Using RNA-Seq to characterize underlying gene expression changes in observed alanine metabolic shifts of ammonia-stressed Chinese hamster ovary (CHO) cell cultures

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Background

Recombinant cell cultures used in the production of biopharmaceuticals naturally produce and accumulate ammonia (NH_3) as a metabolic waste byproduct, largely as a result of glutamine metabolism. At the conclusion of a typical 14-day Chinese hamster ovary (CHO) cell culture, ammonia levels have the potential to reach concentrations as high as 10-20mM. This accumulation of ammonia in the surrounding media is toxic and inhibitory to mammalian cellular metabolism and has been shown to decrease cell growth, protein productivity, and protein quality.

Preliminary metabolite data from parallel fed-batch CHO cells cultured in ambr®250 bioreactors, treated with either 10 mM ammonia or untreated, identified distinctive alanine metabolic profiles due to the treatment. To further examine the changes in the alanine profiles under ammonia-stressed conditions, samples from 0, 3, 6, and 9 days were sequenced via RNA-Seq. The subsequent read data was processed through a bioinformatics pipeline to determine differentially expressed genes and enriched gene sets to characterize transcriptome changes related to alanine metabolism. Since ammonia is also well-known to affect critical quality attributes, glycosylation-related genes were evaluated with particular attention to transcripts associated with sialylation and galactosylation.

Methods

Treatment

Cultures were stressed with 0 mM or 10 mM NH_4Cl in duplicate 12 hours post-inoculation, where media was used to normalize the volume of the 0mM (control) culture to the ammonia-stressed cultures. Amino acid and RNA samples were taken on Days 0, 3, 6, and 9 and quantified by 908 Devices and Novogene, respectively.

Design and Bioreactor Setpoints

Parameter	Setpoint
Vessel Type	ambr 250mL mammalian pitch blade no inlet filters
Cell line	Recombinant CHO-K1 expressing anti-HIV antibody VRC01 (IgG_1)
Seeding Density	0.4 x E6 cells/mL
Working Volume/ Media	210mL Actipro media (GE Healthcare)
Feed	Actipro Cellboost 7a/7b (GE Healthcare)
Feeding Strategy	Pyramid; Cellboost 7a (v/v)/Cellboost 7b (v/v) Day 3 to Day 5 - 3%/0.3% Day 6 to Day 7 - 4%/0.4% Day 8 to Day 9 - 5%/0.5%
Glucose	450g/L stock supplemented daily to maintain >6g/L glucose
Base	1M sodium bicarbonate
pH Setpoint	7.00 +/- 0.05; upper PID CO_2; lower PID 1M $NaHCO_3$
pH PID	Upper (CO_2): flow rate (mL/min) = 0-20; kP = 10; tD = 0; tI = 0 Lower (Base): flow rate (mL/h) = 0-10; kP = 10; tD = 0; tI = 0
Stir Speed	300rpm Day 1 to 5; Day 6 begin cascade for DO control
Dissolved Oxygen (DO)	50%; PID Level 1 - O_2 flow + Level 2 - stir speed
DO PID	O_2 Flow: (Day 1) 0-80 mL/min; kP = 0.03; tD = 0; tI = 0 (Day 7) 0-80 mL/min; kP = 0.04; tD = 0; tI = 150 Stir Speed: (Day 6) 300-600rpm; kP = 3.5; tD = 0; tI = 200 (Day 7) 300-900rpm; kP = 3.5; tD = 0; tI = 150 (Day 8) 300-800rpm; kP = 3.5; tD = 0; tI = 150

Bioinformatics Pipeline

Raw Reads	~260bp raw sequence reads returned in .fastq file
Trimmomatic	Remove adapter sequences, fragments ≤ 36bp, low quality bases (Phred < 15)
STAR	Create genome index from annotation file & align sequence reads to reference genome
samtools	Viewing, sorting, indexing SAM/BAM file formats
HTSeq	Expression quantification to selected identifier in annotation file
DESeq2	Differentially expressed gene analysis

Four statistical analyses were conducted in DESeq2 to identify significant differentially expressed genes. The raw counts matrix was first passed through a likelihood ratio test (LRT) analogous to an ANOVA with a false-discovery rate (FDR) < 0.05. Genes below the FDR cutoff were also required to be significant in at least one pairwise Wald comparison between the treated 10 mM NH3 group and control 0 mM NH3 group for days 3, 6, and 9 (FDR < 0.05 and fold change (FC) ≥ 1.1).

