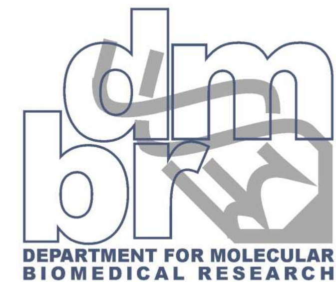
Engineering complex-type N-glycosylation in *Pichia pastoris* using *Pichia* GlycoSwitch™ technology

Nico Callewaert



Which glycans for which therapeutic goal?

Glyco-engineering in biotech

- Goals:
  - Provide increased homogeneity of bio-pharmaceuticals that cannot be made in *E. coli*
  - Provide robust eukaryotic cells with non-immunogenic glycans
  - Tune the glycans to the intended function of the pharmaceutical (e.g. vaccine, mAb, long-circulating molecule)
- Ideally: engineering in GRAS eukaryotes with order-of-magnitude lower production facility costs and turnaround times (micro-organisms, some plants)

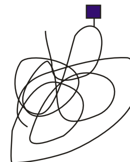
Glyco-engineering in biotech

In vivo/in vitro glyco-engineering?

In vitro	*In vivo*
- Reactions can be driven to completion - No complicated issues with targeting of enzymes - No issues with cellular physiology compatibility - More versatile	- Conversions limited by cellular dynamics - Enzyme targeting very important - Engineering should not affect physiology
- Activated sugar donors needed - GMP-produced enzymes needed - Scaleability has been an issue ⇒ COST!!!!	NONE OF THIS!!!!

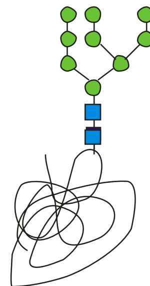
Which glycans for which therapeutic function?

- No/small, homogenous N-glycans
  - Molecules of which the glycan structures don’t matter but should not affect DSP efficiency
  - Things you want to get a crystal structure of
  - Vaccines of which N-glycans hamper immunogenicity
  - Molecules of which pharmacokinetics should not be determined by the glycans.



Which glycans for which therapeutic function?

- High-mannose glycans
  - ADEPT (antibody-dependent enzyme prodrug treatment) and imaging antibody fragments requiring fast clearance (cfr. talk Dr. Chester)
  - Molecules that need a high and acute dosing
  - Drugs that need targeting to the lysosomes of mannose-receptor-expressing cells



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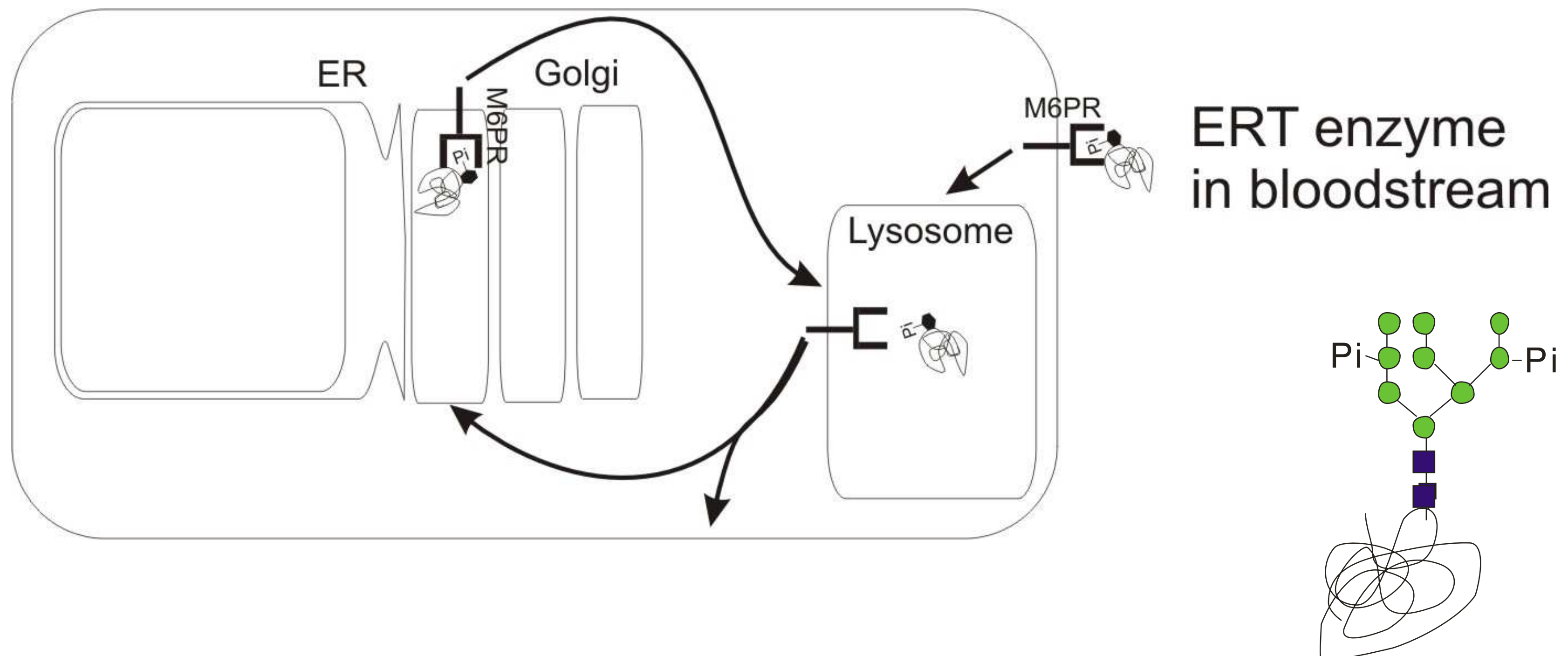
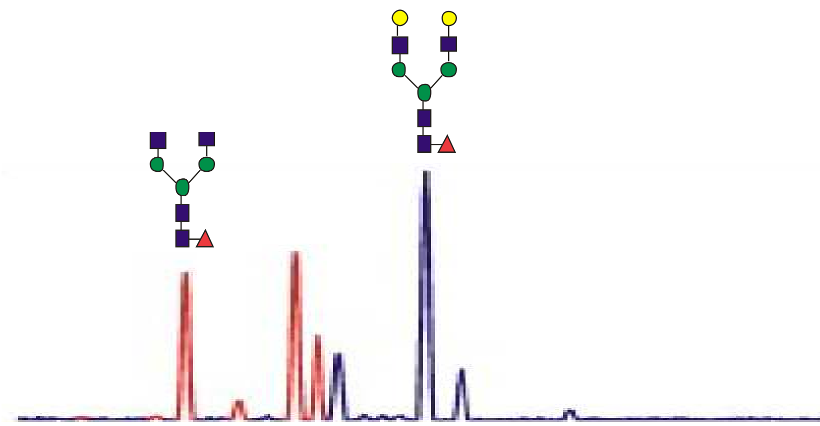
Which glycans for which therapeutic function?

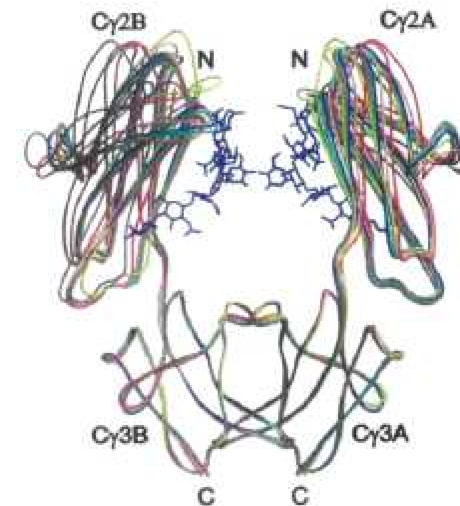
- Phosphorylated N-glycans
  - Lysosomal targeting through Man-6-Pi receptor



Which glycans for which therapeutic function?

