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Molecular characteristics of an immobilization antigen gene of the fish-parasitic protozoan *Ichthyophthirius multifiliis* strain ARS-6

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Abstract

Ichthyophthirius multifiliis (Ich), a ciliated protozoan parasite of fish, expresses surface antigens (i-antigens), which react with host antibodies that render them immobile. The nucleotide sequence of an i-antigen gene of *I. multifiliis* strain ARS-6 was deduced. The predicted protein of 47 493 Da is comprised of 460 amino acids (aa's) arranged into five imperfect repeats with periodic cysteine residues with the structure: $CX_{(19)20}CX_2CX_{16-27}CX_2CX_{20(21)}CX_3$. The N-terminal aa's typify a signal peptide motif while a stretch of C-terminal aa's resemble a glycosyl–phosphatidyl–inositol (GPI)-anchor addition site. The degree of deduced i-antigen aa sequence identity of strain ARS-6 (GenBank accession # ACH87654 and # ACH95659) with other *I. multifiliis* i-antigen sequences present in GenBank ranges from 99% to 36% identity with 52 kDa i-antigens of *I. multifiliis* strain G5 (accession #s AAK94941 and AAK01661 respectively). Immunoblot analysis of i-antigens following exposure of *I. multifiliis* theronts to catfish anti-*I. multifiliis* immune serum did not show any appreciable alteration in i-antigen expression. The mechanism that regulates i-antigen expression in *I. multifiliis* remains a puzzling question.

Keywords: immobilization antigen, gene, nucleotide sequence, *Ichthyophthirius multifiliis*

Introduction

Ichthyophthirius multifiliis (Ich) is a hymenostomatid parasitic ciliate that causes 'white spot' disease in many species of freshwater cultured and/or free living fish. Fish that recover from *I. multifiliis* infection are known to possess both serum and cutaneous mucus antibodies that are capable of immobilizing the parasite *in vitro* (Clark & Dickerson 1997). The antigens on the parasite surface that are involved in the antibody-mediated immobilization are comprised of a class of abundant glycosyl–phosphatidyl–inositol (GPI)-anchored membrane proteins that are aptly designated as immobilization antigens (i-antigens) (Clark, Gao, Gaertig, Wang & Cheng 2001; Clark & Forney 2003). Similar GPI-anchored proteins that demonstrate antibody-mediated immobilization have been observed in a variety of phylogenetically distinct, parasitic and free-living ciliate protozoans that thrive in an aquatic environment (Kusch & Schmidt 2001; Hatanka, Umeda, Yamashita & Hirazawa 2007). Fish-parasitic ciliates, in which the phenomenon of immobilization has been well studied include *I. multifiliis* (Clark & Dickerson 1997; Swennes, Findly & Dickerson 2007), *Cryptocaryon irritans* (Hatanka *et al.* 2007) and *Philasterides dicentrarchi* (Iglesias, Parama, Aivarez, Leiro, Ubeira & Sanmertin 2002; Lee & Kim 2008). *Tetrahymena* and *Paramecium* species are free-living ciliated protists that have served as model systems, in which i-antigens have been extensively studied (Doerder 2000; Simon & Schmidt 2005). Both *Tetrahymena* and *Paramecium* possess a repertoire of paralogous genes that encode alternative forms of i-antigens, the expression of which is under the influence of certain environmental cues such as temperature and medium, in which they

grow (Kusch & Schmidt 2001; Simon & Schmidt 2007). Typically, only one i-antigen is constitutively expressed (invoking mutual exclusion) in any given strain (Doerder 2000; Simon, Marker & Schmidt 2006). Logically, a selection mechanism that results in the expression of a single i-antigen gene from a repertoire must exist, but the precise mechanism whereby one i-antigen gene is selected for transcription is as yet unknown.

