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Development of the filamentous fungus *Myceliophthora thermophila* C1 into a next-generation therapeutic protein production system

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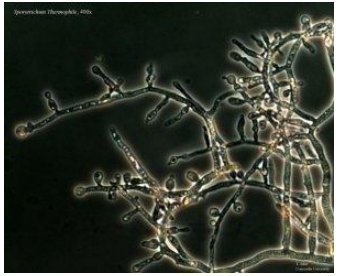
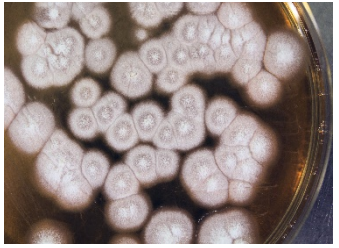
Myceliophthora thermophila (Thermotelomyces heterothallica)

- Thermophilic fungus originally isolated from alkaline soil in Russia
- Designated earlier as *Chrysosporium lucknowense* and currently as *Myceliophthora thermophila*
- Isolated for its ability to produce neutral/alkaline cellulases for use in textile applications
- Dyadic Announced Successful Completion of Sequencing of C1 Fungal Genome (May/2005)
- Genome sequenced 2011 by JGI (Nat. Biotechnol. 2011 Oct 2;29(10):922-7)





- **Dyadic International Inc. has developed an industrially proven expression system based on the fungus *Myceliophthora thermophila*, C1 Expression System**
  - Improved production strains with unique morphology and low viscosity
  - C1 received a Generally Recognized As Safe (GRAS) designation from the FDA, with no viruses detected, no mycoplasmas, and no detectable mycotoxins (FDA, 2009)
  - Highest production level of enzymes >120 g/L
  - Highest production level of an individual recombinant enzyme 80 g/L

DYADIC®

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Our goal

To further develop C1 into efficient gene expression system of biologic vaccines and drugs, to help speed up the development, lower production costs and improve the performance at flexible commercial scales.

Efficient Expression

- Library of promoters, carrier proteins, signal sequences and terminators
- Synthetic Expression System (SES)
- Dual vectors
- Split-marker technology
- Marker recycling
- Site-specific or random integration

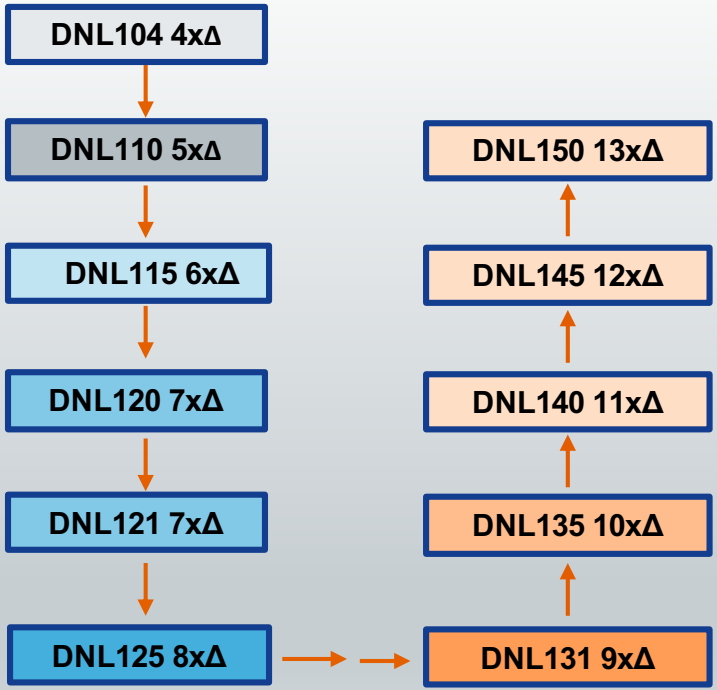
Reducing Proteolytic Activity

- Identification of key proteases
- Deletion of protease genes
- Characterization and utilization of protease deletion strains

Glycoengineering

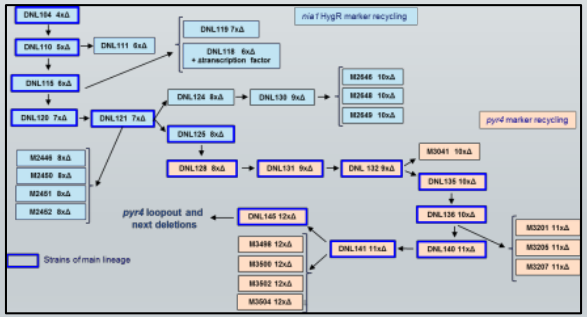
- Generation of humanized protein-glycan structures
- Engineering a G0-glycan producing C1