- Hybrid-type and non-sialylated complex-type glycans
  - IgG Fc N-glycan
  - Different structures modulate the IgG effector function





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Comparison of biological activity among nonfucosylated therapeutic IgG1 antibodies with three different *N*-linked Fc oligosaccharides: the high-mannose, hybrid, and complex types

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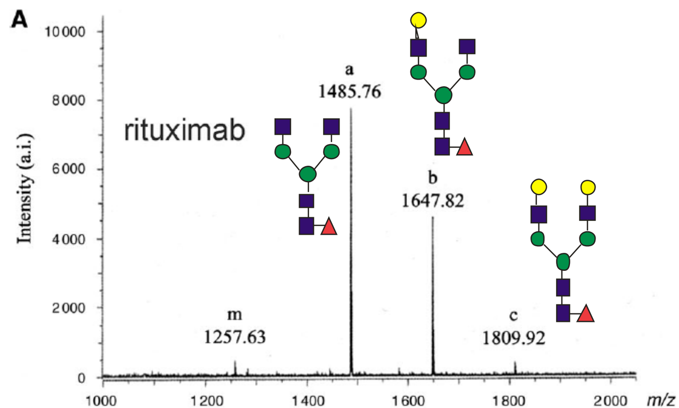
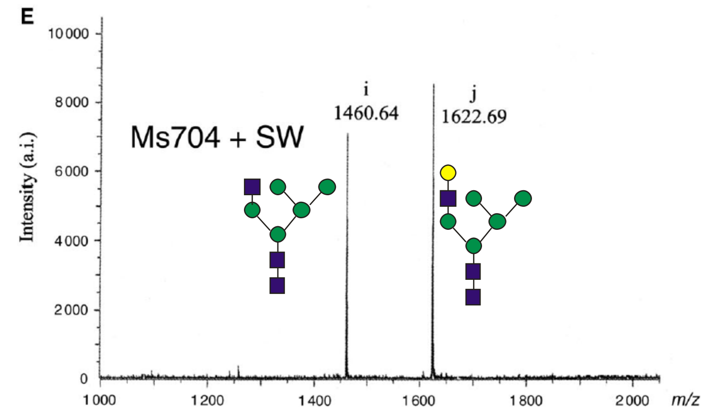
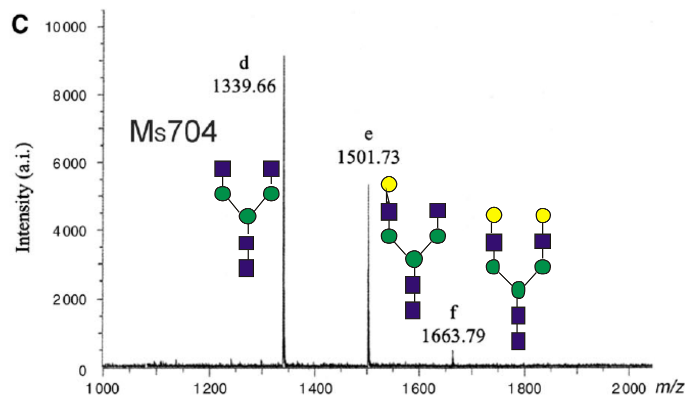
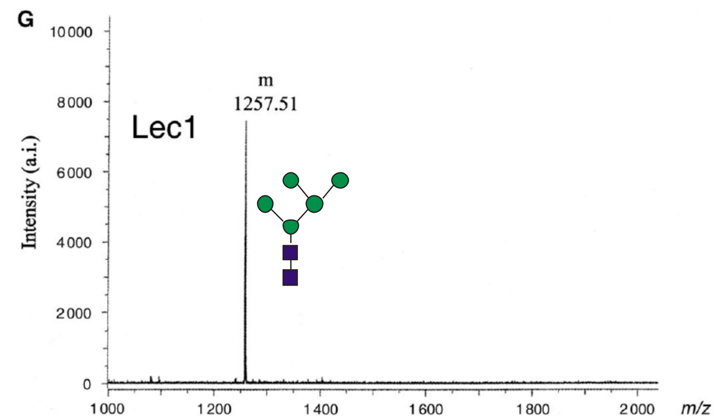
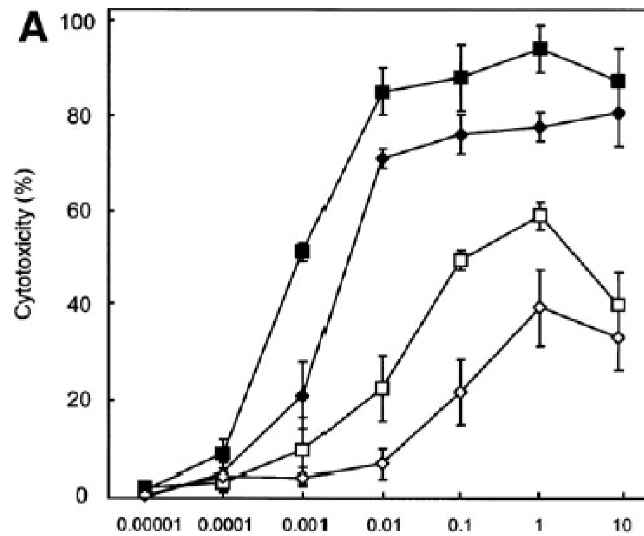


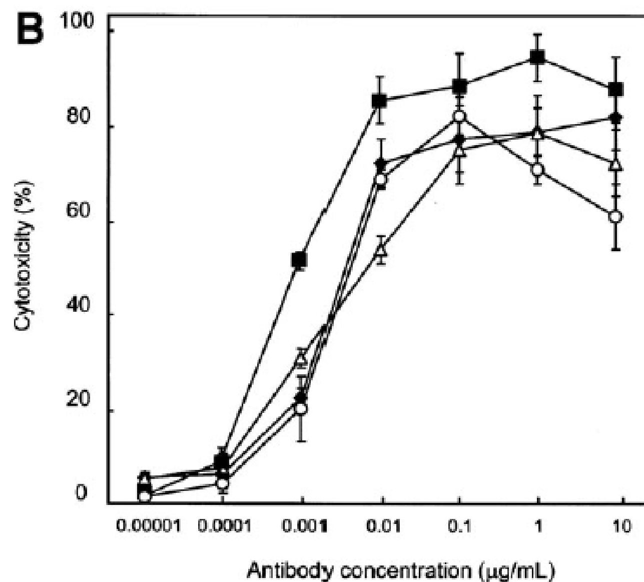


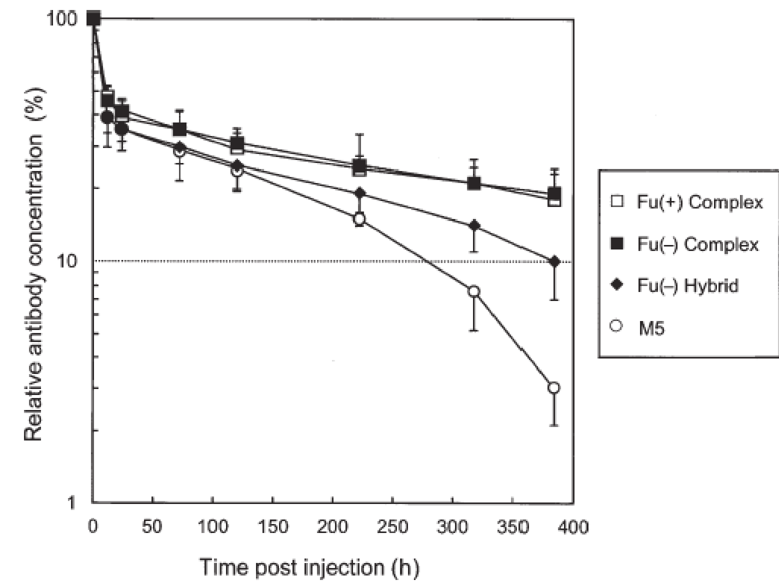




Removal of core fucose: ADCC activity much more potent!

