Identification of differentially expressed genes in parasitic phase *Miamiensis avidus* (Ciliophora: Scuticociliatia) using suppression subtractive hybridization

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ABSTRACT: *Miamiensis avidus*, a causative agent of scuticociliatosis in cultured marine fish, can live not only in seawater as a free-living organism but also in fish as a parasite. In this study, a cDNA library of representative mRNAs more specific to parasitic phase *M. avidus* was generated using suppression subtractive hybridization (SSH), and 520 clones selected from the SSH library were single-run sequenced. The differential gene expression patterns were confirmed by semi-quantitative reverse-transcription PCR. Of the 510 SSH clones, 21 clones of 6 putative genes did not match sequences in the public database. The expectation values (*E*-values) of 117 clones encoding 9 putative genes were greater than 1×10^{-5}. The other 372 clones that met the criterion of *E* value $<1 \times 10^{-5}$ were matched to 26 known sequences in the database. Genes associated with signal transduction, cell proliferation, membrane transportation, protein translocation, and transcription regulation were preferentially expressed in parasitic phase *M. avidus*. The differential gene expression may be needed for the ciliates to survive in the host fish, and the corresponding proteins might be used as antigen candidates for development of scuticociliatosis vaccines.

KEY WORDS: *Miamiensis avidus* · Cultured · Parasitic phase ciliates · Suppression subtractive hybridization · Antigen candidates

INTRODUCTION

The facultative parasitic ciliate *Miamiensis avidus,* which is the synonym of *Philasterides dicentrarchi* reported by Kim et al. (2004) (Jung et al. 2007, Song et al. 2009), is a causative agent of scuticociliatosis, and leads to high cumulative mortalities in cultured olive flounder *Paralichthys olivaceus* in Korea (Kim et al. 2004, Jung et al. 2007). As there are, at present, no effective *in vivo* chemotherapeutants, especially for systemic infections, effective vaccines against scuticociliatosis would be highly desirable.

The external surface of ciliates is mainly covered with a glycosylphosphatidyl inositol (GPI) anchored glycoprotein called surface antigen, also called immobilization antigens (i-antigens). The i-antigens have

been intensively studied as the principal target antigen for vaccine development against *Ichthyophthirius multifiliis*, an obligate parasitic ciliate of freshwater fish (Clark et al. 1995, Wang & Dickerson 2002). However, because of the existence of several serotypes in *I. multifiliis*, i-antigen-mediated immunity is serotype-specific (Dickerson et al. 1993, Dickerson & Clark 1996, 1998, Wang et al. 2002). Generally, free-living ciliates have several different i-antigens, which are alternatively expressed according to ambient environment changes (Caron & Meyer 1989, Simon & Schmidt 2007). Since *Miamiensis avidus* can live not only in seawater as a free-living organism but also in fish as a parasite, the ciliates should experience harsh environmental changes during penetration of fish tissue. Recently, we have demonstrated that sera

of olive flounder immunized with *in vitro* cultured *M. avidus* showed agglutination activity to the cultured ciliates but not to the parasitic-phase ciliates, even though originating from the same cultured ciliate population (Lee & Kim 2008). Moreover, it has been observed that agglutinated scuticociliates following exposure to immune sera escaped from the aggluti-nated mass within a few hours and displayed no adverse effects (Iglesias et al. 2003, Lee & Kim 2008). Therefore, we suggest that other surface antigens expressed constitutively or expressed specifically under the infection state (needed for survival of the ciliates in the host fish) might be more suitable to develop protective subunit vaccines against scutic-ociliatosis (Lee & Kim 2008).

It is reasonable to assume that survival and disease establishment of *Miamiensis avidus* in fish host are closely associated with a set of genes expressed specif-ically in the parasitic phase. However, no information on *M. avidus* genes related to parasitic phase has so far appeared in the literature. The aim of the present study is to find ciliate genes differentially expressed by *M. avidus* in the infectious state using suppression subtractive hybridization (SSH), and discuss the poten-tial of those up-regulated genes as antigen candidates for development of subunit vaccine against scutico-ciliatosis.