C1 lineage of protease deficient strains



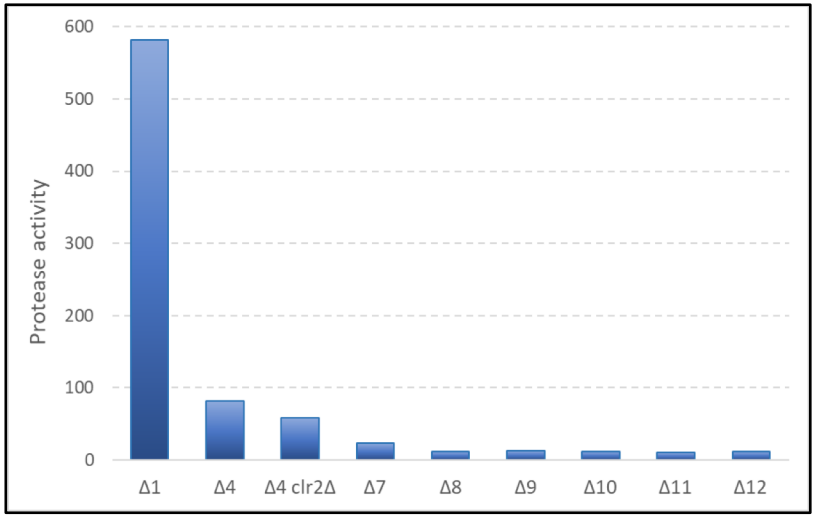
Systematic deletion of protease genes based on:

- Isolation and identification of extracellular proteases
- C1 protease library in *Pichia pastoris*
  - More than 50 proteases
- Effect of different protease inhibitors on protease activity
- mRNA sequencing data
- Protease gene annotation

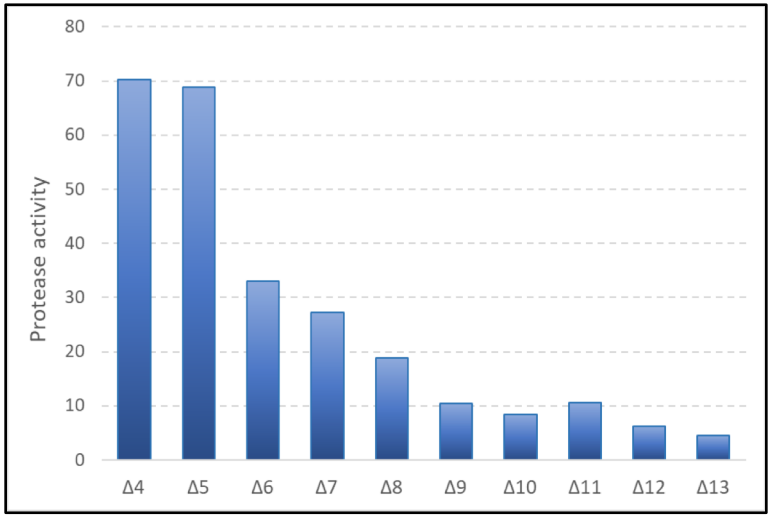


Total extracellular protease activity is greatly reduced in C1 protease deficient strains

- Direct fluoresence-based assay with casein substrate
- The activity of the 12xΔ strain is reduced over 50-fold compared to the 1xΔ strain (fermentation cultures)
- The activity of the 13xΔ strain is reduced over 15-fold compared to the 4xΔ strain (MTP cultures)