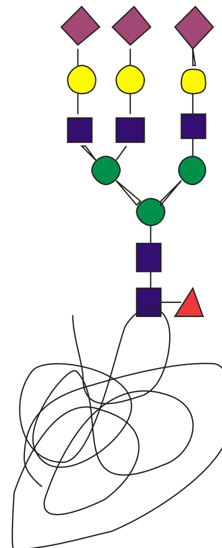






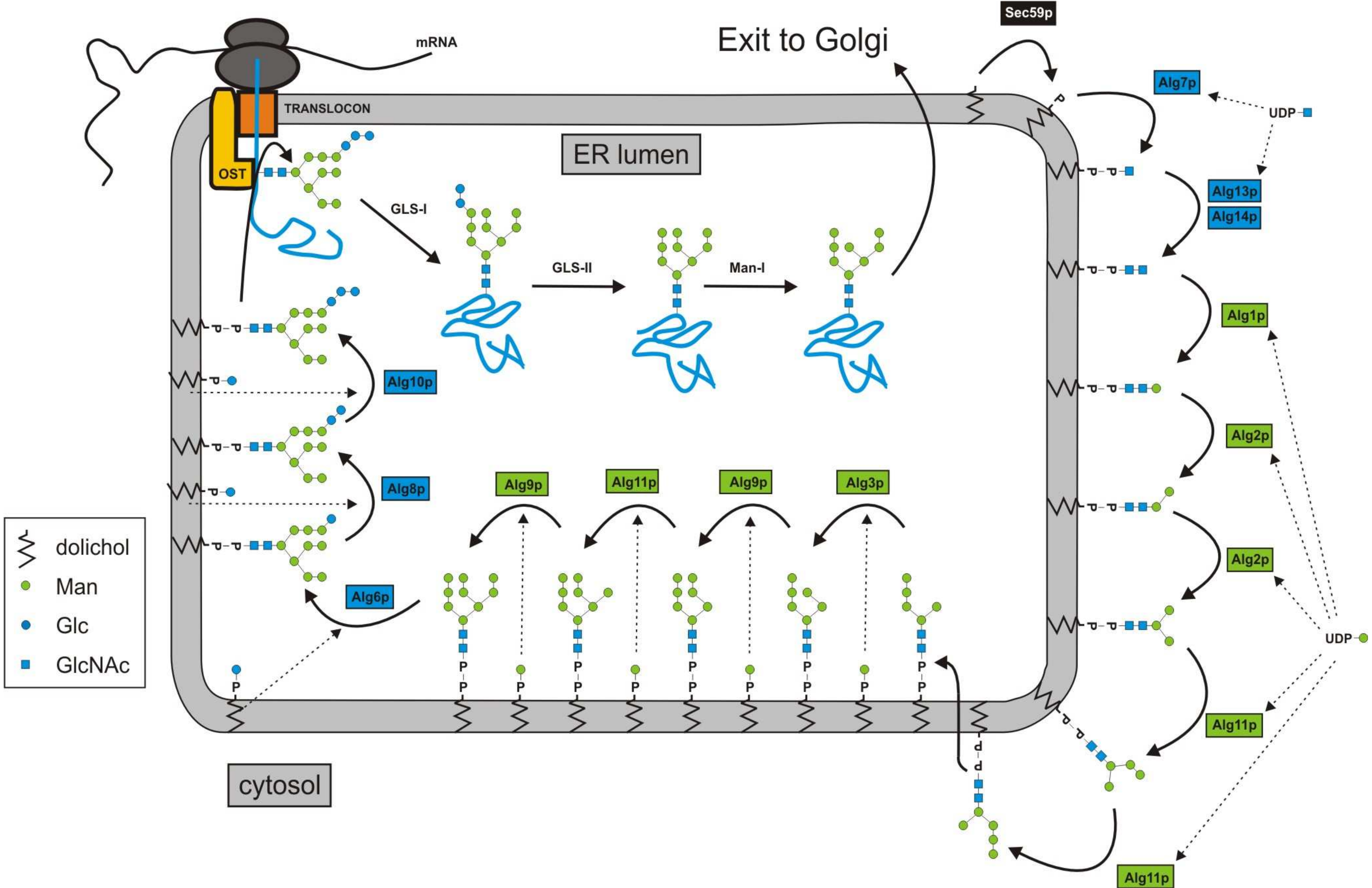
Which glycans for which therapeutic function?

- Sialylated N-glycans
  - Long circulation time in blood (EPO, alpha-1-antitrypsin) (actually: non-Man, non-GlcNAc, non-Gal terminated).
  - Recombinant IVIG Abs (alpha-2,6-sialylation).



