Recombinant protein expression in lower eukaryotes
Yeasts

Lecture 3

MINIREVIEW

Introduction and expression of genes for metabolic engineering applications in *Saccharomyces cerevisiae*

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MINI-REVIEW

Yeast expression platforms

Erik Böer · Gerhard Steinborn · Gotthard Kunze · Gerd Gellissen

Advantages of eukaryotic hosts

- Conservation of many cellular processes between different eukaryotic species
  - Correct S-S bond formation
  - Post-translational modifications
    - O- / N-glycosylation
  - Proteolytic processing of a precursor into active form
    - Selected segments of amino acid sequences are removed to yield a functional protein
  - Secretory pathway (proteins targeted to various organelles or exported for harvesting)

Advantages of yeast as expression system

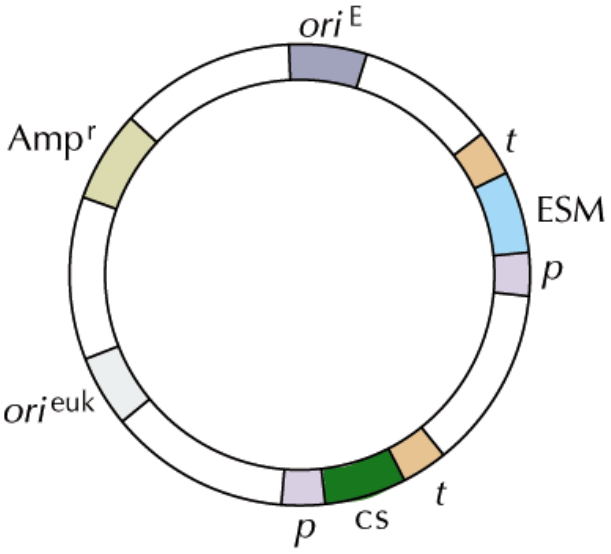
- It is easy and cheap to grow on simple media in small vessels and large scale bioreactors
- secretes few of its own proteins -> product can easily be purified
- Tremendous range of vectors and genetic resources available, including promoters and regulatory systems
- Vectors can be maintained as plasmids or integrated
- We know a lot about *Saccharomyces cerevisae* and also *Pichia pastoris*
- For therapeutics no risks of contaminations with human pathogens

Disadvantages of yeast as expression system

- Slower growth, doubling time of bacteria is 20-30 min while of yeast 1.5-2 hours at 30°C.
- Hyperglycosylation of secreted glycoproteins can be observed
- Post-translational modifications such as glycosylation may be different when compared to human proteins
- Higher number of recombination events (advantage for strain engineering, disadvantage for strain stability)

A general eukaryotic expression vector

- A selectable eukaryotic marker gene (ESM)
- A eukaryotic promoter sequence
- The appropriate eukaryotic transcriptional and translational stop signal
- A sequence that signals polyadenylation of the transcript messenger RNA (mRNA)
- eukaryotic origin of replication (ori^{euk})
- Marker gene (Amp^R) and origin (ori^E) for propagation of vector in *E. coli*



Coding sequence of gene of interest

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Saccharomyces cerevisiae as expression system

Vector and expression strategies

Some general facts about *Saccharomyces cerevisiae*

- Fast growing and robust lower eukaryote of about 5 µm diameter
- Haploid or diploid organism that can reproduce asexually and sexually
- Extensively used as model system which generated a vast knowledge
- First eukaryote being sequenced (1996)
  - Verified ORFs: 4932, uncharacterized ORFs:866, dubious ORFs: 809
- Many tools available for genetic engineering
- generally recognized as safe (GRAS-status)
- Used for production of many recombinant proteins, biochemicals, biofuels and secondary metabolites