Western blots of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) resolved protein profiles of *I. multifiliis* strains have revealed the presence of a single polypeptide band or duplet of polypeptide bands within an apparent molecular mass range of 40–60 kDa (Clark, Lin & Dickerson 1995; Dickerson & Clark 1996). Genes that code for three *I. multifiliis* i-antigens have been cloned and sequenced (Clark, Lin, Jackwood, Sherrill, Lin & Dickerson 1999; Lin, Lin, Wang, Wang, Stieger, Klopfleisch & Clark 2002). One of the genes [designated *IAG48*(*G1*)] encodes a 48 kDa polypeptide of the *I. multifiliis* isolate G1, belonging to serotype A (Clark *et al.* 1999), while two other genes *IAG52A*(*G5*) and *IAG52B*(*G5*), encoding 52 kDa polypetides, are from serotype D (Lin *et al.* 2002). Apparently isolate G1 also contains an additional i-antigen gene that has not been cloned (Clark *et al.* 1999). Comparison of the aligned amino acid (aa) sequences of the three i-antigens from these two (A and D) serotypes, has revealed similar structural features with differences in the copy number of tandemly repetitive aa sequence domains, but the antigens share only ~50% overall sequence identity among them. Given that distinctive serotype differences are distinguishable among isolates, based on i-antigen recognition by antiserum and by Southern blotting (Dickerson, Clark & Leff 1993), it is apparent that different serotypes possess diverse i-antigen genes. Here, we describe an i-antigen of *I. multifiliis* strain ARS-6 and compare the deduced aa sequence with those of presently known aa sequences of the serotypes A and D. Additionally we examined the influence of i-antigen-directed antibody pressure on the expression of *I. multifiliis* surface epitopes *in vitro*.

Materials and methods

Fish and parasite isolate

Channel catfish, *Ictalurus punctatus* (Rafinesque), National Warmwater Aquaculture Center Strain 103 (~ 25–30 g) were reared at the Aquatic Animal Health Research Unit, Auburn, Alabama. Fish were maintained in aquaria with flowing dechlorinated water, constant aeration and water temperature at $23 \pm 2\ ^{\circ}C$. *Ichthyophthirius multifiliis* strain ARS-6, originally isolated from an infected channel catfish from a fish pond at New Hope, AL, USA, was propagated and maintained through serial passage in channel catfish as previously described (Xu, Klesius & Panangala 2006). Theronts developed from a single mature trophont of *I. multifiliis* ARS-6 were harvested, aliquoted, stored frozen in liquid nitrogen and used for all subsequent experiments in this study.

Protein sequence analysis

Whole *I. multifiliis* theronts were lysed in sample buffer (Laemmli 1970) to yield a protein concentration of $\sim 1\ \mu g\ \mu L^{-1}$ (w/v) determined with the bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL, USA). *Ichthyophthirius multifiliis* protein sample was loaded ($30\ \mu L\ well^{-1}$) onto a 10–20% gradient polyacrylamide (Criterion) slab gel (Bio-Rad, Hercules, CA, USA) and electrophoresed at 150 V (constant voltage) for 2 h at 15 °C. A portion of the gel was stained with GelCode Coomassie blue (Pierce Biotechnology) while an excised portion of the same gel was used for locating the position of i-antigen by immunoblotting under non-reducing conditions (Towbin, Staehlin & Gordon 1979). Protein blots were probed with primary serum antibodies from catfish immunized with *I. multifiliis* theronts, followed by a secondary, horseradish peroxidase-conjugated goat-anti catfish IgM (custom prepared by Rockland Immunochemicals, Gilbertsville, PA, USA). Antibody-bound antigens were visualized by an enzyme-substrate (4-chloro-1-naphthol, Bio-Rad) according to instructions provided. The i-antigen protein (apparent molecular mass of ~ 37 kDa) band of *I. multifiliis* was located, excised from the polyacrylamide gel and submitted for microsequence analysis to the mass spectrometry and Proteomics Shared Facility of the University of Alabama at Birmingham, Alabama, USA. Tandem mass spectral data derived by proteolysis, and liquid chromatography-tandem mass spectrometry (LC-MS/MS) were analysed with MASSLYNX MAXENT 3 (Micromass, Manchester, UK) software program. The deduced amino-terminal aa sequence of i-antigen peptides of *I. multifiliis* was used to find matching sequences in the National Center for Biotechnology Information, GenBank database using the Basic Local Alignment Search Tool.

Oligonucleotide primers, amplification of genomic DNA and cDNA, and sequencing

The sequence of the i-antigen coding sequences and flanking sequences (deposited in GenBank under accession # FJ012354 and #FJ19440) was determined from three overlapping PCR products obtained as described below. The central portion, including 1.1 kb of the coding sequence, was determined from a PCR product generated using primers based on i-antigen gene sequence segments in GenBank encoding peptides found in the purified i-antigen peptide of ARS-6. The 5′ portion of the coding sequence and 269 nucleotides (nt) of upstream sequence were determined from a PCR product generated by inverse PCR using primers based on the sequence determined from the central portion. The 3′ portion of the coding sequence plus 49 nt of downstream sequences through the polyadenylation site were determined from an amplified and cloned cDNA fragment generated by a rapid amplification of cDNA ends (RACE) procedure.

