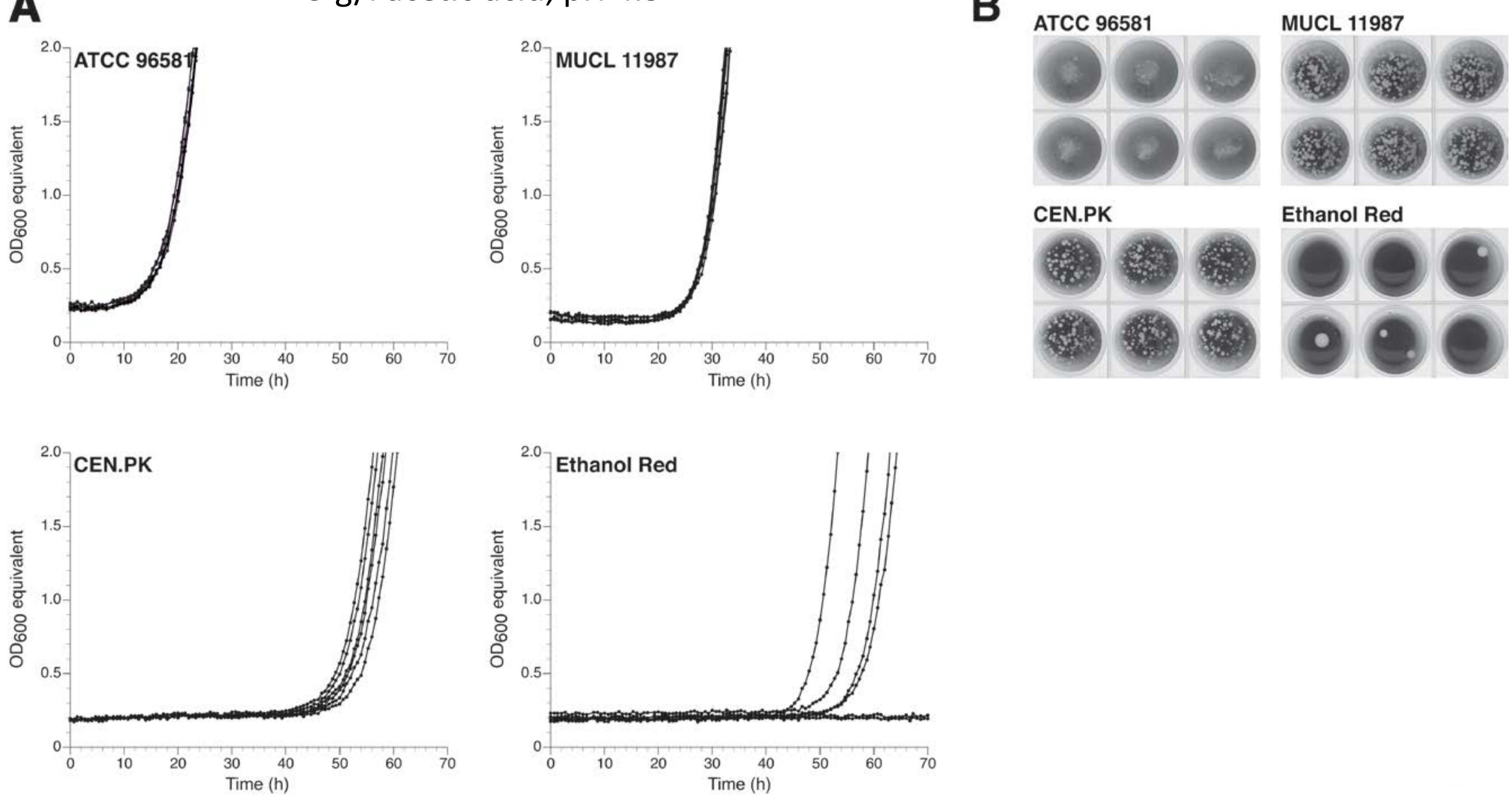
9 g/l acetic acid, pH 4.5



Swinnen *et al.*, submitted

S. cerevisiae strains strongly differ in acetic acid tolerance (particularly lag phase)

9 g/l acetic acid, pH 4.5



Swinnen *et al.*, submitted

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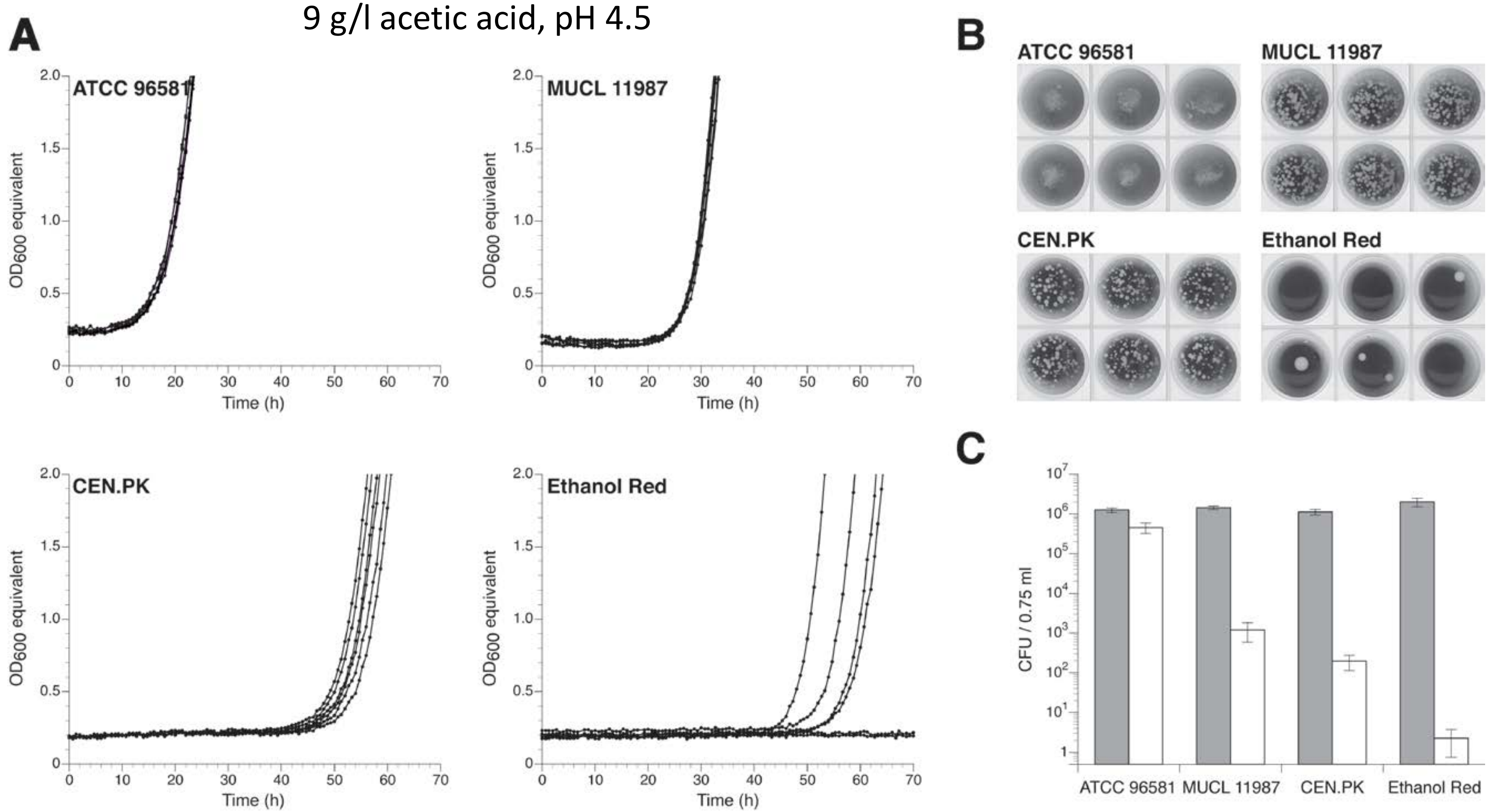
S. cerevisiae strains strongly differ in acetic acid tolerance (particularly lag phase)

