

Eimeria tenella glucose-6-phosphate isomerase: molecular characterization and assessment as a target for anti-coccidial control

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(Received 19 November 2009; revised 5 January 2010; accepted 8 January 2010; first published online 17 March 2010)

SUMMARY

Limitations with current chemotherapeutic and vaccinal control of coccidiosis caused by *Eimeria* species continue to prompt development of novel controls, including the identification of new drug targets. Glucose-6-phosphate isomerase (G6-PI) has been proposed as a valid drug target for many protozoa, although polymorphism revealed by electrophoretic enzyme mobility has raised doubts for *Eimeria*. In this study we identified and sequenced the *Eimeria tenella* G6-PI orthologue (*EtG6-PI*) from the reference Houghton strain and confirmed its position within the prevailing taxonomic hierarchy, branching with the Apicomplexa and Plantae, distinct from the Animalia including the host, *Gallus gallus*. Comparison of the deduced 1647 bp *EtG6-PI* coding sequence with the 9016 bp genomic locus revealed 15 exons, all of which obey the intron-AG-/exon-/GT-intron splicing rule. Comparison with the Weybridge and Wisconsin strains revealed the presence of 33 single nucleotide polymorphisms (SNPs) and 14 insertion/deletion sites. Three SNPs were exonic and all yielded non-synonymous substitutions. Preliminary structural predictions suggest little association between the coding SNPs and key G6-PI catalytic residues or residues thought to be involved in the coordination of the G6-PI's substrate phosphate group. Thus, the significant polymorphism from its host orthologue and minimal intra-specific polymorphism suggest G6-PI remains a valid anti-coccidial drug target.

Key words: G6-PI, coccidiosis, drug target, *Eimeria tenella*.

INTRODUCTION

The *Eimeria* species are apicomplexan parasites that cause the life-threatening disease coccidiosis in all livestock animals, most notably poultry. The annual cost incurred by the *Eimeria* species has been estimated to exceed £2 billion per annum (Shirley *et al.* 2004). Of the *Eimeria* species that infect the chicken, *Eimeria tenella* is considered to be one of the most economically important based upon a combination of prevalence and pathogenicity. Control of the *Eimeria* species is largely achieved through prophylactic chemotherapy, although drug resistance is rife and the portfolio of effective drugs is limited. Live wild-type and attenuated vaccines for the *Eimeria* species that infect chickens are effective but relatively expensive and their use is limited to a small part of the global poultry industry. New, cost-effective anti-coccidial control strategies are required but the high cost of developing novel chemotherapeutics has

proven to be limiting. Importantly, the *Eimeria* species have been adept at rapidly evolving to escape deleterious selective pressures such as those imposed by drug selection. For example, genetic resistance was described within the first year after the introduction of drugs including buquinolate, lasalocid and salinomycin (Chapman, 1997). Nonetheless, chemotherapy remains a valid anti-coccidial approach. A key component in the development of new anti-coccidial drugs will be knowledge defining the rapidity at which resistance can be selected. An assessment of relevant pre-existing genetic diversity will be integral to this process.

The increasing availability of transcriptomic, proteomic and genomic data for pathogens such as the *Eimeria* species now renders rational drug development increasingly feasible (Wan *et al.* 1999; Ng *et al.* 2002; Bromley *et al.* 2003; Shirley *et al.* 2004). Studies with related protozoan parasites including *Toxoplasma gondii* and the Kinetoplastida have highlighted glucose-6-phosphate isomerase (G6-PI) as a novel chemotherapeutic target (Dzierszinski *et al.* 1999; Hardré *et al.* 2000). Three-dimensional protein structure modelling and phylogenetics based upon *T. gondii* G6-PI have implied that it is a plant homologue, while studies using *Trypanosoma brucei*

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G6-PI revealed the possibility of inhibiting this enzyme using novel competitive inhibitors. Thus G6-PI, an enzyme that catalyses the reversible isomerization of D-glucose 6-phosphate to D-fructose 6-phosphate in the glycolysis pathway, could prove valuable as a target for the development of novel anti-coccidial drugs for use in poultry. G6-PI has previously been used in electrophoretic enzyme mobility studies with *Eimeria* as a means of differentiating between strains of species including *Eimeria acervulina*, *Eimeria mitis* and *E. tenella* (Sutton *et al.* 1986; Shirley *et al.* 1989). Although the basis of the observed electrophoretic diversity remains unknown, knowledge that G6-PI is characterized by naturally occurring phenotypic diversity within *Eimeria* species may limit its attraction as a drug target. The occurrence and extent of genetic polymorphism is as yet unknown for *Eimeria* G6-PI prior to drug selection, although the distribution of coding polymorphism across sites which may be considered 'relevant' (e.g. predicted active site) would temper enthusiasm for further development given the elevated possibility of pre-existing inherently resistant genotypes. Thus, identification of the *E. tenella* G6-PI (*EtG6-PI*) orthologous coding sequence and determination of any putative genetic basis for the observed electrophoretic polymorphism between *E. tenella* strains can inform on the suitability of G6-PI for inclusion in the future development of anti-coccidial drug strategies.

MATERIALS AND METHODS

Parasites

The *E. tenella* Houghton (H), Weybridge (Wey) and Wisconsin (Wis) strains were kindly provided by Professor Martin W. Shirley of the Institute for Animal Health, Compton, UK. The H and Wey strains were isolated in the UK and the Wis strain was isolated in the USA (Joyner and Norton, 1969; Jeffers, 1975; Chapman and Shirley, 2003). *E. tenella* parasites were propagated and isolated in accordance with previously outlined procedures (Shirley, 1995).