Pichia pastoris glyco-engineering: GlycoSwitch™

N-glycosylation: conserved ER pathway



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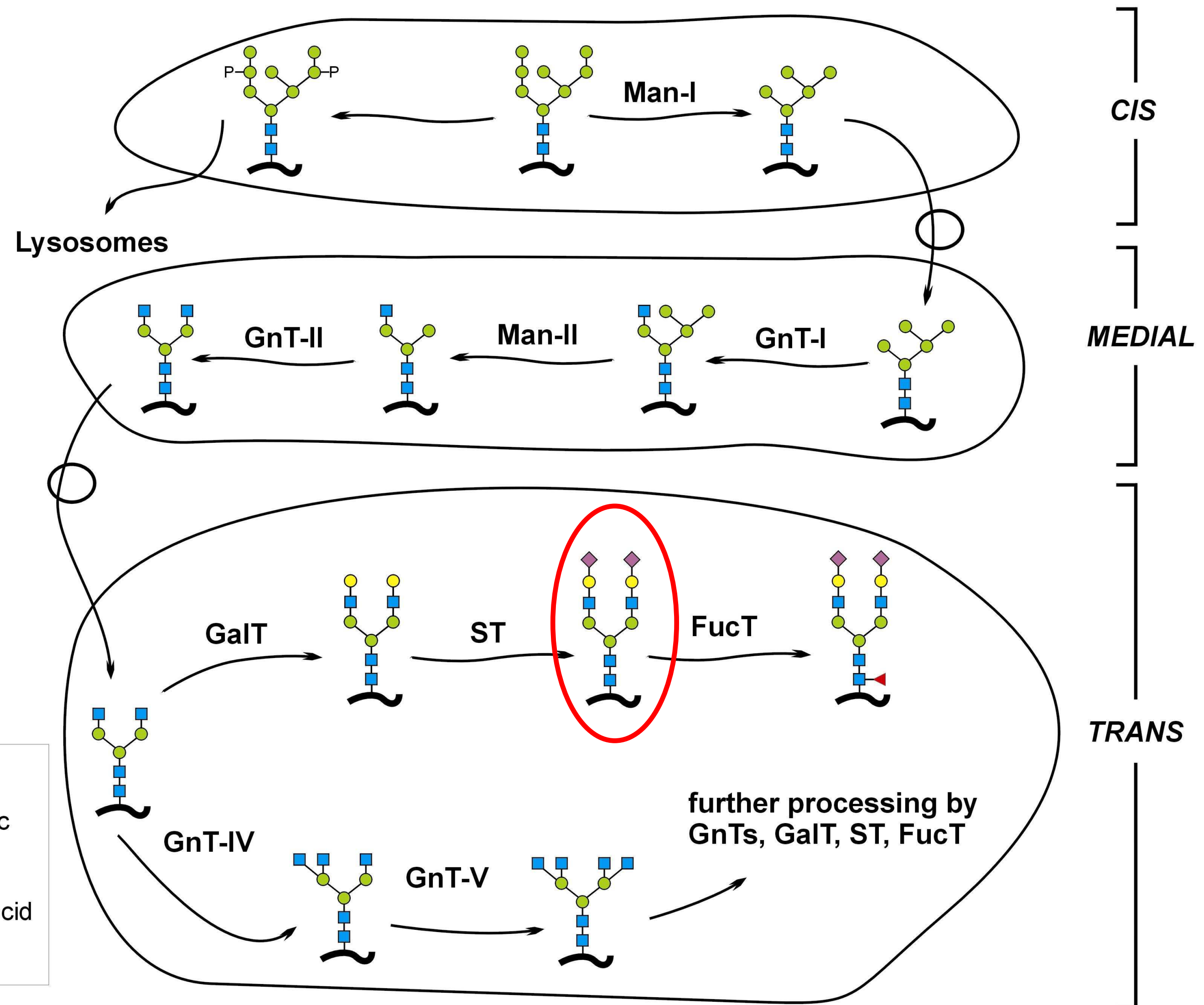
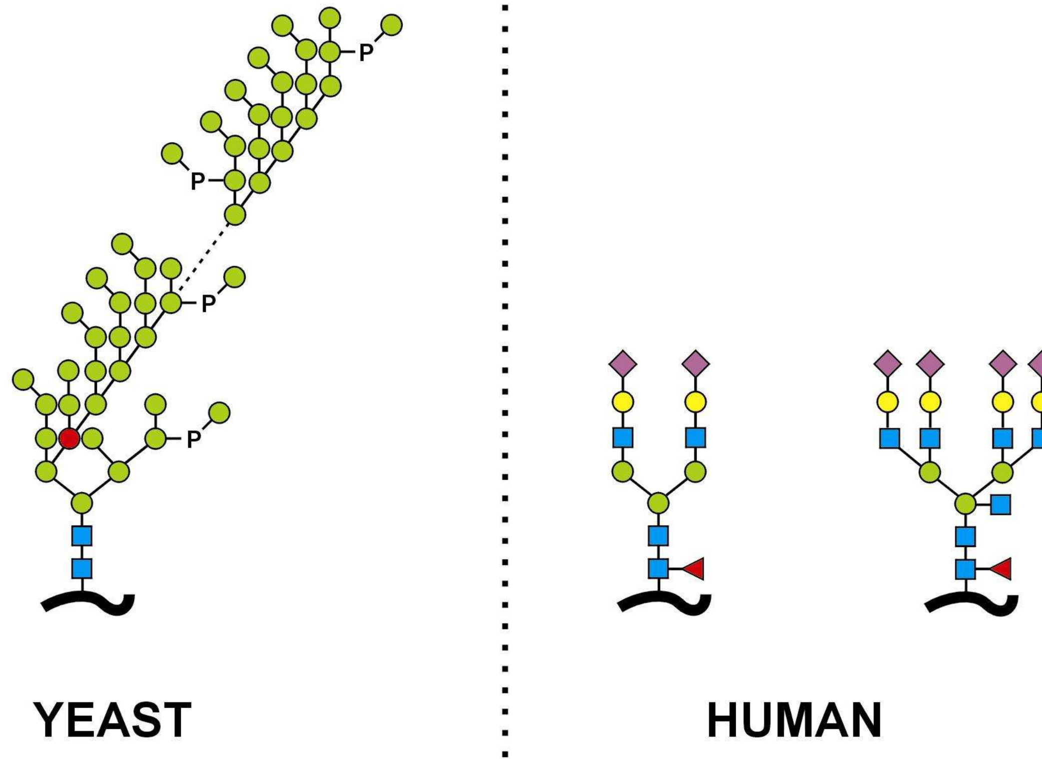
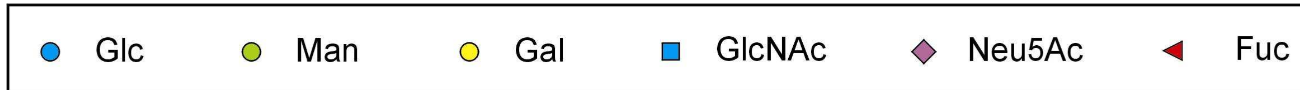
Golgi pathways: divergent amongst eukaryotes, here: human hepatocyte



Yeast versus man





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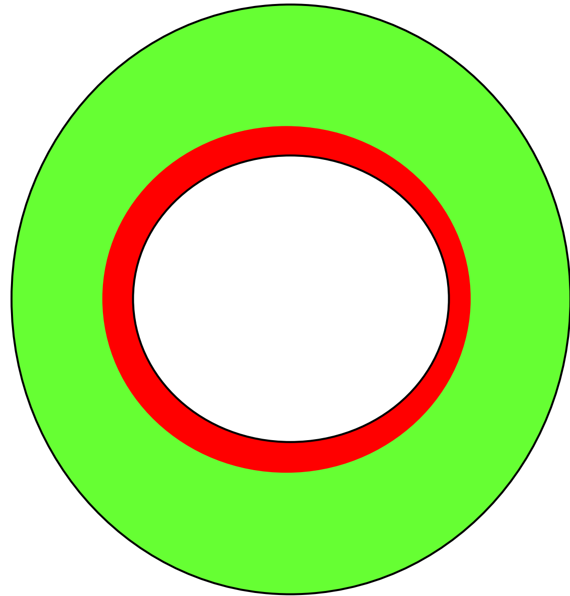
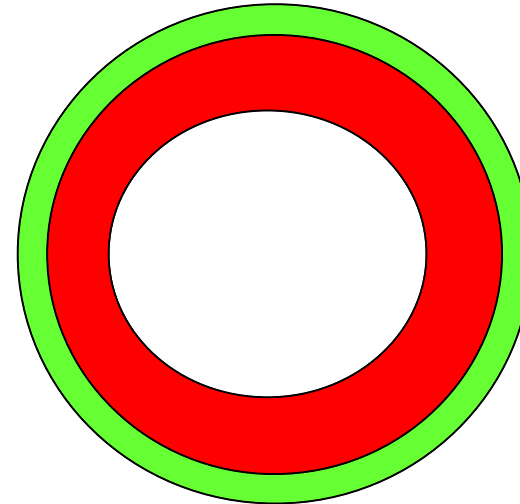
S. cerevisiae