Results – Alanine Biosynthesis

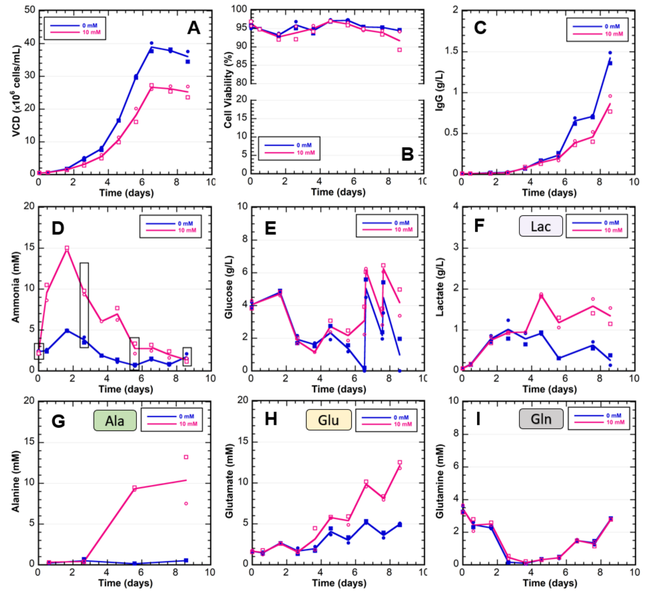

Figure 1. Growth and metabolite profiles for the 0 mM (blue) and 10 mM (magenta) cultures. The lines represent the mean of duplicate cultures; and, all data points are shown. The sample times collected for transcriptome analysis are shown in panel D. **A.** Viable Cell Density (VCD); **B.** Cell viability; **C.** IgG (titer); **D.** Ammonia; **E.** Glucose; **F.** Lactate; **G.** Alanine; **H.** Glutamate; and **I.** Glutamine

Max VCD, exponential growth rates, and final titer differed significantly ($p \leq 0.05$) between the control and treated cultures **(Fig 1A-C)**. The difference in final titer was attributed to the VCD, which was confirmed by specific productivity(q_p) calculations (data not shown).

Glucose was fed as needed to maintain culture concentration between 4 and 6 g/L **(Fig 1E).** Glutamine is not contained in the feeds, where glutamate is in Cellboost 7a and accumulates in the ammonia-stressed cultures **(Fig 1H-I)**. Bolus feed volumes were determined by reactor volume and not VCD, thus certain metabolites accumulated with fewer cells to consume the nutrients.

The observed increase of 9 mM in the ammonia-stressed alanine profile (**Fig. 1G**) between Days 3 and 6 exceeds the amount of alanine fed, which would account for only 1.9 mM. Preliminary analysis of specific consumption rate data (not shown) shows an equimolar rate for alanine production and ammonia consumption suggesting a two-step ammonia detoxification pathway through ammonia fixation to α-ketoglutarate by the action of glutamate dehydrogenase followed by transamination from glutamate to alanine.

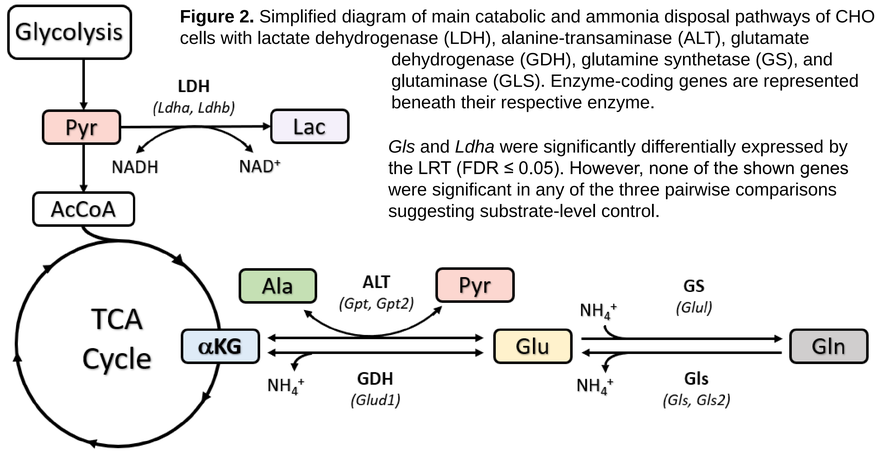

Figure 2. Simplified diagram of main catabolic and ammonia disposal pathways of CHO cells with lactate dehydrogenase (LDH), alanine-transaminase (ALT), glutamate dehydrogenase (GDH), glutamine synthetase (GS), and glutaminase (GLS). Enzyme-coding genes are represented beneath their respective enzyme.

Gls and *Ldha* were significantly differentially expressed by the LRT (FDR ≤ 0.05). However, none of the shown genes were significant in any of the three pairwise comparisons suggesting substrate-level control.

