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Microarray Analysis of Differentially Expressed Genes between Cysts and Trophozoites of *Acanthamoeba castellanii*

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Abstract: *Acanthamoeba* infection is difficult to treat because of the resistance property of *Acanthamoeba* cyst against the host immune system, diverse antibiotics, and therapeutic agents. To identify encystation mediating factors of *Acanthamoeba*, we compared the transcription profile between cysts and trophozoites using microarray analysis. The DNA chip was composed of 12,544 genes based on expressed sequence tag (EST) from an *Acanthamoeba* ESTs database (DB) constructed in our laboratory, genetic information of *Acanthamoeba* from TBest DB, and all of *Acanthamoeba* related genes registered in the NCBI. Microarray analysis indicated that 701 genes showed higher expression than 2 folds in cysts than in trophozoites, and 859 genes were less expressed in cysts than in trophozoites. The results of real-time PCR analysis of randomly selected 9 genes of which expression was increased during cyst formation were coincided well with the microarray results. Eukaryotic orthologous groups (KOG) analysis showed an increment in T article (signal transduction mechanisms) and O article (posttranslational modification, protein turnover, and chaperones) whereas significant decrement of C article (energy production and conversion) during cyst formation. Especially, cystein proteinases showed high expression changes (282 folds) with significant increases in real-time PCR, suggesting a pivotal role of this proteinase in the cyst formation of *Acanthamoeba*. The present study provides important clues for the identification and characterization of encystation mediating factors of *Acanthamoeba*.

Key words: *Acanthamoeba*, encystation, microarray, cystein proteinase

INTRODUCTION

Acanthamoeba is the causative agent of granulomatous amoebic encephalitis, a fatal disease of the central nervous system, and amoebic keratitis, a painful sight-threatening disease of the eyes [1]. The life cycle of *Acanthamoeba* can be considered in 2 stages, trophozoites and cysts. Under unfavorable conditions, the trophozoite forms a cyst with high resistance to various biocides, pH, and high temperature [2]. Encystation is one of the most common causes of treatment failure in *Acanthamoeba* infection.

Recently, to understand the encystation mechanism of cyst-forming protozoa, encystation mediating factors have been investigated. Especially in *Acanthamoeba*, the mechanism of the cyst-specific protein 21 during differentiation of *Acanthamoeba* was reported [3], a serine proteinase mediating encystation of *Acanthamoeba* was characterized [4], and the major role of cysteine proteases during the early phase of *Acanthamoeba* encystment was suggested [5]. The autophagy related protein 8 (Atg8) mediating autophagy formation, and autophagy related protein 3-mediated lipidation of Atg8, involved in encystation of *Acanthamoeba*, were characterized [6,7]. To examine the gene expression profile during encystation of *Acanthamoeba castellanii*, differentially expressed gene (DEG) screening was performed [8], and an expressed sequence tag (EST) analysis and eukaryotic orthologous groups (KOG) assignment of encystation induced genes were investigated [9,10]. In addition, EST database of *Acanthamoeba* cysts and trophozoites for comparative gene studies was constructed [11]. These previous studies could help researchers understand the encystation mechanisms of encysting protozoa, including *Acanthamoeba*. However, the exact comparison of the expression profile of cysts and trophozoites has yet to be elucidated. Further investigation to understand the encystation mechanisms requires exact data of DEGs

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during encystation to find the encystation mediating factors.

In this study, we report on cDNA microarray analysis containing 12,544 genes of *Acanthamoeba* by comparing the transcriptome of cysts and trophozoites. Microarray analysis can monitor simultaneously gene expression levels of a large number of genes [12]. Previously, we have reported ESTs analysis and KOG assignment of *Acanthamoeba* cysts and trophozoites [9,10]. Based on ESTs from our *Acanthamoeba* ESTs database [11], *Acanthamoeba* ESTs of TBest DB [13], and other *Acanthamoeba* related genes in the NCBI, cDNA microarray chip was constructed and differentially expressed genes between cysts and trophozoites were analyzed. This is the first report in cyst forming protozoa, including *Acanthamoeba* to use the cDNA microarray technique to research encystation mediating genes.