P. pastoris, Y. lipolytica, higher fungi

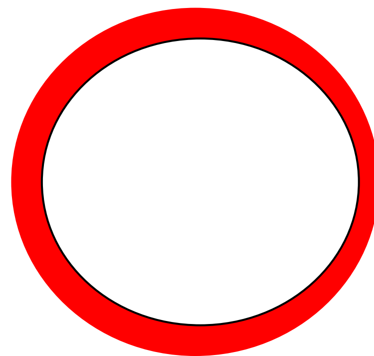
Green: mannoproteins

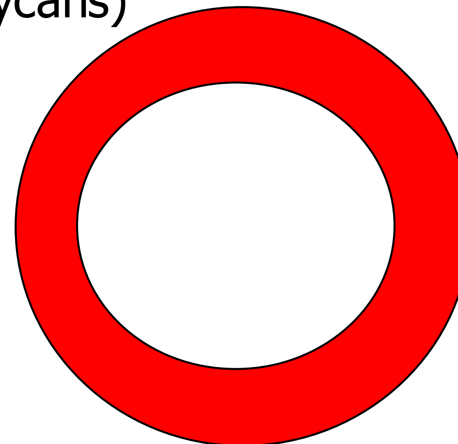
Red: chitin and beta-glucans



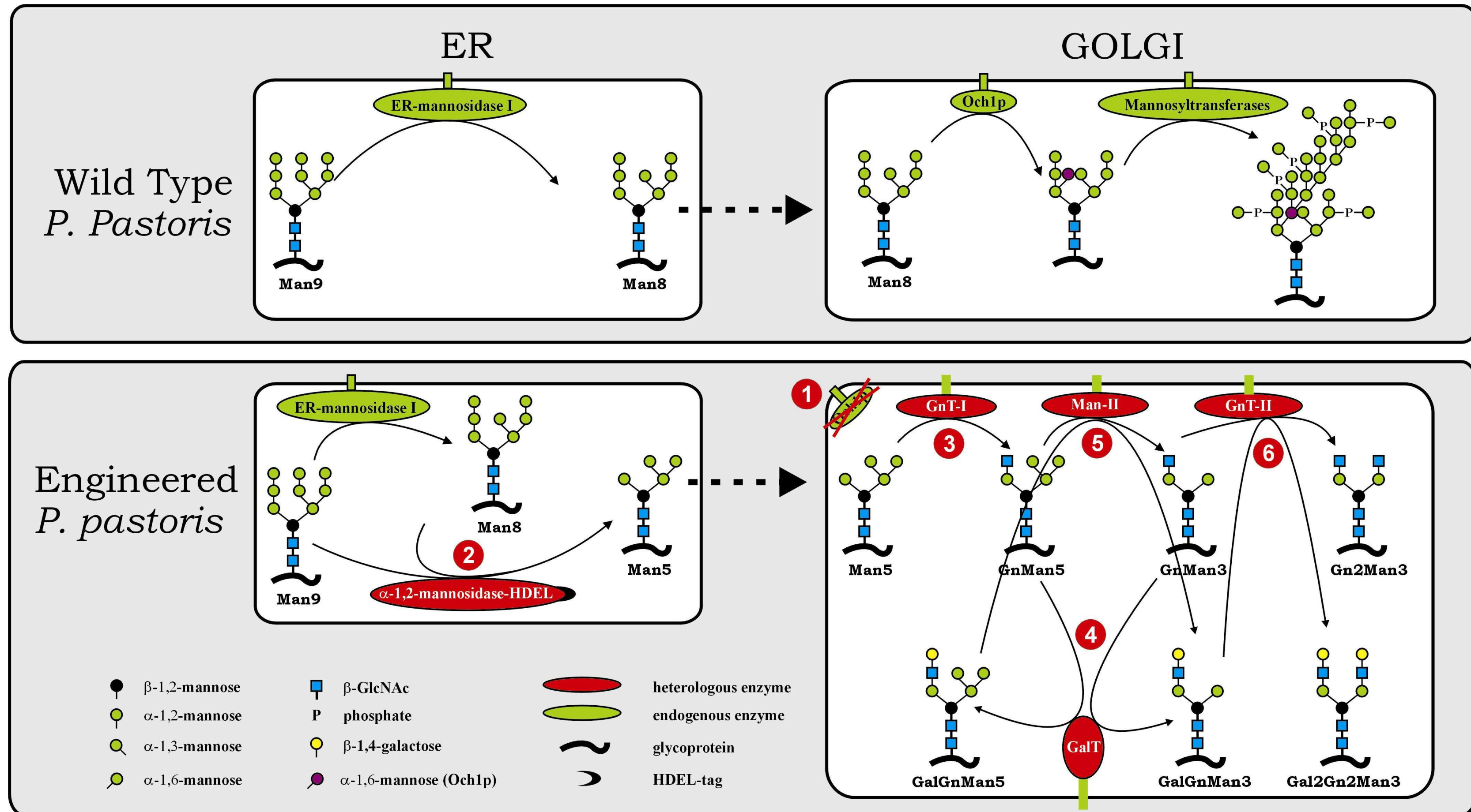


och1 (or other manipulations that remove hypermannosyl-glycans)





Glyco-engineering strategy



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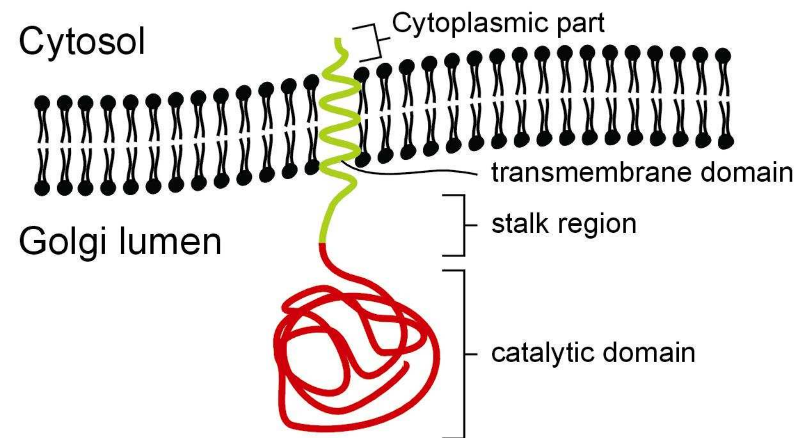
Glyco-engineering strategy

- Fusion protein
  - localization signal of yeast endogenous protein
  - catalytic domain of heterologous enzyme



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Plasmids for humanisation of Pichia N-glycosylation pathway

pGlycoSwitchM5-AOX1-URA3 7526 bp

pGlycoSwitchM5-URA3 7281 bp

pPICHYG MNN2DmManII (AMR2fragment) 9291 bp

pGAPHYGMnn2DmManII (AMR2fragment) 8557 bp

pPICADEKrecoGnTI 7476 bp

pGAPADEKrecoGnTI 6856 bp

pPICARG4MNN2rGnT-II 8154 bp

pGAPARG4MNN2rGnT-II 7707 bp

pGAPNORMnn2SpGal10hGalTr 5827 bp

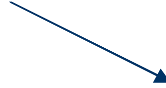
pAOX1NORMnn2SpGal10hGalTr 6275 bp

5 transformation steps to achieve bi-antennary galactosylated N-glycans in *P. pastoris*

