Purine salvage in the apicomplexan *Sarcocystis neurona*, and generation of hypoxanthine-xanthine-guanine phosphoribosyltransferase-deficient clones for positive-negative selection of transgenic parasites

SRIVENY DANGOUDOUBIYAM*, ZIJING ZHANG *and* DANIEL K. HOWE

M. H. Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, KY 40546, USA

(*Received 24 January 2014; revised 27 March 2014; accepted 28 March 2014; first published online 13 June 2014*)

SUMMARY

Sarcocystis neurona is an apicomplexan parasite that causes severe neurological disease in horses and marine mammals. The Apicomplexa are all obligate intracellular parasites that lack purine biosynthesis pathways and rely on the host cell for their purine requirements. Hypoxanthine-xanthine-guanine phosphoribosyltransferase (HXGPRT) and adenosine kinase (AK) are key enzymes that function in two complementary purine salvage pathways in apicomplexans. Bioinformatic searches of the *S. neurona* genome revealed genes encoding HXGPRT, AK and all of the major purine salvage enzymes except purine nucleoside phosphorylase. Wild-type *S. neurona* were able to grow in the presence of mycophenolic acid (MPA) but were inhibited by 6-thioxanthine (6-TX), suggesting that the pathways involving either HXGPRT or AK are functional in this parasite. Prior work with *Toxoplasma gondii* demonstrated the utility of HXGPRT as a positive-negative selection marker. To enable the use of HXGPRT in *S. neurona*, the Sn*HXGPRT* gene sequence was determined and a gene-targeting plasmid was transfected into *S. neurona*. SnHXGPRT-deficient mutants were selected with 6-TX, and single-cell clones were obtained. These SnΔHXG parasites were susceptible to MPA and could be complemented using the heterologous *T. gondii HXGPRT* gene. In summary, *S. neurona* possesses both purine salvage pathways described in apicomplexans, thus allowing the use of HXGPRT as a positive-negative drug selection marker in this parasite.

Key words: *Sarcocystis neurona*, Apicomplexa, HXGPRT, purine salvage, transfection.

INTRODUCTION

The apicomplexan *Sarcocystis neurona* is an important pathogen causing severe neurological disease in horses and marine mammals. The normal life cycle of *S. neurona* alternates between opossums, the definitive host, and a variety of small mammals such as raccoons, skunks, cats and armadillos that can serve as intermediate hosts (Dubey *et al*. 2000, 2001*a*,*b*; Cheadle *et al*. 2001, 2002). Similar to these natural intermediate hosts, horses become infected when they ingest food and water contaminated with *S. neurona* sporocysts shed in the opossum faeces. In horses, *S. neurona* may reach the central nervous system, resulting in a clinical disease called equine protozoal myeloencephalitis (EPM) that is endemic in the Americas (Beech, 1974; Dubey *et al*. 2001*a*). In *S. neurona*-infected horses, clinical signs and severity of EPM are dependent on the area of the CNS affected. Most common clinical symptoms include head tilt, depression, muscle atrophy, paralysis, lameness and seizures. Despite treatment of affected horses, complete recovery does not always occur and potential lifelong debility and risk of EPM relapse exists (Fenger, 1998; MacKay, 2006).

Apicomplexans are purine auxotrophs that fulfil their purine requirements by salvaging from their host cells. Purine salvage is accomplished by complementary pathways involving either hypoxanthine-xanthine-guanine phosphoribosyltransferase (HXGPRT) or adenosine kinase (AK), neither of which is essential for parasite survival (Krug *et al*. 1989). *Toxoplasma gondii* deficient in AK activity can grow in the presence of the adenine analogue adenine arabinoside (Ara-A) (Pfefferkorn and Pfefferkorn, 1978). Similarly, *T. gondii* lacking functional HXGPRT are capable of growing in the presence of 6-thioxanthine (6-TX), a toxic analogue of xanthine (Pfefferkorn and Borotz, 1994). Importantly, parasites that lack functional HXGPRT are sensitive to mycophenolic acid (MPA), which blocks inosine-5′-monophosphate (IMP) dehydrogenase and prevents purine salvage by the AK pathway. Consequently, the *HXGPRT* gene can be used for positive-negative selection of transgenic parasites (Donald *et al*. 1996; Donald and Roos, 1998).