MATERIALS AND METHODS

Amoebae cultivation

A. castellanii Castellani (ATCC #30011) was obtained from the American Type Culture Collection and was grown axenically in PYG (Peptone-Yeast-Glucose) at 25℃ [9].

Induction of encystation and preparation of total RNA

Encystment was induced according to the procedure of Bowers and Korn [14]. After 3 days, the cells were harvested for extraction of the total RNA. Total RNAs of trophozoites and cysts were purified using TRIzol reagent (Gibco BRL, Rockville, Maryland, USA).

Table 1. KOG analysis of cyst and trophozoite genes up-regulated more than 2 folds

KOG category		No. of genes (%)	
		Cyst-high	Troph-high
Information storage and processing			
J	Translation, ribosomal structure and biogenesis	28 (3.99)	135 (15.72)
A	RNA processing and modification	27 (3.85)	22 (2.56)
K	Transcription	10 (1.43)	11 (1.28)
L	Replication, recombination and repair	1 (0.14)	4 (0.47)
B	Chromatin structure and dynamics	3 (0.43)	9 (1.05)
Metabolism			
C	Energy production and conversion	14 (2.00)	67 (7.80)
G	Carbohydrate transport and metabolism	12 (1.71)	26 (3.03)
E	Amino acid transport and metabolism	9 (1.28)	25 (2.91)
F	Nucleotide transport and metabolism	17 (1.40)	13 (1.51)
H	Coenzyme transport and metabolism	0 (0.00)	11 (1.28)
I	Lipid transport and metabolism	12 (1.71)	23 (2.68)
P	Inorganic ion transport and metabolism	7 (1.00)	5 (0.28)
Q	Secondary metabolites biosynthesis, transport and metabolism	5 (0.71)	12 (1.40)
Cellular process and signaling			
D	Cell cycle control, cell division, chromosome partitioning	7 (1.00)	5 (0.58)
Y	Nuclear structure	0 (0.00)	1 (0.12)
V	Defense mechanisms	3 (0.43)	2 (0.23)
T	Signal transduction mechanisms	36 (5.14)	23 (2.68)
M	Cell wall/membrane/envelope biogenesis	3 (0.43)	0 (0.00)
N	Cell motility	0 (0.00)	0 (0.00)
Z	Cytoskeleton	6 (0.86)	39 (4.54)
W	Extracellular structures	16 (2.28)	12 (1.40)
U	Intracellular trafficking, secretion, and vesicular transport	25 (3.57)	15 (1.75)
O	Posttranslational modification, protein turnover, chaperones	39 (5.56)	30 (3.49)
Poorly characterized			
R	General function prediction only	33 (4.71)	37 (4.31)
S	Function unknown	7 (1.00)	13 (1.51)
Not classified		381 (54.35)	319 (37.14)
Total		701 (100.00)	859 (100.00)

Target preparation for microarray

The MessageAmp™ II-Biotin Enhanced (Ambion, Austin, Texas, USA) Single Round aRNA Amplification Kit is based on the RNA amplification protocol developed in the laboratory of James Eberwine [15]. The procedure consists of reverse transcription with an oligo (dT) primer bearing a T7 promoter using ArrayScript™, a reverse transcriptase (RT) engineered to produce higher yields of first strand cDNA than wild type enzymes. ArrayScript catalyzes the synthesis of virtually full-length cDNA, which is the best way to ensure production of reproducible microarray samples. The cDNA then undergoes a second strand synthesis and clean-up to become a template for in vitro transcription in a reaction containing biotin-modified UTP and T7 RNA polymerase. To maximize biotin-labeled aRNA yield, an optimized mixture of biotin-labeled and unlabeled NTPs was supplied with kit, and Ambion's proprietary MEGAscript® in vitro transcription (IVT) technology was used to generate hundreds to thousands of antisense RNA copies of each mRNA in a sample.

Microarray hybridization

Hybridization was performed with 5 μg of a labeled target sample per 1 CustomArray™ 12K microarray. Hybridizations were performed for 16 hr at 45°C with gentle rotation. Thereafter, the arrays were washed with 6X SSPE, 0.05% Tween -20 for 5 min, 3X SSPE, 0.05% Tween -20 for 1 min, 0.5X SSPE, 0.05% Tween -20 for 1 min, and 2X PBS, 0.1% Tween -20 for 1 min. Biotin-labeled targets were hybridized for 30 min with gentle rotation. Thereafter, the arrays were washed with 2X PBS, 0.1% Tween -20 for 1 min, and 2X PBS for 1 min. The LifterSlip™ was laid at an angle onto the microarray so that it was centered over the semiconductor area. First the Imaging Solution was touched with one side of the LifterSlip™, and then slowly the slip down was lowered, taking care not to introduce air bubbles.