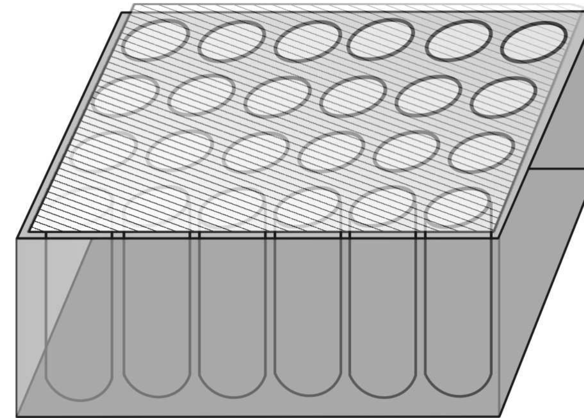
Clonal N-glycan screening after each step

Clone screening

Transformation



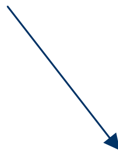
Clonal selection

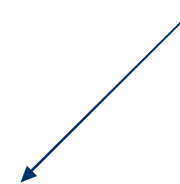


Growth and induction in 24-deepwell with gas-permeable membrane, mini-fed batch

Cells: extraction of hot buffer-solubilizable, cold MeOH-precipitatable compounds

Culture medium





N-glycan profiling after dot blotting on PVDF in 96-well plate

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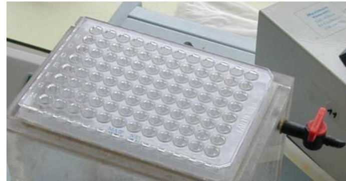
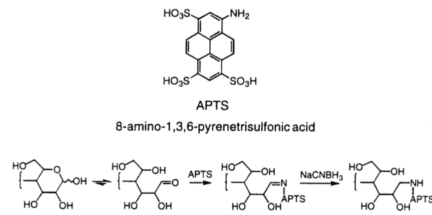
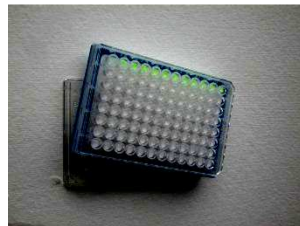
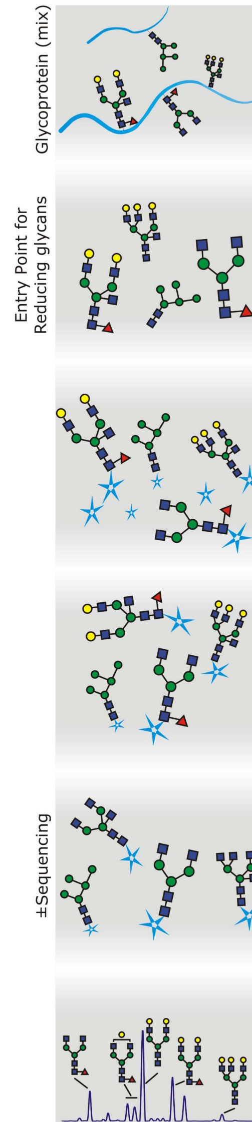
Glycan analysis

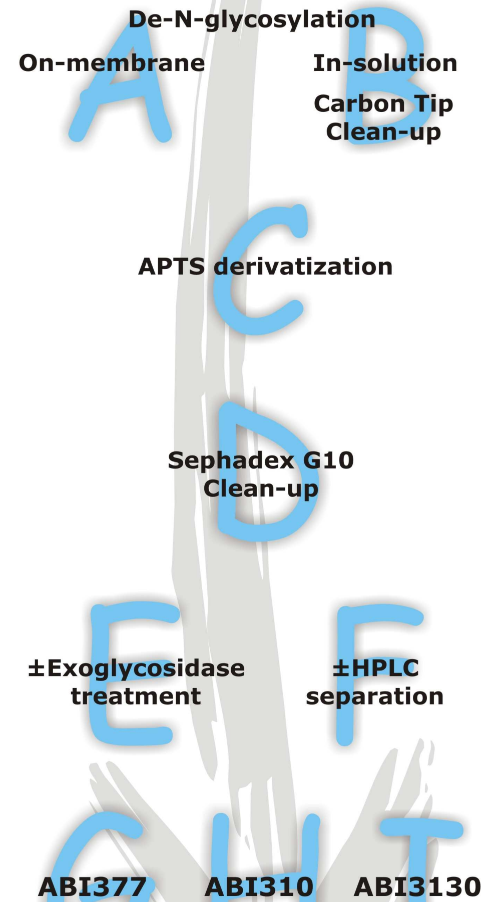






LAROY et al. FIGURE 1





Important clonal variation upon transformation of glycan engineering constructs.

Thus: multiple clones must be glyco-profiled rapidly.

