研究成果の刊行に関する一覧表（平成２４年度）

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濱野真二郎	アメーバ症		今日の治療指針 2014	医学書院	東京	2014	255
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Makiuchi, T., Mi-ichi, F., Nakada-Tsukui, K., and Nozaki, T	Novel TPR-containing subunit of TOM complex functions as cytosolic receptor for *Entamoeba* mitosomal transport	Scientitfic Reports	3	1-7	2013
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井上幸次、大橋裕一、浅利誠志ほか	感染性角膜炎診療ガイドライン（第２版）	日本眼科学会雑誌	117	467-509	2013
Laura Biller, Jenny Matthiesen, Vera Kuhne, Hannelore Lotter, Ghassan Handal, Tomoyoshi Nozaki, Yumiko Saito-Nakano, Michael Schumann, Thomas Roeder, Egbert Tannich, Eberhard Krause, and Iris Bruchhaus	The cell surface proteome of *Entamoeba histolytica*	*Molecular and Cellular Proteomics*	13 (1)	132-44	2014
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Ⅲ．研究成果の刊行物・別刷

Transcriptome Analysis of Encystation in *Entamoeba invadens*

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Abstract

Encystation is an essential differentiation process for the completion of the life cycle of a group of intestinal protozoa including *Entamoeba histolytica*, the causative agent of intestinal and extraintestinal amebiasis. However, regulation of gene expression during encystation is poorly understood. To comprehensively understand the process at the molecular level, the transcriptomic profiles of *E. invadens*, which is a related reptilian species that causes an invasive disease similar to that of *E. histolytica*, was investigated during encystation. Using a custom-generated Affymetrix platform microarray, we performed time course (0.5, 2, 8, 24, 48, and 120 h) gene expression analysis of encysting *E. invadens*. ANOVA analysis revealed that a total of 1,528 genes showed ≥3 fold up-regulation at one or more time points, relative to the trophozoite stage. Of these modulated genes, 8% (116 genes) were up-regulated at the early time points (0.5, 2 and 8h), while 63% (962 genes) were up-regulated at the later time points (24, 48, and 120 h). Twenty nine percent (450 genes) are either up-regulated at 2 to 5 time points or constitutively up-regulated in both early and late stages. Among the up-regulated genes are the genes encoding transporters, cytoskeletal proteins, proteins involved in vesicular trafficking (small GTPases), Myb transcription factors, cysteine proteases, components of the proteasome, and enzymes for chitin biosynthesis. This study represents the first kinetic analysis of gene expression during differentiation from the invasive trophozoite to the dormant, infective cyst stage in *Entamoeba*. Functional analysis on individual genes and their encoded products that are modulated during encystation may lead to the discovery of targets for the development of new chemotherapeutics that interfere with stage conversion of the parasite.

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Introduction

Amebiasis is common among individuals exposed to unsanitary health conditions in developing countries. Amebiasis is also seen in developed countries among men who have sex with men and mentally handicapped people [1,2]. In both cases, the infection is established through ingestion of the cysts in feces, or fecal contaminated food and water [1]. Although *in vitro* cultivation and *in vivo* passage of the reference strains and recent clinical isolates of *E. histolytica* led to identification and characterization of the virulence mechanisms associated with amebiasis [3], the molecular mechanisms of differentiation from the invasive trophozoite to the dormant, infective cyst stage, called encystation, remains largely unknown. This is in part due to the lack of *in vitro* or *in vivo* systems that allow differentiation of *E. histolytica* [4]. To overcome this, *E. invadens*, which is a related reptilian species that causes an invasive disease similar to that of *E. histolytica*, has been used as a model system for encystation as *E. invadens* trophozoites can be induced to encyst in axenic

conditions [5–7]. The morphology, the life cycle consisting of binary stages, the sites of encystation, invasiveness to the colonic epithelium, and potential dissemination from the intestine into other organs through the portal vein are similar between the two species.