9 g/l acetic acid, pH 4.5

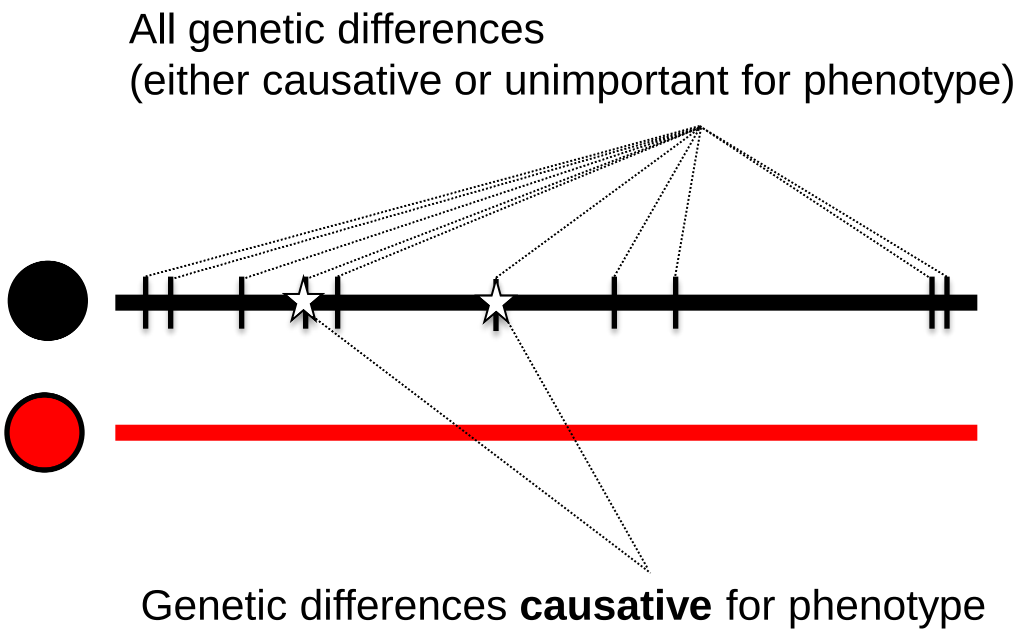


Swinnen *et al.*, submitted

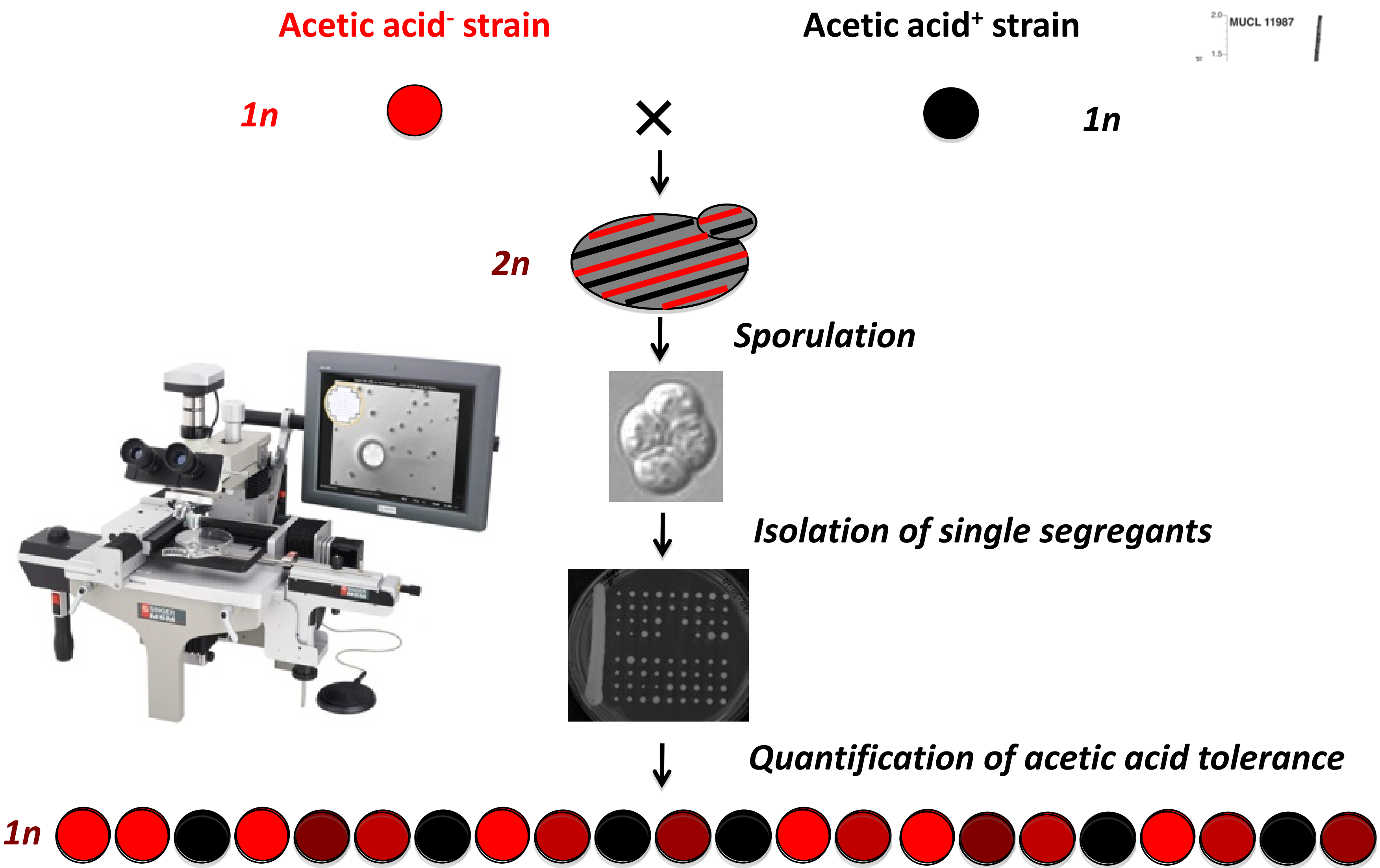
The major challenge in reverse engineering: How to identify the causative genetic differences?

Strain with desirable phenotypic trait

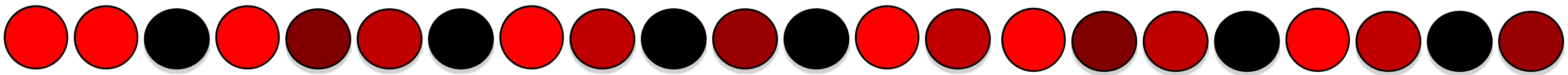
Reference strain without the desirable trait



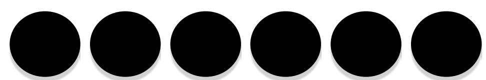
Acetic acid tolerance is a quantitative trait



Identification of the crucial genetic determinants

1n 

Select only segregants with acetic acid⁺ phenotype



Pooled segregant whole genome analysis

Significant genetic association?

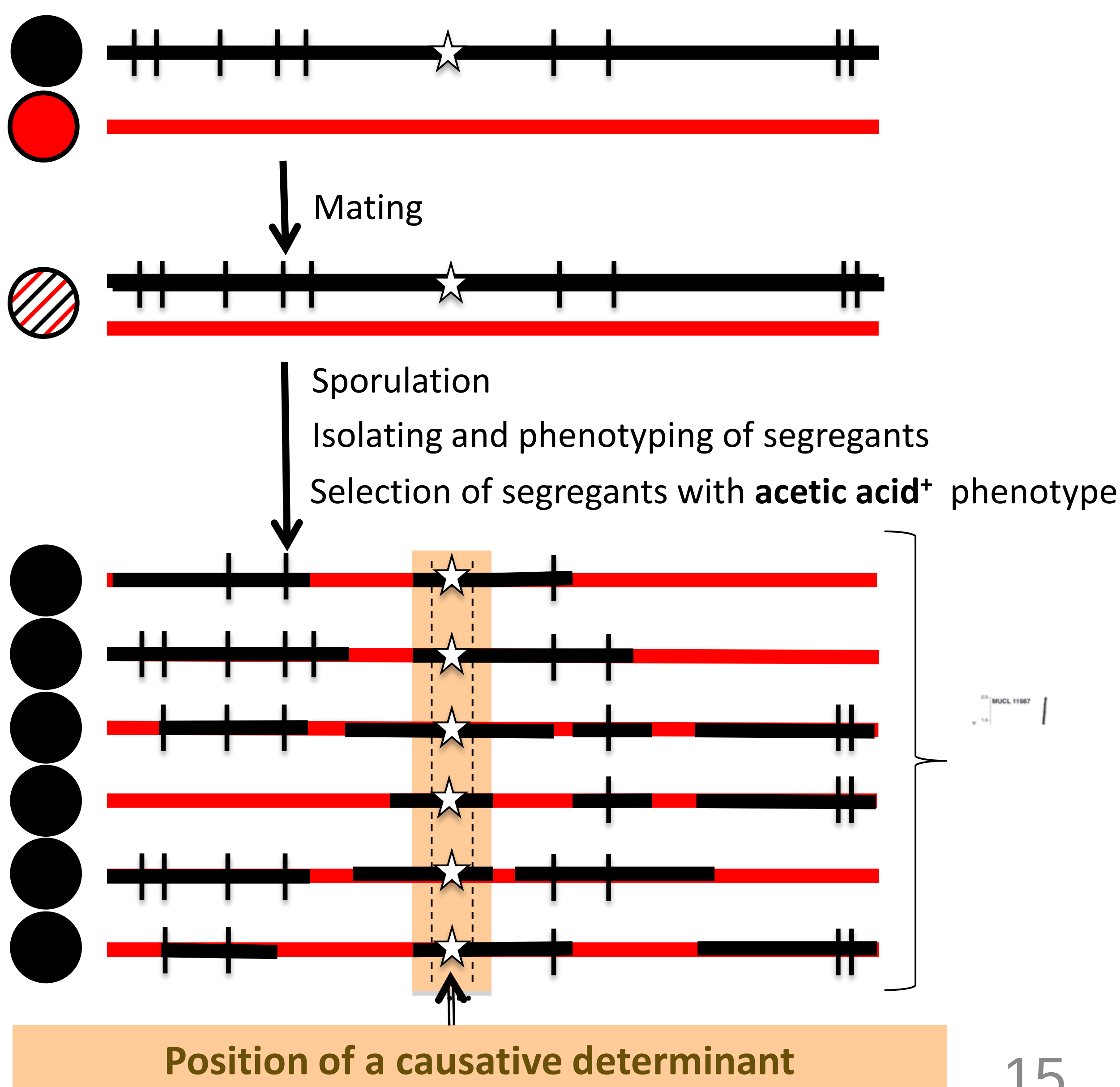
Genome-wide genetic association analysis