Generation of the *E. tenella* G6-PI cDNA sequence

Total RNA was isolated from sporulated oocysts using TRI Reagent (Molecular Research Center Inc., USA) for use as a template in 5' and 3' Rapid Amplification of cDNA Ends (RACE) reactions performed with the SMART RACE cDNA Amplification kit (Clontech, USA). The forward (GSP2: 5' GAT TTG CGT GGG GAT TGG GGG AAG TTA 3') and reverse (GSP1: 5' CCA TTG GTC CCT GGT TCT CCG AAG TAA 3') RACE primers were designed based on the predicted *EtG6-PI* coding sequence (GenBank Accession no. AM269894). The PCR temperature and cycling

conditions were as follows: 5 cycles of 94 °C for 30 s and 72 °C for 3 min; 5 cycles of 94 °C for 30 s, 70 °C for 30 s and 72 °C for 3 min; and 25 cycles of 94 °C for 30 s, 68 °C for 30 s and 72 °C for 3 min. PCR products were then purified, cloned into the pTZ57R vector (Fermentas, Lithuania), and sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, USA) on the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA). The most probable coding sequence for *EtG6-PI* was predicted using NCBI ORF Finder, while the similarity search was performed against the GenBank non-redundant database using blastx (Altschul *et al.* 1990).

Phylogenetic analysis

Multiple orthologous G6-PI amino acid sequences were retrieved by PSI-BLAST (Altschul *et al.* 1997) with a minimum match cut-off value of $E \leq e^{-100}$. A total of 32 unique taxa containing 682 characters were selected for further analyses. Sequences were aligned using MUSCLE (Edgar, 2004*a,b*) followed by visual inspection and manual adjustment. Phylogenetic analyses were conducted using PHYLIP (Felsenstein, 1989). Phylogenetic trees were constructed using the Neighbor-Joining (NJ) method (as implemented in NEIGHBOR) from distance matrixes calculated by the Jones-Taylor-Thornton model (using PROTDIST). The Maximum Parsimony (MP) method was also used (using PROTPARS). The parsimony settings for these analyses were ordinary parsimony, with non-weighted sites. The robustness of the trees was evaluated by bootstrap analysis of 1000 random iterations (using SEQBOOT). All trees were then viewed and edited using TREEVIEW 1.6.6 (Page, 1996) with *Mycobacterium tuberculosis* and *Nocardia farcinica* being rooted as outgroups.

Amplification and sequencing of the *EtG6-PI* genomic locus

The *EtG6-PI* genomic locus was PCR amplified using the enhanced proof-reading hybrid Expand Long Template PCR System kit (Roche Applied Science, Germany). Primers (5'UTR-F: 5' ACA CAC CTT TGC GTT TCG AGC CCC T 3' and 3'UTR-R: 5' CTG GCG CCC CGA ATC AAT TGT CTA A 3') were designed in the untranslated regions flanking *EtG6-PI* identified through comparison of the *EtG6-PI* cDNA with the *E. tenella* chromosome 1 sequence (GenBank Accession no. NC008685; Ling *et al.* 2007*b*). Genomic DNA was extracted from sporulated oocysts as previously described (Shirley, 1995). The PCR temperature and cycling conditions were 94 °C for 2 min, followed by 10 cycles of 94 °C for 10 s, 63 °C for 30 s and 68 °C for 8 min; 25 cycles of 94 °C for 15 s, 63 °C for 30 s

and 68 °C for 8 min + 5 s per cycle; and a final step of 68 °C for 7 min. PCR products were subsequently purified, cloned into the pCR-XL-TOPO vector (Invitrogen Corporation, USA) and sequenced as described above.

EtG6-PI structure homology modelling

The *EtG6-PI* H strain coding sequence was used to identify the equivalent Wey and Wis strain coding sequences from the sequenced genomic loci. All 3 coding sequences were translated and subjected to automated protein structure homology modelling using the SWISS-MODEL workspace (Arnold *et al.* 2006) via the ExPASy web server and studied using Invitrogen VectorNTI Advance 11.0 3D Molecule Viewer.

RESULTS

Identification and characterization of the EtG6-PI coding sequence

EtG6-PI is predicted to be encoded in 1647 bp, yielding a polypeptide of 549 amino acids (GenBank Accession no. GU117732). Blastx analysis of the coding sequence revealed the highest degree of similarity to the *T. gondii* G6-PI sequence (E value 0.0, 61% identity). All 100 of the top hits were annotated as, or similar to, G6-PI (E values $\leq e^{-166}$). The *EtG6-PI* predicted amino protein sequence was of a comparable size to each of these hits.

Alignment of the predicted *EtG6-PI* amino acid sequence with G6-PI amino acid sequences identified from multiple diverse taxonomic ranks revealed conserved G6-PI superfamily residue patterns (Fig. 1). Among the most notable residue patterns were [DENSEA]-x-[LIVM]-[GP]-G-R-[FY]-[ST]-[LIVMFSTAP]-x-[GSTA]-[PSTACM]-[LIVMSA]-[GSAN] (PROSITE Accession no. PS00765) at residues 289-302 and [GSA]-x-[LIVMCAYQS]-[LIVMFYWN]-x-(4)-[FY]-[DNTH]-Q-x-[GA]-[IV][EQST]-x-(2)-K (PROSITE Accession no. PS00174) at residues 528-545. Consideration of the proposed *EtG6-PI* translation start site with consensus sequence from *T. gondii* translational start sites revealed conservation of the 3 most favoured nucleotides (C⁻⁴, A⁻³, A⁻³; Seeber, 1997). Identification of serine as the second amino acid was also consistent with previous reports from *T. gondii* (Matrajt *et al.* 2002).