Several studies focused on identifying genes involved in the stage conversion of *Entamoeba*. It has been shown that galactose/N-acetylgalactosamine, proteasome, beta-adrenergic components, and transcription factors Myb affect stage switching [4,8–12]. Protein kinase C inhibitors and short chain fatty acids have also been linked to encystation [13–15]. In *Giardia lamblia*, cysteine proteases (CP) and UDP-N-acetylglucosamine pyrophosphorylase have been shown to be key enzymes during encystation [16,17]. Similarly, an *E. invadens* CP isotype was found elevated in encysting cells [18]. In the social ameba *Dictyostelium discoideum*, cyclic AMP is used as an autocrine factor for sporulation [19].

Availability of the whole genome sequence of *E. histolytica* has facilitated production of custom-made DNA microarray necessary for identification and classification of genes related to virulence [20,21], the response against oxidative and nitrosative stresses [22], and stage conversion [12]. A Myb transcription factor in *E. histolytica* was also found to regulate transcription of stage-specific genes [23]. Here, we present the whole genome transcriptional profiling of *E. invadens* during encystation. Genes modulated during encystation and their patterns are examined to identify genes and pathways that are involved in encystation.

Materials and Methods

Cultivation and encystation of *E. invadens*

Axenic cultures of *E. invadens* strain IP-1 trophozoites were maintained in BI-S-33 medium at 26 °C. To induce encystation, in two independent experiments in triplicate, trophozoites were harvested in the late logarithmic phase and the cells were transferred to a seven 36 ml flasks with 47% LG medium at a final concentration of 5 x 10^5/ml [8]. Cells were collected at seven time points: 0, 0.5, 2, 8, 24, 48, and 120 h after exposure to the encystation medium. After incubation in 47% LG medium, total numbers of cells were counted under a microscope. One portion of the cells was saved for RNA extraction and another portion was used for the differentiation of trophozoites and cysts. For the determination of cysts, the cells were resuspended in PBS containing 0.05% sarkosyl, and allowed to sit for 20 min at room temperature [24,25]. After lysed cells were stained with 0.22% trypan blue (Wako Pure Chemical Industries Ltd., Japan), intact cysts were counted and the encystation efficiency was measured by dividing the number of cysts resistant to 0.05% sarkosyl (Sigma-Aldrich, St. Louis MO, USA) with the total number of cells suspended in PBS without sarkosyl, in two independent experiments performed in triplicate.

RNA extraction

For isolation of RNA, the cells, harvested at various time points and tested for the sarkosyl sensitivity as described above, were washed three times with 1X PBS and collected by centrifugation at 1, 500 rpm for 5 minutes after induction to wash off the encystation medium. The collected cell pellets were resuspended in 1 ml of Trizol reagent (Invitrogen, Carlsbad, CA, USA) and lysed using a Dounce homogenizer (approximately 300 strokes) until majority of the cysts were lysed as previously described [39]. The RNA concentration for each sample was measured using a Nanodrop Spectrophotometer 1000 (Thermo Scientific, Wilmington, DE, USA). RNA integrity was checked using Bio-Rad's Experion Automated Electrophoresis System (RNA StdSens analysis kit).