Glucose Units

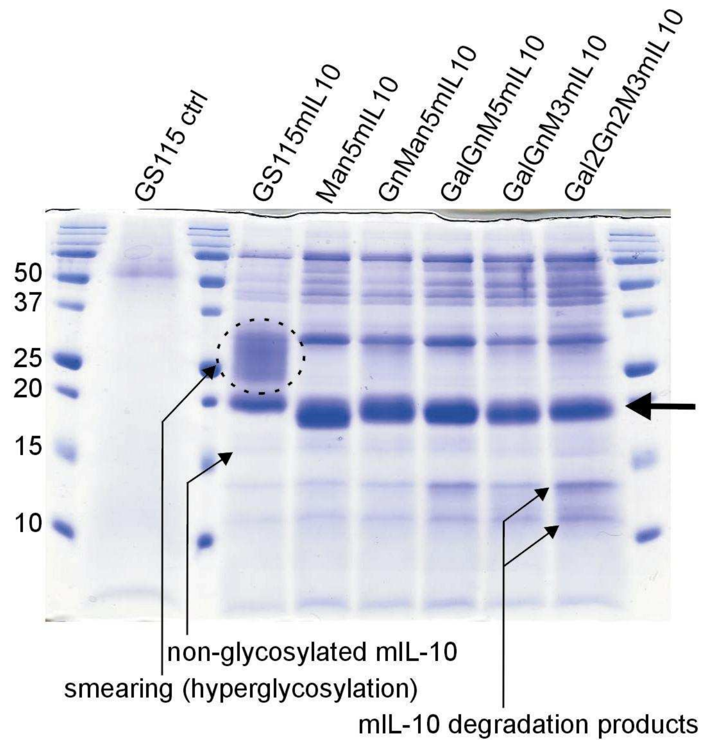
5

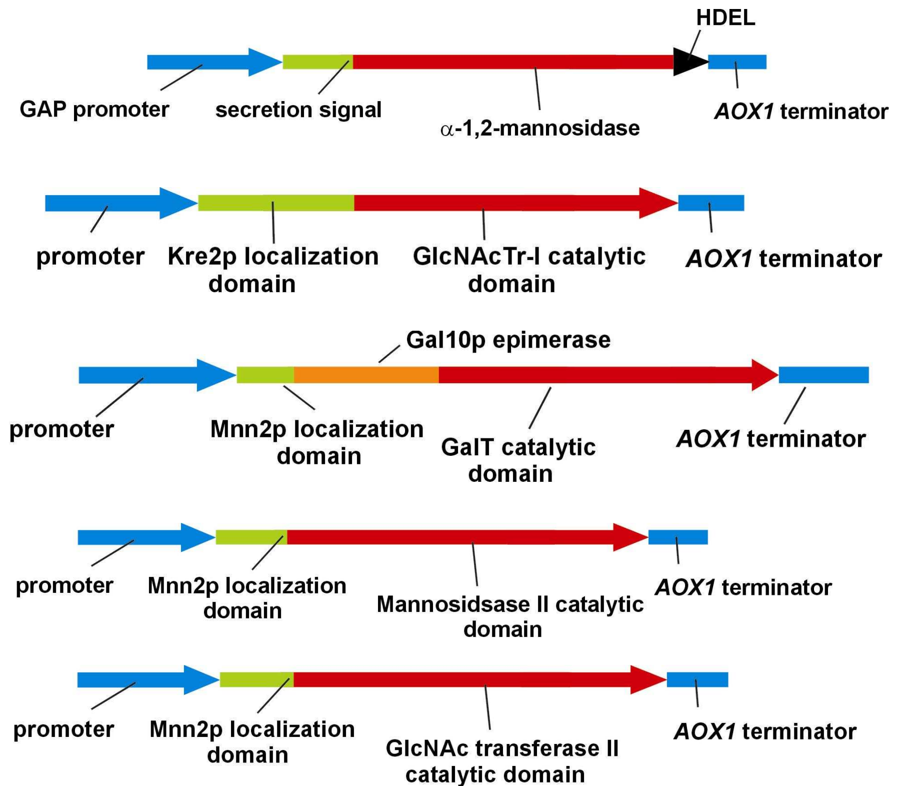
15

Relative Fluorescence Units

1000
0
800
0
410
0
260
0
360
0
440
0
240
0
330
0
270
0
600
0
420
0
230
0
380
0

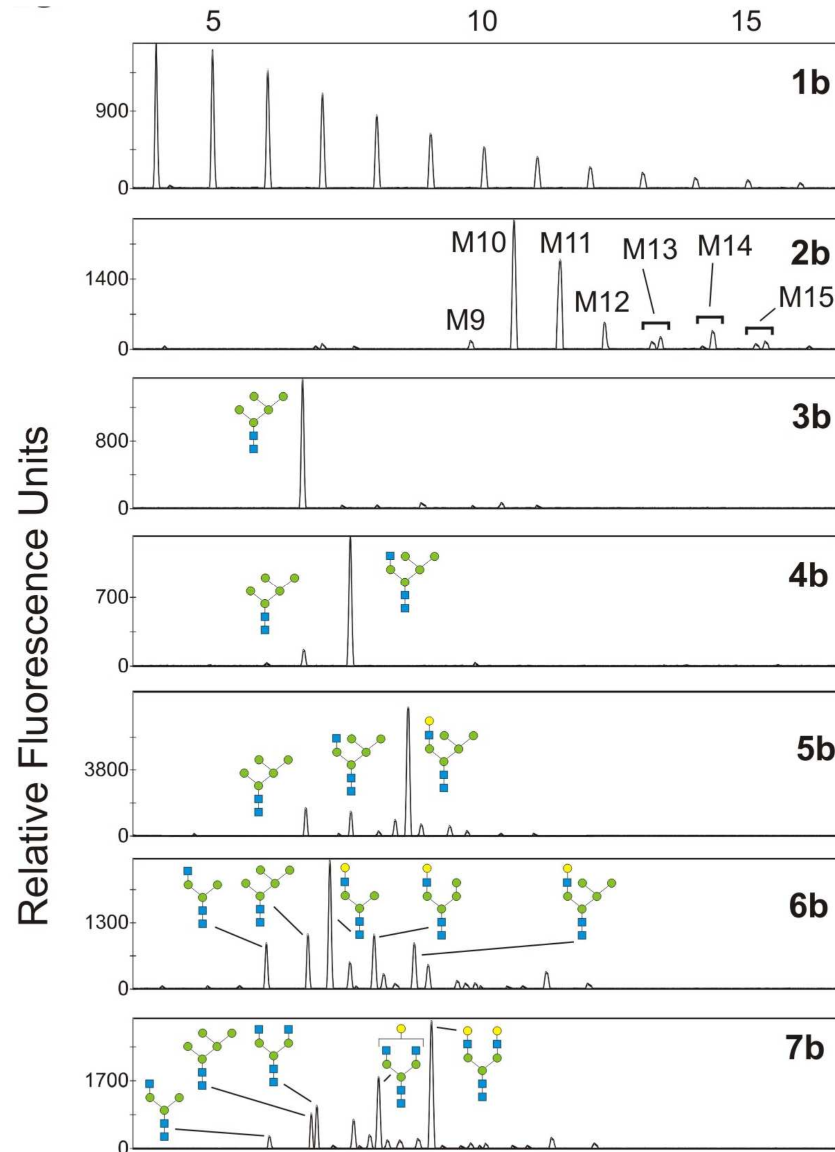
Case I: mIL10, expression level 5-10 mg/l





mIL-10

From hypermannosylation to complex-type human N-glycans



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What's the effect of all of this on growth?

Strain	Introduced enzyme	% Conversion	Doubling time (hours)	Maximum growth rate μ (h^{-1})
GS115 (*his4*)	None	NA	2.40	0.0048
GS115mIL-10	None	NA	2.36	0.0049
M5mIL-10	Man-I	ND[1]	2.31	0.0050
GnM5mIL-10	GnT-I	89.5%	2.51	0.0046
GalGnM5mIL-10	GalT	84.5%	2.82	0.0041
GalGnM3mIL-10	Man-II	90.8%	3.61	0.0032
Gal2Gn2M3mIL-10	GnT-II	95.5%	4.62	0.0025

