Transformation of mutant *Saccharomyces cerevisiae* strains and establishing a disease model for ALG12-CDG

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BACKGROUND

Congenital disorders of glycosylation (CDG) are a group of rare multisystem disorders characterized by inherited defects in enzymes involved in the glycosylation pathway. Glycosylation is the cellular process of linking oligosaccharides to proteins. This process is necessary for the proper folding, functioning and transport of proteins which play a critical role in cellular and organism biology. There are two major types of CDG (type I & type II) and many additional subtypes classified according to the specific dysfunctional enzyme. Type I pertains to glycosylation defects found in the endoplasmic reticulum while type II deals with glycosylation deficiencies relating to processes in the Golgi apparatus. Common symptoms associated with CDG type I patients include failure to thrive, developmental delay, hypotonia and seizures. However, symptoms and severity can vary even within the individual subtypes of the disorder, which poses difficulties in diagnosing patients simply through clinical examination. Table 1 lists the various genes and corresponding enzymes involved in the assembly of dolichol-linked oligosaccharides and the associated disorder subtypes observed in cases of CDG type I.[1] Among the approximate 1000 individuals diagnosed with CDG type I, there have been eight known cases of the ALG12-CDG (CDG-Ig) subtype worldwide.[1] This particular subtype is differentiated by a deficiency in the mannosyltransferase 8 enzyme, responsible for transferring the eighth mannose from dolichol-P-Man to the expanding dolichol-linked oligosaccharide as shown in Figure 1. [2,3] Clinical features such as facial dysmorphism, psychomotor retardation, developmental delay, hypotonia, and coagulopathy have been observed in the majority of diagnosed patients. ALG12-CDG is extremely rare, though has been observed in patients of various nationalities and is likely underdiagnosed. Currently, there is no direct treatment for the disorder. Seizures can be managed and function can be optimized through supportive therapies, however, disease models are needed in order to investigate potential targeted therapeutics.

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.[1]

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

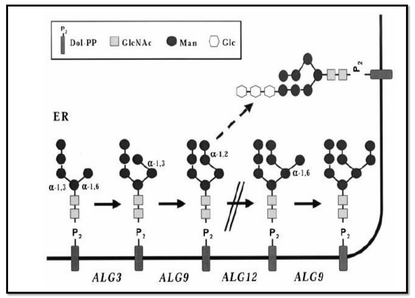

Figure 1. Sequential addition of four mannose residues at the luminal side of the endoplasmic reticulum.[2]

Yeast / Plasmid combinations	Growth Characteristics
YG840 / no plasmid	Temp sensitive 30°C; uracil auxotroph
YG840 / pALG12	Temp sensitive 30°C; uracil prototroph
YG840 / HSALG12	Temp sensitive 30°C; uracil prototroph
YG840 / HSALG12 T67M	Temp sensitive 30°C; uracil prototroph
YG840 / HSALG12 R146Q	Temp sensitive 30°C; uracil prototroph
YG843 / no plasmid	Temp sensitive 23°C; uracil auxotroph
YG843 / pALG12	Temp sensitive 23°C; uracil prototroph
YG843 / HSALG12	Temp sensitive 23°C; uracil prototroph
YG843 / HSALG12 T67M	Temp sensitive 23°C; uracil prototroph
YG843 / HSALG12 R146Q	Temp sensitive 23°C; uracil prototroph

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

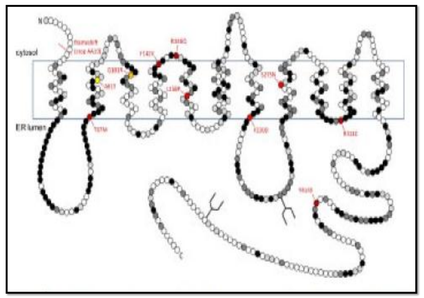

Figure 2. Model of mannosyltransferase 8 (ALG12).[1]

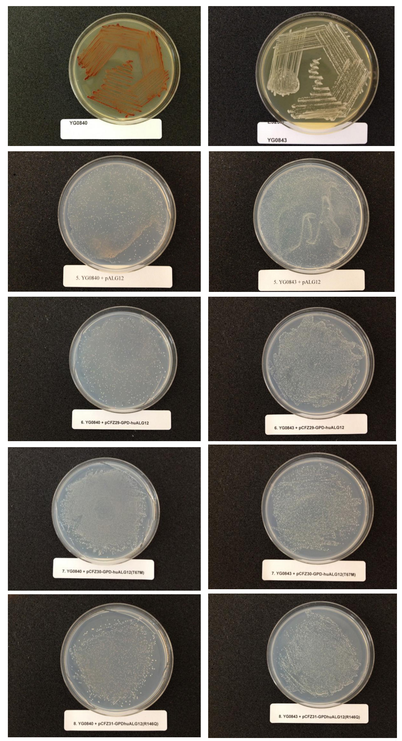

Figure 3. *S. cerevisiae* strains YG840 & YG843: Pre- and post-transformation.

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 YG840 and YG843. YG840 (SS328 w/ Dalg12::KanMX) has the deletion of the ALG12 gene, which encodes for mannosyltransferase 8: the deficient enzyme associated with ALG12-CDG (Figure 2).[1] YG843 (SS328 w/ wbp1-2 Dalg12::kanMX4) lacks the ALG12 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 contained uracil synthesis pathways. pALG12 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.[4,5] Plasmid HsALG12 contains human genomic DNA incorporating the human ALG12 gene, while HsALG9 T67M and HsALG9 R146Q plasmids contain the human ALG12 gene with single nucleotide polymorphism mutations known to correlate with reported clinical ALG9-CDG cases.[6]

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. coli*, 1-5 ml was pelleted and all of the medium was 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 at room temperature. 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.

RESULTS

Uracil auxotrophic strains YG840 and YG843 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 ALG12 sequences including relevant mutations desired for disease modeling. Subsequently, further research will strive to characterize the role of ALG12 in organism development and function through comparison of mutant strains, transformed strains and wild type strains. Identification of biomarkers may assist in the development of rescue experiments with the future goal of identifying potential therapeutics for this rare disorder.

Acknowledgements: We thank Prof. Markus Abei of the Swiss Federal Institute of Technology Zurich in Switzerland for kind provision of yeast strains and plasmids; Thanks to Stefanie Materniak for research services support; Thank you to Dr. John Steeves and Dr. Frank McCarthy for use of the Dalhousie Medicine New Brunswick Laboratory and assorted reagents.

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