Affymetrix Microarray Hybridization

All reagents and protocols used in this study were as described in GeneChip® Expression Analysis Technical Manual (Affymetrix, Inc. Santa Clara, CA, USA). Five arrays were used for five independently isolated RNA samples corresponding to 2 biological replicates (3 arrays for the first set and 2 arrays for the second set; two sets of encystation experiments were carried out > 1 year apart in-between), were used for each time point. Using the One-Cycle cDNA synthesis kit, 5 g of total RNA was reverse transcribed using T7-Oligo (dT) primer in the first strand cDNA synthesis. After the second strand synthesis, the double-stranded cDNA template was used for *in vitro* transcription (IVT), in the presence of biotinylated nucleotides (GeneChip IVT labeling kit) to produce Biotin-labeled cRNA. Unincorporated NTPs were removed from the biotinylated cRNA (GeneChip® sample cleanup module) and then purified, quantified and fragmented. Hybridization cocktail of eukaryotic hybridization controls and fragmented, labeled cRNA (GeneChip® Hybridization, Wash and Stain Kit) were hybridized for 16 hours at 45 °C (Hybridization Oven 640, Affymetrix) to custom-generated Affymetrix platform microarray (49-7875) with probe sets consisting of 11 probe pairs each representing 12, 384 *E. invadens* open reading frames (Eh_Eia520620F_Ei) and 9, 327 *E. histolytica* (Eh_Eia520620F_Eh). The array chips were washed and stained (GeneChip® Hybridization, Wash and Stain Kit) with Streptavidin–phycoerythrin Biotinylated anti-streptavidin antibody using a GeneChip® Fluidics Station 450 (Affymetrix) for 1.5 hours. After washing and staining, the GeneChip® arrays were scanned using the Hewlett-Packard Affymetrix Scanner 3000.

Analysis of microarray data

Raw probe intensities were generated by the GeneChip Operating Software (GCOS) and GeneTitan Instrument from Affymetrix. Normalized expression values for each probe set were obtained from R 2.7.0 downloaded from the BioConductor project (http://www.bioconductor.org) using robust multiarray averaging with correction for oligosequence (gcRMA). Standard correlation coefficients were calculated using GeneSpring GX 10.0.2. Reproducibility of the experiments was determined by Pearson's correlation coefficient and confirmed by principal component analysis. Only genes that were considered 'present' by GCOS at least one of three arrays at any time points were used in further analysis. One-way ANOVA analysis with Tukey's Post Hoc test was performed to extract

differentially expressed genes. Gene probe sets were considered differentially expressed between time points if they had at least a 3 fold change compared against the value at 0 hour and a p-value < 0.05, calculated using Welch's t-test, after multiple test correction by the Benjamini–Hochberg method. A post-hoc test using Tukey's Honestly Significant Difference test was conducted to determine significant differences between samples. The data presented in this publication have been deposited in NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE33312.

Annotation of Rab small GTPases and cysteine proteases

We searched the *Entamoeba* genome database using *E. histolytica* Rab small GTPases and cysteine proteases as query [3,26]. One hundred twenty one Rab and 64 EiCPs were annotated after CLUSTAL W alignment, manually edited using BioEdit and phylogenetic tree created using MEGA4 software [27]. For details, see references [28,29].

Results and Discussion

Kinetics of morphological differentiation

In order to identify and characterize genes and gene cascades involved in encystation, we examined the transcriptional profiles at 7 time points (0, 0.5, 2, 8, 24, 48, and 120 h) during encystation of the reptilian ameba *E. invadens*. At 8 h post-induction of encystation by transferring trophozoites to the differentiation medium of low osmolarity containing no glucose, trophozoites became highly motile as compared with those maintained in the BI-S-33 medium, and only 0.9-2.8% of cysts were formed (Figure 1A). At 24 h after induction, the trophozoites rounded up, became immobile, and formed clusters, and the percentage of cysts increased to 19.7%. At 48 h of encystation, large multicellular aggregates were formed, and 50.7% of the total cells transformed into cysts. At 120h post induction 91.5% of cells transformed into cysts (Figure 1A).

Overview of transcriptional changes during encystation

Among 12, 384 probe sets (Table S1) corresponding to *E. invadens* open reading frames (including 1, 272 probe sets that had been removed from NCBI), approximately 6, 014 genes were found to be expressed, i.e. had a "present" call in at least one of the five experimental replicates, at least one time point. We did not only choose genes that were found "present" in all five replicates because this will narrow down the size of genes to be analyzed during the course of encystation (Figure 1B and Table S2). These genes were filtered to extract genes whose probe sets represent a single gene (a probe set name contains suffix "_at") or that were so highly similar in sequence to other genes as to make it impossible to design a unique probe set (a probe set with a suffix "s_at"). We set significant levels of changes to 3 fold, similar to that used in the previous work [12], where cyst-specific genes were identified in recent clinical isolates of *E. histolytica*. Furthermore, much higher numbers of