MATERIALS AND METHODS

Ciliates. Ciliates were isolated from ascitic fluid of an infected olive flounder *Paralichthys olivaceus* col-lected from a local fish farm in Korea and were iden-tified as *Miamiensis avidus* (syn. *Philasterides dicen-trarchi*) using species-specific oligonucleotide primers (Kim et al. 2004). Chinook salmon embryo (CHSE)-214 cells, incubated at 20°C in Eagle's minimum essential medium (MEM; Sigma) supplemented with 10% heat-inactivated fetal bovine serum, were used as grazing material to grow the ciliates in axenic cul-ture condition.

To obtain parasitic phase ciliates belonging to the same population of the above cultured ciliates, 1×10^5 cells of the CHSE-cultured ciliates were injected into intraperitoneal cavity of juvenile olive flounder weigh-ing 10 to 15 g. After 3 to 5 d following injection, the parasitic-phase ciliates were isolated from ascitic fluid of the fish (showing severe abdominal expansion) using a syringe, and incubated for 1 h at 20°C to let the ciliates graze contaminating fish cells present in the ascitic fluid. The ciliates were then harvested by cen-trifugation at $200 \times g$ for 5 min, and washed 3 times by centrifugation at $150 \times g$ for 5 min in 10 ml of Hanks' balanced salt solution (Sigma).

RNA preparation. Total RNA from ciliates harvested from CHSE culture or from artificially infected floun-der was prepared using RNAiso (Takara) according to the manufacturer's instructions. Poly A+ RNA from the total RNA was isolated using PolyATract mRNA isola-tion system IV (Promega). After total and poly A+ RNA isolation, RNA's integrity was determined by ultravio-let (UV) spectrophotometry (Ultrospec® 3100 *pro*, Amersham Phamacia Biotech) and formaldehyde gel electrophoresis.

SSH. SSH was performed using the PCR-Select™ cDNA Subtraction Kit (Clontech) according to the manufacturer's instructions. Briefly, 2 µg of poly A+ RNA from both parasitic-phase and CHSE-cultured *Miamiensis avidus* were reverse transcribed into sin-gle-stranded cDNA in a reaction containing 1 µM cDNA synthesis primer, 1× first-strand buffer, 1 mM dNTP, and 20 units AMV reverse transcriptase. The second-strand cDNAs were synthesized using second-strand enzyme cocktail containing DNA polymerase I, RNase H, and *Escherichia coli* DNA ligase. The blunt-ending of DNA ends was performed by T4 DNA poly-merase. The cDNAs were then phenol-extracted, ethanol-precipitated, and digested by 15 units of *Rsa*I restriction enzyme to generate shorter, blunt-ended, double-stranded cDNA fragments, which are optimal for subtraction and required for adaptor ligation. The *Rsa*I-digested cDNA of parasitic phase ciliates (tester cDNA) was separated into 2 pools that were ligated to different adaptors (adaptor 1 and adaptor 2R). The *Rsa*I-digested cDNA (1.5 µl) of the CHSE-cultured ciliates (driver cDNA) and 4× hybridization buffer (1 µl) were added to each of 2 seperate tubes contain-ing 1.5 µl of adaptor 1- and 2R-ligated *Rsa*I-digested cDNA of parasitic phase ciliates (tester cDNA). The samples were denatured at 98°C for 1.5 min after overlaying with one drop of mineral oil, and then allowed to hybridize at 68°C for 8 h in a thermal cycler. After this first hybridization, the 2 samples were combined, and 1 µl of freshly heat-denatured driver cDNA (cDNA of the CHSE-cultured ciliates) was added, and then the sample was allowed to hybridize at 68°C overnight in a thermal cycler. After 2-step hybridization of adaptor-ligated tester cDNA and an excess of driver cDNA as above, the parasitic phase *M. avidus* specific, double-stranded cDNA with both adaptors was generated. To amplify this parasitic phase *M. avidus*-specific, ds cDNA with both adap-tors, 2 rounds of PCR were performed with primers specific for the adaptor sequence. The reverse sub-traction was performed simultaneously following the above protocol. In the reverse subtraction, the cDNA of CHSE-cultured ciliates was used as a tester and the cDNA of parasitic phase ciliates was used as a driver. Aliquots from the primary and secondary PCR reac-