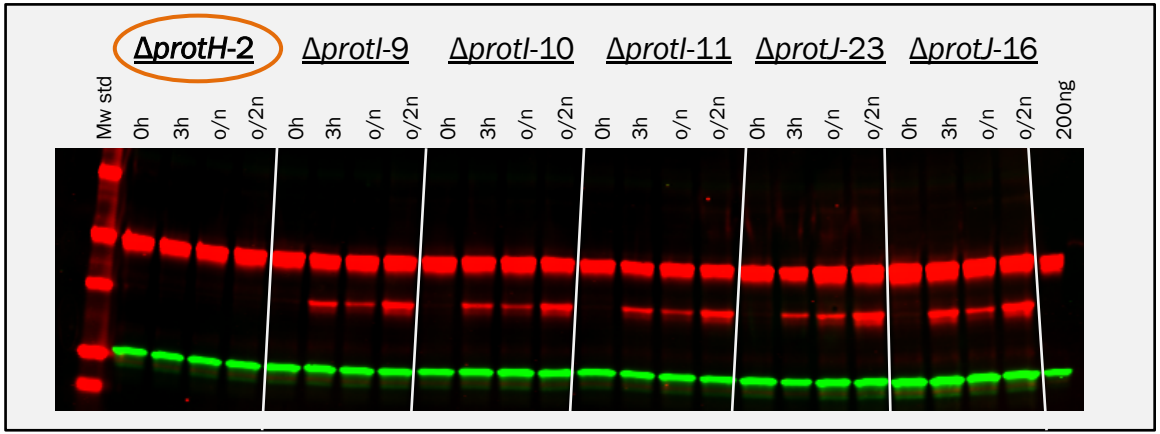
Fermentation culture



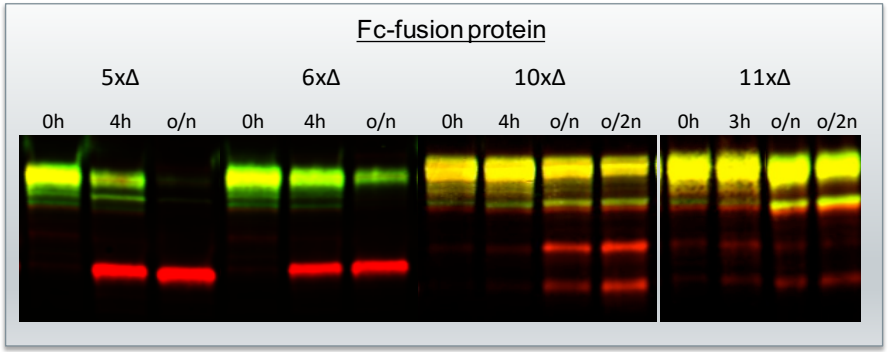
MTP culture

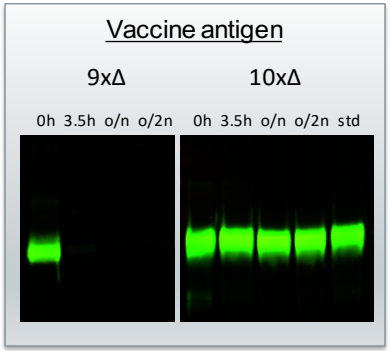
Protease deletions are improving stability of target proteins

Target proteins were spiked into the culture supernatants of the different protease deletion strains



ΔprotH has positive effect on stability





Increased stability with more advanced strains

Fc-fusion protein:

Yellow full-length
Red degradation product

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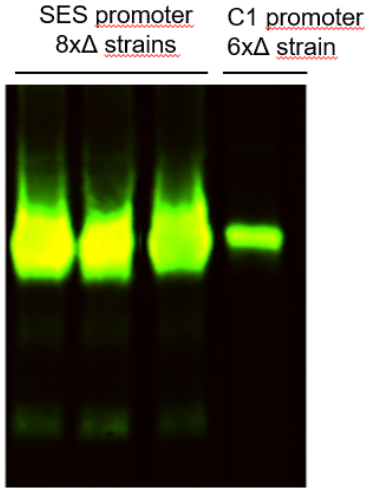
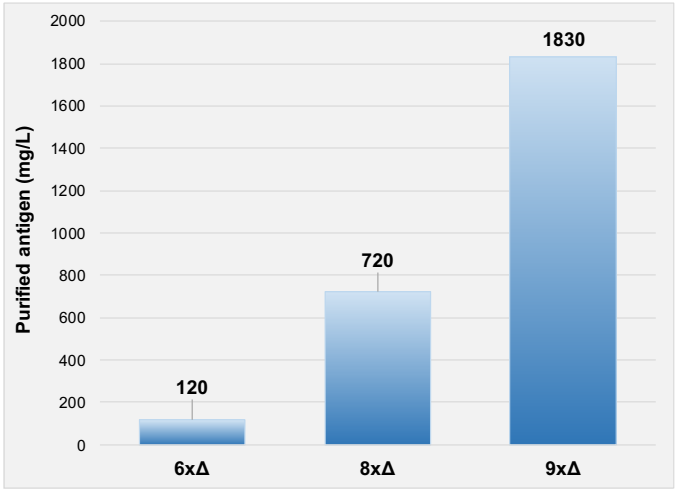
Production of Biologics in C1 - Success in expressing high level of ZAPI antigen