To amplify a genome segment containing nearly 80% of the i-antigen coding sequence, oligonucleotide primers IAg FP1A and IAgRP2A (Table 1) were designed based on portions of a previously described sequence in GenBank (accession # AF405431) encoding peptides identified by sequencing of purified i-antigen peptides. Primers were synthesized at the IA State University, DNA Sequencing and Synthesis Facility, Ames, Iowa, USA. Genomic DNA from *I. multifiliis* theronts was isolated and purified with a DNeasy Tissue Kit (Qiagen, Valencia, CA, USA) following the protocol for purification of genomic DNA from cultured animal cells. Nucleic acid was quantified with a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA) spectrophotometer. Polymerase chain reaction amplifications were performed in a final volume of 25 μL, with 1.0 μL ($\sim$ 20–30 ng μL^{-1}) of *I. multifiliis* DNA, 12.5 μL of 2 × Master Mix (Promega, Madison, WI, USA), 1.25 μL of each (10 μM) forward (IAgFP1A) and reverse (IAgRP2A) primer (Table 1), and 9.0 μL sterile-distilled water. Amplification was performed in a T gradient thermocycler (Whatman Biometra, Göttingen, Germany). The cycling parameters consisted of an initial denaturation at 95 °C for 30 s, followed by 27 cycles at 95 °C for 30 s, annealing at 65–45 °C (decreasing 1 °C each cycle for the first 20 cycles) for 30 s, 72 °C for 1 min and a final extension at 72 °C for 10 min. Amplified product was examined by gel electrophoresis on a 1.5% agarose gel stained with ethidium bromide. A 100 bp DNA ladder (Fisher Scientific, Pittsburg, PA, USA) was used as molecular size standards. Amplicons of about 1.1 kbp were excised from the gel and purified using QIAquick (Qiagen) gel extraction and purification kit according to the manufacturer's instructions and the products were directly sequenced at the Iowa State University, DNA Sequencing and Synthesis Facility using primers IAgFP1A, IAgRP2A, IAgFP4 and IAgRP5 (Table 1).

An inverse-PCR technique described by Keim, Williams and Harwood (2004) was used to obtain the remaining sequences of the i-antigen gene 5′ to the PCR product. Briefly, 1.5 μg of purified *I. multifiliis* theront-DNA was digested with 10 U *Taq*I (Promega) according to the protocol supplied, extracted with phenol/chloroform, and precipitated with ethanol. The DNA fragments were self-ligated in a total volume of 21 μL containing 10 μL of DNA (4 ng μL^{-1}), 10 μL of 2 × ligation buffer, and 1 μL T4 DNA-ligase

Table 1 Primers used for polymerase chain reaction (PCR) or sequencing

Name	Use	Sequence*	Location†	Orientation
IAgFP1A	PCR/sequencing	ggctGCaGTTAATTGTCCTaATGGwGC	327–349	Forward
IAgRP2A	PCR/sequencing	gcggtaccGCyTAACATTTAGTACATTCACTTGC	1434–1459	Reverse
IAgFP4	Sequencing	GTAATCCTACAGGTCAGG	673–690	Forward
IAgRP5	Sequencing	TAGCCTCAAGACCTGATC	1129–1146	Reverse
IAgRACE	3′-RACE	GGTTCAGCATCTGTTCCAGGTAATAGTGC	1323–1351	Forward
IAgQ-F	Inverse PCR	TGAGAATCGGCTTTGTGTACTTGG	503–526	Reverse
IAgS-R	Inverse PCR	GGATCACCTACTTTTACTTAATCCCTCAC	603–631	Forward
IAgR	Sequencing	CCACCTTATCTGTGTTGAGAATCGG	517–541	Reverse
IAgT-S	Sequencing	GCTCCTGTATCAGATTATCCATTC	359–382	Reverse

*Lowercase letters indicate primer nucleotides differing from gene sequence; w = A or T; y = T or C.
†Nucleotide numbering according to GenBank # FJ012354.