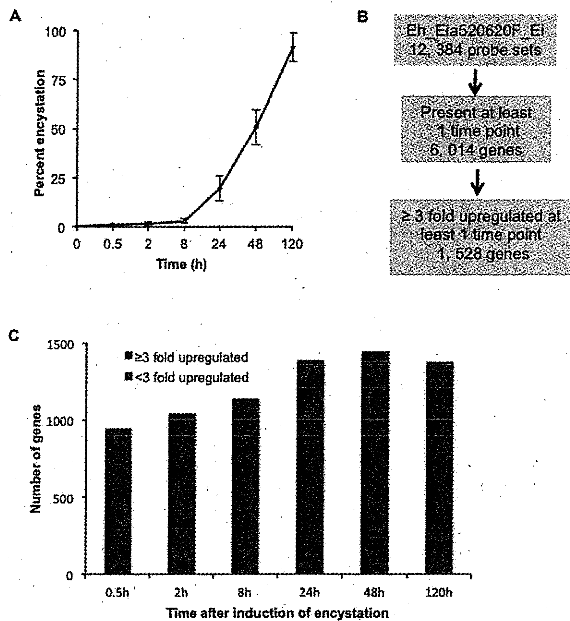

Figure 1. Overview of transcriptomic analysis. (A) Kinetics of differentiation. The percentages of the amoebae resistant to 0.05% sarkosyl during encystation are shown. Values are presented as % encystation and represent the mean ± S.D. of two independent experiments conducted in triplicate. (**B**) **Flow of analysis**. Microarray data were obtained in triplicates from *E. invadens* exposed to 47% LG medium for 0, 0.5, 2, 8, 24, 48, or 120 h, and genes expressed in at least one time point were selected for further analysis. The second data set of two biological replicates are shown as a representative. (**C**) The number of genes that were proven to be statistically significantly up-regulated by ≥3 or <3 fold at each time point of encystation.

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genes were selected when lower fold (e.g., two fold) changes were used, which made description of modulated genes very lengthy. To validate the reproducibility of the results, we compared the transcriptomic data from the two biological replicates at different time points during encystation (Figure S1). The two data sets showed reasonable Pearson correlation coefficients (R values ranging from 0.7451 to 0.9021). We selected for the further analysis only genes that were modulated by ≥3 fold in both sets of biological replicates.

In general, the number of up- and down-regulated genes increased as encystation proceeded. We mainly focused on the up-regulated genes during encystation. The number of genes that were up-regulated ≥3 fold at any time points was 1, 528 (Table 1 and Table S3). Among the up-regulated genes, the number and proportion of the genes up-regulated ≥3 fold, compared to up-regulated < 3 fold, tends to increase at the later time points of encystation (Figure 1C) with the highest number of up-regulated genes noted at 48 h of encystation. A total of 2841 genes were down regulated by ≥3 fold at one or

Table 1. Grouping and distribution of 1,528 genes that were up-regulated ≥3 fold at least one time points during encystation.

Category	0.5h	2h	8h	24h	48h	120h	Number of genes
1	+						11
2		+					30
3			+				10
4	+	+					26
5	+		+				1
6		+	+				15
7	+	+	+				23
8				+			85
9					+		4
10						+	57
11				+	+		89
12					+	+	358
13				+		+	7
14				+	+	+	362
15	+	+	+	+	+	+	54
16		+		+			3
17	+	+	+	+			12
18	+	+	+	+	+		13
19	+			+			3
20		+	+	+			18
21		+	+	+	+		19
22		+	+	+	+	+	81
23		+				+	2
24			+	+			16
25			+	+	+		23
26			+			+	2
27			+	+	+	+	107
28	+		+	+	+	+	4
29	+	+		+	+	+	4
30	+	+	+		+	+	1
31	+	+			+		1
32	+	+		+			3
33	+	+				+	3
34	+				+	+	10
35		+			+	+	17
36			+		+	+	6
37	+			+	+		1
38		+		+	+	+	20
39	+			+	+	+	12
40		+	+		+	+	3
41	+	+			+	+	11
42	+	+		+	+		1
Total							1528

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more time points during encystation (Table S1). At each time point, 263, 528, 543, 1325, 1835, and 1998 genes were downregulated by ≥3 fold at 0.5, 2, 8, 24, 48, and 120 h, respectively.