Disadvantages of S. cerevisiae

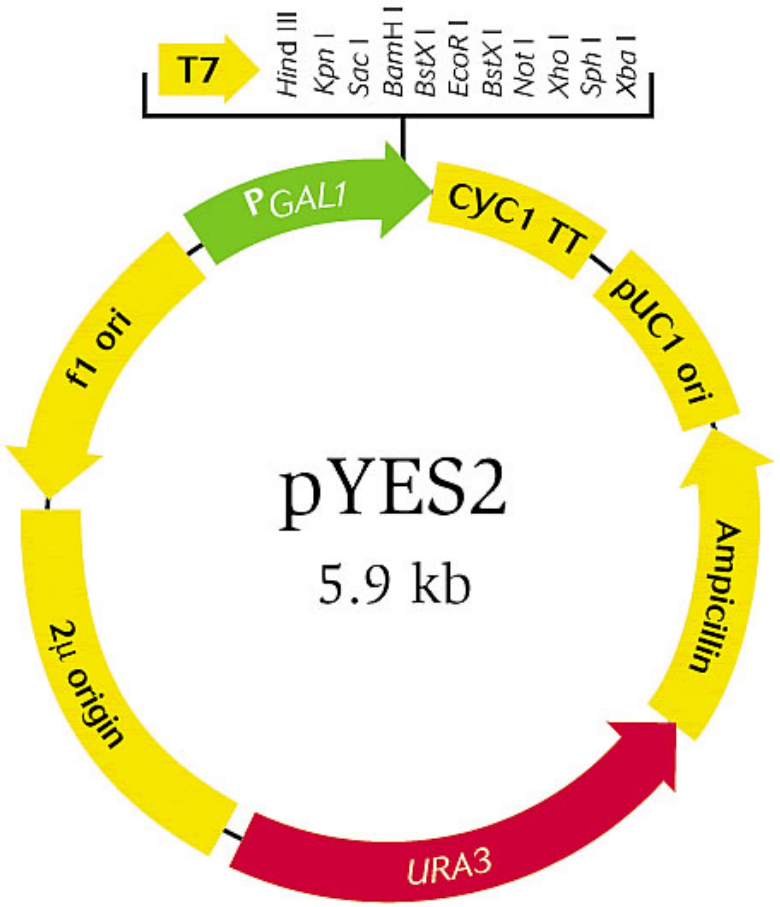
- Evolved to ferment sugars into EtOH
  - Even under aerobic conditions, in presence of excess sugar produces EtOH up to levels inhibiting growth

S. cerevisiae expression vectors

- Episomal plasmid vectors or *E. coli*/yeast shuttle plasmids
  - High copy number (25-200 copies per cell) based on 2μ origin of replication
  - Low copy number (1-3) based on CEN/ARS origin of replication
- Integrative vectors
- Yeast artificial chromosomes (YACs)

2μ based plasmid

- 2μ origin of replication, copy number 25-200
- Yeast promoter and terminator
- Yeast selectable markers
  - G418, zeocin resistance
  - **Auxotrophic marker genes**: *URA3, HIS3, TRP1, LEU2*, *ADE2*
- *E. coli* origin of replication and selection marker
- 2μ plasmids can be unstable at large scale (>10 liters)



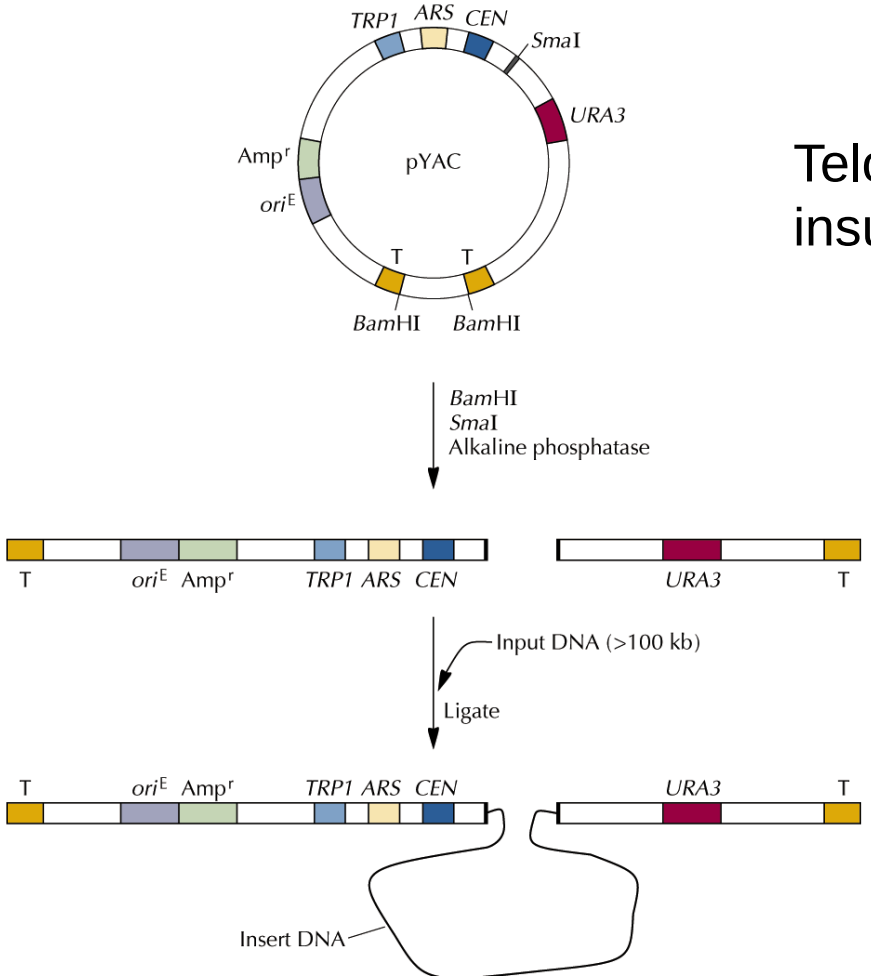
CEN/ARS based plasmid

- low copy number vectors (1 to 3 copies) based on ARS and CEN sequences
  - vectors replicate as though they were small chromosomes
  - Copy number is stably maintained
- Centromere (CEN) is required for segregation of chromosomes during mitosis
- ARS (autonomous replicating sequence) is the origin of replication in the yeast genome
- Other elements as in 2μ plasmids