(400 U μL^{-1}; Biolabs, Atlanta, GA, USA). The ligation reaction was carried out at 20 °C for 30 min, followed by 25 °C for an additional 30 min and terminated by heating at 68 °C for 15 min. Inverse PCR amplification was performed with the ligated DNA in a 25 μL volume that contained 12.5 μL 2 × FailSafe PreMix Buffer-C (Epicenter Biotechnologies, Madison, WI, USA), 0.5 μL (50 μM) of each primer IAgQ-F and IAgS-R (Table 1), 1.25 μL of template DNA, and 10.25 μL of sterile-distilled water. The cycling parameters were: initial denaturation at 95 °C for 2 min, followed by 30 cycles of 95 °C for 30 s, annealing 58–49 °C (decreased by 0.3 °C each cycle) for 45 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. The amplified product was analysed by electrophoresis on a 1% agarose gel with ethidium bromide. A ∼ 600 bp PCR product was excised from the gel and the DNA extracted using a QIAquick (Qiagen) gel-extraction kit according to instructions provided. The purified DNA was submitted (Iowa State University, DNA Sequencing and Synthesis Facility) for sequencing with the primers IAgR and IAgT-S (Table 1).

Sequences at the 3′end of the i-antigen gene were obtained from cDNA generated by a reverse transcription template switching procedure (Zhu, Machleder, Chenchik, Li & Siebert 2001) using a SMART™ RACE cDNA amplification kit (Clontech, Mountain View, CA, USA) according to the manufacturer's protocol. Briefly, using 1 μg of total RNA from *I. multifiliis* theronts as template, the poly(A^+) mRNA was reverse transcribed using an oligo-dT primer, a 3′-CDS primer-A with 3′dC-tailing activity, and Moloney Murine Leukemia Virus reverse transcriptase (Promega). The first-strand cDNA generated through this reaction was used as template for 3′ RACE-PCR with a 5′gene-specific primer (IAgRACE; Table 1) and a Universal Primer A Mix (UPM) primer. Thermal cycling conditions were programmed according to a standard touchdown PCR protocol (Don, Cox, Wainwright, Baker & Mattick 1991). The resulting product was analysed on a 1.2% agarose gel with ethidium bromide to identify a single specific band. The amplicon (∼ 405 bp) was extracted and purified using a QIAquick (Qiagen) gel extraction kit. The product was cloned using a standard T/A-cloning strategy into the pGEM-T Easy vector (Promega) and independent clones sequenced in both directions using T7 and SP6 primers. Sequencing was performed in an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and the sequence of the 3′ end of the transcript through the poly-A tail was obtained.

Evaluation of serum antibody effect on *I. multifiliis* theronts

Channel catfish infected with mature *I. multifiliis* trophonts were anaesthetized with 100 mg L^{-1} tricaine methanesulphonate (Argent Chemical, Richmond, WA, USA) and the skin was gently scraped to dislodge the parasites. Mucus containing the parasite was mixed with water and filtered through a 0.45 μm filter to trap the mucus and sloughed skin, and the extruded parasites collected in 140 × 20 mm Petri dishes and allowed to attach. The water in the Petri dishes was renewed three times and the trophonts were incubated at 22–24 °C in fresh water for 24 h. Theronts were harvested in 40 mL water, washed in PBS (pH 7.4) and concentrated by centrifugation (Beckman Coulter, Miami, FL, USA) at 228 *g* for 5 min. The theronts resuspended in fresh water were enumerated using a Sedgewick-Rafter cell (VWR Scientific Products, Atlanta, GA, USA). The threonts (∼ 6900 theronts mL^{-1}) were divided into six aliquots in 50 mL tubes each containing approximately equal numbers of theronts. Theronts in six tubes (45 mL $tube^{-1}$) were divided into three experimental groups A, B and C (two tubes per group). Theronts in group A were treated with catfish anti-*I. multifiliis* serum at a final dilution of 1:200 while those in group B were treated with a final anti-serum dilution of 1:400. The anti-serum was previously collected from channel catfish immunized with *I. multifiliis* theronts and had a theront-immobilization titre of 1:1024. The group C theronts were maintained without any antibody treatment as controls. All tubes were held at 22–24 °C with oscillation and at 7 and 14 h post treatment, theronts from each group were collected by centrifugation at 1250 *g* for 5 min. The supernatant in each tube was discarded and the theront pellet transferred to a 1.5 mL microcentrifuge tube. This experiment was repeated three times.

Theronts were disrupted for 30 s with a Virsonic 600 (Virtis, Gardiner, NY, USA) sonifier, mixed (1:2 v/v) with Laemmli sample buffer (Bio-Rad) under non-reducing conditions and boiled for 5 min. Each sample ∼ 40 μL was loaded into the wells of a precast Criterion (Bio-Rad) 10–20% linear gradient polyacrylamide slab gel and electrophoresed at 175 V (constant voltage) at 15 °C for 2 h. Electrophoretically resolved proteins were transferred onto PVDF membranes

and the protein blots were probed with catfish anti-*I. multifiliis* antibodies as described above.

