Transformation of mutant *Saccharomyces cerevisiae* strains and establishing a disease model for ALG9-CDG

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BACKGROUND

First described in 1980 by Dr Jaak Jaeken, congenital disorders of glycosylation (CDG) compose a rare group of genetic disorders that result in faulty protein glycosylation as a result of an enzymatic deficiency. Glycosylation is the cellular process of adding sugar chains to proteins. The glycoproteins produced by this multi-step enzymatic glycosylation pathway are necessary for the normal growth and function of cells, tissues and organs. CDG has two types (type I & type II) and many subtypes. Where the enzyme deficiency pertains to the glycosylation pathway associated with the endoplasmic reticulum, this is termed CDG type I (Figure 1). Where the glycosylation deficiency is found further upstream in the golgi apparatus this is termed CDG type II. Each CDG subtype is defined by the specific abnormal glycosylation enzyme that results from a specific genetic abnormality (Table 1). There are many clinical features common to various CDG sub-types such as failure to thrive, developmental delay, hypotonia and seizures, though some subtypes have unique clinical features including liver disease, clotting disorders and immunodeficiency. Approximately 1,000 individuals worldwide have been diagnosed with CDG type I and fifteen subtypes of CDG type I have been identified to date. There have been three cases of ALG9-CDG (CDG-1L) diagnosed internationally and two of these three children are Canadian.[1,2,3] Over the past 30 years, great strides have been made in terms of gaining a greater understanding of the cellular pathophysiology of CDG as well as diagnostic capabilities. With the exception of MPI-CDG, however, there are currently no therapies for this disorder outside of supportive management. Establishing a disease model is critical in order to begin work that focuses on identifying potential therapeutic options. This current research describes the seminal work in Atlantic Canada where efforts are underway to establish disease models for ALG9-CDG using *Saccharomyces cerevisiae* and *Danio rerio*. This presentation describes the progress to date with regards to the *S. cerevisiae* model.

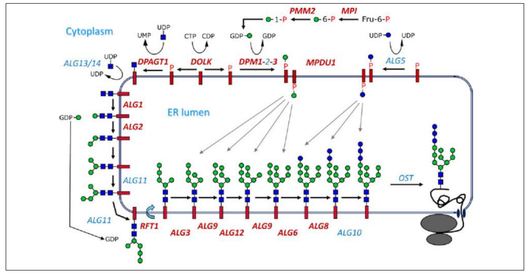


Figure 1. Pathway of dolichol-linked oligosaccharide biosynthesis.[4]

Gene	OMIM[a]	Enzyme	Disorder[b]	Disorder[c]	OMIM[a]	Mutations	Patients
PMM2	601785	Phosphomannomutase 2	CDG-Ia	PMM2-CDG	212065	103	> 800
MPI	154550	Mannose phosphate isomerase	CDG-Ib	MPI-CDG	602579	18	> 25
DPAGT1	191350	GlcNAc-1-P transferase	CDG-Ij	DPAGT1-CDG	608093	3	3
ALG1	605907	Mannosyltransferase 1	CDG-Ik	ALG1-CDG	608540	4	7
ALG2	607905	Mannosyltransferase 2	CDG-Ii	ALG2-CDG	607906	2	1
ALG3	608750	Mannosyltransferase 6	CDG-Id	ALG3-CDG	601110	9	11
ALG9	606941	Mannosyltransferase 7–9	CDG-Il	ALG9-CDG	608776	2	3
ALG12	607144	Mannosyltransferase 8	CDG-Ig	ALG12-CDG	607143	11	8
ALG6	604566	Glucosyltransferase 1	CDG-Ic	ALG6-CDG	603147	20	> 36
ALG8	608103	Glucosyltransferase 2	CDG-Ih	ALG8-CDG	608104	12	9
DOLK	610746	Dolichol kinase	CDG-Im	DOLK-CDG	610768	2	4
DPM1	603503	Dolichol-P mannosyltransferase 1	CDG-Ie	DPM1-CDG	608799	6	8
DPM3	605951	Dolichol-P mannosyltransferase 3	CDG-Io	DPM3-CDG	612937	1	1
MPDU1	604041	Man-P-dolichol utilization defect 1	CDG-If	MPDU1-CDG	609180	5	5
RFT1	611908	RFT1 homolog (*S. cerevisiae*)	CDG-In	RFT1-CDG	612015	5	6

