Isotope Labeling of Human Blood Group B Galactosyltransferase for NMR Experiments

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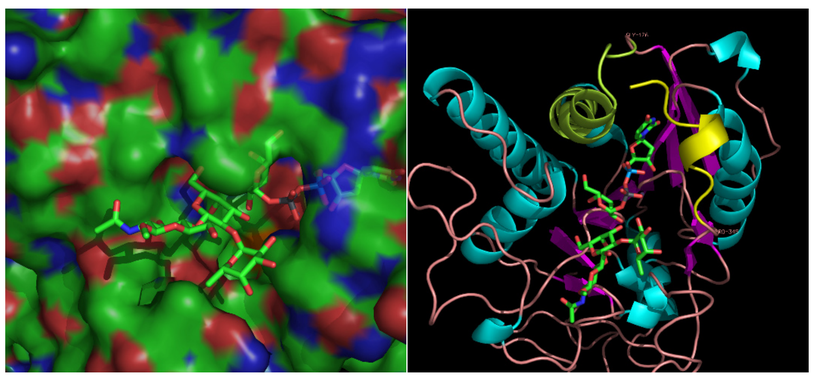
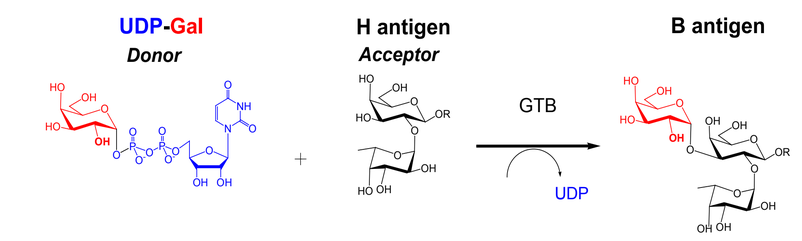
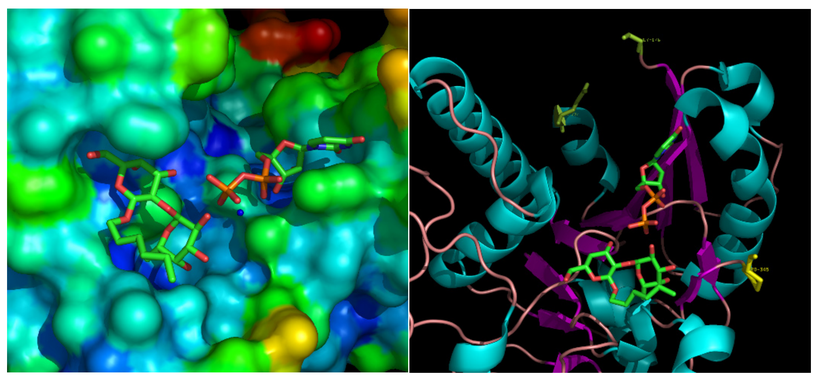
Human Blood Group B Galactosyltransferase (GTB)







GTB is a retaining glycosyltransferase that transfers galactose from UDP-Gal to acceptor saccharides (center). The crystal structure (right panel, [2]) does not reveal the position of the galactose residue and is missing a loop (residues 177-196) and the C-terminal end (residues 346-354). A homology model (left panel, [4]) shows that UDP-Gal should be completely buried in the donor binding pocket.

Open Questions

- **What is the role of the C-terminal loop for the catalytic activity of GTB? How does the catalytic residue Glu303 interact with the donor substrate?**
- **In order to address these questions by NMR, uniformly or selectively ^{13}C,^{2}H,^{15}N- (CDN)-isotope labeled GTB has to be used.**
- **Strategies for selective labeling of important amino acids in the catalytic site and in the loop are required.**
- **The labeling techniques have to yield high expression rates of GTB to minimize the costs.**

New Achievments

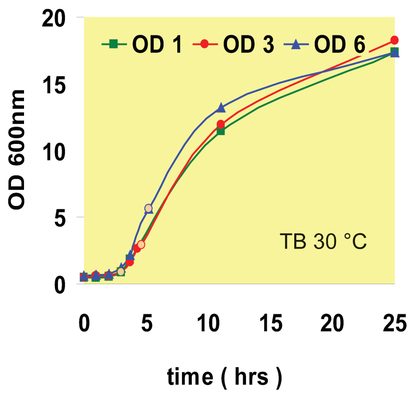
Here we describe for the first time:

- **Expression of uniformly ^{2}H,^{15}N-labeled GTB (DN-GTB) in high yield with a low cost labeling technique [3].**
- **Expression of uniformly ^{15}N-labeled GTB (N-GTB) in high yield.**
- **700 MHz NMR (cryo probe): ^{1}H,^{15}N HSQC and ^{1}H,^{15}N TROSY spectra of these GTB-samples.**

Optimization of GTB Expression in *E.coli*

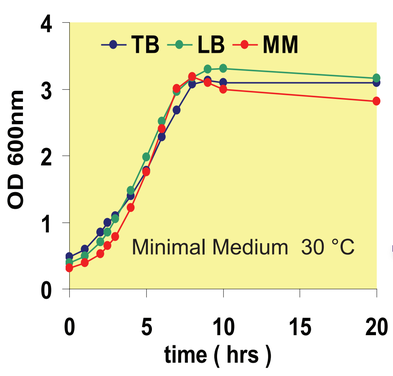
The GTB clone produces in TB full medium as well as in minimal medium 4 mg GTB / 1 g pellet. With standard protocolls for protein expression in minimal medium about 30% of the cell mass, compared to that in full medium, are produced, resulting in a lower yield of recombinant protein. To get high yields of protein an optimized clone as well as optimized conditions for protein expression in full medium are necessary to get the same yield of protein in in minimal medium as in full medium.

Induction



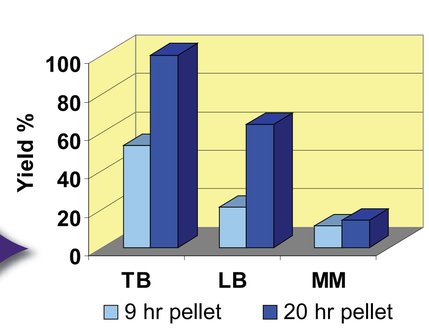
- The expression on rich medium should be optimized.
- Expression should not depend on OD of induction. The yield of GTB did not decrease until induction at OD 4

Starter culture

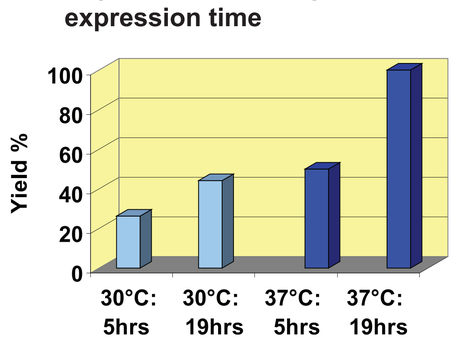


- The yield of recombinant protein in minimal medium depends on the starter culture used and on the optimized supplements to the minimal medium. The growth curves in minimal medium do not show any difference although different starter cultures were used.

Yield in minimal medium

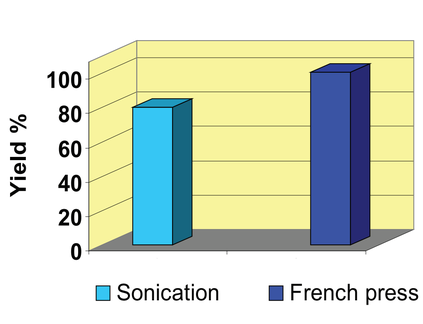


Dependence on temperature and expression time



- The temperature for expression in minimal medium can be totally different from the temperature in full medium.
- The time of expression has to be checked carefully. Some clones produce more than 60% of recombinant protein during the stationary phase.
- French Press lyses gives about 20% more yield of protein than ultrasonication.