Yeast artificial chromosome (YAC)

- The YAC is maintained as a separate linear chromosome in the host yeast cell and is highly stable
- In addition to CEN/ARS sequences YAC contain terminal telomer sequences
- The YAC is designed to clone large fragments of DNA (100-1000kb)

Construction of yeast artifical chromosomes (YAC)



Telomere sequence required to insure stability of YAC ends

DNA fragment can range from 100 to 1000kb

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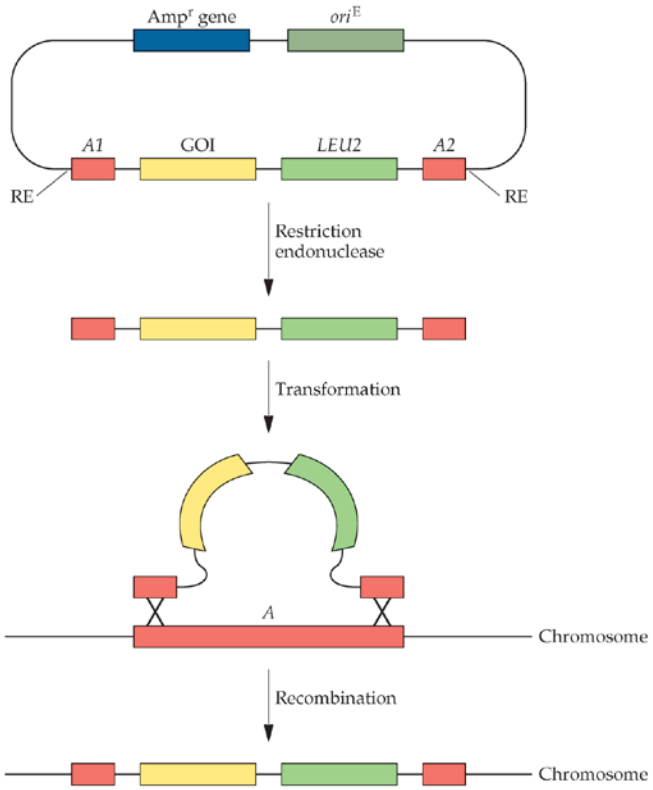
Integration into the chromosome by homologous recombination

- Ability of complementary sequences to align and exchange fragments in a double crossover event
- High efficiency with linear fragments, low efficiency with circular plasmids
- Targeted integration of gene of interest or deletion of interfering gene
- Site-specificity and high recombination frequency of *S. cerevisiae* is major advantage over other yeast systems

Expression in S. cerevisiae
Use of integrative systems



- Yeast integrative plasmid lacks eukaryotic origin of replication!
- Homologous region of 40-50 bp on each side typically sufficient for integration

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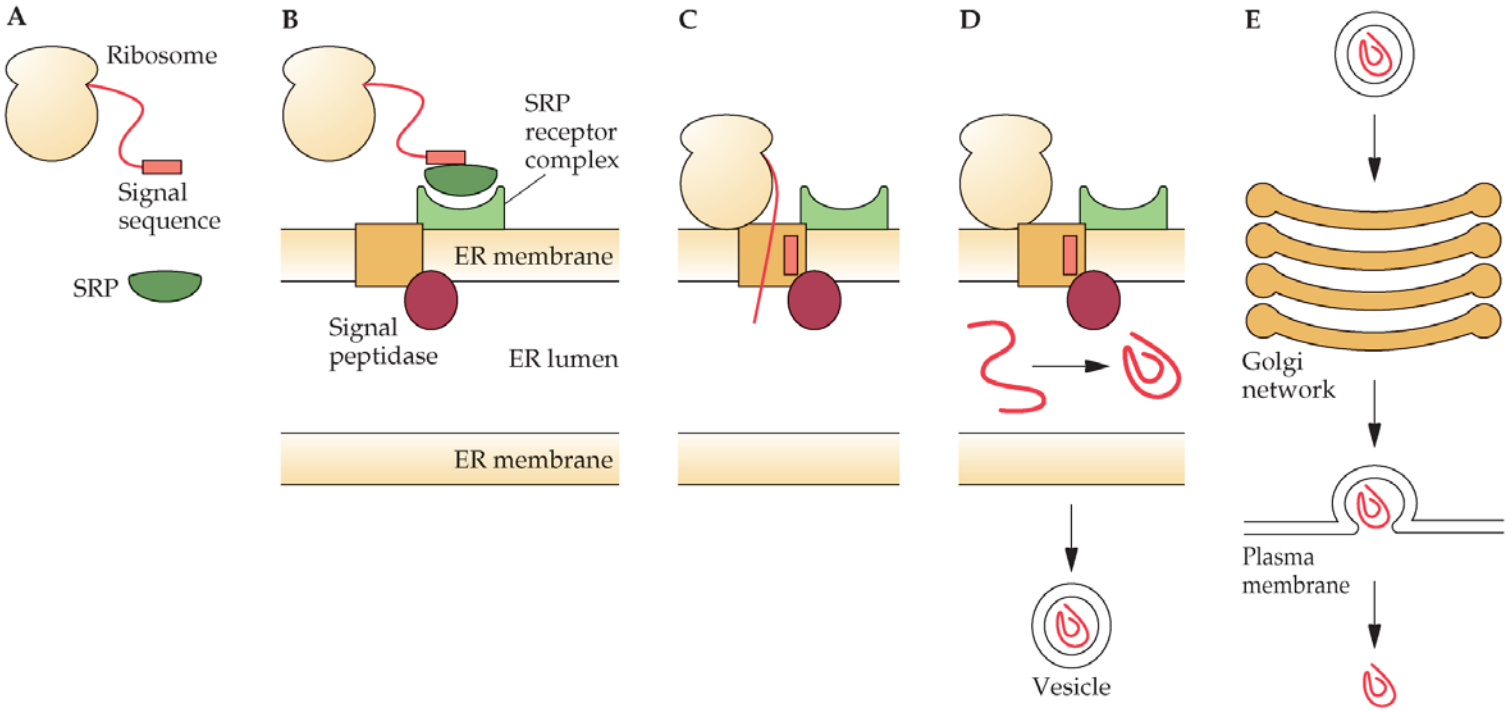
Recombinant proteins produced in *S. cerevisiae*