Table 1. Gene defects leading to deficient assembly of dolichol-linked oligosaccharides in CDG type I.[4]

[a]http://www.ncbi.nlm.nih.gov/sites/entrez?db = OMIM.
[b]According to the recommended nomenclature of 1999 [Aebi et al., 1999]. [5]
[c]According to the recommended nomenclature of 2008 [Jaeken et al., 2008]. [6]

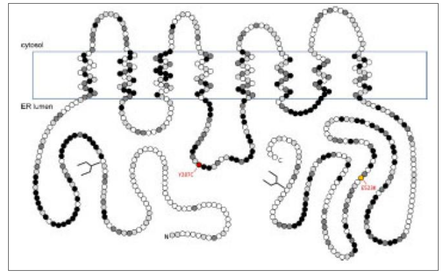


Figure 2. Model of mannosyltransferase 7-9 (ALG9).[4]

METHODS

Yeast Strains: Mutant strains of *S. cerevisiae* derived from the temperature-sensitive, uracil-auxotroph SS328 strain (genotype: Mata ade2-101 ura3-52 his3D200 lys2-801) were obtained. Yeast strains included YG414 and YG415. YG414 (SS328 w/ Dalg9::KanMX) has deletion of the ALG9 gene, which encodes for mannosyltransferase 7-9, the deficient enzyme associated with ALG9-CDG (Figure 2).[7] YG415 (SS328 w/ wbp1-2 Dalg9::kanMX4) lacks the ALG9 gene and also has additional down-regulation of the oligosaccharyl transferase protein.

Plasmids: Mutant yeast strains were transformed with four different plasmids in separate experiments with the yeast/plasmid combinations shown in Table 2. All plasmids used in this study contain uracil synthesis pathways. pALG9 is a derivative of YEp352 and contains a 2.5 kbp *Bgl*II-*Kpn*I fragment of yeast genomic DNA encompassing the *ALG9* ORF plus 0.5 kbp upstream sequence in the *Bam*HI-*Kpn*I sites.[8,9] Plasmid HsALG9 contains human genomic DNA incorporating the human ALG9 gene, while HsALG9 E523K and HsALG9 Y286C plasmids contain the human ALG9 gene with single nucleotide polymorphism mutations known to correlate with reported clinical ALG9-CDG cases.[1,2,3]

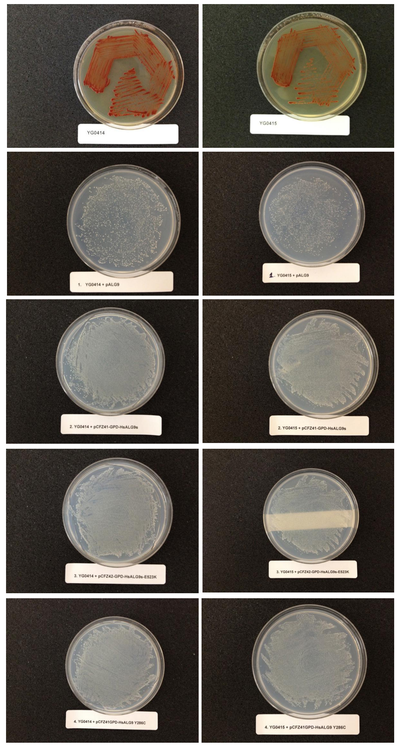
Plasmid Preparation : The PureLink™ Quick Plasmid Miniprep Kit (Invitrogen, Carlsbad, California) was used as per instructions 250789 Version C; 07 July 2005. Using an overnight culture of *E. col*, 1-5 ml was pelleted and all medium thoroughly removed. The pellet was then resuspended in 250 µL resuspension buffer (R3; 50mM Tris-HCl, pH 8.0; 10 mM EDTA) with RNase A ensuring no cell clumps remained. Following addition of 250 µl lysis buffer (L7; 200mM NaOH, 1% w/v SDS) the capped tube was inverted 5 times and then incubated for exactly 5 minutes. Next, 350 µl precipitation buffer (N4) was added and the tube inverted until the solution was found to be homogenous. The mixture was then put into a microcentrifuge (at ~ 12,000 x g) for 10 minutes to clarify the lysate from the lysis debris. The supernatant was loaded onto a spin column which was then placed in a 2-ml wash tube and into the microcentrifuge for 1 minute. The flow-through was then discarded and the column placed back into the wash tube and 500 µl wash buffer (W10) with ethanol added, incubated for 1 minute and then centrifuged for an additional minute. The flow-through was again discarded and the column placed back in the wash tube. Finally, the column 700 µl of wash buffer (W9) was added, the column was centrifuged for 1 minute and the flow-through discarded. One additional minute of centrifuging was done to ensure any residual wash buffer was removed. The wash tube was then discarded. In order to elute the DNA, the spin column was placed into a clean 1.5 ml recovery tube and 75 µl of TE buffer (10mM Tris-HCl, pH 8.0; 0.1mM EDTA) pre-heated to 65-70°C was added to the center of the column. The mixture was then incubated for 1 minute at room temperature and then centrifuged for 2 minutes to produce the purified plasmid DNA.