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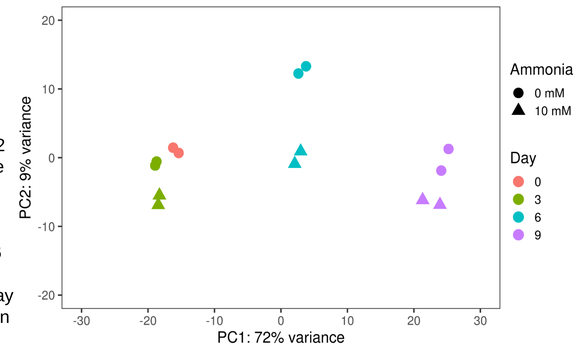
Results – Transcriptomics & Glycosylation

Figure 3. Principal Component Analysis (PCA) plot of the 12,520 genes returned from the LRT. PCA analysis reveals two significant dimensions. PC1 is attributed to the growth phase due to samples clustering vertically by day, and PC2 is attributed to the NH_3 stress by the same logic. Interestingly, the transcriptomes of the control and stressed cultures were more tightly clustered on Day 9 relative to Day 6 by PC2. This follows the ammonia profiles in **Fig. 1D** converging on Day 6 and implies a transient response in gene expression to the stress.



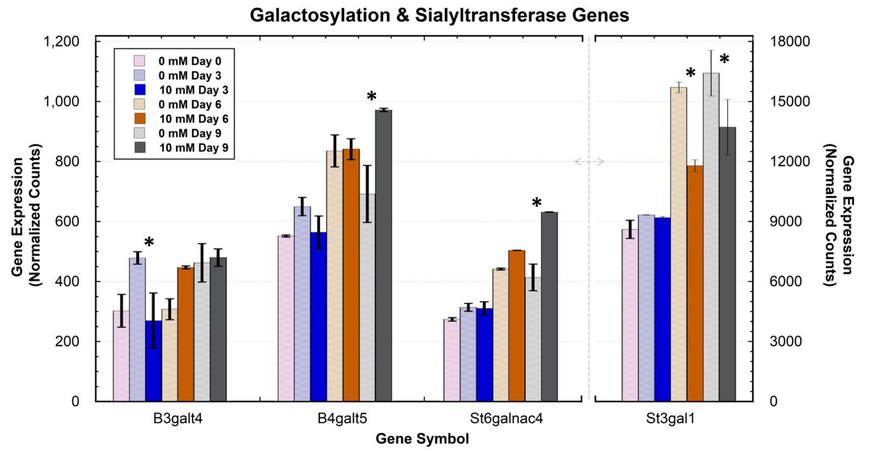


Figure 4. Expression plots for two significant galactosyltransferase genes (*B3galt4*, *B4galt5*) and two significant sialyltransferase genes (*St6galnac4*, *St3gal1*). Asterisks denote pairwise significance (FDR ≤ 0.05, |FC| ≥ 1.1). It is known that ammonia reduces sialylation and galactosylation levels, and a decrease in sialylation is likely attributed to the observed reduction in the sialyltransferase genes, primarily *St3gal1*, in the ammonia-stressed cultures. While the primary galactosyltransferase gene, *B4galt1*, and galactosidase genes were not differentially expressed, two analogs, *B3galt4* and *B4galt5,* were found to be significantly perturbed with reduced expression on Day 3 and increased expression on Day 9, respectively.

A total of 25 significant glycosylation-related genes were found through this analysis including genes related to mannosidases, mannosyltransferases, GlcNAc-transferases, lysosomal enzymes, N-glycans-transferase, nucleotide sugar transporters and nucleotide synthesis. The aggregate effect of these differentially expressed genes likely played a role in the glycosylation profile along with post-translational regulatory effects.

Conclusion

There is currently limited understanding of the transcriptomic and metabolomic changes underlying CHO cell ammonia response using Next Generation Sequencing (NGS). **Figure 3** and overall low fold changes observed in the dataset imply ammonia inflicts a relatively low impact on gene expression.

Previous experiments showed alanine may serve as an early indicator of stress prior to increases in lactate concentration. This experiment aimed to verify alanine as a potential biomarker in response to ammonia-stress and explore the transcriptional landscape driving its high production rate. Interestingly, genes encoding alanine transaminase are not differentially expressed suggesting regulation through substrate-level control. While further analysis is needed, specific consumption rate data also suggests the use of the GDH and ALT pathways as a two-step ammonia-detoxification pathway as seen in the equimolar ammonia consumption and alanine production.

Ammonia is known to affect glycosylation profiles. Decreased sialylation levels are likely attributed to significant reduction in *St3gal1*, although it is likely the combined effect of the 25 significantly differentially expressed genes is responsible for a shift in glycan levels.