VACCINES
- Hepatitis B virus surface antigen
- Malaria circumsporozoite protein
- HIV-1 envelope protein

DIAGNOSTICS
- Hepatitis C virus protein
- HIV-1 antigens

HUMAN THERAPEUTIC AGENTS
- Epidermal growth factor
- Insulin
- Insulin-like growth factor
- Platelet-derived growth factor
- Proinsulin
- Fibroblast growth factor
- Granulocyte-macrophage colony-stimulating factor
- α_1 antitrypsin
- Blood coagulation factor XIIIa

Reminder: Secretion of proteins



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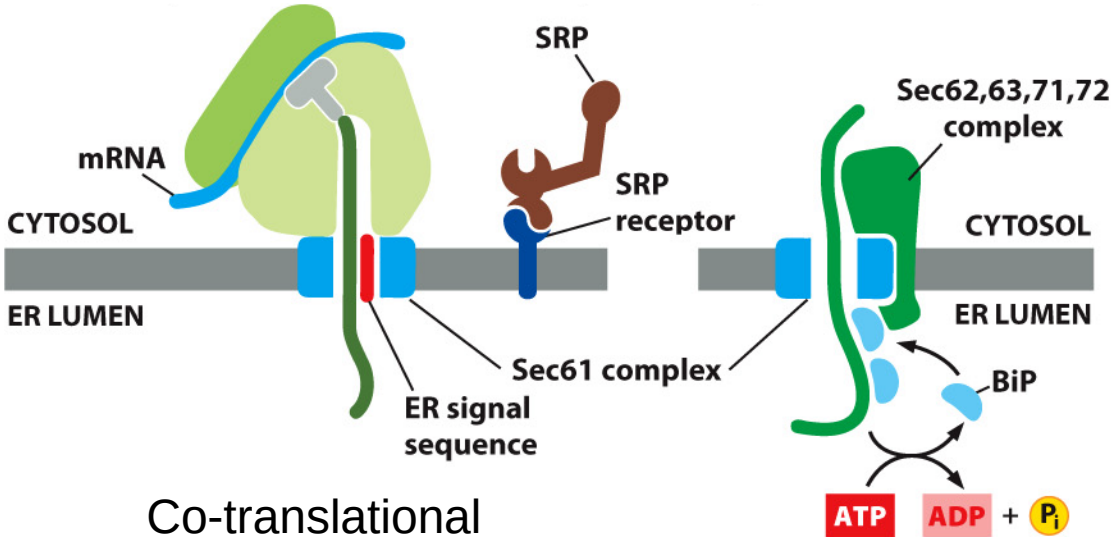
Reminder: Secretion of proteins

- Protein secretion is basically the same in all eukaryotic organisms
- Signal sequence of protein is recognized by signal recognition particle (SRP)
- SRP directs secretory protein to the translocation machinery on the ER-membrane
- After translocation proteins undergo:
  - Folding
  - Post-translational modifications
  - Is transported through secretory pathway
  - Released into the medium