Microarray data acquisition

Hybridized microarrays were scanned with PMT 250-270, Pixel size 5, Focus position 130, using a GenePix 4200A microarray scanner (Axon Instruments, Union City, New Jersey, USA). After completion of scan, the resultant image was examined by the eye and proceeded for data extraction (https://webapps.combimatrix.com).

Microarray data analysis

After data extraction, background for individual samples was calculated. For the background calculation, factory built control probes with low intensities (lowest 5-30%) was used, and then their median signal intensity that can be used for subtraction was calculated. The microarray data for individual samples were normalized by a mean-shift normalization using the probes with signal value greater than zero, signal value

Table 2. Real-time PCR analysis of selected genes based on the microarray results

ID	Probe comment	Fold difference		Primer sequence
		Microarray	Real-time PCR	
NC0115-T3	Cysteine proteinase; ACCP2	282.38	1,384	F: 5′-AAC AGC ACG CTC GTT TCC CTC T-3′ R: 5′-GTA GTT GGC CTC CGT CAT GAG CTT-3′
ACL00008706	Catalase related cluster	56.30	11.0	F: 5′-ATC GCA CAC ACA CCA CAC TTT C-3′ R: 5′-CAA CGG AGC ACA AGA GAA CAA A-3′
NC0392-T3	Proteophosphoglycan ppg4	31.21	137.19	F: 5′-TCA TCA TCA CCT CGC TAC ACC A-3′ R: 5′-TAG TTG CAG GTG CGT TCG AGA-3′
NC0903-T3	Arginase II	9.27	20.25	F: 5′-CTC AAG GAG CTA GAC GGT GGA A-3′ R: 5′-TAG GTG TCG AGA AGG CCA GAG A-3′
AC-T0782-T3	Cyst specific protein CSP21	5.19	24.95	F: 5′-GAG AAG TAC GGC AAC GTG GAG A-3′ R: 5′-GCA TAG AGC AGG CGT TCA ACA-3′
ACL00008536	Dipeptidyl peptidase IV cluster	4.64	4.06	F: 5′-ACT GGC GAC ACA CCG TCT ATC T-3′ R: 5′-TGC GCG AGG AAT CAA CAA A-3′
NC0224	Protein serine/threonine kinase	3.85	8.17	F: 5′-CCA CAT CAA CCA AAT CAC CGA-3′ R: 5′-CGC ATA ACC GAA CAT CAT CCA-3′
ACL00001979	Ubiquitin	3.69	13.27	F: 5′-ACA CAC AAG CCA GCA ATG CA-3′ R: 5′-AAG TGG TGG AGG AAA CAC GCT-3′
AC-C0063	Heat shock protein	2.8	3.41	F: 5′-GAC CGA CGG CAA TAA ACT CGT-3′ R: 5′-TCG CTG CAC CAA CAG ATT CTC-3′

Table 3. The list of genes that showed high expression changes more than 10 fold in cysts