- Schmallenberg virus antigen coupled with Spytag - difficult-to-express protein in animal cells and microbial systems
- First strain had a native C1 promoter in 6xΔ protease strain
- Using synthetic promoter (SES) for expression, higher copy number and 8x protease deletion strain increased production six-fold
- Development of the protease deficient strain and process conditions increased the amount even further
- The C1 produced protein formed efficiently nanoparticles with Spycatcher and showed good immunogenicity in cattle





- The new strain using SES promoter system in improved protease deletion background **significantly increased the production and stability of the target antigen**
- **Up to 1.8 g/L** was purified by affinity chromatography

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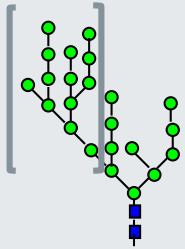
Advantage of C1 over Yeast and CHO

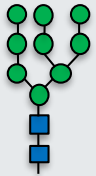
- C1 glycan structure is more mammalian like than typically in yeasts
  - The native C1 glycans are mostly high mannose type (Man3-Man9) including some hybrid glycans
  - Less engineering steps needed for C1
  - Stable genome - defined glycan structure is stable from culture to culture and batch to batch
- We aim at defined mammalian glycan forms G0, G0F, G2 and G2F in our glycoengineering efforts

Typical Yeast Glycan Structure



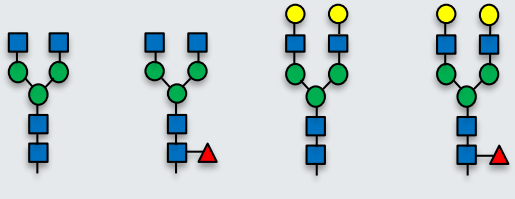
Man_{30-50}

Dyadic C1 Glycan Structure



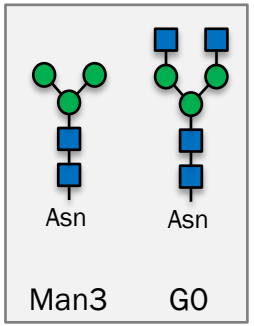
Man_{3-9}

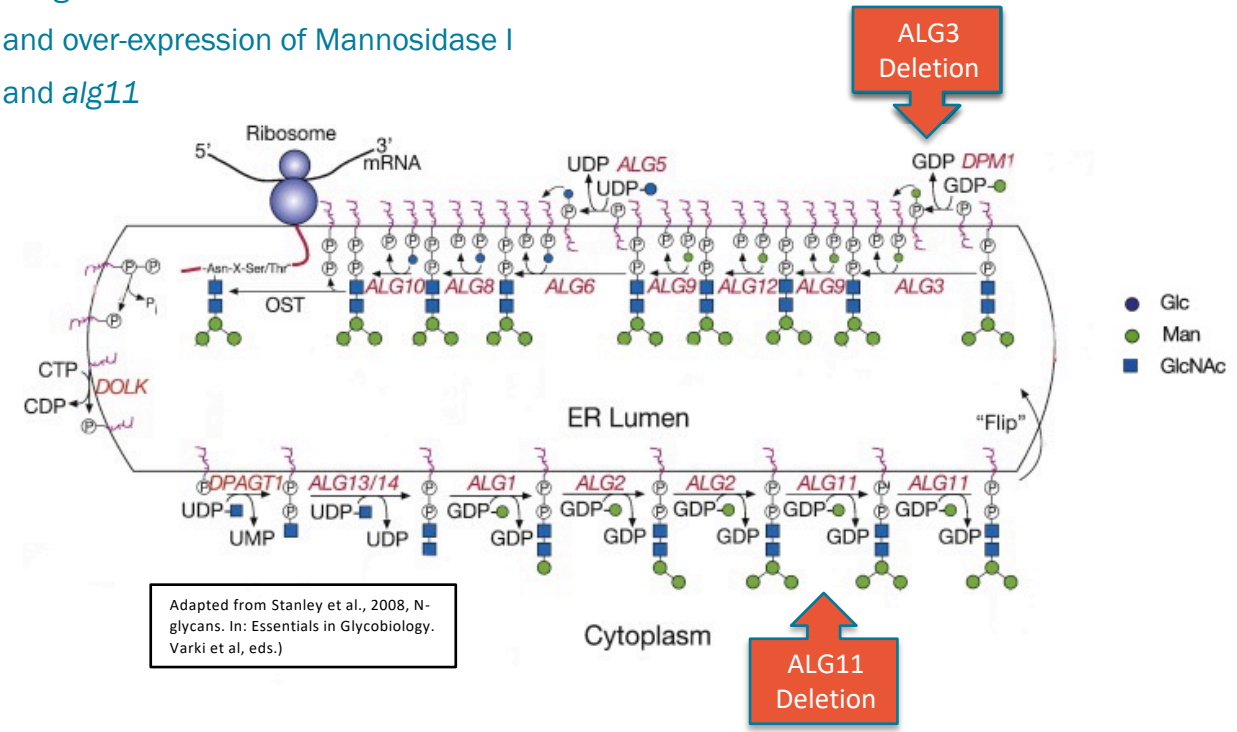
Targeted Mammalian Glycoform Structures



Two main approaches to gain high Man3

- Man3 is the important precursor of G0 glycans
- Two approaches for high Man3:
  - Deletion of *alg3* and over-expression of Mannosidase I
  - Deletion of *alg3* and *alg11*





Adapted from Stanley et al., 2008, N-glycans. In: Essentials in Glycobiology. Varki et al, eds.)

The effect of *alg3* deletion in C1

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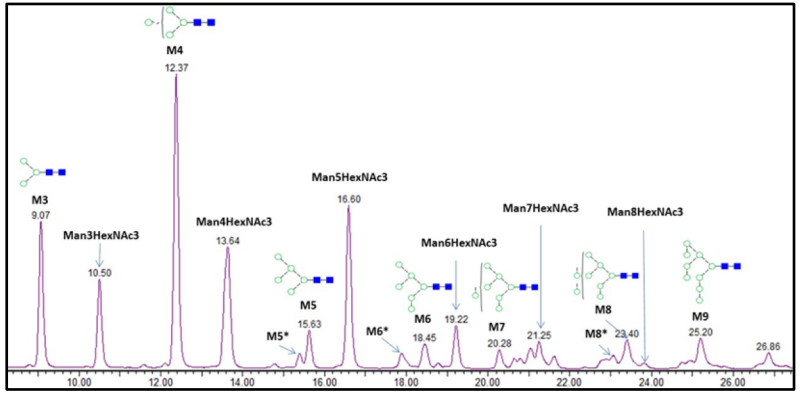
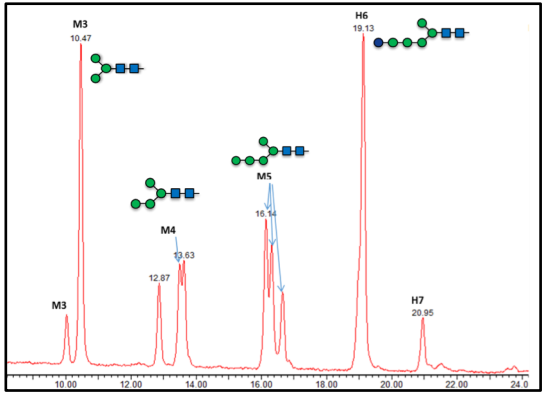
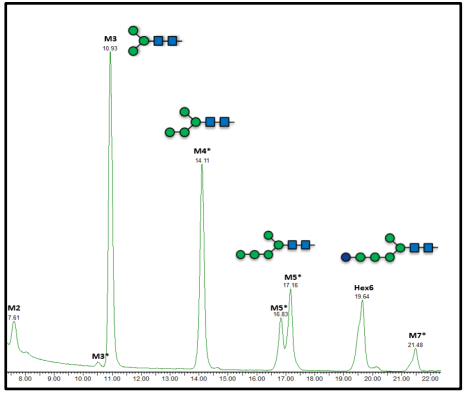
Wild type strain
- native proteins



alg3 deletion strain
– native proteins



alg3 deletion strain
– Nivolumab



- The *alg3* gene was deleted by replacing it with a marker gene
- Glycans on native proteins and on purified Nivolumab from shake flask cultures were analyzed
- **Deletion of *alg3* gene significantly simplified the glycan pattern both on native proteins and on Nivolumab**
  - All higher Mw glycans and hybrid glycans were omitted
  - Substantial amounts of Hex6, Man5 and Man4 glycans remained
- Further engineering along this strategy is in progress