Acetic acid^{+} strain

Acetic acid^{-} strain

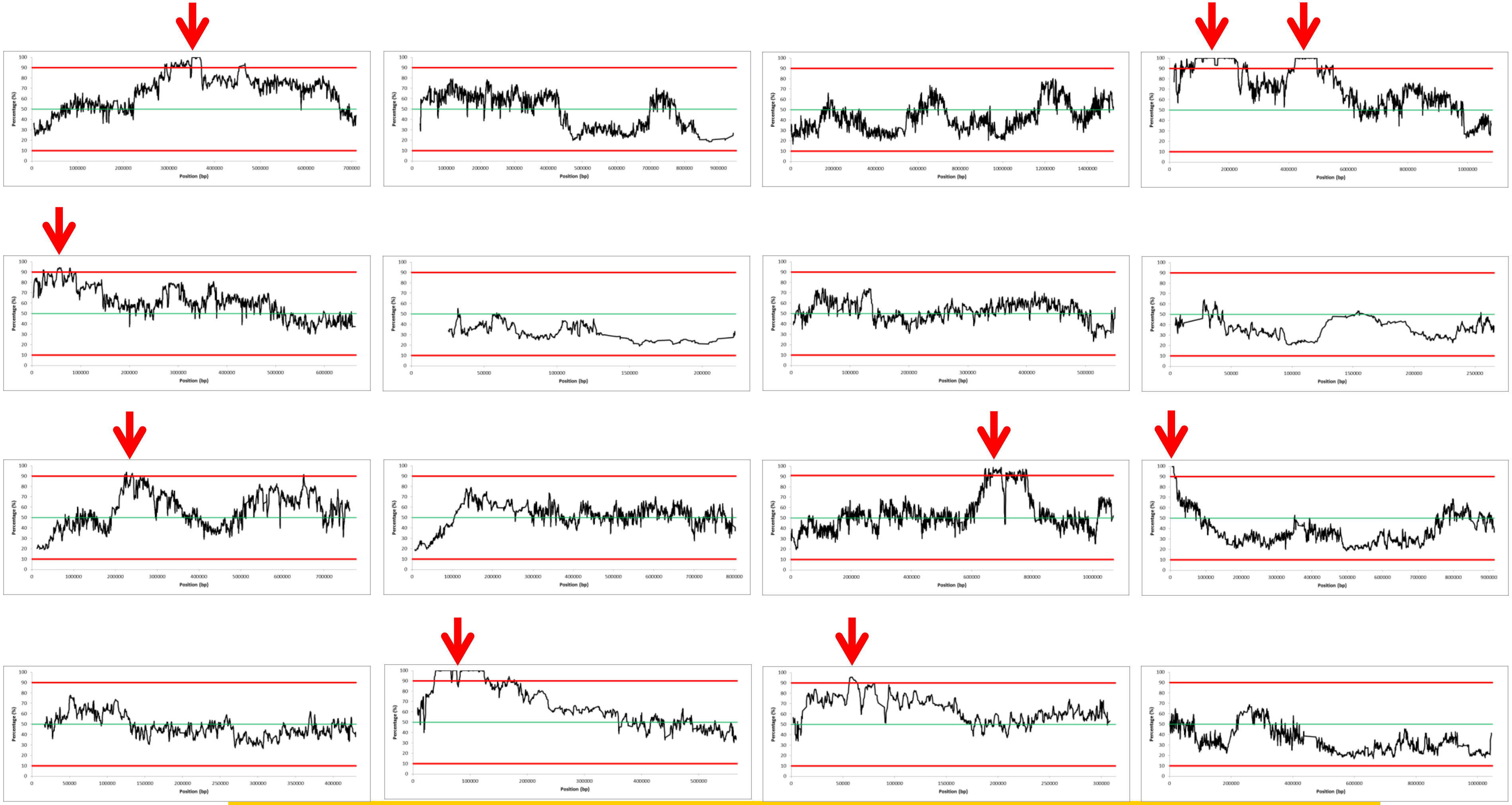
Diploid hybrid strain

Segregants with acetic acid^{+} phentotype



Method reviewed by Swinnen et al. (2012) *FEMS Yeast Res.*

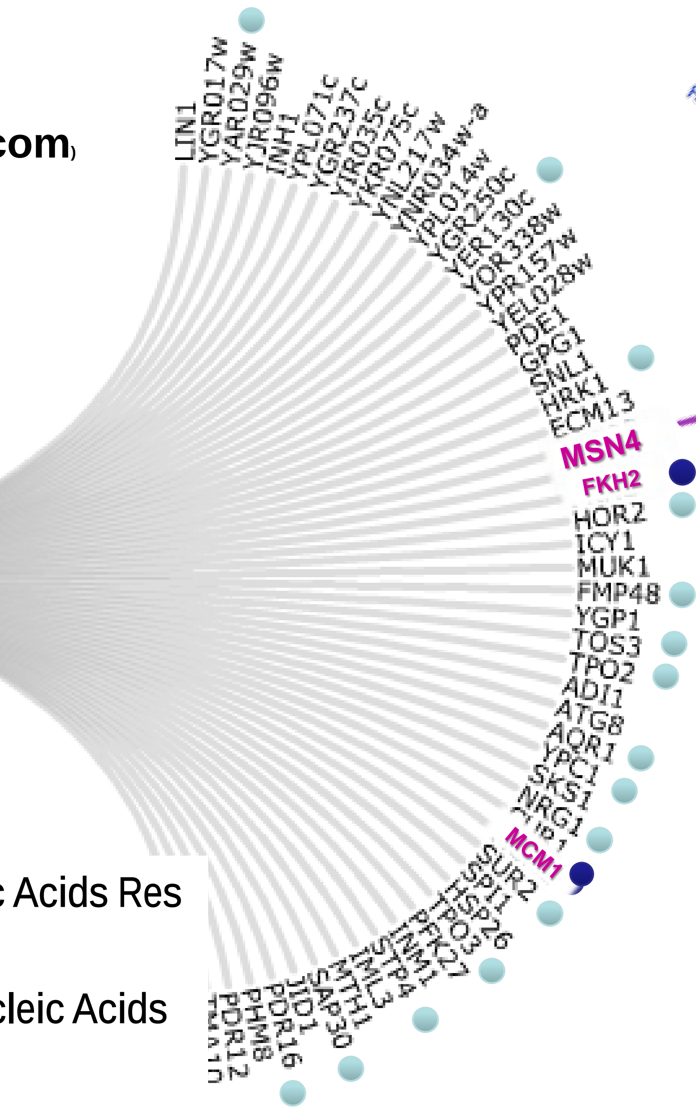
Genome-wide genetic association mapping of the two strains with different acetic acid tolerance

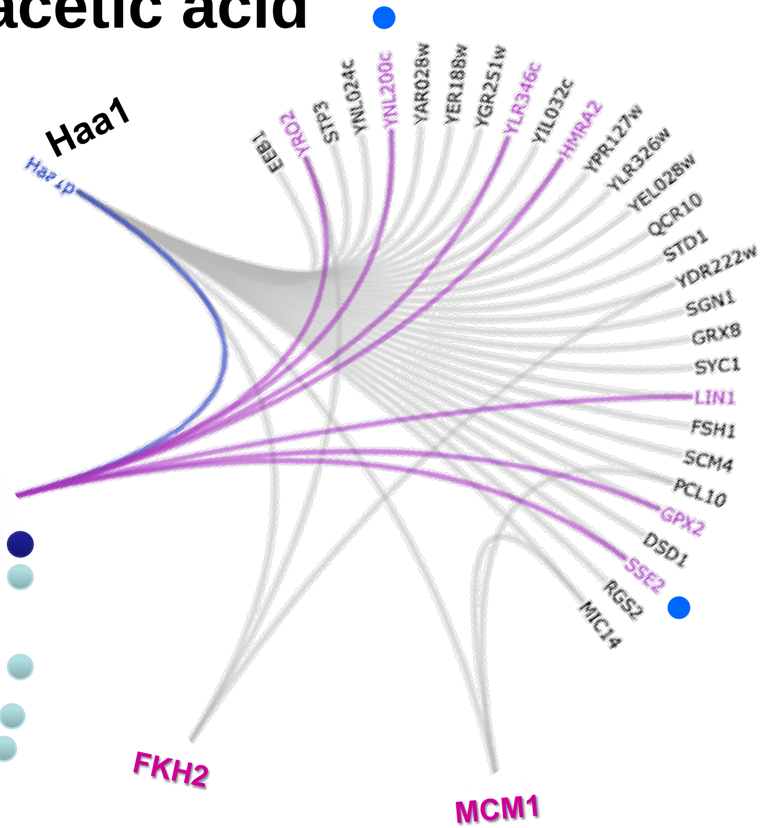


→ Analysis of determinant regions ongoing

Role of Haa1 and the Haa1-regulon in yeast response and resistance to acetic acid

(www.yeastract.com)



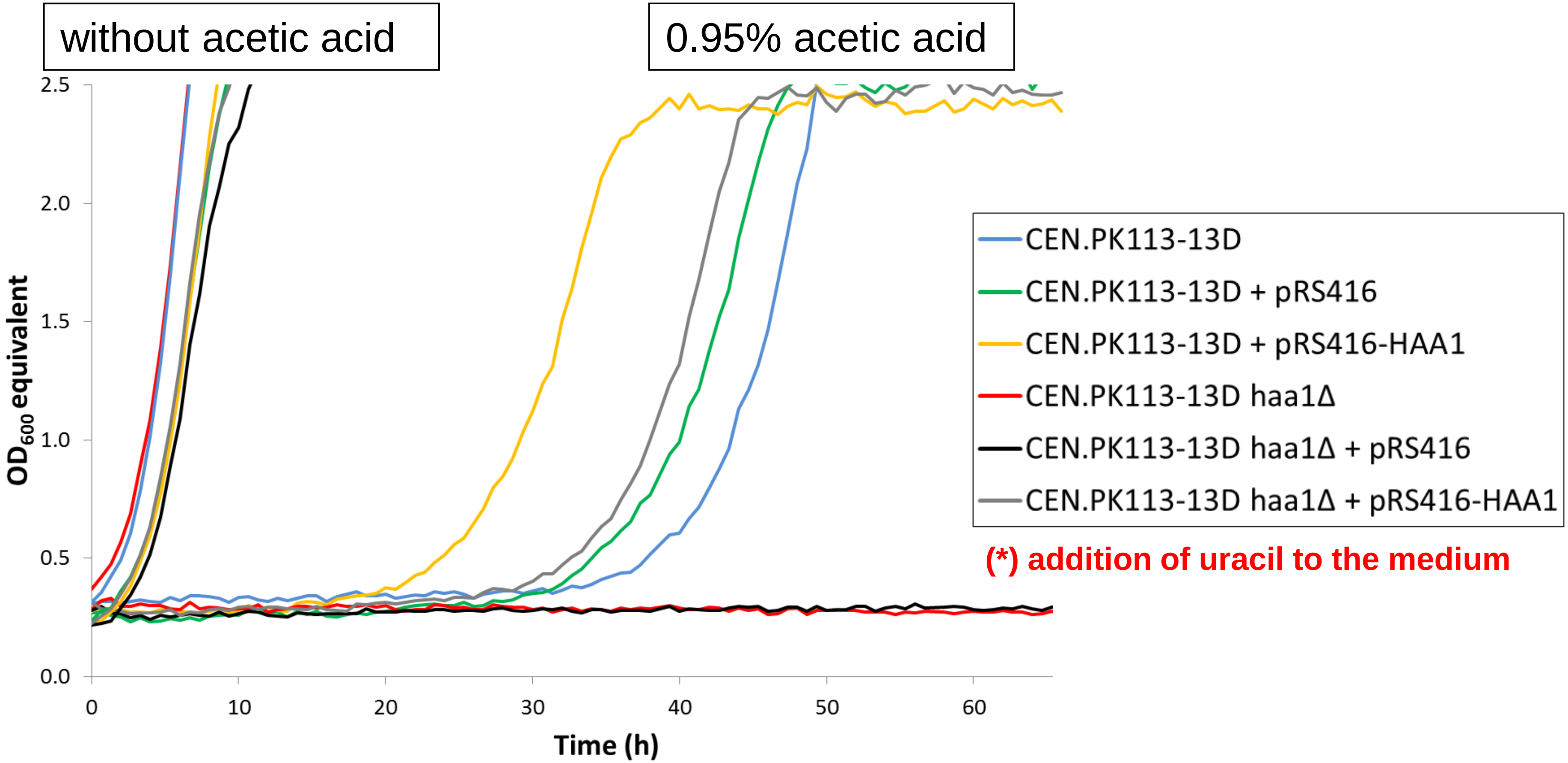


Mira *et al.* (2011) Nucleic Acids Res 39(16): 6896-907

Teixeira *et al.* (2014) Nucleic Acids Res 42(1): D161-6

- Acetic acid-resistance genes indirectly regulated by Haa1
- 16 acetic acid-resistance genes directly regulated by Haa1
- TFs regulated by Haa1

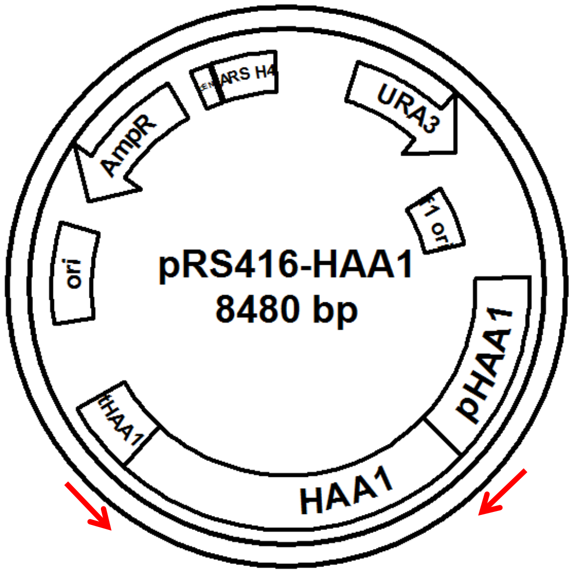
Transcription Factor Engineering



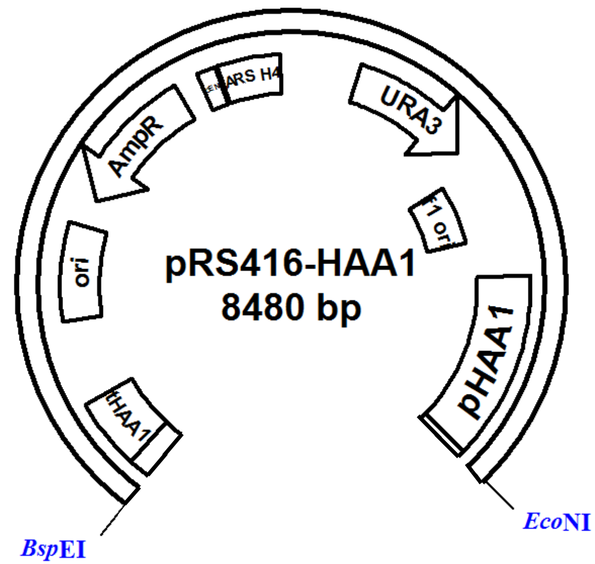
Screening for growth on Synthetic medium with 0.95% acetic acid (pH 4.5)

Transcription factor engineering

1. Error prone PCR



2. Restriction of pRS416-*HAA1*



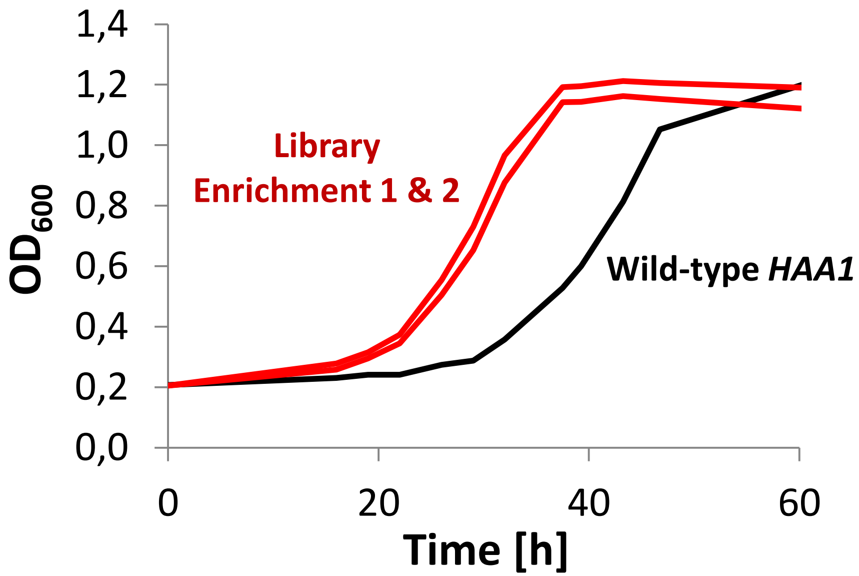
Homologous recombination in CEN.PK113-13D and CEN.PK113-13D *haa1*Δ