Results and discussion

Peptide sequence analysis of purified i-antigen of *I. multifiliis* strain ARS-6 revealed 11 peptides with sequences corresponding to the predicted aa sequence of the protein encoded by the gene designated *IAG52B*[*G5*] (GenBank accession # AAK9491), the major i-antigen expressed by *I. multifiliis* strain G5 (Lin *et al.* 2002). These peptides (underlined in Fig. 1) range in size from nine to 56 aa's and together represent over 50% of the *IAG52B*[*G5*]-encoded aa sequence. None of the peptides exactly match i-antigens encoded by genes other than *IAG52B*[*G5*] in GenBank. The deduced aa sequence of the entire *I. multifiliis* strain ARS-6 i-antigen polypeptide, determined from the sequence of the gene initially identified using primers based on the sequenced peptides is also extremely similar (99% identity) to the entire aa sequence of the protein encoded by the gene designated *IAG52B*[*G5*]. The deduced aa sequence of the ARS-6 strain i-antigen is much less similar to available predicted aa sequences of other *I. multifiliis* strains, with 54% identity with strain G1 (GenBank accession # AAD31283) and 42% identity with strain NY4 (aa sequence predicted from nucleotide sequence derived from overlapping sequences in the GenBank EST database). The deduced aa sequence of the ARS-6 i-antigen displays only 36% identity with the sequence of an over 100-fold less abundantly expressed i-antigen (GenBank accession # AAK01661) encoded by a second gene, designated *IAG52A*[*G5*], of *I. multifiliis* strain G5 (Lin *et al.* 2002). We did not determine whether strain ARS-6, like strains G1 and G5, contains an additional i-antigen gene(s). However, the predicted aa sequence of the gene we identified matches the peptides found in purified i-antigen, suggesting that the gene identified encodes the most abundant i-antigen of ARS-6 even if the ARS-6 genome encodes more than one i-antigen. When the proteins of *I. multifiliis* ARS-6 were resolved by one-dimensional SDS-PAGE, only a single protein band with an apparent molecular mass of $\sim$ 37 kDa was excised from the gel. However, aa sequence analysis

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ARS-6     MKFNILIILIISLFINELRAVNCPNGAAIANGQSDTGAADINTCTHCQKHFYFNGGNPAGQAPGAGQFNP 70
AAK94941  MKFNILIILIISLFINELRAVNCPNGAAIANGQSDTGAADINTCTHCQKHFYFNGGNPAGQAPGAVQFNP 70
                              *                     *  *

ARS-6     GVSQCIACQVHKADSQHRQGGDANLAAQCSNLCPAGTAVEDGSPTFTQSLTQCVNCKPNFYFNGGNPTGQ 140
AAK94941  GVSQCIACQVHKADSQHRQGGDANLAAQCSNLCPAGTAVEDGSPTFTQSLTQCVNCKPNFYFNGGNPTGQ 140
          *  *                  *   *                     *  *

ARS-6     APGAGQFDPTQLIANPDLANNPEVPNVSSPNGQCVACQVNKSDSQLRPGAQANLATQCNNECPTGTAIQD 210
AAK94941  APGAGQFDPTQLIANPDLANNPEVPNVSSPNGQCVACQVNKSDSQLRPGAQANLATQCNNECPTGTAIQD 210
                                    *  *                  *   *

ARS-6     GAIFIYTQSISQCTFCKVDFYFNGGNPSAQNPGNGQFTPGQLIANPDAATSAQIPMVPGPNSKCVACESK 280
AAK94941  GAIFIYTQSISQCTFCKVDFYFNGGNPSAQNPGNGQFTPGQLIANPDAATAAQIPMVPGPNSKCVACESK 280
                    *  *                                                  *  *

ARS-6     KTNSQSRSGLEANLAAQCGTECPAGTLVTDGVTPTYTVSLSQCVNCKAGFYQNSNFEAGKSQCNKCAVSK 350
AAK94941  KTNSQSRSGLEANLAAQCGTECPAGTLVTDGVTPTYTVSLSQCVNCKAGFYQNSNFEAGKSQCNKCAVSK 350
                         *   *                  *  *                  *  *