Reminder: Co- and postranslational translocation



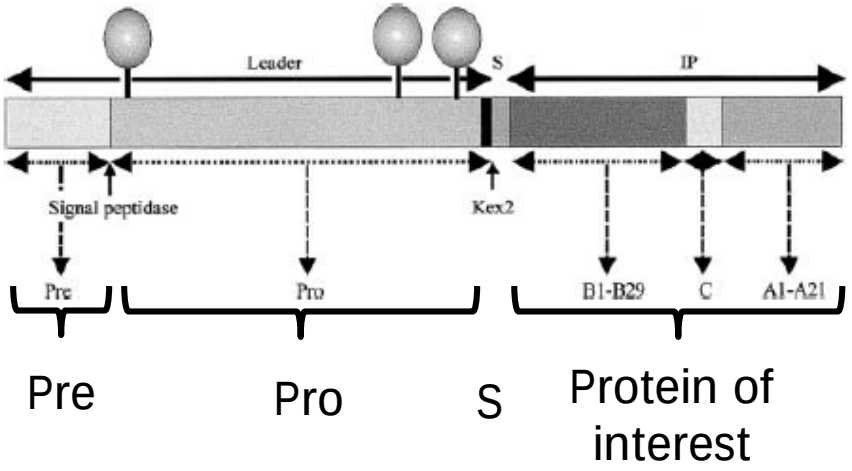
Co-translational translocation

Post-translational translocation

- In mammals, mostly cotranslational translocation is used
- In yeast, co-translational and post-translation translocation routes are used
  - Route chosen depends on the strength of the interaction between signal sequence and SRP (stable interaction or not)

Mat-alpha leader sequence driving protein expression in yeast

- Mating factor alpha (Matα) leader sequence is most used signal to drive protein secretion in yeasts (Baker's yeast and *P. pastoris*)
- Matα leader consist of a pre- and prosequence
- Presequence functions as signal sequence (=ER import signal)
  - it drives post-translational translocation
- The pro-peptide, a hydrophobic protein (binding to chaperones) is thought to slow down and ensure proper folding of the entire protein
- Proteolytic processing occurs during translocation (pre-sequence) and when leaving the Golgi (pro-sequence)



Kjeldsen T. 2000

Promoter used for expression in *S. cerevisiae*

Table 7.1 Promoters for *S. cerevisiae* expression vectors

Promoter	Expression conditions	Status
Acid phosphatase (*PH05*)	Phosphate-deficient medium	Inducible
Alcohol dehydrogenase I (*ADHI*)	2–5% Glucose	Constitutive
Alcohol dehydrogenase II (*ADHII*)	0.1–0.2% Glucose	Inducible
Cytochrome c_1 (*CYC1*)	Glucose	Repressible
Gal-1-P Glc-1-P uridyltransferase	Galactose	Inducible
Galactokinase (*GAL1*)	Galactose	Inducible
Glyceraldehyde-3-phosphate dehydrogenase (*GAPD, GAPDH*)	2–5% Glucose	Constitutive
Metallothionein (*CUP1*)	0.03–0.1 mM copper	Inducible
Phosphoglycerate kinase (*PGK*)	2–5% Glucose	Constitutive
Triose phosphate isomerase (*TPI*)	2–5% Glucose	Constitutive
UDP galactose epimerase (*GAL10*)	Galactose	Inducible

Pichia pastoris as expression system

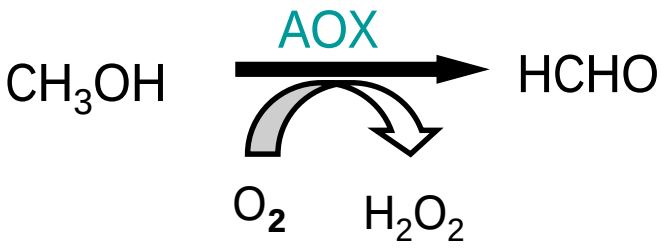
Vector and expression strategies

Comparison of expression system *S. cerevisiae – P. pastoris*

- Problems with production in *S. cerevisiae*:
  - For some proteins production level low
  - Hyperglycosylation (more than 100 mannose residues in N-glycosylation)
  - Sometimes secretion not good -> protein retained in cell wall
  - Produces high amounts of EtOH -> toxic for the cells
- Advantages of production in *Pichia pastoris*:
  - Highly efficient promoter, tightly regulated (alcohol oxidase -> AOX, induced by MeOH)
  - Produces no EtOH -> very high cell density can be achieved
  - Secretes very few of its own proteins
  - 10-100 fold higher heterologous protein expression levels