Selection of plasmid-containing transformants

Screening of library for acetic acid tolerance

Transcription factor engineering

1. A mutant *HAA1* library has been enriched in acetic acid containing medium



2. Several transformants expressing a mutated *HAA1* gene showed an improved acetic acid tolerance (in terms of the duration of the latency phase) as compared to the strain expressing the wild type *HAA1* gene

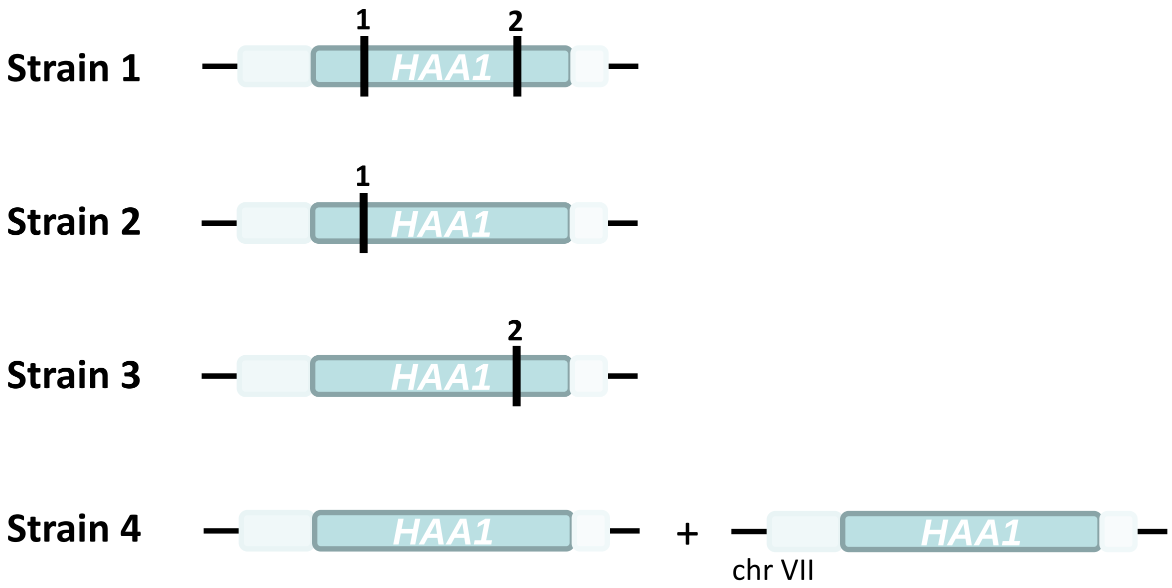
3. Focus on the *HAA1* allele with the lowest number of mutations

- **<u>Introduction of the mutations in the genome of CEN.PK113-7D:</u>**

Purpose → Eliminate any possible effects of the plasmid and auxotrophic background

→ Determine the individual effect of each mutation

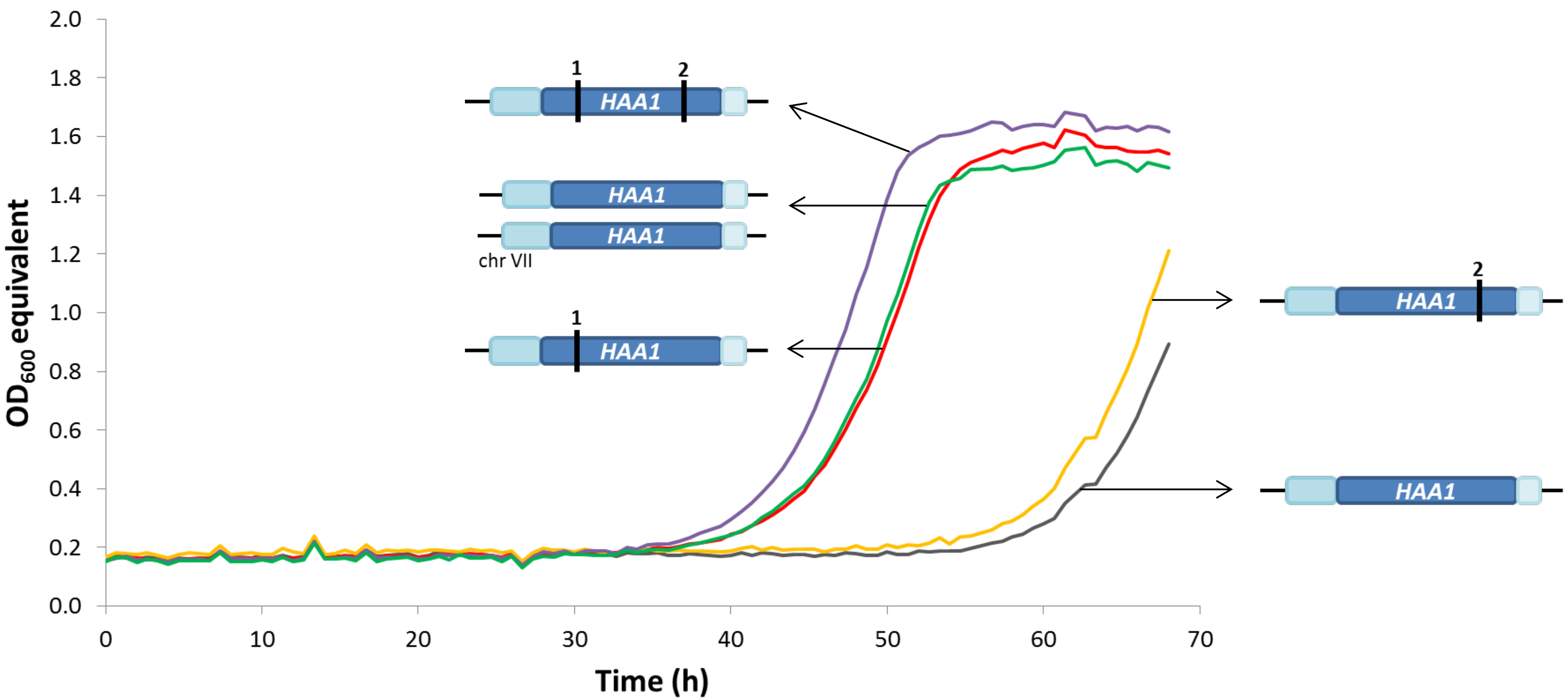
Strains



- **Introduction of the mutations in the genome of CEN.PK113-7D:**

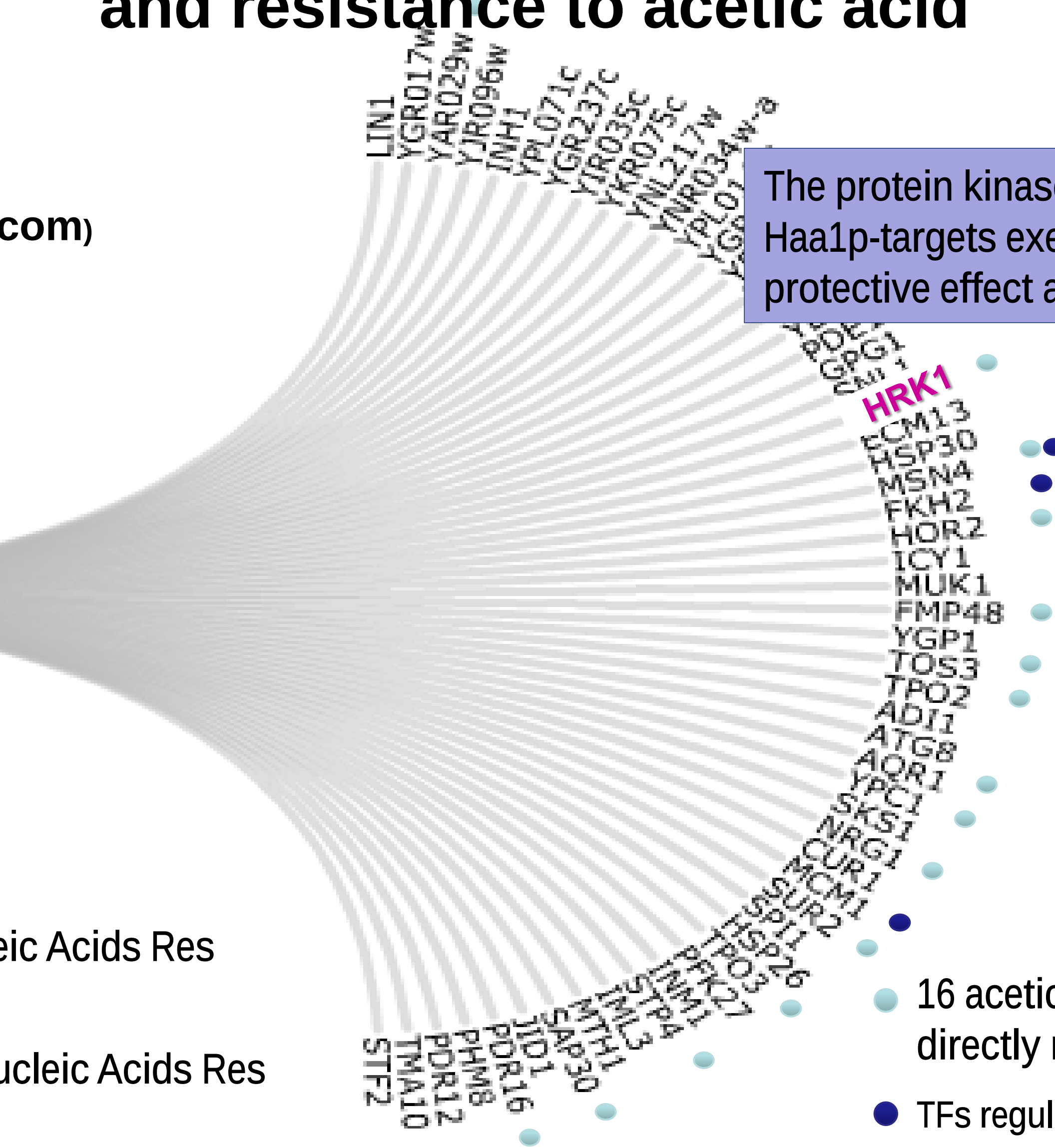
Screening of the mutant strains for acetic acid tolerance

→ 160 mM – pH 4.5



Role of Haa1 and the Haa1-regulon in yeast response and resistance to acetic acid

(www.yeastract.com)