ID	Probe comment	KOG	Fold (Cyst/troph)
NC0115-T3	Cysteine proteinase; ACCP2	O	282.38
NC0961-T3	Cysteine proteinase; ACCP2	O	273.54
AC-T0581-T3	Similar to elaC homology	R	80.82
ACE00000784	-	-	73.39
ACL00003940	Herpes_BLLF1 multi-domain protein	-	60.94
ACL00008706	Catalase related cluster	AR	56.30
NC0692-T3	Extracellular endoglucanase	WV	54.94
NC0680-T3	-	-	53.32
ACL00002157	-	-	48.23
NC0642-T3	-	-	41.34
NC0757-T3	-	-	38.91
ACL00003760	-	-	36.86
ACL00009010	-	-	36.09
ACL00004491	-	-	33.26
NC0678-T3	-	-	31.73
NC0457-T3	-	-	31.72
NC0392-T3	Proteophosphoglycan ppg4	-	31.21
AC-C0617-T3	SJCHGC01975 protein	O	29.79
ACL00008637	Novel protein related cluster	U	27.80
NC0271-T3	Salivary gland secretion 1 CG3047-PA	-	27.04
NC0289-T3	-	-	26.25
NC0423-T3	Proteophosphoglycan 5	-	23.32
ACL00004246	-	-	23.31
NC0956-T3	Similar to serine/arginine repetitive matrix 1	-	22.54
NC0802-T3	Proteophosphoglycan 5	WV	21.72
ACL00002714	Dynamin B precursor related cluster	UR	21.23
NC0276-T3	Similar to conserved hypothetical protein	T	20.90
ACE00001346	Similar to molecular chaperone (HSP90 family)	O	20.34
NC0283-T3	Proteophosphoglycan ppg4	-	19.68
ACL00008639	Herpes_gp2 multi-domain protein	-	19.22
NC0994-T3	Proteophosphoglycan 5	-	19.13
NC0807-T3	Antifreeze glycoprotein	-	18.43
NC0791-T3	Hypothetical protein Kpol_1072p54	DO	17.66
ACL00002613	-	-	17.13
NC1140-T3	Proteophosphoglycan ppg4	-	16.94
NC0582-T3	-	-	16.92
ACL00002232	-	-	16.50
ACL00002108	Transcription factor TFIIF	K	15.49
NC0747-T3	Pseudogene	-	15.27
NC0325-T3	Proteophosphoglycan ppg4	-	15.07
NC1103-T3	Proteophosphoglycan ppg4	-	14.95
AC-T0529-T3	-	-	14.77
ACL00002483	-	-	14.58
AC-T0879-T3	Iron-sulfur cluster assembly fam...	CU	14.54
NC0896-T3	Proteophosphoglycan ppg4	-	14.53
ACL00001119	trEST Trithorax protein related cluster	R	13.83
NC0987-T3	Palmitoyl-protein thioesterase 3	IO	13.77
NC0591-T3	Similar to scavenger receptor class F, member 2, partial	WV	13.70
NC0780-T3	Hemerythrin HHE cation binding domain-containing protein	-	13.63
ACL00008833	Extensin_2 multi-domain protein	T	12.98

(*Continued to the next page*)

Table 3. (*Continued from the previous page*) The list of genes that showed high expression changes more than 10 fold in cysts

ID	Probe comment	KOG	Fold (Cyst/troph)
ACL00008542	-	-	12.47
ACL00009103	-	-	12.34
ACL00009220	-	-	11.76
NC0721-T3	LigA	-	11.57
NC0626-T3	Proteophosphoglycan 5	A	11.57
ACL00000192	Ubiquitin	OR	11.03
NC1049-T3	Antifreeze glycoprotein	-	10.89
ACL00008353	Tymo_45kd_70kd multi-domain protein	WV	10.73
NC0528-T3	BTB/POZ domain-containing protein	-	10.62
ACL00002099	Putative 5-3 exoribonuclease related cluster	LA	10.47
ACL00008536	Dipeptidyl peptidase IV related cluster	O	10.46
ACL00003598	trEST Herpes_UL56 multi-domain protein	-	10.35
ACL00004676	-	-	10.27
AC-T0408-T3	Histone H3	B	10.16
NC0574-T3	Hypothetical protein MGG_00576	-	10.10
ACL00000775	Unknown (eubacterial similarity)	-	10.05

lesser than 60,000 (saturation value), and signal value greater than the lowest 5% of each sample' signal value. A total of 7,825 probes (or 7,862 probes) were used in the final analysis. LPE-test *P*-value of < 0.05 and Fold-change (> 2, < -2) were applied to determine differentially expressed sets of genes across 3 experimental groups. We also performed complete linkage Hierarchical clustering based on Euclidean distance measure of samples using the normalized significant genes. We looked into the patterns of expressed changes for groups. We used ArrayAssist® 5.5.1 (Stratagene, La Jolla, California, USA) as statistical software.