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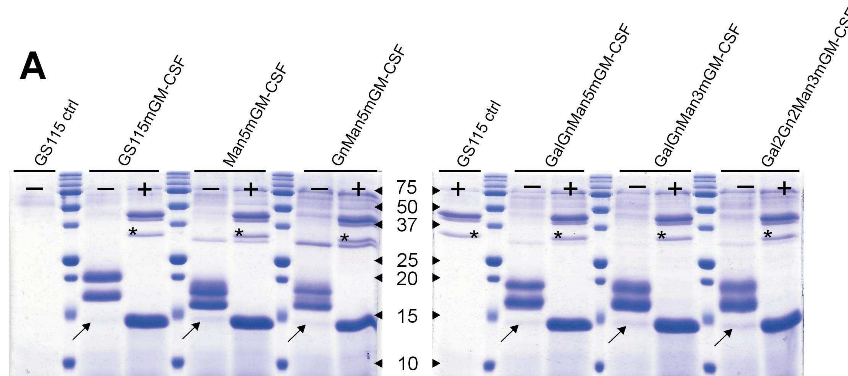
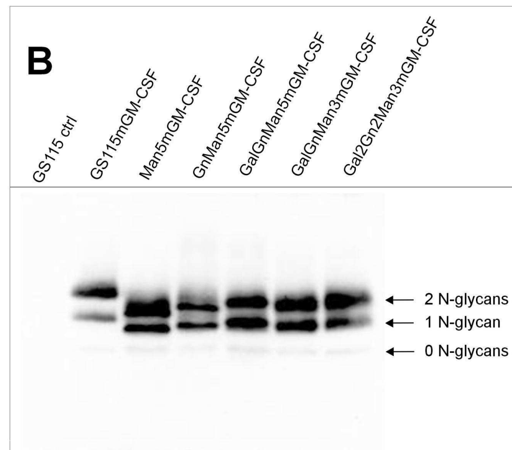
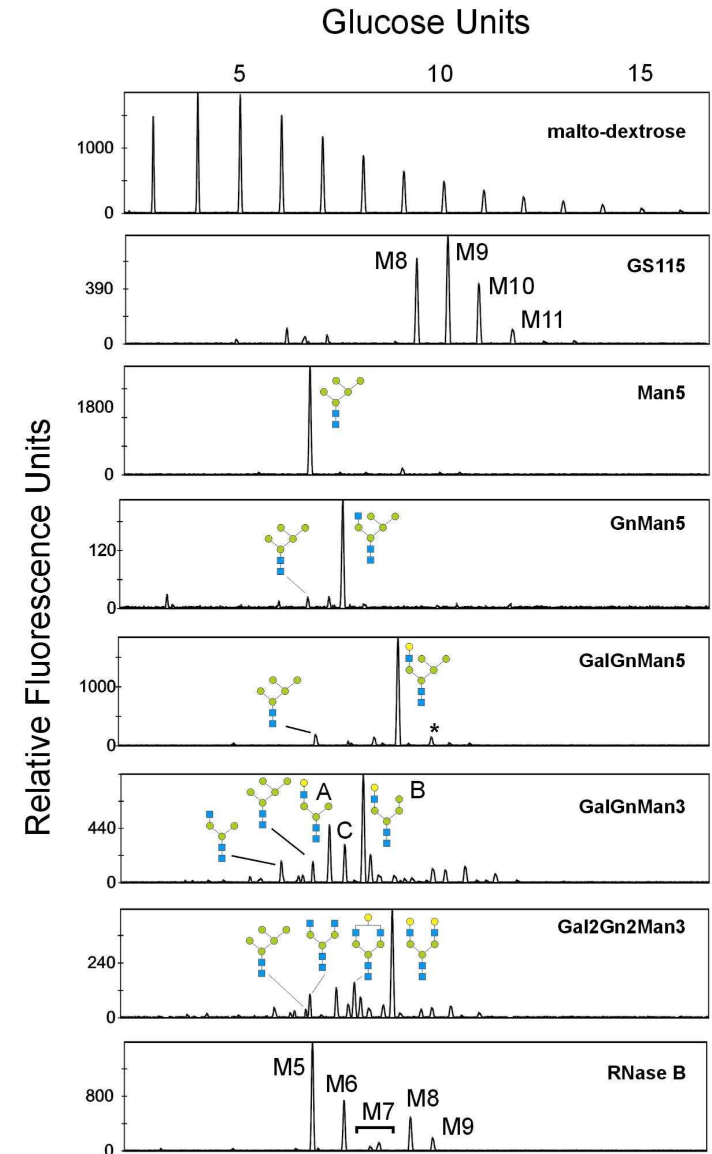
mGM-CSF

How about highly expressed proteins?

- Expression level: 200 mg/l in shake flasks
- 2 N-sites







Read all about it:

PROTOCOL

Engineering complex-type N-glycosylation in *Pichia pastoris* using GlycoSwitch technology

Pieter P Jacobs[1,2], Steven Geysens[1,2], Wouter Vervecken[1,2], Roland Contreras[1,2] & Nico Callewaert[1,3]

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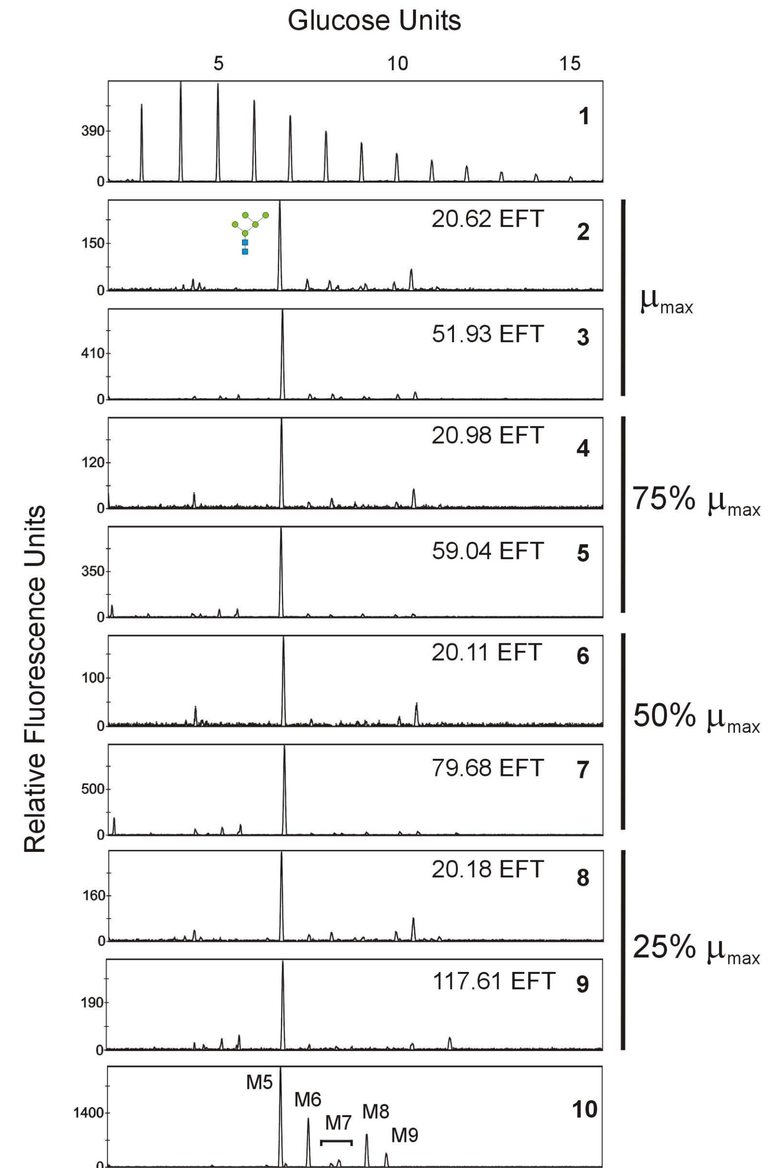
Here we provide a protocol for engineering the N-glycosylation pathway of the yeast *Pichia pastoris*. The general strategy consists of the disruption of an endogenous glycosyltransferase gene (*OCH1*) and the stepwise introduction of heterologous glycosylation enzymes. Each engineering step results in the introduction of one glycosidase or glycosyltransferase activity into the *Pichia* endoplasmic reticulum or Golgi complex and consists of a number of stages: transformation with the appropriate GlycoSwitch vector, small-scale cultivation of a number of transformants, sugar analysis and heterologous protein expression analysis. If desired, the resulting clone can be further engineered by repeating the procedure with the next GlycoSwitch vector. Each engineering step takes ~3 weeks. The conversion of any wild-type *Pichia* strain into a strain that modifies its glycoproteins with $Gal_2GlcNAc_2Man_3GlcNAc_2$ *N*-glycans requires the introduction of five GlycoSwitch vectors. Three examples of the full engineering procedure are provided to illustrate the results that can be expected.

GM-CSF

Fermentation study

- Collaboration with Drs. M. Meagher and Mehmet Inan
- Man5 mGM-CSF strain
- Growth at different growth rates
- N-glycan analysis: OK
- mGM-CSF yields
  - Highest yields at 50% μ_{max}
  - ~1.2 g/l



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Future perspectives

- Fully defining upscaling parameters, cell banking procedures etc.
- Optimizing other post-translational modifications
- Utilizing the system to produce biopharmaceuticals with novel properties

VIB

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- Research Corporation Technologies: co-funding *Pichia* work
- FWO, IWT, VIB
- Michael Meagher, Mehmet Inan
- Pieter Jacobs, Steven Geysens, Roland Contreras.