Phylogenetic analysis

The *E. tenella* G6-PI amino acid sequence was aligned with previously published orthologous sequences from other apicomplexan species and other major lineages including Plantae, Fungi, Animalia and Kinetoplastida. The topology of the NJ and MP

trees generated from these sequences supported previously described taxonomic relationships associating the Plantae with the Apicomplexa in a distinct cluster more distant to sequences derived from fungi, animals and kinetoplastids (Fig. 2; Dzierszinski *et al.* 1999). Similarly, within the apicomplexan clade, *E. tenella* G6-PI was more closely related to the *T. gondii* G6-PI orthologue than those of *Cryptosporidium parvum*, *Cryptosporidium hominis*, *Plasmodium falciparum* and *Plasmodium yoelii* (>75% bootstrap confidence support). The separation of the *Cryptosporidium* species from the other coccidian genera was also statistically reliable (bootstrap value, >85%).

Structural organization of the EtG6-PI locus

Amplification and sequencing of the *EtG6-PI* genomic locus yielded a contig of approximately 9 kb in length (GenBank Accession no. GU117733). Sequence comparison between the genomic and cDNA *EtG6-PI* sequences demonstrated that the gene consisted of 15 exons and 14 introns (Table 1, Fig. 3). All exons were found to obey the adopted intron-AG-/exon/-GT-intron splicing rule (Mount, 1982). The average length of exons and introns was 110 bp and 527 bp respectively.

Intra-specific EtG6-PI coding sequence comparison

Multiple sequence alignment analysis of the predicted *EtG6-PI* genomic sequence from the *E. tenella* H, Wey (GenBank Accession no. GU117735) and Wis (GenBank Accession no. GU117734) strains revealed a panel of insertions/deletions (indels) and SNPs. From a total of 14 indels, 7 specifically characterized the H strain while 5 and 2 defined the Wey and Wis strains respectively (Fig. 3). No deletion was shared among the strains investigated here. In all but 2 examples, a base deleted from one strain was non-polymorphic in the other strains. The exceptions occurred at base positions 581 and 600 (581: H[-], Wey[G], Wis[T]; 600: H[C], Wey[-], Wis[A]).

In addition to the indels, 33 SNPs were detected. Among these SNPs, 14 were found to involve purine substitutions (A and G bases) and 13 involved pyrimidine substitutions (C and T bases) (Fig. 3). Base substitutions between the purine and pyrimidine groups occurred at 6 sites. No single site was found to be polymorphic between all 3 strains. Only 3 SNPs were located within exonic regions, 1 within exon 1 and 2 within exon 10 (Fig. 3). All 3 exonic SNPs incurred non-synonymous substitutions.

EtG6-PI structure homology modelling

SWISS-MODEL protein structure homology modelling revealed a best hit to PDB Chain 1t10A for all 3 *EtG6-PI* proteins (E value 0.0). Based upon this