Gene expression analysis based on real-time PCR

Nine target genes were selected for real-time PCR, and 18s rDNA was used for the reference gene (sense 5′-TCCAATTTTCTGCCACCGAA and antisense 5′-ATCATTACCCTAGTCCTCGCGC). Target gene-specific primers were defined for each transcript with the ABI primer express software (Table 2). Total RNA was purified using Trizol reagent (Gibco BRL, Rockville, Maryland, USA), and cDNA synthesis was conducted using a RevertAid first-strand cDNA synthesis kit (Fermentas, Hanover, Maryland, USA). Real-time PCR was performed with the ABI PRISM® 7000 sequence detection system (Applied Biosystems, Foster City, California, USA), using the default thermocycler program for all genes; 10 min of preincubation at 95°C, followed by 40 cycles of 15 sec at 95°C, and 1 min at 60°C. Individual reactions were carried out in 20 µl volumes in a 96-well plate containing 1×buffer, 3.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, different concentrations of sense and antisense primers (Table 2), 0.025 U/µl enzyme, and SYBR green. All reaction mixtures were made using SYBR Premix Ex Taq (Takara, Otsu, Shiga, Japan). The experiments were performed in triplicate.

RESULTS

Features of cDNA chip and expressed cluster analysis

A cDNA microarray was constructed with 12,544 genes based on *Acanthamoeba* ESTs database [11], TBest DB [13], and all *Acanthamoeba* related genes in the NCBI, and spotted in duplicate on each microarray slide. To check the reproducibility between samples, the scatter plot comparisons of gene expression pattern between samples were performed (data not shown). Between cyst and cyst, and trophozoite and trophozoite, samples had a similarity value of 1 approximately. This showed the perfect positive correlation. However, between cysts and trophozoites, samples had a similarity value of 0.9 approximately. These identified genes appeared to be differentially expressed between cysts and trophozoites. Hierarchical clustering of the data obtained from 12,544 elements was analyzed (data not shown). Red areas indicate up-regulated transcripts and green areas indicate down-regulated transcripts in the cyst than in the trophozoite. The color saturation degree corresponded to the gene expression ratio. Gene transcripts that were up-regulated or down-regulated more than 2-folds were considered to be differentially expressed. Comparison of the signals ob-

tained using the microarray appreciated the fold change in gene expression for 1,560 genes. Up-regulated transcripts in the cyst compared to the trophozoite were 701 genes, while down-regulated transcripts were 859 genes.

KOG analysis of differentially expressed genes

A general functional classification of 1,560 differentially expressed genes was obtained by a BLASTX similarity search against the KOG database (Table 1). A (RNA processing and modification), P (inorganic ion transport and metabolism), D (cell cycle control, cell division, and chromosome partitioning), V (defense mechanisms), T (signal transduction mechanisms), W (extracellular structures), U (intracellular trafficking, secretion, and vesicular transport), and O (posttranslational modification, protein turnover, and chaperones) articles were also more highly populated in cyst genes than those of trophozoites. Comparison of the KOG analysis between microarray results and previous ESTs analysis [9,10] revealed higher percentages of cyst genes related to D, T, and O articles than those of trophozoites. Especially, KOG analysis of microarray showed a higher percentage of cyst genes to T and O articles, and a lower percentage of cyst genes related to J (translation, ribosomal structure, and biogenesis) and C (energy production and conversion) articles than those of trophozoites (Fig. 1). Microarray analysis also suggests that signal molecules and protein modification related proteins are important in encystation of *Acanthamoeba*.

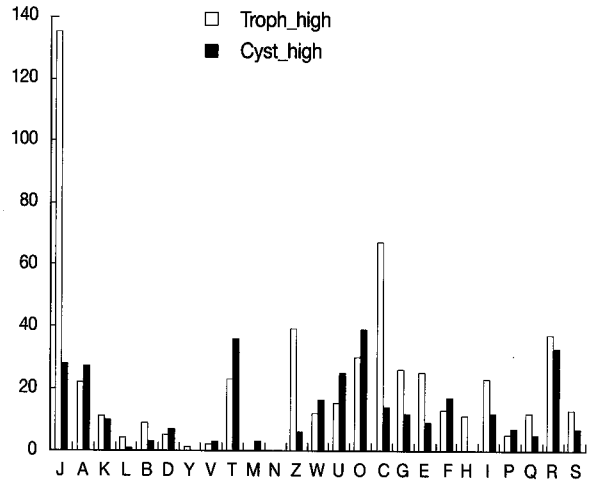

Fig. 1. Comparison of gene expression by microarray and KOG analysis. Cyst up-regulated genes showed higher percentages in A, D, T, W, U, O, and F articles. In J, C, and Z (cytoskeleton) articles, cyst genes were down-regulated.