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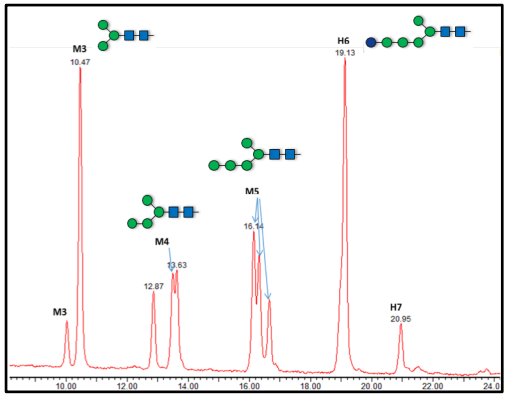
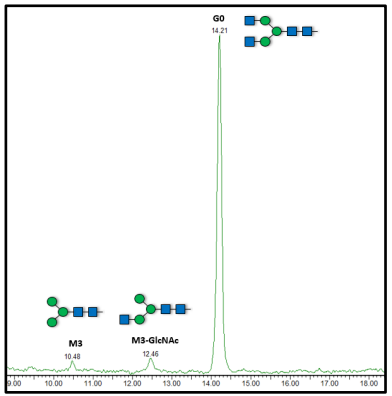
Applying *alg3-alg11* deletion strategy to produce G0 glycans

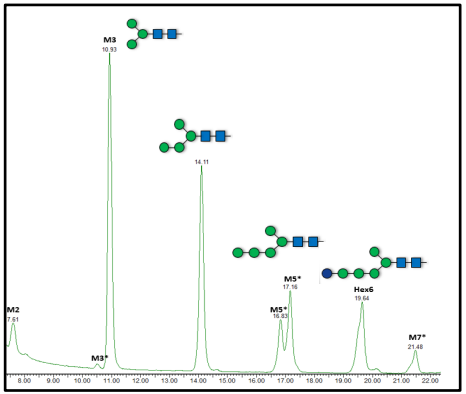
alg3 deletion strain – native proteins



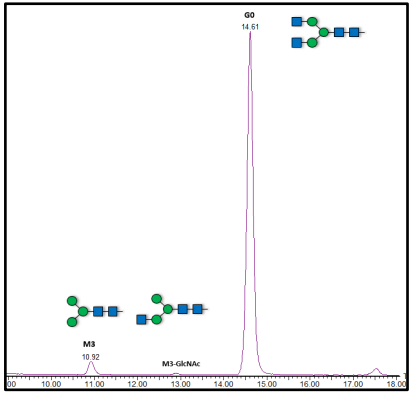
alg3-alg11 deletion with GNT1+2 - native proteins



alg3 deletion strain – Nivolumab



alg3-alg11 deletion with GNT1+2 - Nivolumab



- The *alg11* gene was deleted from an *alg3* deletion strain. Simultaneously heterologous GNT1 and GNT2 were expressed from the *alg11* locus.
- **Glycan levels with 94% of G0** have been reached. In addition to G0, only Man3 and GlcNAcMan3 remain in the glycan pattern.
- G0 glycan levels of over 95% have been reached with this strategy on native proteins.

Glycan	Relative amount
M3	3.64%
M3-GlcNAc	0.45%
G0	94.11%

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The effect of flippase expression

No flippase overexpression

Glycan	Area (Abundance)	Relative amount
M3	69.34	7.05%
G0	914.85	92.95%
Sum of peaks	984.19	100%