Yield after lysis

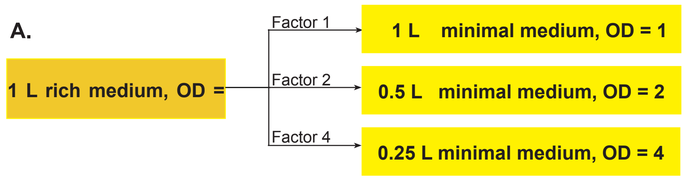


Labeling Strategy

A: The labeling technique after Marley et al. [3]:

• High yields of labeled protein, significant reduction in costs for labeled compounds, isotopic incorporation >90%.
• Cell mass is generated in unlabeled rich media allowing rapid growth to high cell densities (but cells have to be still in the log-phase!).
• The cell pellet from unlabeled rich medium is used to inoculate isotopically defined labeled minimal medium a higher cell densities, optimized for maximal protein expression.
• The labeling technique can be adapted to all uniform CDN-isotope labeling. No adaption of bacteria to D_2O is necessary.

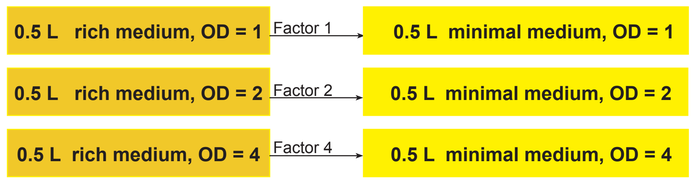
A.

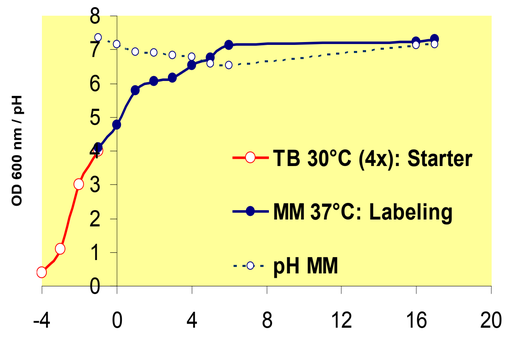


B: Modified labeling protocoll

• When the concentration factor after Marley et al. is 4 and a high concentration of the labeled protein is needed, it can be difficult to manage the high volumes of rich medium with an OD of 1 to produce the cell mass needed for inoculation in minimal medium.
• If the clone accepts an induction at OD 4 without any loss in yield it is possible to inoculate the same volume of minimal medium with the bacterial pellet at an OD

B.



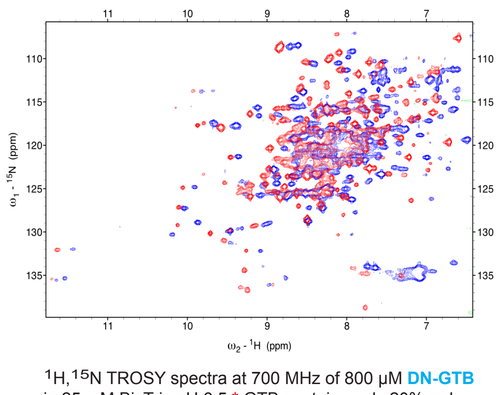


• For the production of DN-GTB the pellet from 500 ml TB medium at an OD of 4 is used to inoculate 500 ml DN-minimal medium (concentration factor 4x).
• The concentration of the antibiotic is doubled to increase the selection pressure in D_2O.
• Expression in minimal medium is performed at 37°C, after one hour growing the bacteria are induced. Seven hours after induction the temperature is set to 30°C and protein expression is stopped after 16 hours.

DN-minimal medium composition	final conc.	important
KD_2PO_4/Na_2DPO_4	50 mM	
NaCl	0.05%	
$^{15}ND_4Cl$	0.10%	
D7-glucose	0.40%	
D8-glycerol	0.40%	++
thiamin	20 µg/ml	+
$MgSO_4$	4 mM	+++
$MgCl_2$	1 mM	+
$CaCl_2$	0.1 mM	
$FeSO_4$, $CoCl_2$	1 µM	+
$ZnCl_2$	0.2 mM	+
vitamin solution BME, Sigma	1x	++
DN-uniform labeled full medium	10%	++
Amp	200 µg/ml	+
pH	7.2 - 7.5	+
temperature	37°C	+++
time of expression	18 hrs	+++
air supply		++