ARS-6     TGSASVPGNSATSATQCQNDCPAGTVVDDGTSTNFVALASECTKCQANFYASKTSGFAAGTDTCTECSKK 420
AAK94941  TGSASVPGNSATSATQCQNDCPAGTVVDDGTSTNFVALASECTKCQANFYASKTSGFAAGTDTCTECSKK 420
                        *   *                  *  *                  *  *

ARS-6     LTSGATAKVYAEATQKAQCASSTFAKFLSMSLIFISFYLL 460
AAK94941  LTSGATAKVYAEATQKAQCASSTFAKFLSMSLIFISFYLL 460
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Figure 1 ClustalW amino acid (aa) sequence alignment of the predicted i-antigen polypeptide of *Ichthyophthirius multifiliis* strain ARS-6 (GenBank accession nos. ACH87654 and ACH95659) with aa sequence of i-antigen IAG52B of *I. multifiliis* strain G5 [GenBank accession no. AAK94941 (Lin *et al.* 2002)]. Amino acids 1-452 of the ARS-6 i-antigen was derived from genomic DNA sequence (GenBank accession no. FJ012354) and aa 26–460 was derived from cDNA (GenBank accession no. FJ194440). Arrows indicate positions at which aa differ. Periodic cysteine residues that coincide are indicated by asterisks. These cysteine residues are conserved among all five *I. multifiliis* i-antigen aa sequences in GenBank. Vertical dotted lines indicate beginning of repeat units. Underlining indicates portions represented by peptides identified during microsequence analysis of purified i-antigen of *I. multifiliis* strain ARS-6.

of predicted protein specified a molecular mass of 47.5 kDa with an isoelectric point of 5.12. Lin *et al.* (2002) found the i-antigen of strain G5, with 99% aa sequence identity and identical in length to that of ARS-6, migrated with an apparent molecular mass of approximately 52 kDa. The increased electrophoretic mobility we observed might be a result of the non-reducing denaturation conditions we used, which might affect the migration of the i-antigen if its numerous cysteine residues are involved in intramolecular disulphide bonds. The apparent difference in molecular mass size of the proteins could also be the result of post-translational modification as has been previously mentioned (Clark, McGraw & Dickerson 1992).

Like the sequences 5′ to other *I. multifiliis* i-antigen genes characterized, the sequences 5′ to the ARS-6 i-antigen coding sequences are extremely A–T rich (93% A+T), while the coding sequences themselves are only 64% A+T. The nucleotide sequence of the i-antigen coding sequences of *I. multifiliis* strain ARS-6 and the i-antigen gene *IAG52B*[*G5*] (GenBank accession no. AF405431) of strain G5 differ in only 3 nt, which resulted in two aa substitutions. Amino acids 1–452 of the ARS-6 i-antigen was derived from genomic DNA sequence (GenBank accession no. FJ012354) and aa 26–460 was derived from cDNA (GenBank accession no. FJ194440). Considering the low aa sequence conservation (50% or less) among i-antigens encoded by previously characterized *I. multifiliis* genes, it is remarkable that the i-antigen genes from ARS-6 and strain G5 are so similar. Two of the 3 nt differences result in aa differences. Although the number of differences is small, the high proportion of nucleotide substitutions resulting in aa changes (non-synonymous mutations) is consistent with positive selection of differences. In strain ARS-6, glycine (G) at position 66 and serine (S) at position 270 (polar aa) are substituted at corresponding positions by valine (V) and alanine (A) (hydrophobic aa), respectively, in strain G5 (Fig. 1). Both of these substitutions fall within the region of i-antigen repeat units between adjacent C–X_2–C motifs, the region where Lin *et al.* (2002) noted the most differences in both sequence and length among i-antigens and speculated that those differences were responsible for antigenic differences. Thus, although the high degree of similarity between ARS-6 and G5 i-antigen genes suggests that ARS-6, like G5, belongs to the most common *I. multifiliis* serotype, the sequence differences noted might result in antigenic differences. To determine whether ARS-6 was serotype D or showed any antigenic difference, an immobilization assay was conducted for ARS-6 theronts using a murine monoclonal antibody (Mab G3-61, kindly provided by Dr Harry Dickerson, University of Georgia, GA, USA) specific for serotype D. The theronts were immobilized by the Mab G3-61 indicating ARS-6 shares the same epitope with *I. multifiliis* serotype D (data not shown).