tion were analyzed by 2% agarose gel electrophore-sis. And the secondary PCR products were diluted and applied to an additional PCR reaction to evaluate the subtraction efficiency using a primer set specific for β-tubulin gene (GenBank accession number GQ342956.1) of *M. avidus*. The nucleotide sequences of β-tubulin gene-specific primers are shown in Table 1. PCR was performed on the forward-sub-tracted cDNA, unsubtracted tester control cDNA for the forward subtraction reaction, reverse-subtracted cDNA, and unsubtracted tester control cDNA for the reverse subtraction reaction, respectively.

Cloning and sequence analysis. For the forward-subtracted cDNA, the final PCR products from the SSH procedure was ligated with pGEM-T easy vector (Promega) and transformed into *Escherichia coli* DH5α. *E. coli* colonies containing the plasmid vec-tors with inserted PCR products were selected by spreading the transformants onto a Luria-Bertani (LB) agar plate containing ampicillin, 5-bromo-4-chloro-3-indolyl-D-galactoside (X-Gal), and isopropyl 1-thio-β-D-galactopyranoside (IPTG). Plasmid DNA were iso-lated from overnight culture of the randomly picked individual white colonies using GeneALL Plasmid miniprep kit (General Biosystem) according to the manufacturer's instruction. DNA sequencing was performed using automatic sequencer ABI3730xl (96-capillary, Applied Biosystems) according to the proto-col of ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kits version 3.1 at the Macrogen. To identify a putative set of genes ex-pressed in infection state of *Miamiensis avidus*, 520 clones isolated from the SSH library were single-run sequenced. After removing the vector sequence and eliminating clones with a poor sequence quality, nucleotide sequences of the remaining 510 clones were compared to previously reported sequences in the EMBL/GenBank databases using the BLASTX search analysis algorithm of National Center for Biotechnology Information (NCBI).

Table 1. Primer sequences and conditions used in the semi-quantitative reverse-transcription PCR (RT-PCR) and mRNA expression. PPMa: parasitic phase *Miamiensis avidus*; F: forward; R: reverse