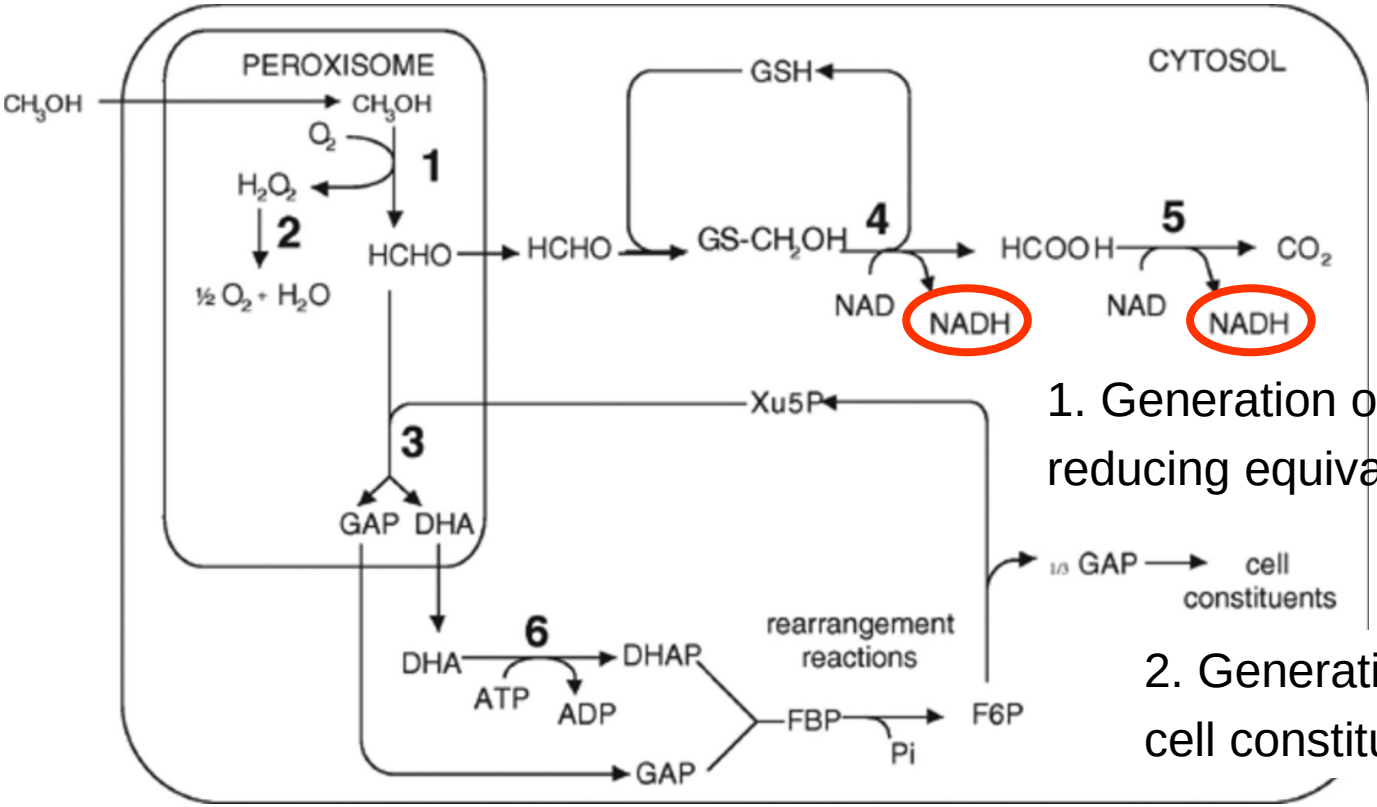
Methanol utilization by *P. pastoris*

- *Pichia* is a methylotrophic yeast (can metabolize MeOH)
- 2 genes encoding alcohol oxidase (AOX1 and AOX2)
  - AOX1 responsible for majority of AOX activity



- Reaction proceeds in specialized compartment (peroxisome)
  - During methanol oxidation, H_2O_2 is produced
  - H_2O_2 is detoxified by catalase

Methanol metabolism in *P. pastoris*



1. Generation of reducing equivalents

2. Generation of cell constituents

1 – **alcohol oxidase**, 2 – **catalase**, 3 – dihydroxyacetone synthase, 4 – formaldehyde dehydrogenase, 5 – formate dehydrogenase, 6 – dihydroxyacetone kinase, GSH – glutathione, Xu5P – xylulose-5-phosphate, FBP – fructose-1,6-bisphosphate.