Serotypic variability among i-antigens of different *I. multifiliis* isolates has been previously observed and this antigenic polymorphism has been ascribed to possible allelic variations within strains (Dickerson *et al.* 1993). Similar allelic variations in i-antigens have been observed among the taxonomically related free-living ciliates *Tetrahymena thermophila* (Doerder, Arslanyolu, Saad, Kaczmarek, Mendoza & Mita 1996) and *Paramecium tetraurlia* and *Paramecium primaurelia* (Beale & Preer Jr 2008). The i-antigens of *I. multifiliis* were first isolated and characterized by Dickerson, Clark and Findly (1989) and Lin and Dickerson (1992). Clark *et al.* (1992, 1999) sequenced and analysed the gene designated *IAG48*[*G1*] encoding for a 48 kDa i-antigen of *I. multifiliis* strain A and elegantly characterized the protein as a parasite coat–protein composed of tandem repeats with distinct periodic cysteine residues. Subsequent studies have shown that i-antigens are attached to the cell surface by a GPI membrane anchor that likely plays an important role in signal transduction (Clark *et al.* 2001). Essentially, all of the features that have been previously described related to the aa sequences encoding *IAG48*[*GI*] (Clark *et al.* 1992), *IAG52A*[*G5*] and *IAG52B*[*G5*] (Lin *et al.* 2002) are found in the i-antigen aa sequence of *I. multifiliis* strain ARS-6. Briefly, the predicted polypeptide consists of 460 aa and is rich in alanine, glycine, serine and asparagine. Salient characteristics include an amino-terminal stretch of predominately hydrophobic aa that constitutes a putative signal peptide typified by an isoleucine at the seventh position. The aa are arranged into five tandem repeats punctuated by periodic cysteine residues, with each repeat unit having the following sequence: $CX_{(19)-20}CX_2CX_{16-27}CX_2CX_{20-(21)}CX_3$ (see Fig. 1). The cysteine residues of each repeating unit possibly participate in tertiary structure configuration through disulphide linkages reminiscent of the variant surface glycoproteins (VSG) of *Trypanosoma brucei* (Chattopadhyay, Jones, Nietlispach, Nielsen, Voorheis, Mott & Carrington 2005). At the C-terminus is a stretch of predominately hydrophobic residues characteristic of the GPI attachment site as described previously (Clark *et al.* 2001).

The *IAG52B*[*G5*] sequence in GenBank contains only 40 nt 5′-flanking sequence and all 40 nt are identical to the ARS-6 sequence. The ARS-6 sequence has a 48 nt 3′ untranslated region before the poly A tail. This sequence is identical to that of the first part of the 73 nt 3′ untranslated region of the IAG52B[G5] cDNA. The shorter 3′ untranslated region we found is possibly due to misannealing of the oligo-dT primer to the A-rich 3′ portion of the 3′ untranslated region during cDNA synthesis. Alternatively, the 3′ RACE product we sequenced could represent an mRNA that was polyadenylated at a different site than the one represented by the IAG52B[G5] cDNA sequence in GenBank.