Target	Primers	Sequence	Product size (bp)	PCR cycles
Clone				
PPMa-1	1F	5′-AGT GAT ATG TGG AAT GAG GAA CAC ACA ATG-3′	240	30
	1R	5′-TAT GAT GGA GAC TAA AGA GAT TAT ATG CAG-3′		
PPMa-5	5F	5′-GAA AGA ACA CCG TTA TCA AGA AAG CTA TTG-3′	134	23
	5R	5′-TCC ATA TAA AGA TTC TAA TTC GGG CAT AGG-3′		
PPMa-6	6F	5′-ACA TTA ATA CAA TTT AGA AAG ACT TAC C-3′	167	30
	6R	5′-ATC CAT GTT TGA ATC CTT GTG ATC AAT ATC-3′		
PPMa-8	8F	5′-TTT ATA CAG TCT TAA ATC CAA GCA TTA CAC-3′	121	30
	8R	5′-ATT GTC ATC AGG GTA ATA AGT GAA GAG AGG-3′		
PPMa-9	9F	5′-TGG AGA AAG TTC AAG ATA AGT TAC AAA TG-3′	383	30
	9R	5′-AGC TAT AAA TAT CAC CAG CCT GAG TAG G-3′		
PPMa-10	10F	5′-AAA GAA TTA CTC TCG ATG AAG TAT TAG AAC-3′	149	25
	10R	5′-AAA TTT GTT CCT TAT TGC TCA TTA TTC C-3′		
PPMa-11	11F	5′-CCT ACG AGG CTC AAC AAC TCA TAA GGT CTT G-3′	222	29
	11R	5′-CGA ATT CTA GTT GTT GTT GTT GCT ATT CGA AG-3′		
PPMa-15	15F	5′-ATG CTC TCC TCT ATG CCT TAC TTG TTC TAC-3′	239	25
	15R	5′-TTT ATG TAA CTC CTG GGT CAG TAT CTA TGC-3′		
PPMa-19	19F	5′-ATC CAA ATT CGT CAA TAA CAT TCC CAT TG-3′	223	25
	19R	5′-GTG AAT AAT ACC CAA TCC TCC ACA TCT AGC-3′		
PPMa-23	23F	5′- ATT CGG AAC CAG ATA TGG AGC CGC TTT AAG-3′	261	29
	23R	5′-TCG GCA TCG GCA GCA GCG GCG GCT TC-3′		
PPMa-24	24F	5′-TTG CAG AAG GGA TTA CCT ACA TAA GTG AAG-3′	197	28
	24R	5′-TGC AAT AAC TAC AGA ACA TAC AAA GAA TCC-3′		
PPMa-26	26F	5′-CTG TTC GGA AGA ATG AAG GGT CTT ACA G-3′	357	25
	26R	5′-TCC GAG ACA CTA GAA TTG TCC GTT AAT C-3′		
PPMa-28	28F	5′-AGC TCA GCT GCT AAG ACA AAG AGG TGT AGA GG-3′	193	20
	28R	5′-CTA TAC TTT AGA CTC AGC AGC AGC TTT AGC C-3′		
PPMa-29	29F	5′-ATG ATC ATG CAA TGA CAT ATG GAT ATT CCA G-3′	155	31
	29R	5′-TAT AAG CAT TAT TTT AGG ATC AAC AAC AGG AAC-3′		
PPMa-37	37F	5′-ACT AAT GGA AGA AGT ATA ATC GAT AGG GAG-3′	233	32
	37R	5′-CAA TGA GTA GAG TCC ATG CAG CAA TAT GGC C-3′		
PPMa-39	39F	5′-CTT AAG CCG ATG CAC CTA ATG CTG CCA C-3′	208	25
	39R	5′-TTG ACA TTT TCT GCG GCT CCC CCT TCA GC-3′		
Gene				
β-tubulin	BTUF	5′-GGA CCT TTC GGA CAA CTC TTC AG-3′	684	28
	BTUR	5′-TCT GGG ATC GGC GGC GCA CAT CAT G-3′		

Verification of SSH results by semi-quantitative reverse transcription PCR (RT-PCR). To further verify differential expression of the isolated genes, semi-quantitative RT-PCR was performed. Total RNAs were isolated from the ciliates from CHSE-culture and artificially infected flounder. cDNA was synthesized from 500 ng of total RNA using Moloney murine leukemia virus (M-MLV) reverse transcriptase and Oligo(dT)$_{15}$ Primer (Promega) were used in RT-PCR. Primer pairs for randomly selected clones were designed from the sequences obtained by random sequencing of SSH clones (Table 1). PCR was performed using Takara r-Taq premix and 1 μl of 10-fold diluted cDNA in 20 μl reaction. For normalization, the β-tubulin gene was used as the control. Thermal cycling conditions were 1 cycle of 3 min at 95°C (initial denaturation), followed by 28 cycles (for β-tubulin) or 20 to 32 cycles (for each SSH gene) of 30 s at 95°C, 30 s at 55°C, 30 s at 72°C. The size of PCR products and optimal PCR cycles used in this study are shown in Table 1. Optimal PCR cycles of selected genes were determined after confirmation by agarose gel electrophoresis of PCR product taken at every 2 or 3 cycles from 15 to 33 cycles.

RESULTS AND DISCUSSION

To identify a putative set of genes involved in the parasitic phase of *Miamiensis avidus*, 510 individual clones were single run sequenced. The efficiency of the present subtraction was evaluated by PCR with a primer set specific for β-tubulin gene of *M. avidus*. As a result, PCR amplification of β-tubulin gene shows that it appeared after 18 cycles when using the unsubtracted tester cDNA as a template, but was not detected until after 28 cycles when using the subtracted cDNA as a template (Fig. 1). This result suggests that cDNA shared by both tester and driver was reduced efficiently.