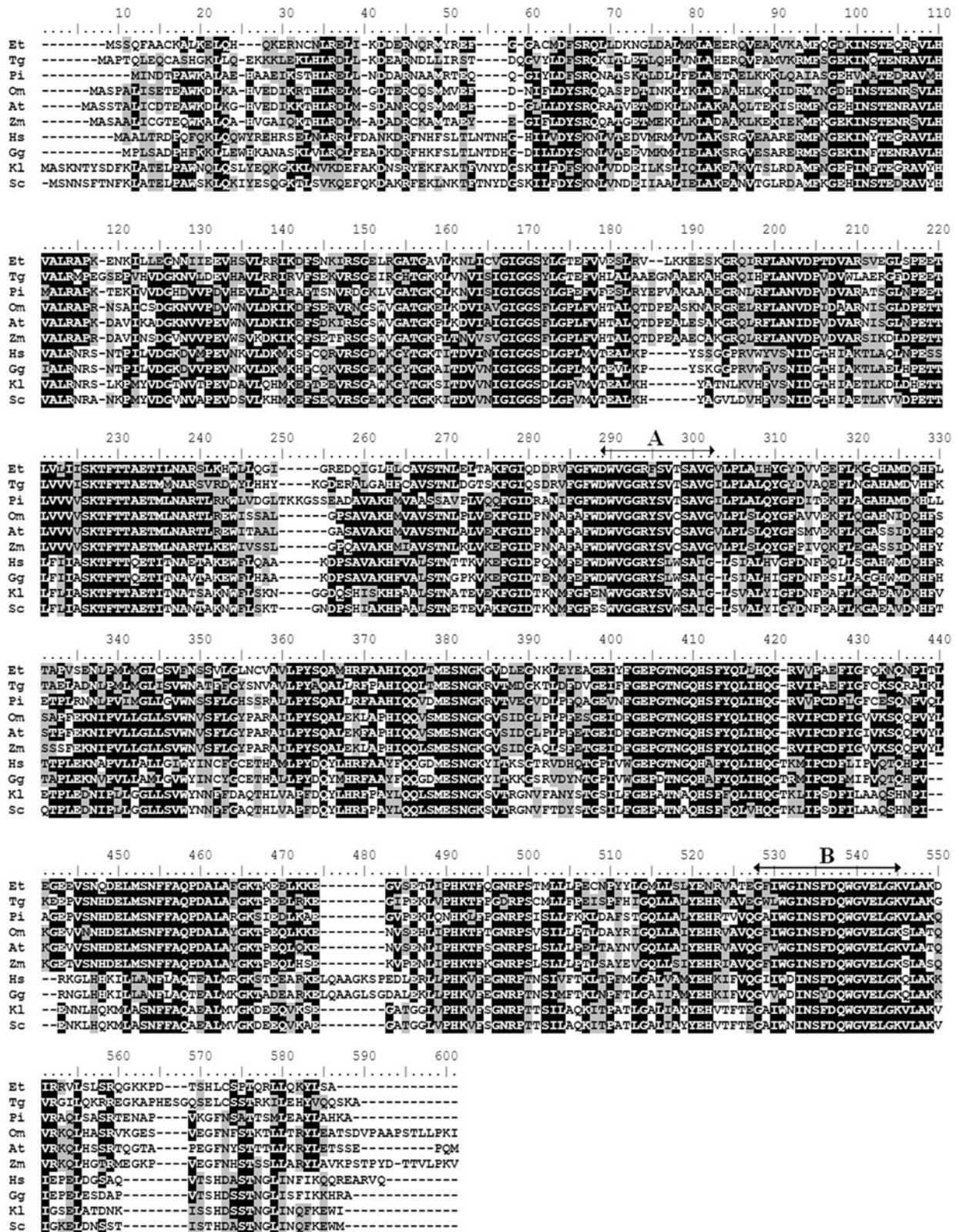


Fig. 1. Multiple sequence alignment of the *EtG6-PI* coding sequence with G6-PI sequences of other organisms. Black-shaded areas show conserved amino acid residues while grey-shaded areas show similar residues. Gaps (–) formed for optimum alignment. A and B denote conserved residue patterns of the G6-PI superfamily. (Et) *Eimeria tenella*; (Tg) *Toxoplasma gondii*; (Pi) *Phytophthora infestans*; (Om) *Oenothera mexicana*; (At) *Arabidopsis thaliana*; (Zm) *Zea mays*; (Hs) *Homo sapiens*; (Gg) *Gallus gallus*; (Kl) *Kluyveromyces lactis*; (Sc) *Saccharomyces cerevisiae*.

model intra-specific *EtG6-PI* secondary structure polymorphism was only predicted to be associated with coding SNP 1 (Fig. 4), suggesting an extended

α -helix associated with the Wey strain compared to the H and Wis strains (residues 7-19 compared to residues 6-13).