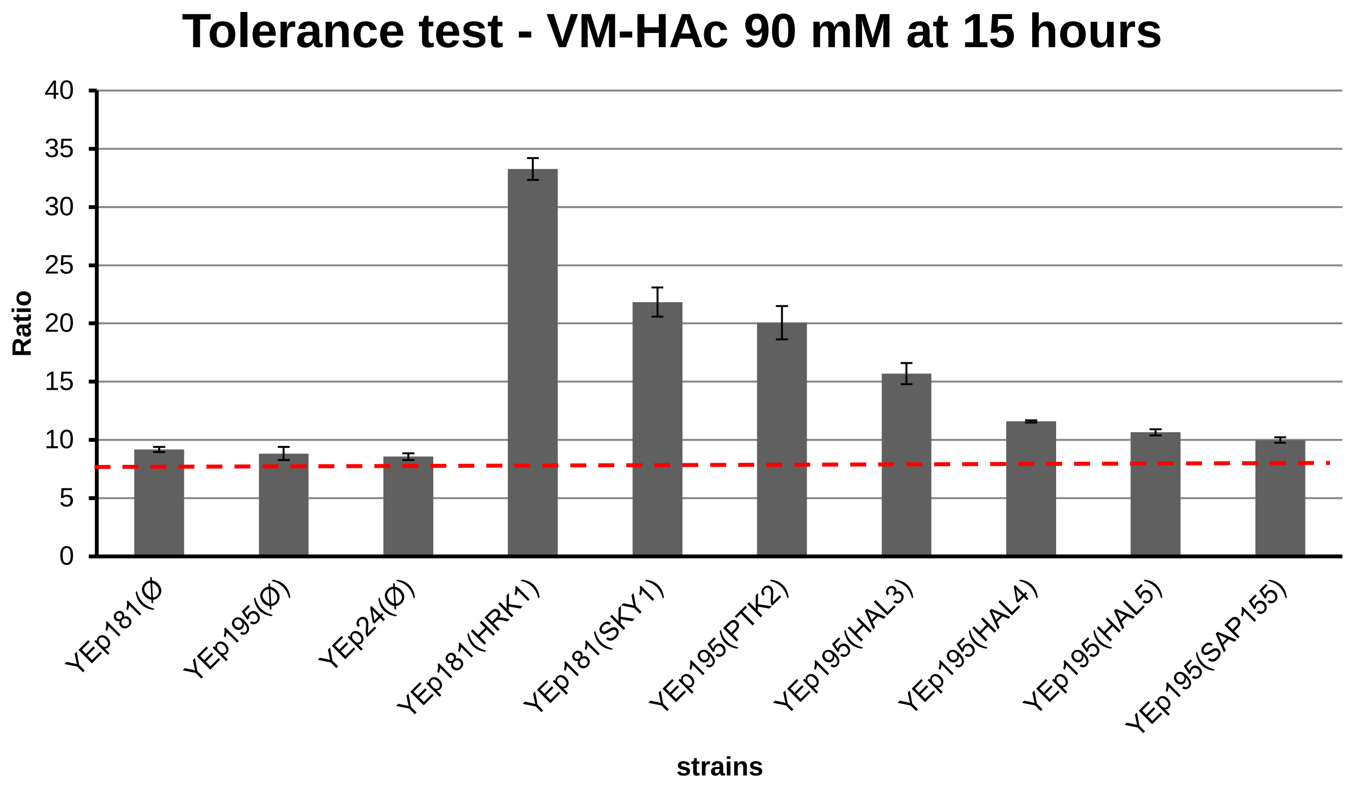
The protein kinase Hrk1 is among the Haa1p-targets exerting the strongest protective effect against acetic acid

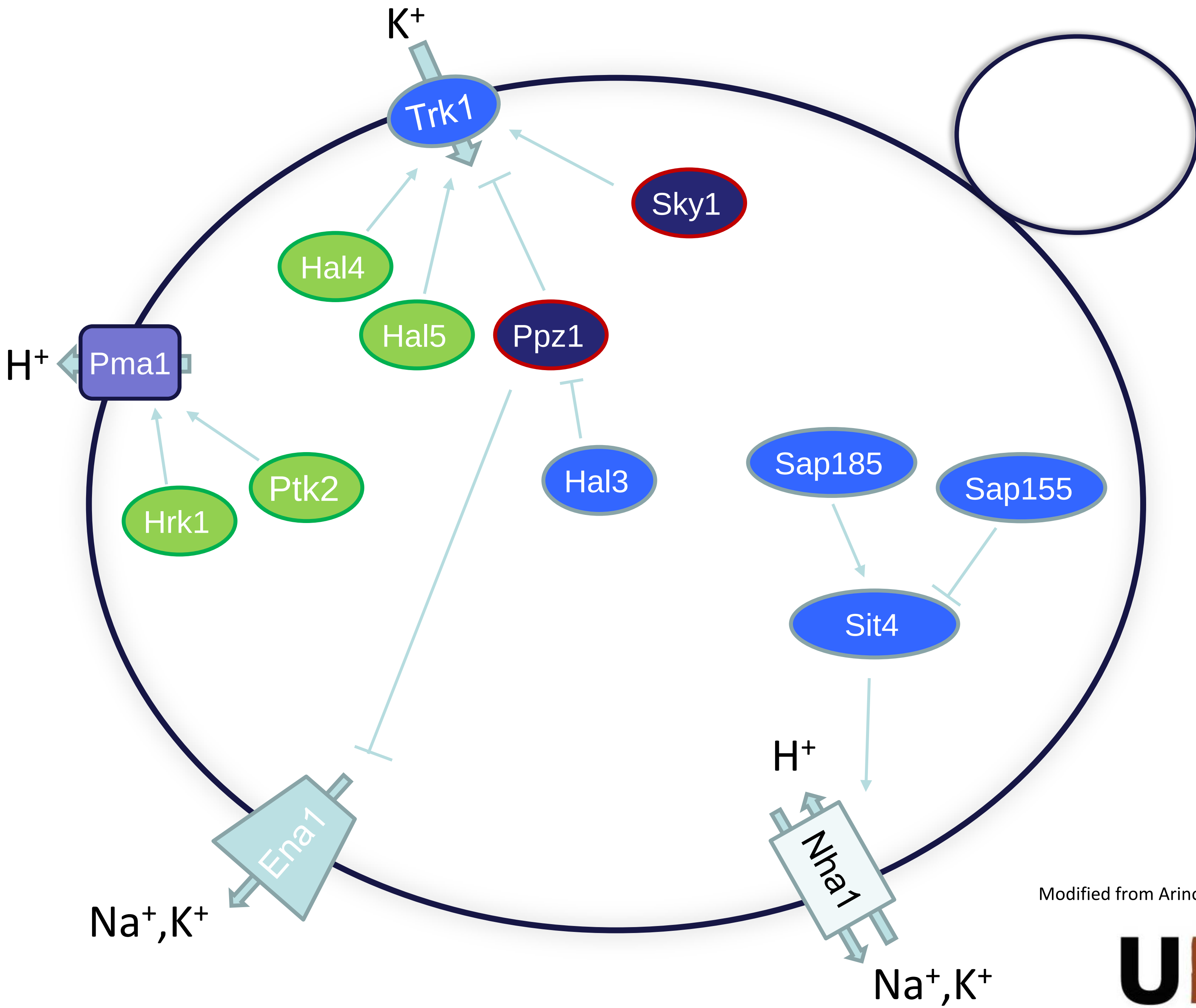
16 acetic acid-resistance genes directly regulated by Haa1

TFs regulated by Haa1

Mira *et al.* (2011) Nucleic Acids Res 39(16): 6896-907

Teixeira *et al.* (2014) Nucleic Acids Res 42(1): D161-6


Tolerance test - VM-HAc 90 mM at 15 hours
Ratio
40
35
30
25
20
15
10
5
0
YEp181(Ø
YEp195(Ø)
YEp24(Ø)
YEp181(HRK1)
YEp181(SKY1)
YEp195(PTK2)
YEp195(HAL3)
YEp195(HAL4)
YEp195(HAL5)
YEp195(SAP155)
strains

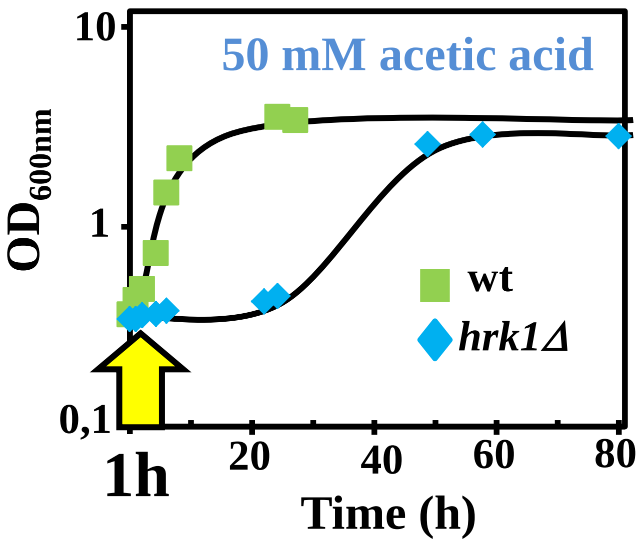


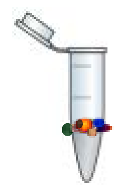
Modified from Arino et al. 2010

UAB

The role of the Haa1p regulon in yeast response and resistance to acetic acid stress

Proteome-wide yeast response to acetic acid stress: role of Hrk1





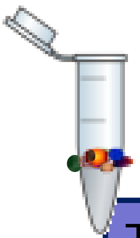
Proteome extraction

-Hrk1p is the Haa1p-target exerting the strongest protective effect against acetic acid

-Hrk1p belongs to a family of kinases involved in the regulation of plasma membrane transporters

The phosphoproteome of a membrane-enriched fraction obtained from WT and *hrk1Δ* cells cultivated in the presence of acetic acid was compared (iTRAQ)

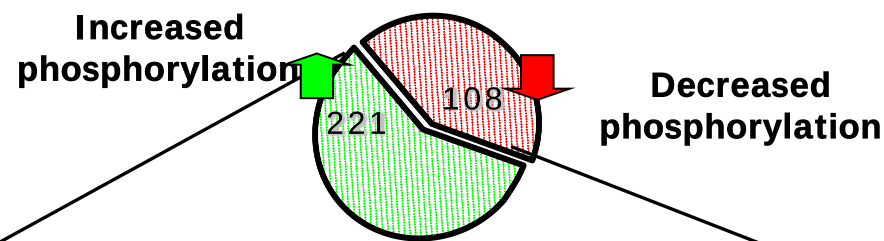
Proteome profiling of acetic acid stressed yeast strains – phosphoproteomic analysis to elucidate Hrk1 biological activity



The phosphoproteomes of acetic acid stressed and unstressed parental strain *S. cerevisiae BY4741* and *hrk1*Δ cells were compared using iTRAQ

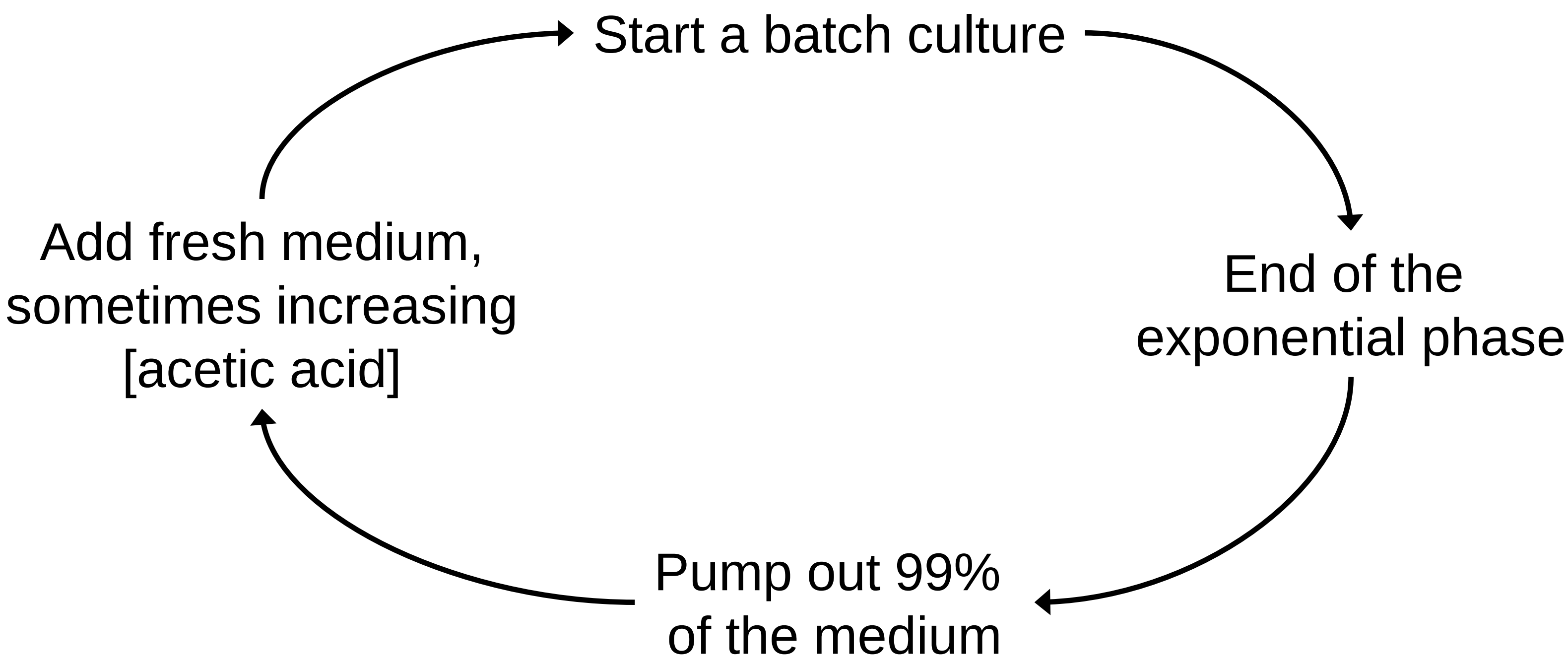
- Phosphate metabolism
- Translation
- Cellular transport
- Stress response
- (…)