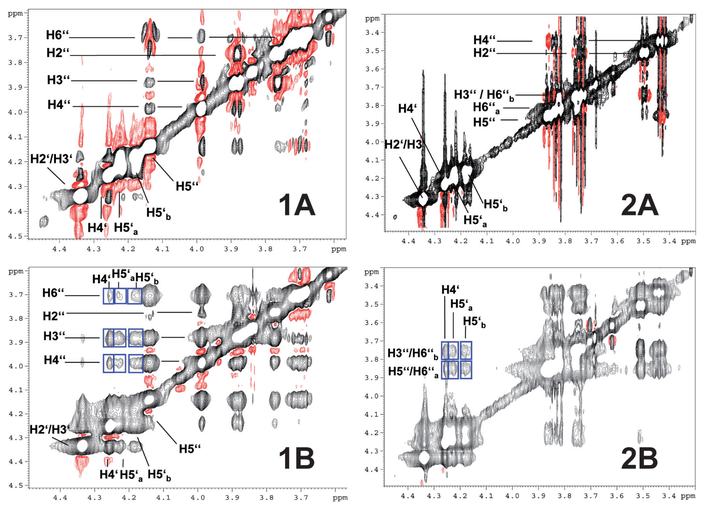
Yield and costs of labeled GTB				
Medium	TB	N-MM	DN-MM	Standard DN-labeling
Volume	1000 ml	1000 ml	1000 ml	1000 ml
€ / L	40 €	60 €	2,800 €	2,800 €
mg / L	100 mg	100 mg	120 mg	28 mg
€ / mg	0.40 €	0.60 €	23 €	100 €

NMR Experiments



^{1}H,^{15}N TROSY spectra at 700 MHz of 800 µM DN-GTB in 25 mM BisTris pH 6.5* GTB contains only 20% α–helix and 20% β–sheet. Red: 50°C, blue: 25°C.

• In order to eliminate spin diffusion via protein protons we used perdeuterated DN-GTB for the transferred NOE experiments. From MALDI-TOF spectra we estimated the extent of deuteration to be larger than 95%.
• The NOESY spectra obtained for UDP-Gal and UDP-Glc in the presence of perdeuterated DN-GTB at 700 MHz unambiguously show negative NOE cross peaks.
• It is well documented that bivalent cations such as Mg^{2+} and Mn^{2+} have a significant impact on the function of glycosyltransferases. With NMR we have directly observed the conformational transitions that occur upon binding of the donor ligands, UDP-Gal and UDP-Glc to GTB in the presence of Mg^{2+} [5].
• NOEs across the pyrophosphate bridge (blue squares) are only observed for the bound states of UDP-Gal and UDP-Glc.
• In the absence of Mg^{2+} ions UDP-Gal and UDP-Glc are bound to GTB but no NOEs across the pyrophosphate bridge are observed.

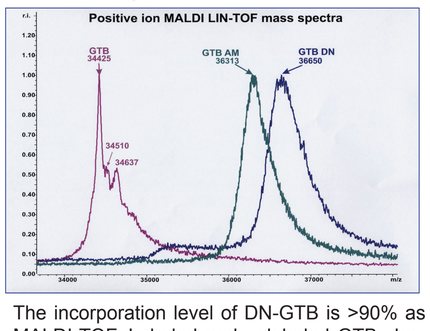


^{1}H-NOESY spectra of a complex of DN-GTB with UDP-Gal (1) and UDP-Glc (2) in the absence (A) and in the presence (B) of Mg^{2+} ions in 25 mM BisTris, pH 6.5* at 700 MHz. The protein : ligand ratio is 1:2.

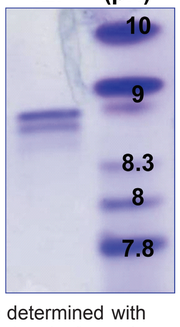
* 25 mM BisTris, pH 6.5 (RT), 50 mM NaCl, 10 mM $MgCl_2$, 5 mM 2ME

Protein Analysis

MALDI-TOF



IEF (pH)



The incorporation level of DN-GTB is >90% as determined with MALDI-TOF. Labeled and unlabeled GTB show two isoforms in the isoelectrofocussing gel.

References:
[1] M.M. Palcic, N.O.L. Seto, O. Hindsgaul (**2001**) *Transf. Med.* 11, 315-323
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[5] J. Angulo, B. Langpap, A. Blume, T. Biet, B. Meyer, N.R. Krishna, H. Peters, M.M. Palcic, T. Peters (2006), submitted

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