Of the 510 SSH clones, 21 clones of 6 putative genes did not show homology to any sequences in the public database, which suggested that they could be novel genes or represent the variable 5′ or 3′ untranslated region (UTR) of the genes (Table 2). The expectation values (*E*-values) of 117 clones encoding 9 putative genes were $>1 \times 10^{-5}$. The other 372 clones that met the criterion of *E*-value $< 1 \times 10^{-5}$ were matched to 26 known sequences in the database, and among these clones, 73 clones of 5 putative genes showed homology to putative proteins derived from *Tetrahymena thermophila* nucleotide sequences. We have deposited the partial sequences of putative genes on the EST database of GenBank. Semi-quantitative RT-PCR of cDNA synthesized from both CHSE-cultured and parasitic-phase *Miamiensis avidus* was performed to confirm
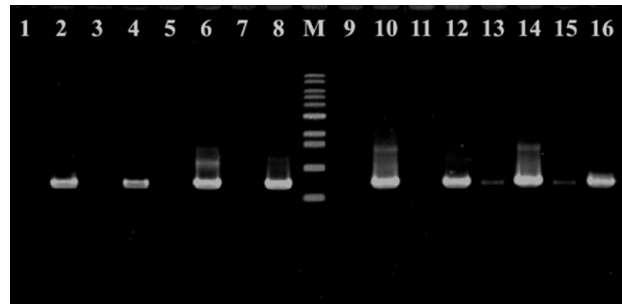

Fig. 1. Subtraction efficiency was evaluated by PCR using β-tubulin gene-specific primers of *Miamiensis avidus*. PCR was performed on the secondary-nested PCR products of the forward-subtracted cDNA (Lanes 1, 5, 9, 13), unsubtracted control cDNA for forward subtraction (Lanes 2, 6, 10, 14), reverse-subtracted cDNA (Lanes 3, 7, 11, 15), and unsubtracted control cDNA for reverse subtraction (Lanes 4, 8, 12, 16). A total of 5 μl of PCR products were removed after 18 cycles (Lanes 1 to 4), 23 cycles (Lanes 5 to 8), 28 cycles (Lanes 9 to 12), and 33 cycles (Lanes 13 to 16) and electrophoresed on a 1% agarose gel. M: 1kb DNA ladder (Bioneer)

differentially expressed genes in parasitic phase *M. avidus*. As shown in Fig. 2, the tested genes were differentially expressed in parasitic phase *M. avidus*, whereas the β-tubulin gene was evenly expressed in both cultured and parasitic phase ciliates.

Of the 372 clones (*E*-value $< 1 \times 10^{5}$), 106 clones showed homology to the acidic ribosomal P0 protein. The acidic ribosomal P0 protein is associated with the translocation step of protein synthesis, along with other ribosomal proteins P1/P2 (Liljas 1991). The conserved C-terminal domain of the P proteins is very antigenic, and the protective or immunological role of antibodies to this domain has been reported in several pathogenic protozoans of mammals (Skeiky et al. 1994, Soto et al. 1995, Chatterjee et al. 2000a,b, Zhang et al. 2007). Furthermore, the surface expression of the P0 protein on apicomplexan patrasites, such as *Plasmodium falciparum* and *Toxoplasma gondii*, has been demonstrated (Chatterjee et al. 2000a,b, Singh et al. 2002). Although the antigenic potential of P0 protein in ciliates has not been reported, the present result suggests that the ribosomal P0 protein of *Miamiensis avidus* might be used as an antigen candidate for a subunit vaccine against scuticociliatosis.

In the present study, many of the differentially expressed clones corresponded to genes that are involved in cellular signaling such as delta 8-sphingolipid desaturase, inosine monophosphate dehydrogenases (IMPDH)/guanosine monophosphate reductase (GMPR) domain-containing protein, protein kinase domain-containing protein, and epidermal growth factor (EGF)-like domain-containing protein. The putative delta 8-sphingolipid desaturase encoding clones were