Yeast Transformation: A yeast transformation kit was utilized (YEAST-1; Sigma, St. Louis, Missouri) and protocol followed as per Sigma Technical Bulletin No. MB-425. *S. cerevisiae* cells were transferred from agar into a microcentrifuge tube, resuspended in 0.5 mls of transformation buffer and then spun for 5 seconds in a microcentrifuge. Following removal of the supernatant leaving 50-100 µl of buffer, 10 µl of 10 mg/ml salmon testes DNA was pipetted into each tube. Subsequently 1 µg of plasmid DNA was added and then vortexed for 10 seconds before adding 600 µl of plate buffer. The mixture was vortexed and then incubated for 16 hours at room temperature. The incubating cells then underwent heat shocking for 15 minutes in a 42 °C water bath. The mixture was then centrifuged and the supernatant removed. Cells were then resuspended in 500 ul of sterile water prior to plating of 100 µl aliquots on selective uracil-deficient YPD media. Plates were incubated face down at 30°C for 72 hours.

Yeast / Plasmid combinations	Growth Characteristics
YG414 / no plasmid	Temp sensitive 30°C; uracil auxotroph
YG414 / pALGP	Temp sensitive 30°C; uracil prototroph
YG414 / HSALG9	Temp sensitive 30°C; uracil prototroph
YG414 / HSALG9 E523K	Temp sensitive 30°C; uracil prototroph
YG414 / HSALG9 Y286C	Temp sensitive 30°C; uracil prototroph
YG415 / no plasmid	Temp sensitive 23°C; uracil auxotroph
YG415 / pALG9	Temp sensitive 23°C; uracil prototroph
YG415 / HSALG9	Temp sensitive 23°C; uracil prototroph
YG415 / HSALG9 E523K	Temp sensitive 23°C; uracil prototroph
YG415 / HSALGP Y286C	Temp sensitive 23°C; uracil prototroph

Table 2. *S. cerevisiae* strain and plasmid combinations utilized.

Figure 3. *S. cerevisiae* strains YG414 & YG415: Pre- and post-transformation.



RESULTS

Uracil auxotrophic strains YG414 and YG415 were observed to grow on YPD media, however, did not grow on uracil-deficient media. Following the transformation procedure utilizing these strains, at 48 hours incubation, a slight haze of growth was observed on uracil-deficient YPD media. At 72 hours, heavy growth of budding yeast were identified as shown in Figure 3. Successful growth in the absence of uracil provides phenotypic evidence of successful transformation with uptake of plasmids and subsequent uracil prototrophy.

DISCUSSION

In addition to phenotypic analysis post-transformation, molecular sequencing will be employed to verify transformation and documentation of relevant ALG9 sequences including relevant mutations desired for disease modeling. Subsequently, further research will strive to characterize the role of ALG9 in organism development and function through comparison of mutant strains, transformed strains and wild type strains. Transcriptome analysis employing RNA microarray is currently underway. Identification of biomarkers may assist in the development of rescue experiments with the future goal of identifying potential therapeutics for this rare disorder.

References:

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