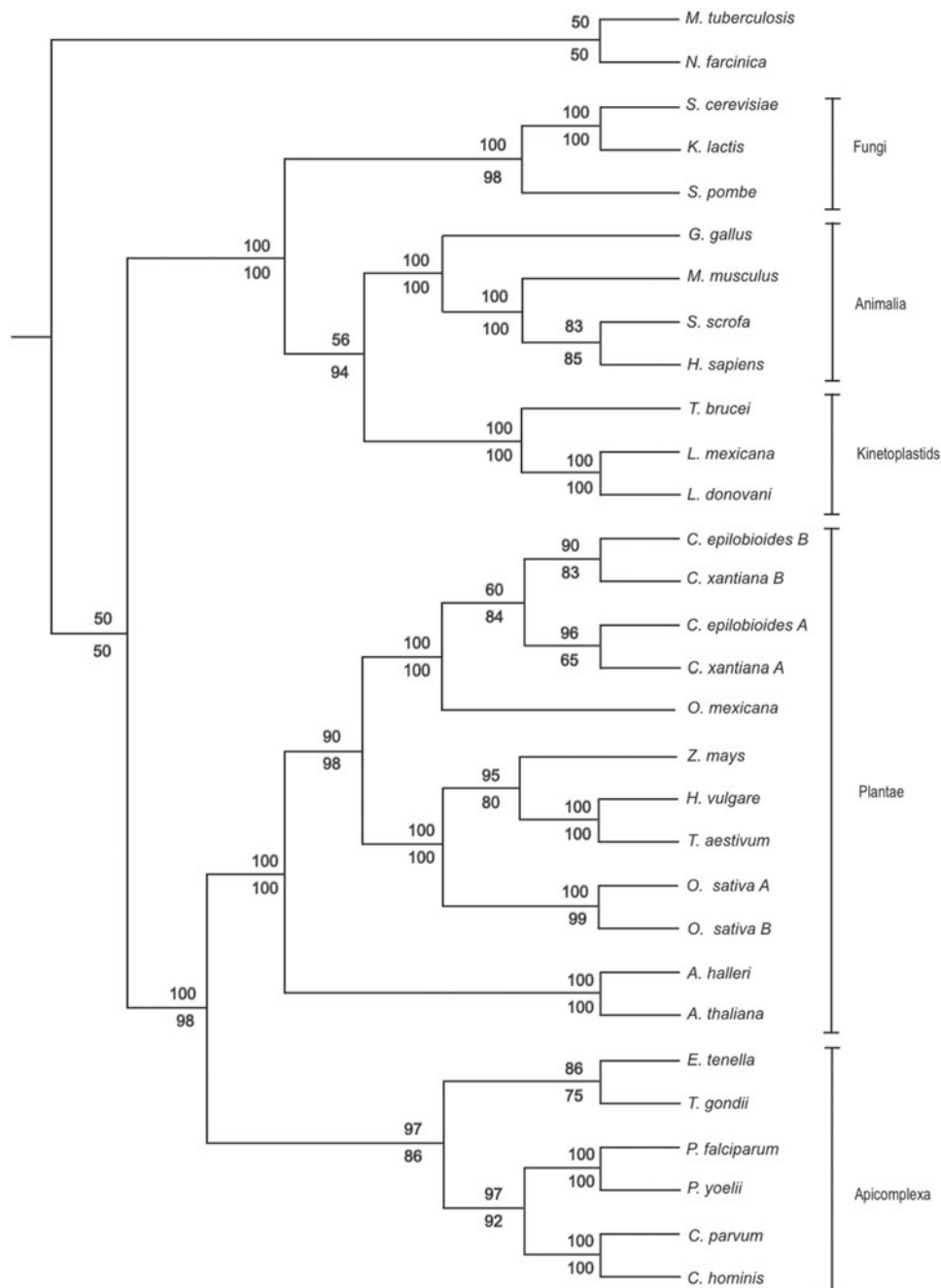


Fig. 2. Neighbor-joining (NJ, upper figure) and maximum parsimony (MP, lower figure) results illustrating the phylogenetic relationships between complete G6-PI amino acid sequences.

DISCUSSION

G6-PI has previously been identified as a candidate drug target for novel strategies to control protozoan parasites including *T. gondii* and the Kinetoplastida (Dzierszinski *et al.* 1999; Hardré *et al.* 2000). By comparison, it can be considered a valid target for anti-coccidial drug development. Historically, resistance has been observed soon after the introduction of every new anti-coccidial drug arising through a combination of apparent genomic plasticity and inadequate application against manufacturers' guidelines (Chapman, 1997). Thus, defining the

potential for resistance has become a key criterion in rational drug development. Previous studies with eimerian G6-PI have revealed polymorphic intra-specific electrophoretic mobility (Sutton *et al.* 1986; Shirley *et al.* 1989), although deficiencies in our knowledge of this molecule have hindered understanding the cause of the observed polymorphism.

The putative *EtG6-PI* described here has been shown to have a high percentage identity and similar sequence length to previously identified G6-PI coding sequences, supporting annotation as a genuine G6-PI orthologue. The identification of

Table 1. Features of exon-intron structure of *EtG6-PI* (H strain)

Exon no.	Nucleotide sequence around exon-intron boundaries				Nucleotide position of exon	Exon size (bp)
	5'acceptor/start codon	5'exon sequence	3'exon sequence	3'donor/stop codon		
1	ATG	ATGTCT...TCGGAG		GTATCT..	0..127	127
2	..GCTCAG	GCGCCT...CGACAG		GTGCCT..	374..453	80
3	..GGGCAG	GTGGAG...GCTCCG		GTAAAC..	657..843	187
4	..GCTCAG	GGGAGC...AATAAG		GTGCGC..	1615..1753	139
5	..CTGCAG	GTTTCT...AAATTG		GTGAGC..	3664..3837	173
6	..GTGCAG	GCCTGC...TGGGTT		GTAAGG..	4395..4486	92
7	..GTTTCAG	GGCGGG...CTGAAC		GTTTCGC..	4711..4797	87
8	..GCGCAG	GGCTGC...TCCCAG		GTCTGC..	5195..5329	135
9	..TTGCAG	GCGATG...GCAACG		GTAAAC..	5639..5690	52
10	..CCCCAG	GCAAAG...ACACAG		GTCTGC..	5806..5893	88
11	..GCATAG	CTTTTA...TTGGAG		GTACCT..	6384..6468	85
12	..GATTAG	GGCGAG...CACCAT		GTGAGG..	6637..6794	158
13	..CTACAG	GCTGCT...GTGGGG		GTAAGG..	7160..7273	114
14	..TTTTAG	AGTCGA...CAAAAA		GTCAGA..	7512..7580	69
15	..GTTTCAG	GCCCGA...GCCTAA		TAA	8953..9016	64

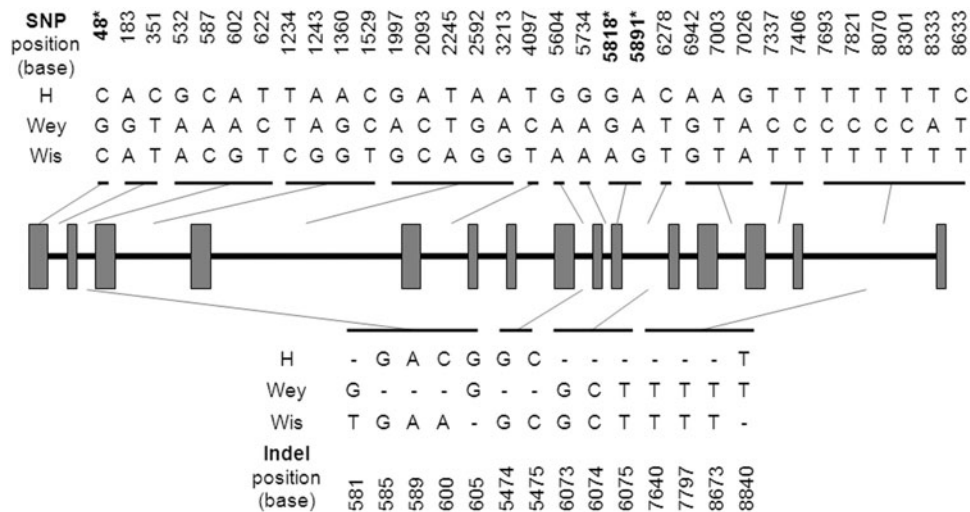


Fig. 3. SNP and indel positions identified within the *EtG6-PI* locus of the *Eimeria tenella* H, Wey and Wis strains. SNPs are shown in the upper panel, indels are shown in the lower panel. Asterisk (*) denotes positions located within coding (exonic) sequence.