Notwithstanding the importance of i-antigens in the host–parasite interactions, the precise mechanism underlying the regulation of i-antigen expression in *I. multifiliis* remains an enigma. In both *T. thermophila* and *Paramecium tetraurelia*, it has been observed that variation in temperature or salt stress could induce alternative expression of one or another of i-antigen genes from a repertoire (Smith & Doerder 1992; Doerder 2000; Matsuda & Forney 2005; Beale & Preer Jr 2008). However, attempts to induce i-antigenic variation by exposure of theront and trophont stages of *I. multifiliis* to different temperatures have resulted in little or no change in two major i-antigen transcripts (Clark *et al.* 1992). On the other hand, developmental regulation of *I. multifiliis* i-antigen expression has been observed. One i-antigen mRNA is expressed at 50-fold greater levels in theronts than in trophonts (Clark *et al.* 1992). Antigenic variation in surface epitopes of a variety of parasitic protists has been observed to be mediated by antibody selective pressure to facilitate evasion of the host immune response (Turner 1992; Mackinnon & Read 2004; Dzikowski & Deitsch 2006; Marcello & Barry 2007; Kyes, Kraemer & Smith 2007). Because *I. multifiliis* is an obligate parasite and antibodies play a significant role in protective immunity (Lin, Clark & Dickerson 1996; Clark & Dickerson 1997; Buchmann, Sigh, Nielsen & Dalgaard 2001; Clark & Forney 2003), it is logical that antibodies play a role in modulating the expression of i-antigens in *I. multifiliis*. Furthermore, exposure to immobilizing antibodies triggers a physiological response in *I. multifiliis* both *in vitro* and *in vivo*, possibly via signal transduction (Clark, Dickerson, Gratzek & Findly 1987; Clark, Lin & Dickerson 1996; Clark & Dickerson 1997). Perhaps antibody triggers the down-regulation of i-antigen mRNA expression observed in trophonts compared with theronts. To examine the possible antibody-mediated regulation of i-antigen expression we cultured *I. multifiliis* theronts in the presence of catfish anti-*I. multifiliis* serum *in vitro* for a period of 14 h. Because incubation of theronts beyond a period of 14 h resulted in gradual disintegration of the organisms, the maximum time that the theronts could be exposed to antibodies to assess any change in i-antigen expression was 14 h in this study. We supposed that this time frame was sufficient to elicit any alteration in antigen expression by an organism-controlled transcriptional event. Indeed, a response to immobilizing antibody *in vivo* (premature exit from host) is evident within 5 h of injection of antibody and is complete within 12 h (Clark *et al.* 1996). However, Western blot analysis of the theronts exposed to two concentrations of anti-*I. multifiliis* serum for 7 or 14 h did not show any change in either the level of i-antigen expression or electrophoretic mobility of i-antigen expressed (Fig. 2). It is possible that the i-antigen protein is sufficiently stable that a reduction in mRNA expression might not affect steady-state protein levels within the time period examined. Furthermore, change to expression of a different i-antigen peptide of similar electrophoretic mobility might not be detectable by Western blotting, because i-antigen epitopes detectable by Western blotting are cross-reactive among immobilization serotypes (Dickerson *et al.* 1993). Previous studies to examine antibody-induced antigenic variation in *Paramecium* species have shown that immobilization by antibody treat-

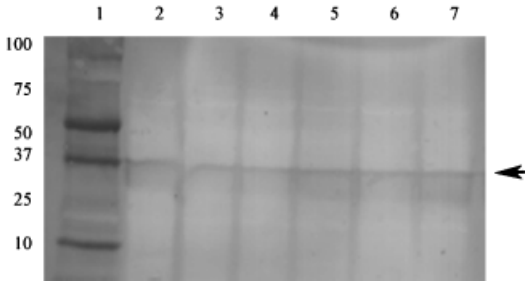

Figure 2 Immunoblot of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) resolved *Ichthyophthirius multifiliis* proteins following exposure of theronts to two (1:200 and 1:400) dilutions of catfish anti-*I. multifiliis* serum for 7 and 14 h. Lanes 2 and 3, theronts treated with no serum (controls); lanes 4 and 5, theronts exposed to antiserum at a dilution of 1:200 for 7 and 14 h, respectively; lanes 6 and 7, theronts exposed to 1:400 dilution of antiserum for 7 and 14 h respectively. Arrow indicates apparent (∼ 37 kDa) molecular mass position of i-antigen protein. Recombinant Precision Plus (Bio-Rad) prestained molecular weight markers are shown in lane 1.

ment did not show any alteration in antigen expression (Finger 1974), while other studies have shown that prolonged growth (beyond 15 h) in antibody containing medium was necessary for any alteration in antigens to occur (Beale & Preer Jr 2008). *Paramecium* and *Tetrahymena* are free-living ciliate protozoans whereas *I. multifiliis* is an obligate parasite that invades the fish epithelium and transiently parasitizes until the trophonts (parasitic stage, $\sim$4–7 days) are shed into the water as tomonts (Clark *et al.* 1987). Both theront and trophont stages of the parasite appear to possess i-antigens because they are immobilized by immune sera from fish and rabbits (Clark *et al.* 1987). The blood-borne protozoan parasites such as *Trypanosoma bruci* (Taylor & Rudenko 2006) and *Plasmodium falciparum* (Horrocks, Pinches, Christodoulou, Kyes & Newbold 2004) remain in the host for longer periods of time and possess vast repertoires of genes that undergo transcriptional switching to one or another of the genes to facilitate evasion of the host immune response. It would be interesting to determine the full-repertoire i-antigen genes in the *I. multifiliis* genome, but the available evidence suggests that as few as two genes may be present (Lin *et al.* 2002). Despite the substantial progress that has been made to understand the biology and survival strategy of this intriguing protozoan parasite, much remains to be known regarding the molecular mechanism by which i-antigen genes in *I. multifiliis* are regulated.

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