To identify genes that are modulated at specific time points during differentiation, we further grouped the genes into 42 categories based on expression profiles (Table 1). About 37% of genes (566) are up-regulated at 0 to 8 h of encystation; 20% (116 genes, categories 1-7) of which are exclusively up-regulated at 0 to 8 h while 80% (450 genes, categories 15-42) of those genes were also up-regulated at later time points (Table 1). List of genes up-regulated at 0.5 and/or 2 h are presented in Table S4. Genes up-regulated at 8 h are listed in Table S5.

For the 962 genes up-regulated at later time points (categories 8-14 Table 1), 9%, 0.4%, and 6% of the genes specifically peak at 24, 48 or 120 h respectively, whereas 37% (358 genes) peak at two time points (48 and 120h), 9% (89 genes) and 0.7% (7 genes) peak at 24/48 h and 24/120 h, respectively. Thirty eight percent of genes (362 genes) are continuously up-regulated at 24 to 120 h. List of genes up-regulated only at 24 h are listed in Table S6 and genes up-regulated at 48 and/or 120 h are listed in Table S7.

Only 31% (469 genes) of the up-regulated genes were annotated (Table S3). Of the 1,059 genes encoding for hypothetical proteins, 18% (187 genes) have orthologs in other organsisms. In the following sections, we summarize the modulated annotated genes based on functional classes.

A: Bacterial surface protein A (BspA) family

Leucine-rich repeat (LRR)-containing proteins, which were initially identified in *Bacteroides forsythus* (BspA), are one of the most abundant multicopy genes in the *E. invadens* genome representing about 1.4% of the total *E. invadens* sequence reads [30]. They are annotated in AmoebaDB as hypothetical proteins with conserved regions. Similarly, 114 genes encoding for BspA-like proteins were identified in *E. histolytica* genome [31]. Homology searches using these *E. histolytica* BspA-like proteins revealed that *E. invadens* contain 149 BspA-like proteins (data not shown). Our transcriptome analysis showed that 26 out of 149 *E. invadens BspA* genes were up-regulated during encystation (Figure 2 and Table S3). About half (11) the genes were up-regulated at 48 and/or 120 h (Table S7), while 5 *BspA* genes were up-regulated at 0.5 and/or 2 h (Table S4), and the other 10 genes were up-regulated at different time points (Figure 2, Table S3). Time-dependent up-regulation of specific subsets of BspA-like genes are intriguing, as BspA was implicated in the attachment and invasion to host cells in *Treponema denticola* and *Tannerella forsythiae* [32,33]. One of *E. histolytica* BspA-like protein, EhLRRP1, has been shown to be localized on the cell surface, but its possible role in interaction with host ligands is not yet established [34]. As proposed in *Trichomonas vaginalis*, where BspA-like proteins might be involved in cell-cell adhesion when *T. vaginalis* forms large aggregates [35], BspA-like proteins may also be involved in a similar phenomenon in *E. invadens* during the early stage of encystation. Despite the similarity in the LRR repeats among *E. invadens*, bacteria, and trichomonads, the lack of the amino-terminal sequence and the transmembrane domain of *E. invadens* LRR suggests that its function is divergent [30]. Thus, up-regulation of *E. invadens* BspA at the late stage of encystation is highly remarkable, as BspA-like proteins were not shown associated with cell differentiation in other organisms [32,33]. It was, however, shown in *T. vaginalis* that transcript level of some BspA proteins change upon exposure