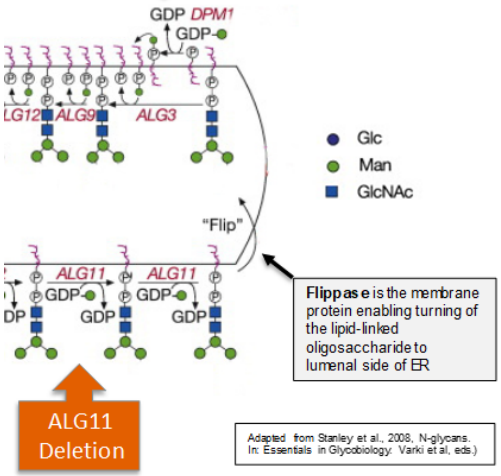
Expression of flippase 1

Glycan	Area (Abundance)	Relative amount
M3	116.77	2.84%
M3-GlcNAc	95.17	2.32%
G0	3894.9	94.84%
Sum of peaks	4106.84	100%

Expression of flippase 2

Glycan	Area (Abundance)	Relative amount
M3	259.86	3.00%
M3-GlcNAc	248.09	2.86%
G0	8162.84	94.14%
Sum of peaks	8670.79	100%

- Strains were made where two different flippase variants were expressed in addition to GNT1 and GNT2 in *alg3-alg11* deletion background
- The total glycosylation level (sum of peaks) in shake flask cultures increased ~4x (flippase 1) or ~9x (flippase 2)
- The glycan pattern remained good with 93-95% G0 glycans on native proteins



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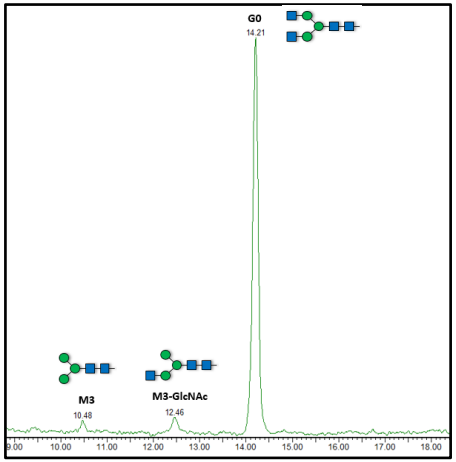
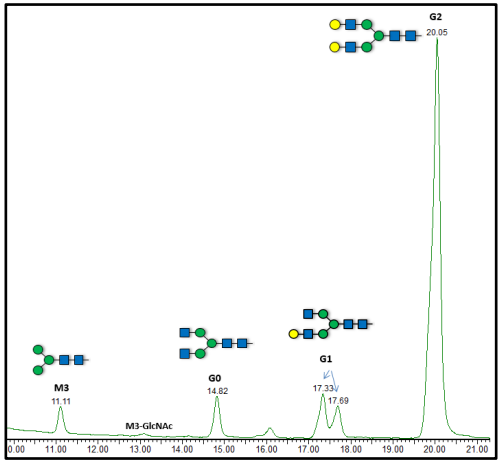
Towards G2 glycans on target proteins

alg3-alg11 deletion with GNT1+2
- native proteins



G0 strain with GalT
- native proteins



Glycan	Relative amount
M3	3.16%
M3-GlcNAc	0.42%
G0	5.81%
G1	6.97%
G1	5.53%
G2	76.38%

- The human GalT1 gene was expressed in a strain with G0 glycan machinery
- Glycans on native proteins from shake flask culture are shown
- **G2 glycan levels over 76%** have been reached with this strategy. In addition to G2, over 10% of G1 glycan forms and some residual G0 and Man3 were detected.

Summary

- Myceliophthora thermophila C1 is an industrialized protein production host that is now developed for therapeutic protein manufacture – with several large biopharma companies entering into collaborations.
- We have identified critical proteases to deal with for therapeutic protein production enhancement and enabled a very significant reduction of the protease load in the production strains.
- Our glycoengineering program aims at high proportions of human glycoforms G0, G2, FG0 and FG2. Excellent G0 levels have been reached through alg3-alg11 deletion strategy.
- Monoclonal antibodies have been produced in C1 with levels reaching 24.5 g/l and rates up to 3.5 g/l/day. Fab fragments have been expressed at levels up to 14.5 g/l and Fc-fusion proteins up to 13.2 g/l in a 7-day process.
- Difficult-to-express proteins have been produced in C1 at superior levels as compared with other production systems.

Product	Production level g/L
Mabs	24.5
Fabs	14.5
Fc-fusion proteins	13.2
Difficult-to-express	
Bispecific antibodies	1.0
Trispecific nanobody	6.6
Viral antigens	1.8
VLPs (extracellular prod.)	2.2
Bacterial vaccine protein	6.0

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THANK YOU!

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