conserved G6-PI superfamily residue patterns, in common with *Bacillus stearotherophilus* and *Entamoeba histolytica*, provided further supporting evidence (Sun *et al.* 1999; Razmjou *et al.* 2006). Comparison with the sequenced *EtG6-PI* genomic locus revealed adherence to the intron-AG-/exon-/GT-intron splicing rule with similar predicted intron and exon sizes to those described previously for the *E. tenella* phosphatidylinositol 4-phosphate 5-kinase gene (PIP5K; Ling *et al.* 2007a).

Phylogenetic comparison of the *E. tenella* G6-PI amino acid sequence supported the previously reported relationship between the Apicomplexa and the Plantae (Köhler *et al.* 1997; Dzierszinski *et al.* 1999; Funes *et al.* 2002; Grauvogel *et al.* 2007). Despite the relatively low support for the distinction

between G6-PI sequences of apicomplexan parasites and their higher order hosts described here, these findings have been supported by others (e.g. Dzierszinski *et al.* 1999), demonstrating their candidacy as specific drug targets. The NJ and MP phylogenetic trees illustrated the taxonomic relationship between *E. tenella* and *T. gondii*, both of which are categorized under the order Eucoccidiorida. Although *Cryptosporidium* spp. are also classified under the order Eucoccidiorida together with *E. tenella* and *T. gondii*, this and previous phylogenetic studies have revealed a greater distance relationship from *E. tenella* to the *Cryptosporidium* spp. than *T. gondii*. The *Cryptosporidium* spp. are now thought to have a closer relationship with apicomplexan parasites under the order Eugregarinorida

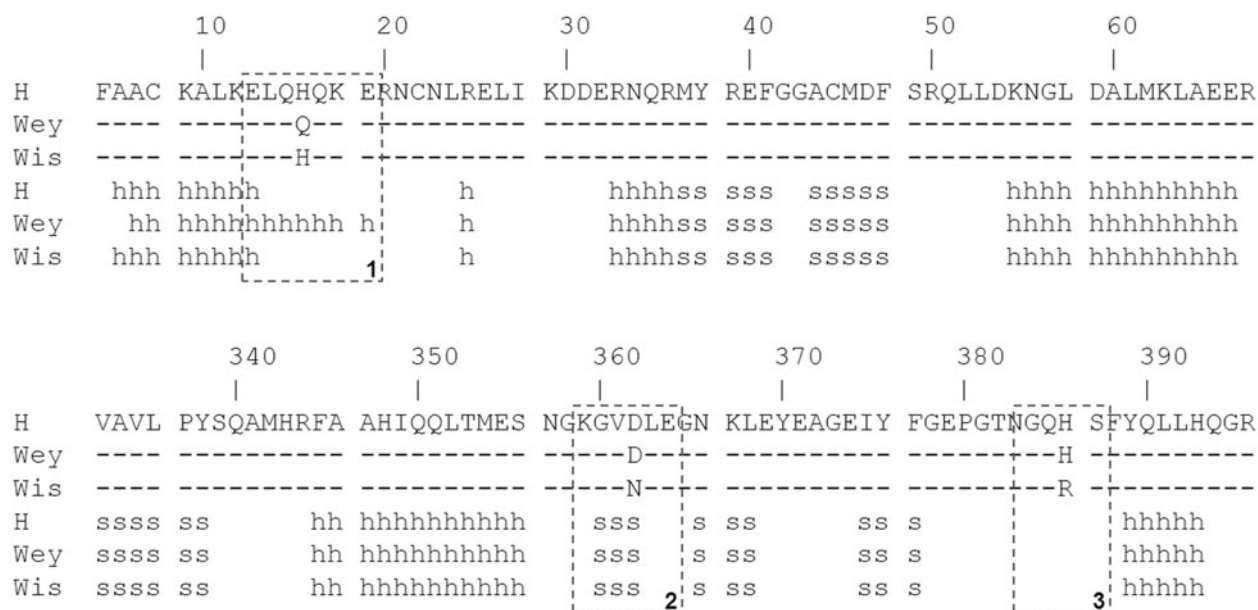


Fig. 4. Influence of exonic SNPs on EtG6-PI secondary structure predicted in the Swiss Model workspace (Arnold *et al.* 2006). Non-polymorphic amino acids are not shown (—), polymorphic amino acids and surrounding regions are shown in boxes 1–3. Only box 1 contains a predicted structural variation. h = α helix, s = β sheet.

than to other members of the order Eucoccidiorida (Carreno *et al.* 1999; Zhu *et al.* 2000; Leander *et al.* 2003).