Table 2. Putative set of genes preferentially expressed in the parasitic phase *Miamiensis avidus* (PPMa). *E*-value: expectation value; EGF: epidermal growth factor; IMPDH: inosine monophosphate deydrogenase; GMPR: guanosine monophosphate reductase; DYHC: dynein heavy chain cytosolic; VSP: variant-specific surface protein

| Name of clone (Accession no.)	Size (bp)	Frequency (%)	Accession no.	Species	Best BLASTX match — Homology to	*E*-value
PPMa-1 (GW397202.1)	625	6.7	CAA11857	*Brassica napus*	Delta 8-sphingolipid desaturase	1×10^{-13}
PPMa-2 (GW397203.1)	511	2.0	ZP_01062224	*Flavobacterium* sp.	Hypothetical beta-lactamase precursor	4×10^{-8}
PPMa-3 (GW397204.1)	598	4.5	YP_913162	*Romanomermis culicivorax*	NADH dehydrogenase subunit 5	9×10^{-3}
PPMa-4 (GW397205.1)	424	2.0	XP_001009203	*Tetrahymena thermophila* SB210	Glyceraldehyde-3-phosphate dehydrogenase, type I family protein	3×10^{-52}
PPMa-5 (GW397206.1)	557	20.8	AAY19283	*Tetrahymena thermophila*	Acidic ribosomal P0 protein	4×10^{-46}
PPMa-6 (GW397207.1)	470	7.3	NP_603105	*Fusobacterium nucleatum* subsp. *nucleatum*	Transcriptional regulatory protein	0.12
PPMa-7 (GW397208.1)	639	2.9	XP_001031037	*Tetrahymena thermophila*	Hypothetical protein TTHERM_00942960	7×10^{-5}
PPMa-8 (GW397209.1)	450	5.5	XP_001024115	*Tetrahymena thermophila*	Hypothetical protein TTHERM_00455090	5×10^{-13}
PPMa-9 (GW397210.1)	841	8.2	XP_001032486	*Tetrahymena thermophila*	Protein kinase domain-containing protein	3×10^{-36}
PPMa-10 (GW397211.1)	410	3.7	XP_001032606	*Tetrahymena thermophila*	Protein kinase domain-containing protein	1×10^{-22}
PPMa-11 (GW397212.1)	234	0.4	XP_001013395	*Tetrahymena thermophila*	Protein kinase domain-containing protein	9×10^{-10}
PPMa-12 (GW397213.1)	197	0.2	XP_001021694	*Tetrahymena thermophila*	Protein kinase domain-containing protein	1×10^{-13}
PPMa-13 (GW397214.1)	314	0.8	XP_001024308	*Tetrahymena thermophila*	Cation channel family protein	4×10^{-6}
PPMa-14 (GW397215.1)	395	3.5	XP_001440548	*Paramecium tetraurelia*	Hypothetical protein	6×10^{-4}
PPMa 15 (GW397216.1)	557	1.0	XP_001013014	*Tetrahymena thermophila*	EGF-like domain-containing protein	4×10^{-22}
PPMa-16 (GW397217.1)	257	0.4	YP_740790	*Tetrahymena paravorax*	Ymf77	2.4
PPMa-17 (GW397218.1)	275	1.2	CAK68281	*Paramecium tetraurelia*	Unnamed protein product	0.072
PPMa-18 (GW397219.1)	616	2.0	XP_001015519	*Tetrahymena thermophila*	Hypothetical protein TTHERM_00382350	1×10^{-37}
PPMa-19 (GW397220.1)	785	4.3	XP_001032893	*Tetrahymena thermophila*	IMPDH/GMPR domain-containing protein	3×10^{-48}
PPMa-20 (GW397221.1)	508	1.2	XP_001012215	*Tetrahymena thermophila*	Hypothetical protein TTHERM_00102650	0.26
PPMa-21 (GW397222.1)	634	2.0	EAA65296	*Aspergillus nidulans*	DYHC	1.3
PPMa-22 (GW397223.1)	517	3.9	AAS93869	*Paramecium tetraurelia*	G-protein beta subunit	2×10^{-25}
PPMa-23 (GW397224.1)	390	3.7	XP_001015607	*Tetrahymena thermophila*	Hypothetical protein TTHERM_00075670	6×10^{-35}