Acetic acid-responsive proteins

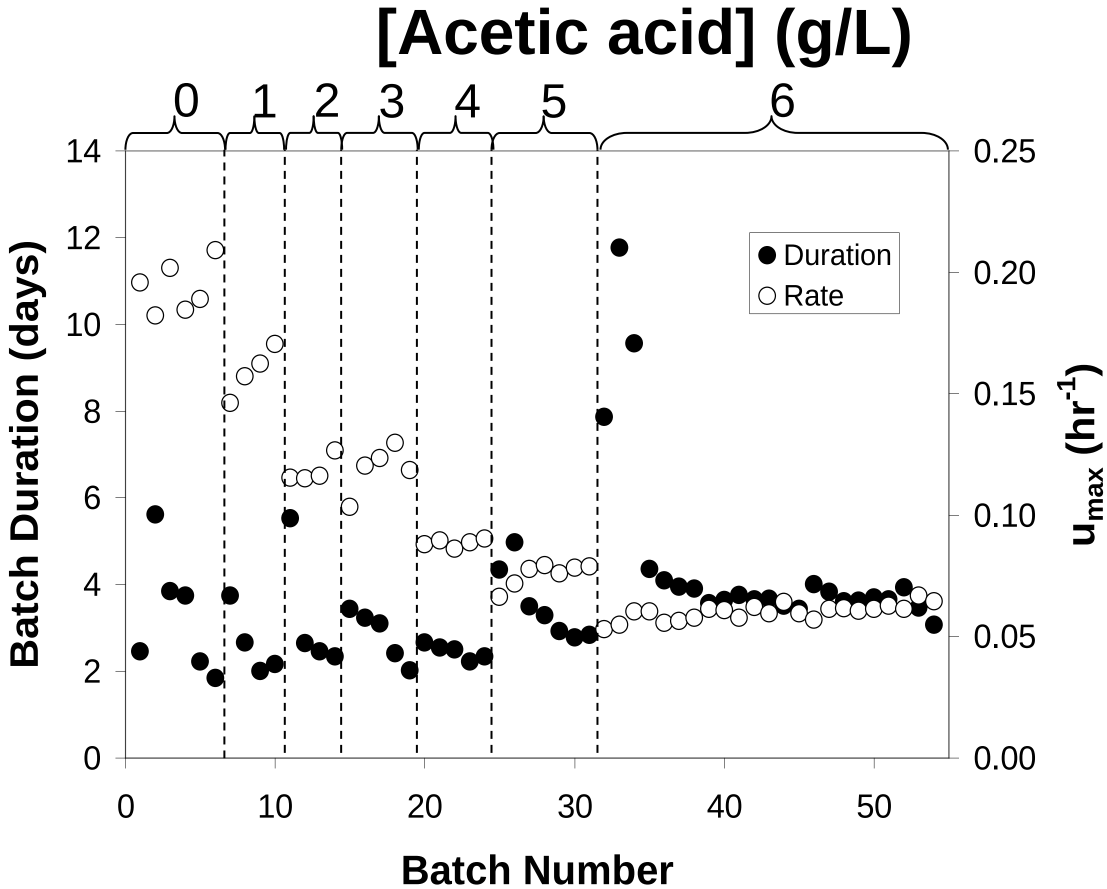


~20% of the proteins with an increased phosphorylation level in parental cells in response to acetic acid stress are Hrk1-dependent

Evolutionary Engineering in Sequential Batch Cultivation



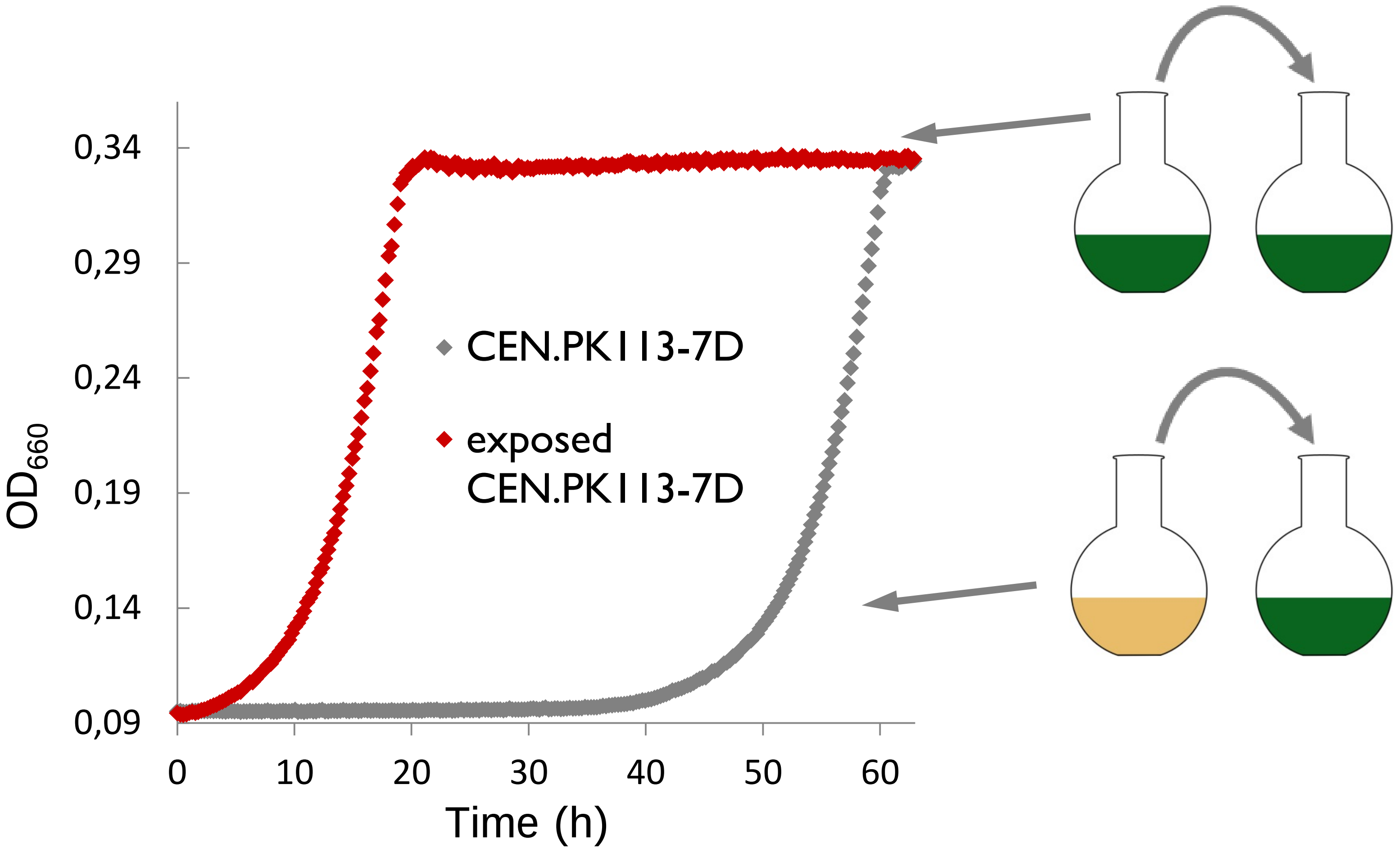
- Ability to grow at higher [acetic acid]
- Faster growth at a given [acetic acid]



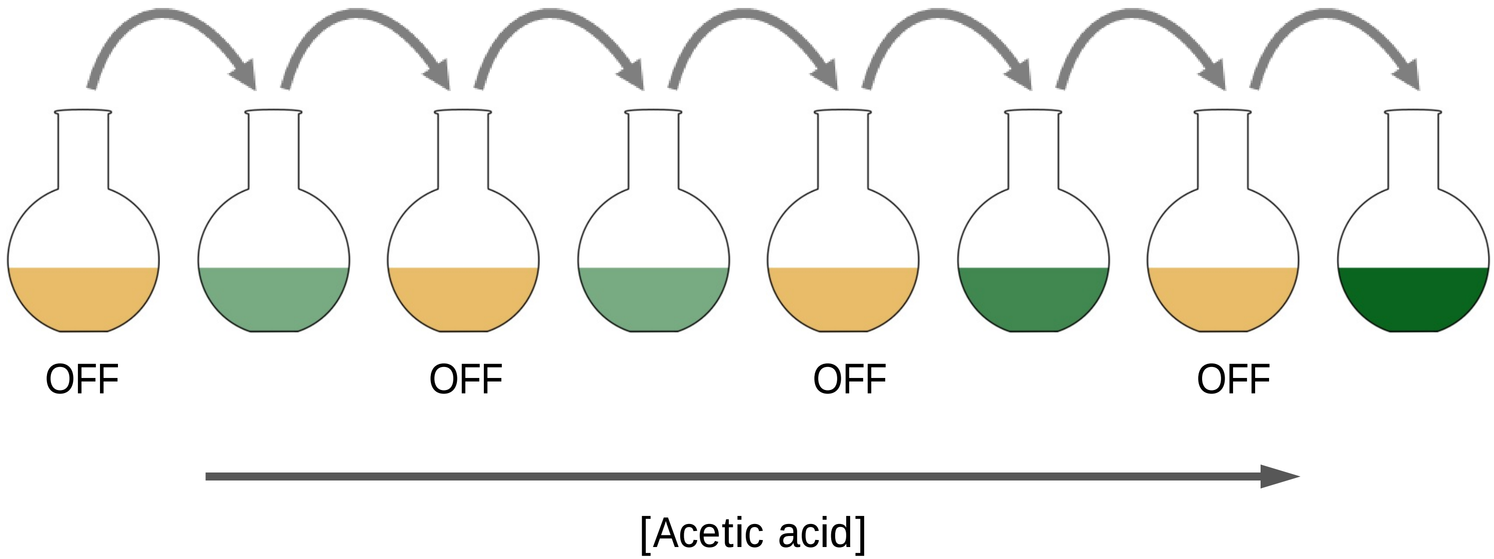
However, acquired phenotype not constitutive, but hyper inducible