* Corresponding author: Department of Veterinary Science, M.H. Gluck Equine Research Center, University of Kentucky, Lexington, KY 40546-0099, USA. E-mail: sriveny.dangoud@uky.edu

Parasitology (2014), **141**, 1399–1405.
doi:10.1017/S0031182014000687

Table 1. *In silico* identification of purine salvage enzymes of *Sarcocystis neurona*

Toxoplasma gondii (query)			Tblastn search of *S. neurona* genome database		Reciprocal blast of identified *S. neurona* purine salvage enzymes to *T. gondii* at Eupathdb.org			
Name of the enzyme	Toxodb_V9.0	Protein length (aa)	Genomic locus scaffold number: position	Protein length (aa)	Score	*e*-value	Identities	Positives
Adenosine kinase	TGME49_250880	465	16: 2467986–2462903	410	621	7.70E-62	153/383 (39%)	207/383 (54%)
Adenylosuccinate synthetase	TGME49_279450	460	08: 2636984–2635491	498	1431	1.10E-147	258/420 (61%)	322/420 (76%)
Adenylosuccinate lyase	TGME49_263190	500	22: 367747–348904	647	1227	4.20E-128	253/485 (52%)	325/485 (67%)
Inosine-5′-monophosphate dehydrogenase	TGME49_233110	551	16: 1016900–1003048	710	1728	3.80E-179	347/502 (69%)	403/502 (80%)
GMP synthase	TGME49_230450	569	05: 433828–441220	632	1619	4.70E-216	311/409 (76%)	351/409 (85%)
Adenosine monophosphate deaminase	TGME49_234280	914	03: 2471158–2496958	1887[a]	2729	0.00E+00	512/686 (74%)	585/686 (85%)
Adenosine deaminase	TGME49_205720	366	18: 1729665–1745624	456	847	5.30E-92	158/312 (50%)	236/312 (75%)
Hypoxanthine-xanthine-guanine phosphoribosyl transferase	TGGT1_060540	279	02:52 03 160–5207925	232	820	6.30E-83	151/230 (65%)	187/230 (81%)
Purine nucleoside phosphorylase	TGME49_107030	247	No orthologue identified	–	–	–	–	–

[a] partial sequence.

Basic methods for DNA transfection and transient expression of reporter molecules such as β-galactosidase, luciferase and yellow fluorescent protein (YFP) have been described previously for *S. neurona* (Gaji *et al*. 2006). Further, selection of stable transformants of *S. neurona* has been accomplished by expressing mutant dihydrofolate reductase-thymidylate synthase (DHFR-TS) to confer resistance to pyrimethamine (Gaji *et al*. 2006). To enhance molecular genetic capabilities for investigating the biology of *S. neurona*, a bioinformatics approach was used to identify sequences in the *S. neurona* genome that encode enzymes of the purine salvage pathway. The Sn*HXGPRT* gene sequence was determined, and SnΔHXG clones of *S. neurona* were developed to enable efficient positive-negative selection of stable transgenic parasites.

MATERIALS AND METHODS

Sarcocystis neurona *strain SN3*

Sarcocystis neurona strain SN3 was propagated in bovine turbinate (BT) cell monolayers. Upon lysis of the infected BT monolayer, the extracellular merozoites were harvested by passing through 23 and 25 G needles and filter-purified to remove the host cell debris (Hoane *et al*. 2003). Freshly isolated merozoites were used for transfection. When not required immediately, merozoites were pelleted and stored at −20 °C until further use.

In silico *identification of purine salvage enzymes*

The *S. neurona* genome has been sequenced and is available through EuPathDB (www.eupathdb.org) and GenBank (Accession # JAQE01000000). A custom searchable database of the draft genome of *S. neurona* was generated using CLC Genomics Workbench version 6.0 (CLC Bio, Cambridge, MA). Apicomplexan orthologues of the enzymes involved in purine salvage were obtained from the eukaryotic pathogen database (EuPathDB.org; Table 1). Tblastn searches of the draft genome sequence of *S. neurona* were conducted using *T. gondii* purine salvage enzyme sequences as the primary queries. Reciprocal searches against the apicomplexan protein sequences in EuPathDB were performed using the newly identified *S. neurona* purine salvage enzyme sequences as queries.

Characterization of the HXGPRT gene and gene locus in S. neurona

The structural organization of the *SnHXGPRT* gene was determined *in silico* and primers were designed to amplify regions of the gene locus (Supplemental Table S1). Total RNA was isolated from SN3 strain merozoites using Trizol reagent (Life Technologies, Grand Island, NY). *SnHXGPRT*-specific primers

https://doi.org/10.1017/S0031182014000687 Published online by Cambridge University Press

were used to generate cDNA, which served as template for PCR. A single *SnHXGPRT* PCR product was obtained and sequenced. The accuracy of the *SnHXGPRT* nucleotide sequence was confirmed by comparison to the *S. neurona* genome sequence.