A panel of 33 SNPs and 14 indels was detected across the *EtG6-PI* locus but only 3 SNPs, at base positions 48, 5818 and 5891, were detected in coding regions. Translation of exon 1 revealed that the H and Wis strains encode the histidine (H) residue while the Wey strain encoded the glutamine (Q) residue. The polar H residue is positively charged while the Q residue, an analogue of the acidic glutamic acid (E), is neutral. Thus, the SNP at base position 48 on exon 1 conferred an overall loss of charge. Two SNPs were detected in exon 10, both of which differentiated the Wis strain from the H and Wey strains. The first SNP in exon 10 resulted in a change from aspartic acid (D; H and Wey strains) to asparagine (N; Wis). The D residue is negatively charged while the N residue is a derivative without charge. The substitution incurs a loss of charge to the amino acid residue. The second SNP in exon 10 resulted in a change from an H residue (H and Wey strains) to arginine (R) residue, both positively charged, incurring no change in charge. Preliminary structural predictions using the SWISS-MODEL workspace (Arnold *et al.* 2006) suggest an extended α -helix associated with the Wey strain-specific exon 1 SNP. Previous study has shown that *E. tenella* H and Wis strain isoenzyme profiles are characterized by the EtG6-PI electrophoretic variant GPI-1, while the Wey strain is characterized by the electrophoretic variant GPI-9 (Shirley *et al.* 1989), a shift possibly induced by this α -helix polymorphism. The SNPs in exon 10 are not predicted to influence secondary protein structure although the charge polymorphism

associated with one of the SNPs could affect tertiary protein structure. Similarly, post-translational modifications such as glycosylation could impact upon electrophoretic mobility. The functional significance of the predicted structural polymorphism is not known. However, although detailed crystallography studies have yet to be conducted on EtG6-PI, *in silico* comparison with *Leishmania mexicana* G6-PI suggests that none of the changes are closely associated with predicted catalytic residues or residues thought to be involved in the coordination of the substrate's phosphate group (Cordeiro *et al.* 2004). A more complete description of the EtG6-PI protein structure is ongoing alongside these studies (manuscript in preparation).

Consideration of the limited EtG6-PI coding and predicted structural polymorphism detected here between *E. tenella* strains isolated on 2 continents, combined with the phylogenetic divergence between the parasite and its host G6-PI, suggest that EtG6-PI remains a valid candidate anti-coccidial drug target. Nevertheless, the finding of only 2 EtG6-PI isoenzyme types to date suggests that it would be prudent to test the efficacy of any candidate anti-EtG6-PI drug on representatives of both types.

FINANCIAL SUPPORT

This work was supported by the IRPA Top Down Grant awarded by the Ministry of Science, Technology and Innovation (MOSTI), Malaysia (R.M., grant number 09-02-02-002 [BTK/TD/003]); the Genomics and Molecular Biology Initiatives Programme of the Malaysia Genome Institute, MOSTI, Malaysia (K.-L.W., grant number

07-05-16-MGI-GMB10); and the Biotechnology and Biological Sciences Research Council (BBSRC), UK (D.P.B., grant number BBE01089X1).