Wright et al. 2011
FEMS Yeast Res. 11: 299-306

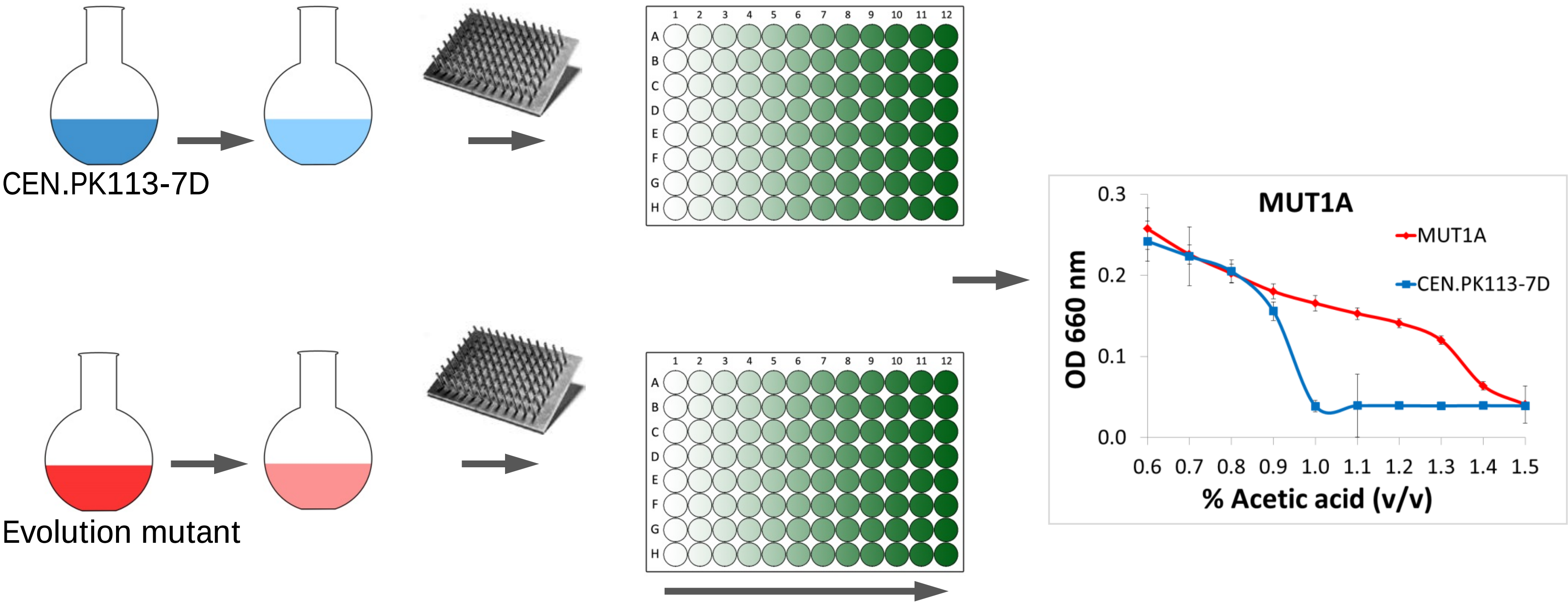
Induction of acetic acid tolerance



Evolutionary ON/OFF approach for constitutive tolerance



Measurements of acetic acid tolerance



Dilute to the same OD_{660}

[Acetic acid]

Measure OD_{660} after 5 days

Aim of the study

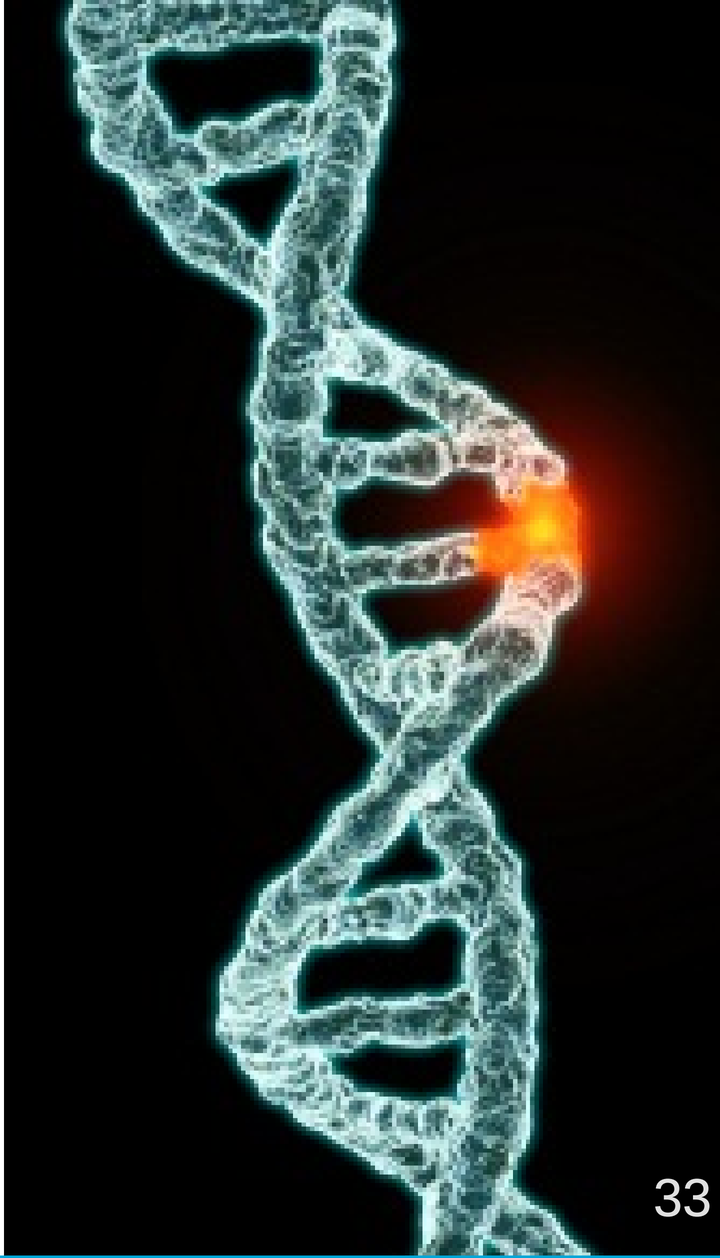
Why are the evolution strains more tolerant to acetic acid?

↓

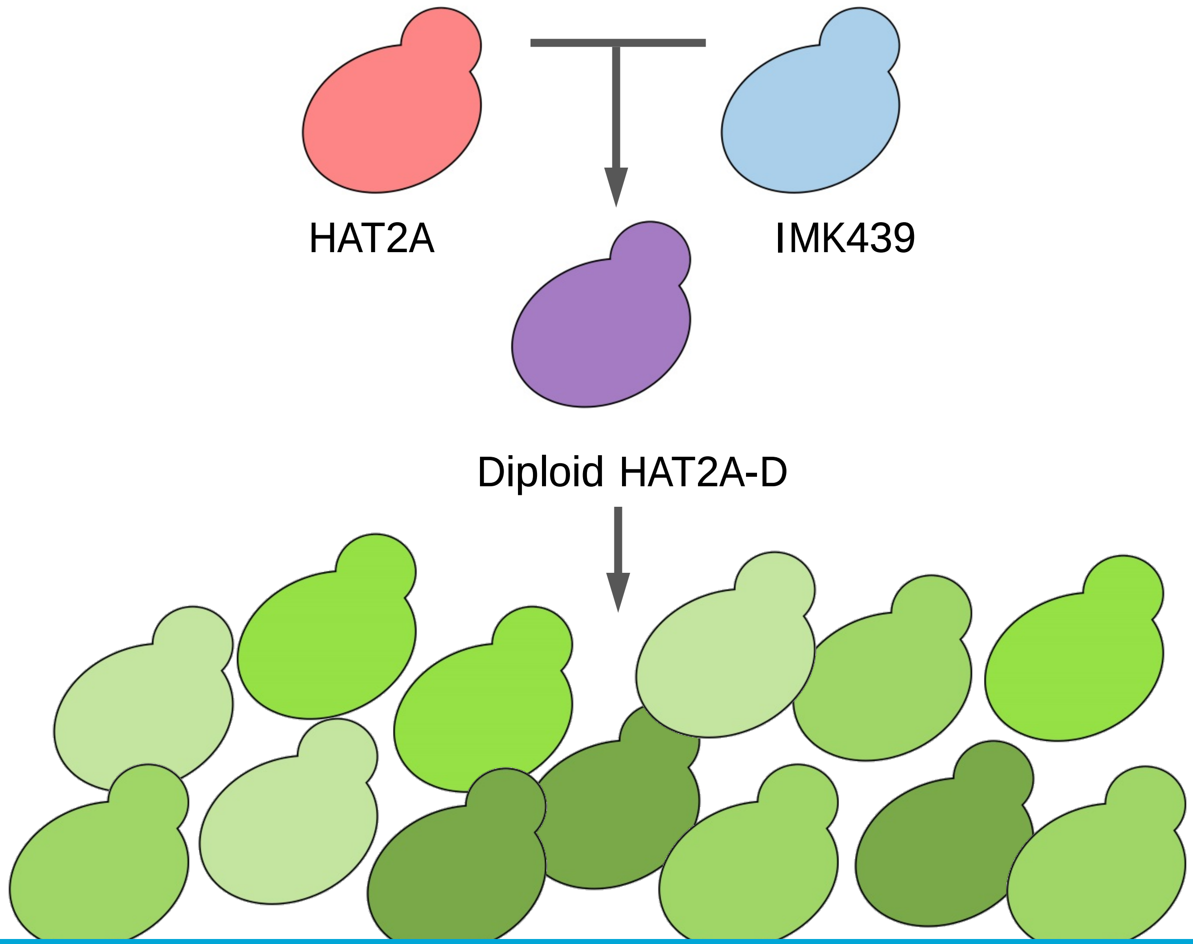
Whole genome sequencing of 6 parallel evolution lines resulting in 10-30 mutations per strain

↓

Crossing and sporulation

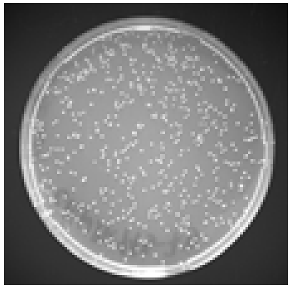


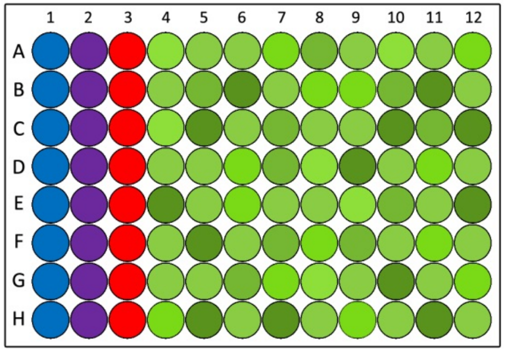
Number of mutations responsible for tolerance? Dominant or recessive?