Gellissen et al. 2005, FEMS yeast research

AOX1 promoter as tool for protein expression

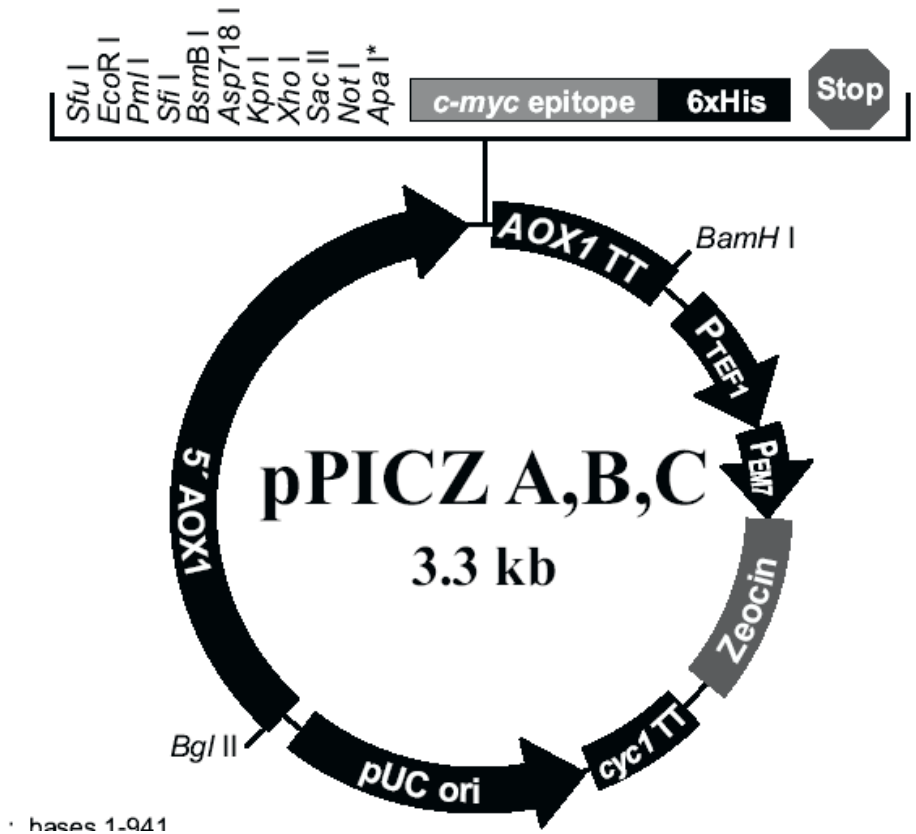
- AOX1 gene inducible by methanol
  - Protein can reach up to 30% total soluble protein in cells grown with methanol
- Expression controlled at transcription level
- Glucose represses transcription, even in presence of methanol -> often Glycerol used as carbon source
- AOX1 promoter powerful tool to drive protein expression

P. pastoris expression host strains

Strain name	Genotype	phenotype
Y-11430		Wild-type
GS115	*his4*	Mut+ His-
KM71	*aox1*::ARG4 *his4 arg4*	MutS His-
MC100-3	*aox1*::ARG4 *aox2*::HIS4 *his4 arg4*	Mut- His+
SMD1168	*pep4 his4*	Mut+ His- protease deficient

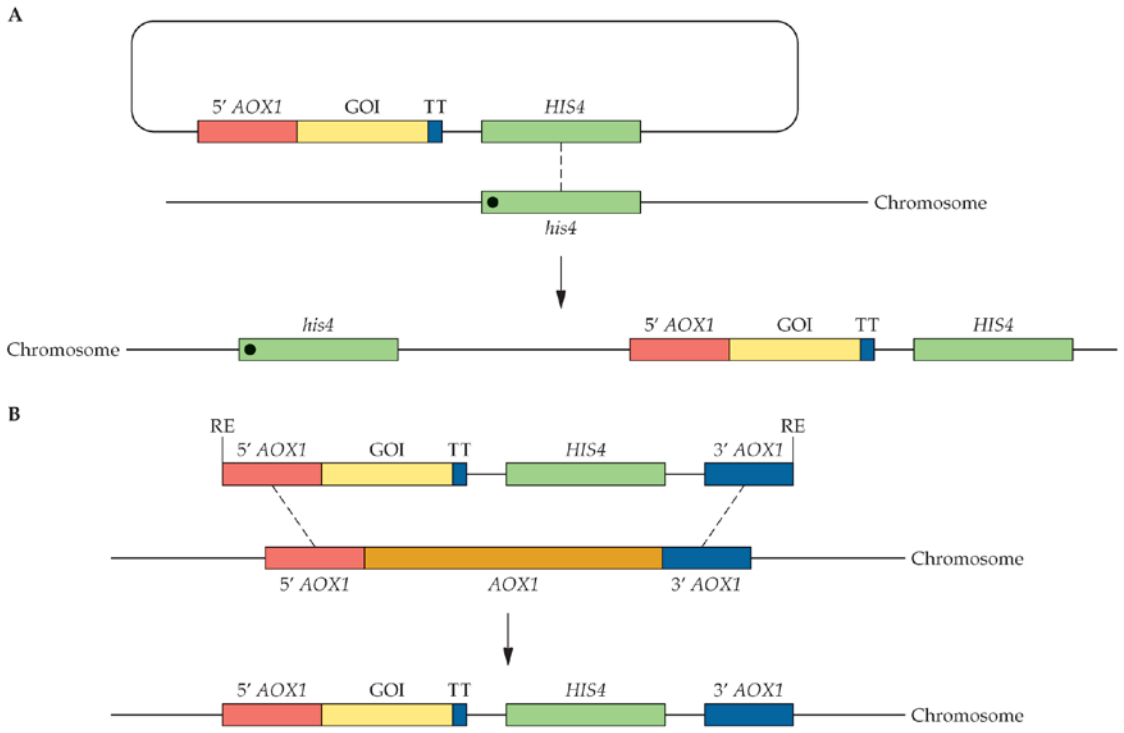
- Mut^{+}: both AOX1 and AOX2 are functional -> methanol as inducer and as carbon source used
- Mut^{S}: only AOX2 functional -> methanol as inducer and just to minor extent as carbon source
- Mut^{-}: none of the AOX are functional -> methanol only as inducer