PPMa-24 (GW397225.1)	395	0.4	XP_001020598	*Tetrahymena thermophila*	Sodium/calcium exchanger protein	3×10^{-20}
PPMa-25 (GW397226.1)	279	2.7	XP_636581	*Dictyostelium discoideum*	Hypothetical protein	6×10^{-4}
PPMa-26 (GW397227.1)	384	1.6	XP_001013009	*Tetrahymena thermophila*	ABC transporter family protein	3×10^{-54}
PPMa-27 (GW397228.1)	342	0.6	BAA86219	*Seriola quinqueradiata*	Alpha hemoglobin B	3×10^{-36}
PPMa-28 (GW397229.1)	248	0.6	YP_592750	*Acidobacteria bacterium*	NmrA-like	4×10^{-14}
PPMa-29 (GW397230.1)	209	0.8	XP_001022031	*Tetrahymena thermophila*	Cation diffusion facilitator family transporter containing protein	3×10^{-13}
PPMa-30 (GW397231.1)	104	1.2			No significant similarity found	
PPMa-31 (GW397232.1)	343	0.8			No significant similarity found	
PPMa 32 (GW397233.1)	90	1.0			No significant similarity found	
PPMa-33 (GW397234.1)	144	0.8			No significant similarity found	
PPMa-34 (GW397235.1)	804	0.2	XP_001013881	*Tetrahymena thermophila*	Cation channel family protein	3.5
PPMa-35 (GW397236.1)	102	0.2			No significant similarity found	
PPMa-36 (GW397237.1)	303	0.2	XP_001024855	*Tetrahymena thermophila*	ChaC-like protein	2×10^{-13}
PPMa 37 (GW397238.1)	352	0.2	XP_001032381	*Tetrahymena thermophila*	Major facilitator superfamily protein	2×10^{-15}
PPMa-38 (GW397239.1)	295	0.2			No significant similarity found	
PPMa-39 (GW397240.1)	228	0.2	AAG37861	*Giardia intestinalis*	VSP	3×10^{-5}
PPMa-40 (GW397241.1)	364	0.2	XP_001017835	*Tetrahymena thermophila*	DnaJ domain-containing protein	1×10^{-15}
PPMa-41 (GW397242.1)	568	0.2	XP_001014988	*Tetrahymena thermophila*	Hypothetical protein TTHERM_00672110	3×10^{-15}
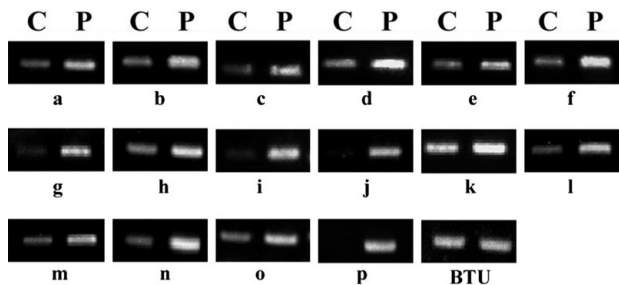
Fig. 2. Semi-quantitative reverse transcription-PCR analysis of differentially expressed genes in parasitic-phase *Miamiensis avidus*. cDNAs used as PCR templates were synthesized from total RNA of the Chinook salmon embryo (CHSE)-cultured ciliates and the parasitic phase ciliates. C: CHSE-cultured *M. avidus*; P: parasitic phase of *M. avidus*: (a) PPMa-1, delta 8-sphingolipid desaturase; (b) PPMa-5, acidic ribosomal P0 protein; (c) PPMa-6, transcriptional regulatory protein; (d) PPMa-8, a hypothetical protein; (e–g) PPMa-9 to 11, protein kinase domain-containing protein; (h) PPMa-15, epidermal growth factor (EGF)-like domain-containing protein; (i) PPMa-19, inosine monophosphate dehydrogenases (IMPDH)/guanosine monophosphate reductase (GMPR) domain containing protein; (j) PPMa-23, hypothetical protein; (k) PPMa-24, sodium/calcium exchanger protein; (l) PPMa-26, ABC transporter family protein; (m) PPMa-28, NmrA-like protein; (n) PPMa-29, cation diffusion facilitator family transporter-containing protein; (o) PPMa-37, major facilitator superfamily protein; (p) PPMa-39, variant-specific surface protein (VSP); BTU: β-tubulin gene used as an internal standard for normalization. PCR products were analyzed on 1.5% agarose gel stained with ethidium bromide