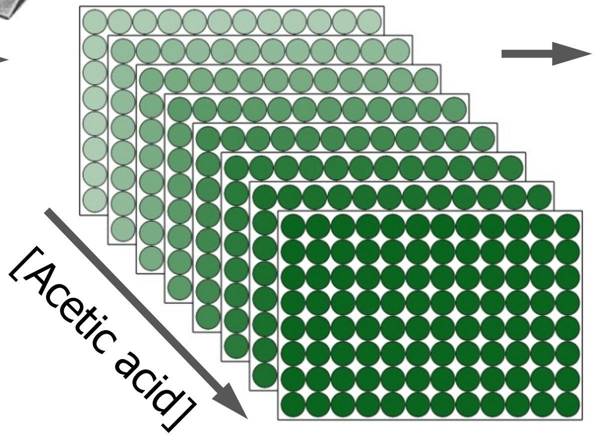
Sporulation and screening

priority targets identified & reverse engineering ongoing



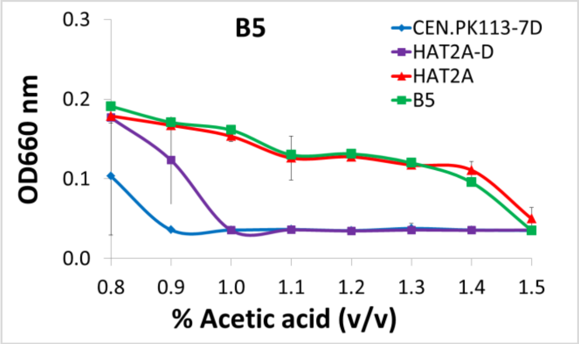


144 haploid segregants



Inoculate without OD_{660} measurement

144x



Measure OD_{660} after 5 days

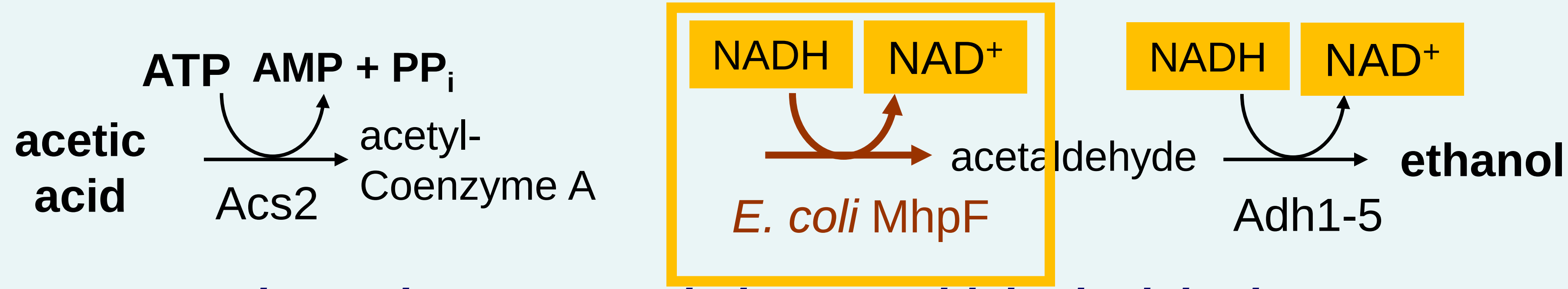
An alternative approach to deal with acetic acid?

Can the inhibitor acetic acid be converted (reduced) to ethanol?

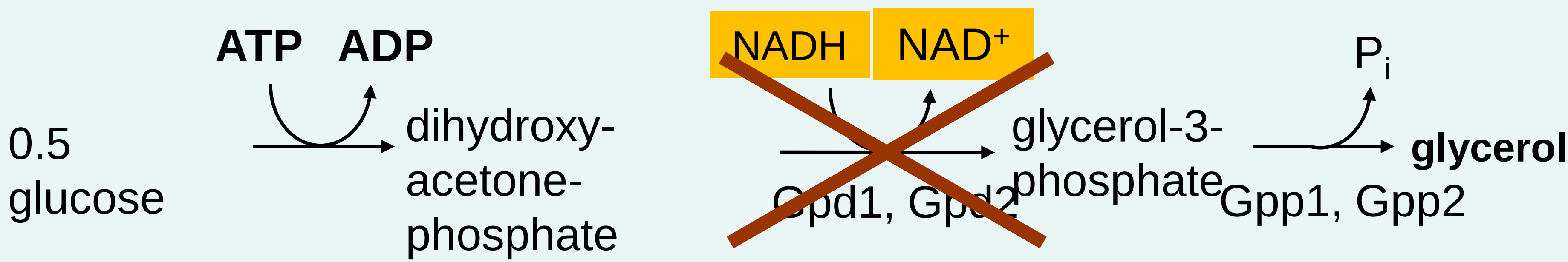
- Attractive option (less acetic acid, more ethanol)
- But where should the reducing equivalents come from?

TU Delft

An engineering strategy to eliminate glycerol production



1. Express heterologous acetylating acetaldehyde dehydrogenase



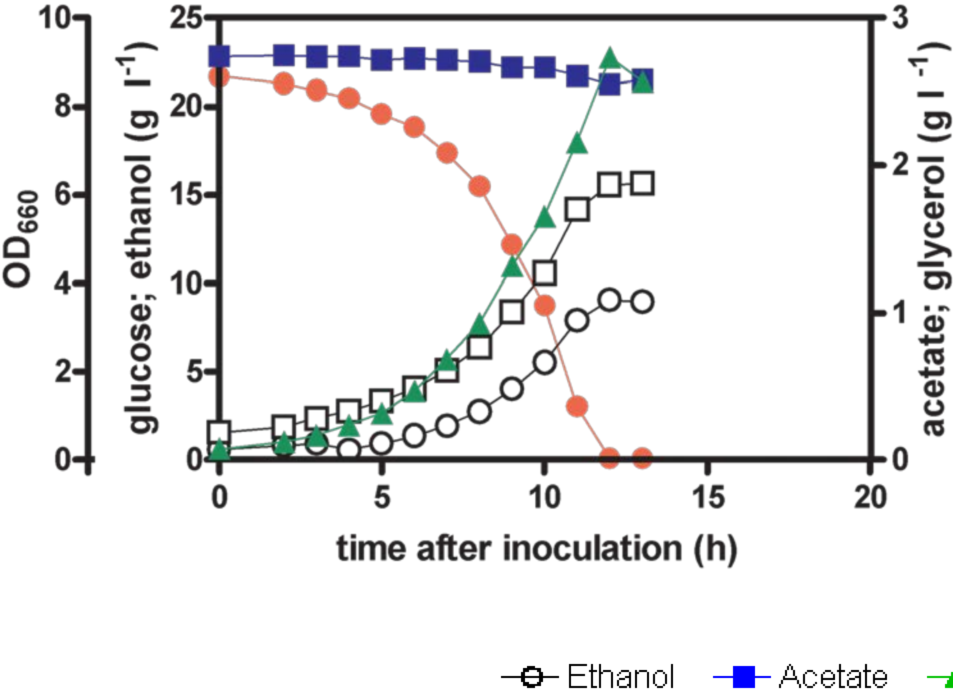
2. Eliminate glycerol production (delete GPD1, GPD2)

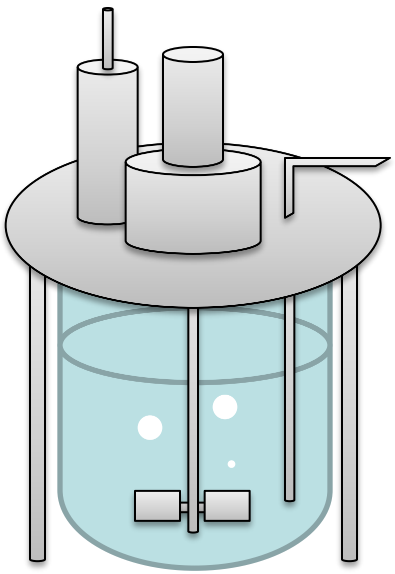
Predicted benefits

- less acetic acid, no glycerol, more ethanol
- 6% higher ethanol yield (industrial conditions)

Strain characterization in Batch

GPD1 GPD2



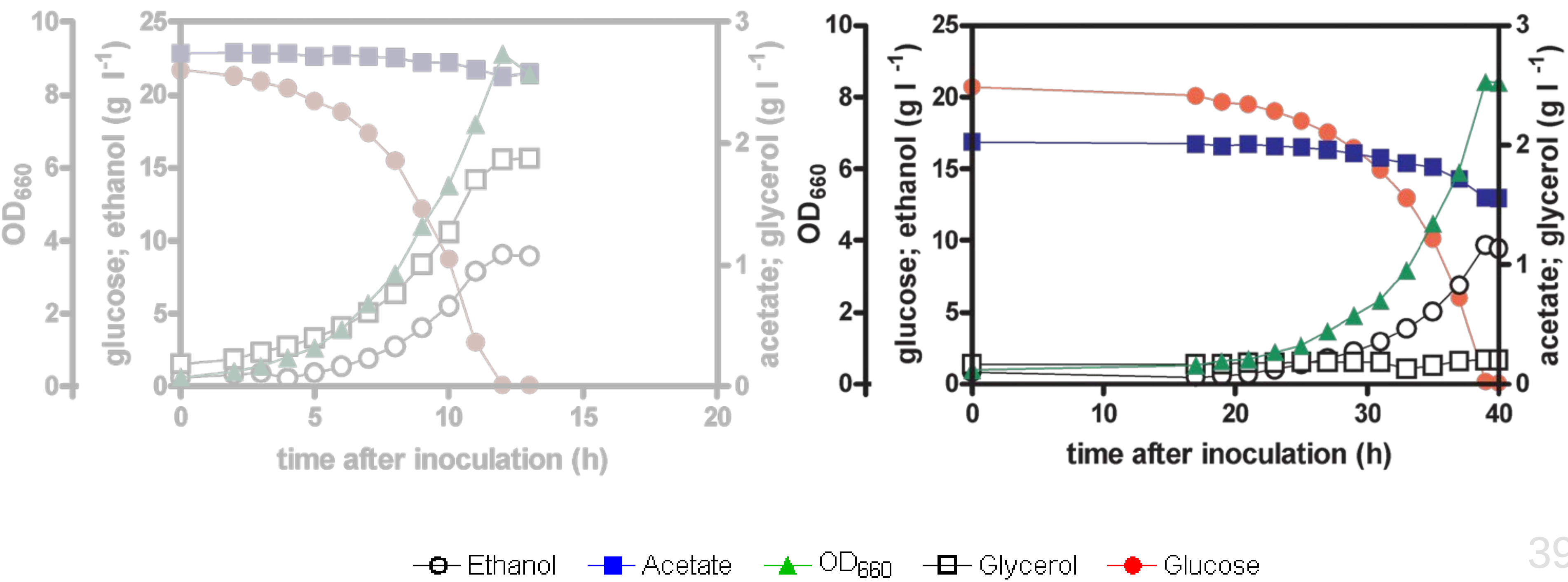


TU Delft

Strain characterization in Batch

13 % increased ethanol yield

GPD1 GPD2 *gpd1 Δ gpd2Δ + mhpF*



Integration and knowledge based engineering of tolerance

Activities Time-table

Please give a diagrammatical representation (block diagram) of the workpackage activities vs. time.

Activity scheme	S1	S2	S3	S4	S5	S6	
WP1 Screening of natural and industrial isolates (Br & L)	■	■					completed
WP2 Evolutionary engineering improved acetic acid tolerance (D)	■	■	■	■			completed
WP3 Identification of relevant genetic loci in tolerant strains (Br & D)			■	■	■		ongoing
WP4 High-copy number screen for genes confering tolerance (Ba)	■	■	■				completed
WP 5 Generation of gTME library & screening transformants (Br&L)	■	■	■	■	■		ongoing
WP6 Proteome & Metabolome profiling (L)	■	■	■				completed
WP7 Characterization of Haa1 regulon (L & Ba)		■	■	■			completed
WP8 Characterization of Rim101p regulon (L & Ba)			■	■	■		deprioritized
WP 9 Identification of acetate exporters (L & D)		■	■	■			ongoing
WP10 Potassium homeostasis in relation to tolerance (Ba & L)	■	■	■	■			ongoing
WP11 Knowledge-based metabolic engineering of tolerance (C)				■	■	■	continued collaboration

(Ba) Barcelona, (Br) Bremen, (D) Delft, (L) Lisbon (C) Consortium. S indicates Semester (half year)

Conclusions

- **Improved understanding on how acetic acid affects processes (single cells, genomics, induction)**
- **Strains with improved tolerance to acetic acid identified**
  → Synthetic biology tools rapidly developed the last 3 years
- **Evolutionary engineering can dramatically improve constitutive tolerance to acetic acid.**
- **Reverse metabolic engineering still ongoing**

?