Real-time PCR analysis of differentially expressed genes based on microarray

To validate the microarray results, quantitative analysis of 9 genes was performed by real-time PCR (Table 2). To correct differences in RNA quality and quantity among the samples, data were normalized using the 18s rDNA. All of 9 genes (Table 2) were highly expressed during encystation. These expression levels were correlated with the microarray analysis.

Highly expressed genes in cysts

Microarray results indicated that 701 genes were highly expressed more than 2-folds during encystation of *Acanthamoeba*. Table 3 shows the list of genes highly expressed more than 10-folds in the cyst than in the trophozoite. As many as 66 genes were highly expressed more than 10-folds in cysts. Especially, cysteine proteinases showed significantly high expression changes, 282 folds in cysts. Real-time PCR also showed high expression levels of cysteine proteinases. On the other hand, among these genes, 22 genes were not identified and 42 genes were not annotated (#N/A). These unidentified or non-annotated genes are needed to be investigated more about functional roles in encystation of *Acanthamoeba*.

DISCUSSION

This comparison of transcriptome between cysts and trophozoites in *A. castellanii* using cDNA microarray demonstrated that 701 genes were highly expressed more than 2-folds in cysts, and 859 genes were expressed lower in cysts than in trophozoites. Among them, cysteine proteinases showed high expression changes (Table 3), and real-time PCR confirmed their high expression levels during encystation (Table 2). Leitsch et al. [5] reported that the protease inhibitors, E64d [(2S,3S)-trans-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester], inhibited the onset of encystment of *A. castellanii*, and described the major role for cysteine proteases during the early phase of encystment [5]. Previously identified cyst specific ESTs [10], such as a cysteine proteinase (NC0115/282-fold increase), ubiquitin (ACL00000192/11-fold increase), subtilisin-like serine proteinase (AC-C0457/5.5-fold increase), calreticulin (AH-0894/2-fold increase), proteophosphoglycan (NC0392/31-fold increase), and cyst specific protein 21 (AC-T0782/5.2-fold increase) were confirmed by this cDNA microarray analysis.

Newly identified various cyst specific genes in this study, such as serine/threonine protein kinase (NC1001/4.6-fold increase),

translation elongation factor (NC0950/4.5-fold increase), tyrosine kinase like protein (ACL00008738/4.4-fold increase), Ras-related small GTP binding protein (ACL00004031/3.9-fold increase), calcium transporting ATPase (ACL00008443/2.1-fold increase), and others were stored on the '*Acanthamoeba* ESTs database' [11]. The list of the fold changes in gene expression for 1,560 genes and their expression levels were stored in an additional file 1 (data not shown). We compared these microarray results with previous reported ESTs analysis data [9,10]. These results commonly indicated that the exhibited high expression changes in cyst genes were significantly enriched for the signal transduction mechanisms (T article) and posttranslational modification, protein turnover, and chaperones (O article). This analysis reveals that signal transduction mechanism is essential for the formation of cysts, and various proteinases required for unnecessary protein degradation or posttranslational modifications. Cysteine proteinases that showed high expression changes in this study may be involved in protein degradation correlated with autophagic procession during encystation.

This is the first report on transcriptional profile comparison between cysts and trophozoites of *Acanthamoeba* using cDNA microarray. A comparison of the Giardia lamblia trophozoite and cyst transcriptome using microarray has been reported with higher expression of 215 genes in the cyst [16]. However, our *Acanthamoeba* microarray analysis showed 701 transcriptomes that could provide information of various encystation mediating genes. Real-time PCR results of randomly selected genes (Table 2) also corroborated microarray data and clearly showed that this microarray data was reliable. This observation of expression differences between cysts and trophozoites could provide many interesting genes related to encystation of *Acanthamoeba*. Furthermore, the microchip produced in this study may provide clues to widen the study of excystation mediating factors and genes related to virulence or other transcriptome analysis of cyst-forming protozoa, including *Acanthamoeba*.

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