the second most abundant clones (34 clones). Sphingolipids are a family of membrane lipids in eukaryotic cells, and consist of a long-chain base (LCB) and a single amide-linked fatty acid (Futerman & Hannun 2004). Sphingolipids and their catabolites are involved in regulating cellular processes, such as cell proliferation, differentiation, migration, apoptosis, and inflammation (Futerman & Hannun 2004, Ogretmen & Hannun 2004). Desaturation of sphingolipid LCB affects the subsequent activity of both the sphingolipid and its derivatives. The essential roles of sphingolipids in infectivity and virulence of a pathogenic protozoan parasite *Leishmania* have been demonstrated (Zhang et al. 2003, Denny et al. 2004). Thus, the role of delta 8-sphingolipid desaturase in infectivity and virulence of *Miamiensis avidus* should be further studied. The clones encoding putative IMPDH/GMPR domain-containing protein were the third most abundant clones (22 clones). IMPDH catalyzes the formation of XMP from IMP, a rate-limiting reaction of de novo GTP biosynthesis, and is associated with cell proliferation and neoplastic cell transformation (Markham et al. 1999), which makes it a target for anti-cancer, antiviral, antimicrobial, antiprotozoan therapies (Franchetti & Grifantini 1999, Hedstrom 1999, Sullivan et al. 2005). GMPR catalyzes the reductive deamination of

GMP to IMP, and plays an important role in maintaining the intracellular balance of A and G nucleotides (Li et al. 2006). The clones encoding protein kinase domain-containing protein were the fourth most abundant clones (21 clones). Many cellular processes including intercellular communication and coordination are controlled by protein phosphorylation, and protein kinases conduct almost all protein phosphorylation in eukaryotic cells (Livneh & Fishman 1997). Protein kinases in many parasitic protozoans have been investigated as drug targets (Doerig et al. 2002, 2005) since protein kinases play important roles through phosphorylating other proteins involved in parasite invasion and survival (Ghosh et al. 2010, Leykauf et al. 2010). Five clones encoding the EGF-like domain-containing protein were isolated from the SSH library. EGF domains are involved in the modulation of cell growth and development through intercellular signaling, which is mediated by EGF-containing ligands and receptors on the cell surface (Kansas et al. 1994). Recently, the EGF-like domain-containing proteins have been identified as vaccine candidate antigens against blood stages of *Plasmodium falciparum* (Burns et al. 2000, Black et al. 2001).

Another group of molecules that we found to be expressed preferentially in the parasitic phase ciliates is related to transporting molecules, such as ABC transporter family protein (8 clones), cation diffusion facilitator family transporter-containing protein (4 clones), and major facilitator superfamily protein (1 clone). In addition, 3 clones of NmrA-like protein, which negatively regulates transcription and is involved in the controlling of nitrogen metabolite repression in fungi, were isolated (Stammers et al. 2001).

Surface proteins of parasites are considered as ideal vaccine candidates because surface-exposed proteins can elicit production of antibodies, which can act as opsonins and/or inhibit colonization and invasion of parasites (Leid et al. 1987, Kedzierski et al. 2004, Mello et al. 2004, Fankhauser et al. 2007). In mammalian protozoan parasites belonging to genus *Plasmodium*, *Trypanosoma*, and *Leishmania*, many vaccination attempts revealed that protective immunity was obtained by immunization with recombinant surface antigen (Musoke et al. 1992, Mello et al. 2004) or plasmid DNA encoding surface antigen (Nielsen et al. 1999, Dumonteil et al. 2004). In the present study, only one clone was matched to a surface protein (acidic ribosomal PO protein), a variant-specific surface protein.

In conclusion, the data reported here identify for the first time a set of genes differentially expressed in the parasitic phase *Miamiensis avidus*. These genes should be further studied to know how the ciliates survive and proliferate in the host fish, and this could provide new strategies for anti-scuticociliates vaccine.

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