REFERENCES

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J.** (1990). Basic local alignment search tool. *Journal of Molecular Biology* **215**, 403–410.
- Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J.** (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**, 3389–3402.
- Arnold, K., Bordoli, L., Kopp, J. and Schwede, T.** (2006). The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. *Bioinformatics* **22**, 195–201.
- Bromley, E., Leeds, N., Clark, J., McGregor, E., Ward, M., Dunn, M. J. and Tomley, F.** (2003). Defining the protein repertoire of microneme secretory organelles in the apicomplexan parasite *Eimeria tenella*. *Proteomics* **3**, 1553–1561.
- Carreno, R. A., Martin, D. S and Barta, J. R.** (1999). *Cryptosporidium* is more closely related to the gregarines than to coccidia as shown by phylogenetic analysis of apicomplexan parasites inferred using small-subunit ribosomal RNA gene sequences. *Parasitology Research* **85**, 899–904.
- Chapman, H. D.** (1997). Biochemical, genetic and applied aspects of drug resistance in *Eimeria* parasites of the fowl. *Avian Pathology* **28**, 221–244.
- Chapman, H. and Shirley, M. W.** (2003). The Houghton strain of *Eimeria tenella*: a review of the type strain selected for genome sequencing. *Avian Pathology* **32**, 115–127.
- Cordeiro, A. T., Michels, P. A., Delboni, L. F. and Thiemann, O. H.** (2004). The crystal structure of glucose-6-phosphate isomerase from *Leishmania mexicana* reveals novel active site features. *European Journal of Biochemistry* **271**, 2765–2772.
- Dzierszinski, F., Popescu, O., Tourse, C., Slomianny, C., Yahiaoui, B. and Tomavo, S.** (1999). The protozoan parasite *Toxoplasma gondii* expresses two functional plant-like glycolytic enzymes. Implications for evolutionary origin of apicomplexans. *The Journal of Biological Chemistry* **274**, 24888–24895.
- Edgar, R. C.** (2004a). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* **32**, 1792–1797.
- Edgar, R. C.** (2004b). MUSCLE: a multiple sequence alignment method with reduces time and space complexity. *BMC Bioinformatics* **5**, 113.
- Felsenstein, J.** (1989). Phylogeny Inference Package (Version 3.2). *Cladistics* **5**, 164–166.
- Funes, S., Davidson, E., Reyes-Prieto, A., Magallón, S., Herion, P., King, M. P. and González-Halphen, D.** (2002). A green algal apicoplast ancestor. *Science* **298**, 2115.
- Grauvogel, C., Brinkmann, H. and Peterson, J.** (2007). Evolution of the glucose-6-phosphate isomerase: the plasticity of the primary metabolism in the photosynthetic eukaryotes. *Molecular Biology and Evolution* **24**, 1611–1621.
- Hardré, R., Salmon, L. and Opperdoes, F. R.** (2000). Competitive inhibition of *Trypanosoma brucei* phosphoglucose isomerase by D-arabinose-5-phosphate derivatives. *Journal of Enzyme Inhibition* **15**, 509–515.
- Jeffers, T. K.** (1975). Attenuation of *Eimeria tenella* through selection for precociousness. *Journal of Parasitology* **61**, 1083–1090.
- Joyner, L. P. and Norton, C. C.** (1969). A comparison of two laboratory strains of *Eimeria tenella*. *Parasitology* **59**, 907–913.
- Köhler, S., Delwiche, C. F., Denny, P. W., Tilney, L. G., Webster, P., Wilson, R. J., Palmer, J. D. and Roos, D. S.** (1997). A plastid of probable green algal origin in apicomplexan parasites. *Science* **275**, 1485–1489.
- Leander, B. S., Clopton, R. E. and Keeling, P. J.** (2003). Phylogeny of gregarines (Apicomplexa) as inferred from small-subunit rDNA and beta-tubulin. *International Journal of Systematic Evolutionary Microbiology* **53**, 345–354.
- Ling, K.-H., Loo, S.-S., Rosli, R., Mariana, N. S., Mohamed, R. and Wan, K.-L.** (2007a). *In silico* identification and characterization of a putative phosphatidylinositol 4-phosphate 5-kinase (PIP5K) gene in *Eimeria tenella*. *In Silico Biology* **7**, 115–121.
- Ling, K.-H., Rajandream, M.-A., Rivallier, P., Ivens, A., Yap, S.-J., Madeira, A. M. B. N., Mungall, K., Billington, K., Yee, W.-Y., Bankier, A. T., Carroll, F., Durham, A. M., Peters, N., Loo, S.-S., Mat-Isa, M. N., Novaes, J., Quail, M., Rosli, R., Mariana, N. S., Sobreira, T. J. P., Tivey, A., Wai, S.-F., White, S., Wu, X., Kerhornou, A., Blake, D., Mohamed, R., Shirley, M., Gruber, A., Berriman, M., Tomley, F., Dear, P. H. and Wan, K.-L.** (2007b). Sequencing and analysis of chromosome 1 of *Eimeria tenella* reveals a unique segmental organization. *Genome Research* **17**, 311–319.
- Matrajt, M., Nishi, M., Fraunholz, M. J., Peter, O. and Roos, D. S.** (2002). Amino-terminal control of transgenic protein expression levels in *Toxoplasma gondii*. *Molecular and Biochemical Parasitology* **20**, 285–289.
- Mount, S. M.** (1982). A catalogue of splice junction sequences. *Nucleic Acids Research* **10**, 459–472.
- Ng, S.-T., Jangi, M. S., Shirley, M. W., Tomley, F. M. and Wan, K.-L.** (2002). Comparative EST analyses provide insights into gene expression in two asexual developmental stages of *Eimeria tenella*. *Experimental Parasitology* **101**, 168–173.
- Page, R. D. M.** (1996). TREEVIEW: An application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* **12**, 357–358.
- Razmjou, E., Haghighi, A., Rezaian, M., Kobayashi, S. and Nozaki, T.** (2006). Genetic diversity of glucose phosphate isomerase from *Entamoeba histolytica*. *Parasitology International* **55**, 307–311.
- Seeber, F.** (1997). Consensus sequence of translational initiation sites from *Toxoplasma gondii* genes. *Parasitology Research* **83**, 309–311.
- Shirley, M. W.** (1995). *Eimeria* species and strains of chickens. In *Biotechnology – Guidelines on Techniques*

- in *Coccidiosis Research* (ed. Eckert, J., Braun, R., Shirley, M. W. and Coudert, P.), pp. 1–24. European Commission, Luxembourg.
- Shirley, M. W., Chapman, H. D., Kucera, J., Jeffers, T. K. and Bedrnir, P.** (1989). Enzyme variation and pathogenicity of recent field isolates of *Eimeria tenella*. *Research in Veterinary Science* **46**, 79–83.
- Shirley, M. W., Ivens, A., Gruber, A., Madeira, A. M. B. N., Wan, K.-L., Dear, P. H. and Tomley, F. M.** (2004). The *Eimeria* genome projects: a sequence of events. *Trends in Parasitology* **20**, 199–201.
- Sun, Y.-J., Chou, C.-C., Chen, W.-S., Wu, R.-T., Meng, M. and Hsiao, C.-D.** (1999). The crystal structure of a multifunctional protein: Phosphoglucose isomerase/autocrine motility factor/neuroleukin. *Proceedings of the National Academy of Sciences, USA* **96**, 5412–5417.
- Sutton, C. A., Shirley, M. W. and McDonald, V.** (1986). Genetic recombination of markers for precocious development, arprinocid resistance, and isoenzymes of glucose phosphate isomerase in *Eimeria acervulina*. *Journal of Parasitology* **72**, 965–967.
- Wan, K.-L., Chong, S.-P., Ng, S.-T., Shirley, M. W., Tomley, F. M. and Jangi, M. S.** (1999). A survey of genes in *Eimeria tenella* merozoites by EST sequencing. *International Journal for Parasitology* **29**, 1885–1892.
- Zhu, G., Keithly, J. S. and Philippe, H.** (2000). What is the phylogenetics position of *Cryptosporidium*? *International Journal of Systematic Evolutionary Microbiology* **50**, 1673–1681.