P. pastoris expression vector



With few exceptions, only integrative vectors available for expression in Pichia

Integrative plasmid systems for *P. pastoris*



- Lower frequency of homologous recombination than in *S. cerevisiae,* due to higher NHEJ activity
- Strain engineering more tedious/random

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Other fungal expression systems

- *Hansenula polymorpha*
- *Kluyveromyces lactis*
- *Schizosaccharomyces pombe*
- *Schwanniomyces occidentalis*
- *Yarrowia lipolytica*
- *Trichoderma reesei*

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Yeast expression platforms

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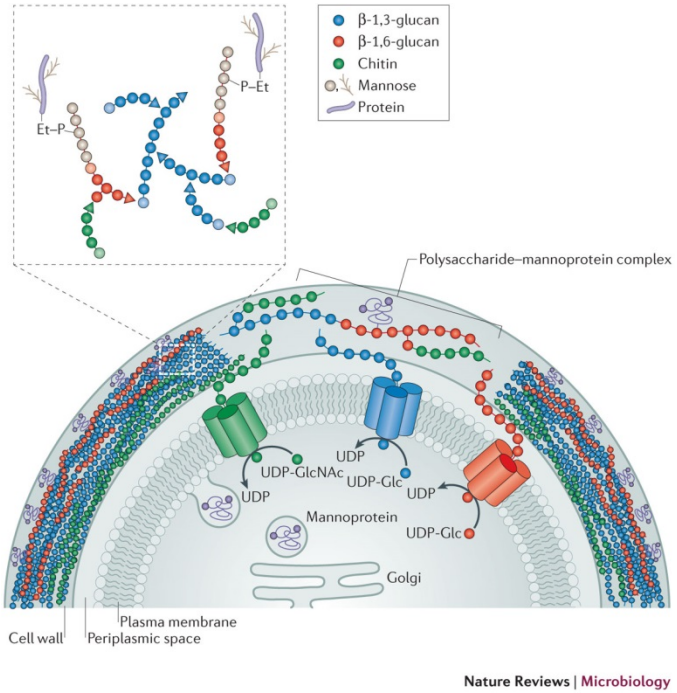
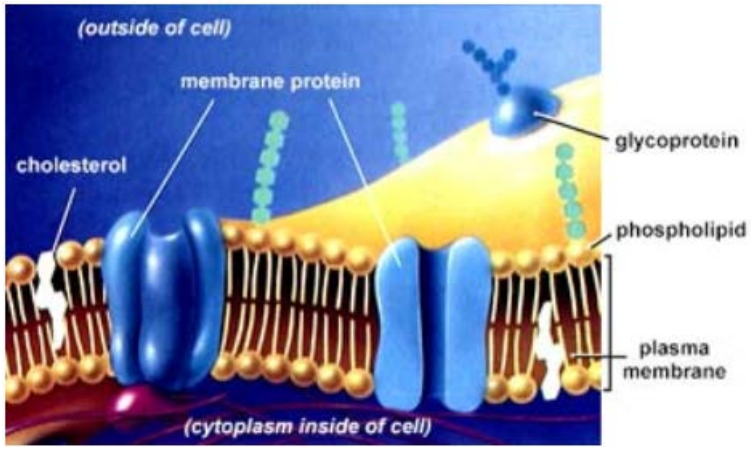
Advantages of yeasts versus other eukaryotic expression systems

- Less expensive, easier to grow, higher throughput
- Shorter cell cycle time than tissue culture (Human embryonic kidney cells – 1 day doubling)
- Higher protein yield
- Protein pharmaceuticals free of human disease
- Fewer regulations compared to tissue culture
- More extensive genetics (CrispR might change this)
- Transformation/DNA manipulations easier

Disadvantage of yeasts versus other eukaryotic expression systems





S. cerevisiae Mammalian cell

- Secretory proteins need to cross the cell wall in yeast!
- Limits secretion of bigger molecules

Summary

- As a unicellular eukaryote, yeast is quick, easy and inexpensive to genetically manipulate and culture
- A wealth of knowledge and tools available for *S. cerevisiae*, and more recently also other fungal expression systems
- High protein yield and ease of industrial scale-up, make yeast/fungal strains useful for protein production