Knockout (KO) plasmid construct for disruption of the SnHXGPRT gene

To generate SnΔHXG parasites, a gene-targeting plasmid consisting of the YFP gene flanked by the UTR regions of *SnHXGPRT* was constructed in pBluescript. The YFP gene amplified from pSnSAG1/YFP-YFP (Gaji *et al*. 2006) was ligated to 3·2 kb of 5′-UTR and 3·4 kb of 3′-UTR of the *SnHXGPRT* locus amplified from *S. neurona* genomic DNA, yielding the plasmid pSnHXG-UTRs-YFP. Primers and restriction enzymes used to construct pSnHXG-UTRs-YFP are listed in Supplemental Table 1. The proper orientation of the YFP gene in pSnHXG-UTRs-YFP was confirmed by restriction enzyme digestion and PCR.

Transfection of S. neurona *merozoites and negative selection using 6-TX*

Sarcocystis neurona merozoites were harvested and resuspended in complete cytomix for use in transfections, as described previously (Howe and Sibley, 1997; Gaji *et al*. 2006). Approximately 40 μg of KpnI-linearized pSnHXG-UTRs-YFP was combined with 2×10^7 merozoites and transfected by electroporation. The transfected merozoites were allowed to recover at room temperature for 10 min and then inoculated into 125-mm culture dishes containing BT cells. At 8 days post-transfection, the Sn*HXGPRT*-deficient population was selected with 80 or 160 μg mL^{-1} of 6-TX in culture medium supplemented with dialysed bovine serum (Donald *et al*. 1996). After 52 days of 6-TX selection, single-cell clones of the surviving parasites were isolated in a 96-well plate.

Confirmation of HXGPRT disruption in S. neurona

The SnΔHXG single-cell clones from the 96-well plate were expanded in a 24-well plate, and genomic DNA was extracted. To confirm disruption of the *SnHXGPRT* gene, a series of PCRs was performed with primer pairs targeting the *SnHXGPRT* locus and the YFP transgene (Supplemental Table S1). Single-cell clones showing disruption of the *HXGPRT* locus were tested for sensitivity to IMP dehydrogenase inhibition by culturing them in the presence of MPA (25 μg mL^{-1}) and xanthine (50 μg mL^{-1}) (Donald *et al*. 1996).

Heterologous complementation of SnΔHXG

The ptubXFLAG::HX plasmid consisting of the *TgHXGPRT* minigene driven by the *T. gondii dhfr* promoter (Bhatti and Sullivan, 2005) was used to complement the SnΔHXG parasites. Briefly, SnΔHXG merozoites were transfected with 40 μg of ptubXFLAG::HX linearized with EcoRV. Post-transfection, the cultures were subjected to MPA selection (25 μg mL^{-1} MPA and 50 μg mL^{-1} xanthine) from day 3 onwards to isolate a Tg*HXGPRT*-complemented *S. neurona* (Sn-TgHXGc) population. After 30 days of selection with MPA, single-cell clones were isolated in a 96-well plate. Amplification primers targeting the expression cassette of ptubXFLAG::HX were used to confirm that MPA-resistant Sn-TgHXGc clones harboured the heterologous *HXGPRT* sequence from *T. gondii*.

Plaque assay

Plaque assays were performed to assess growth of SnΔHXG and Sn-TgHXGc parasites. Approximately 1000 merozoites of wild type SN3 and the transgenic clones were inoculated in triplicate into wells of a 24-well plate containing BT cell monolayers. Parasites were grown either in the absence or presence of MPA, and the plaques were counted 10 days post-inoculation.

RESULTS

Genes for purine salvage enzymes in S. neurona

Sequences encoding enzymes involved in purine salvage by *S. neurona* were identified by Tblastn searches of the custom *S. neurona* genome database (Table 1). The two key enzymes AK and HXGPRT that aid in complementary purine salvage pathways were identified in the *S. neurona* genome. The *S. neurona AK* and *HXGPRT* sequences showed 39 and 65% amino acid identity to their respective *T. gondii* orthologues. Adenosine monophosphate (AMP) and IMP, which are the initial products of the purine salvage pathways, are inter-convertible in *T. gondii* and facilitated by the enzymes AMP deaminase, adenylosuccinate synthetase and adenosuccinate lysase; all three enzymes were identified in the *S. neurona* genome sequence. Similarly, orthologues to IMP dehydrogenase and GMP synthase were identified. Surprisingly, no orthologue for purine nucleoside phosphorylase was identified in the draft sequence of the *S. neurona* genome.

Characterization of the SnHXGPRT *gene*

Based on the draft genome sequence of *S. neurona*, the genomic locus of *SnHXGPRT* spans 4765 bp. Sequence of a PCR product obtained from *S. neurona* merozoite cDNA suggested that SnHXGPRT is a 232 amino acid protein encoded by a 699 bp open reading frame derived from 8 exons of the genomic locus (GenBank Accession No. KF406342). *Sarcocystis neurona* SN3 strain merozoites grew

https://doi.org/10.1017/S0031182014000687 Published online by Cambridge University Press

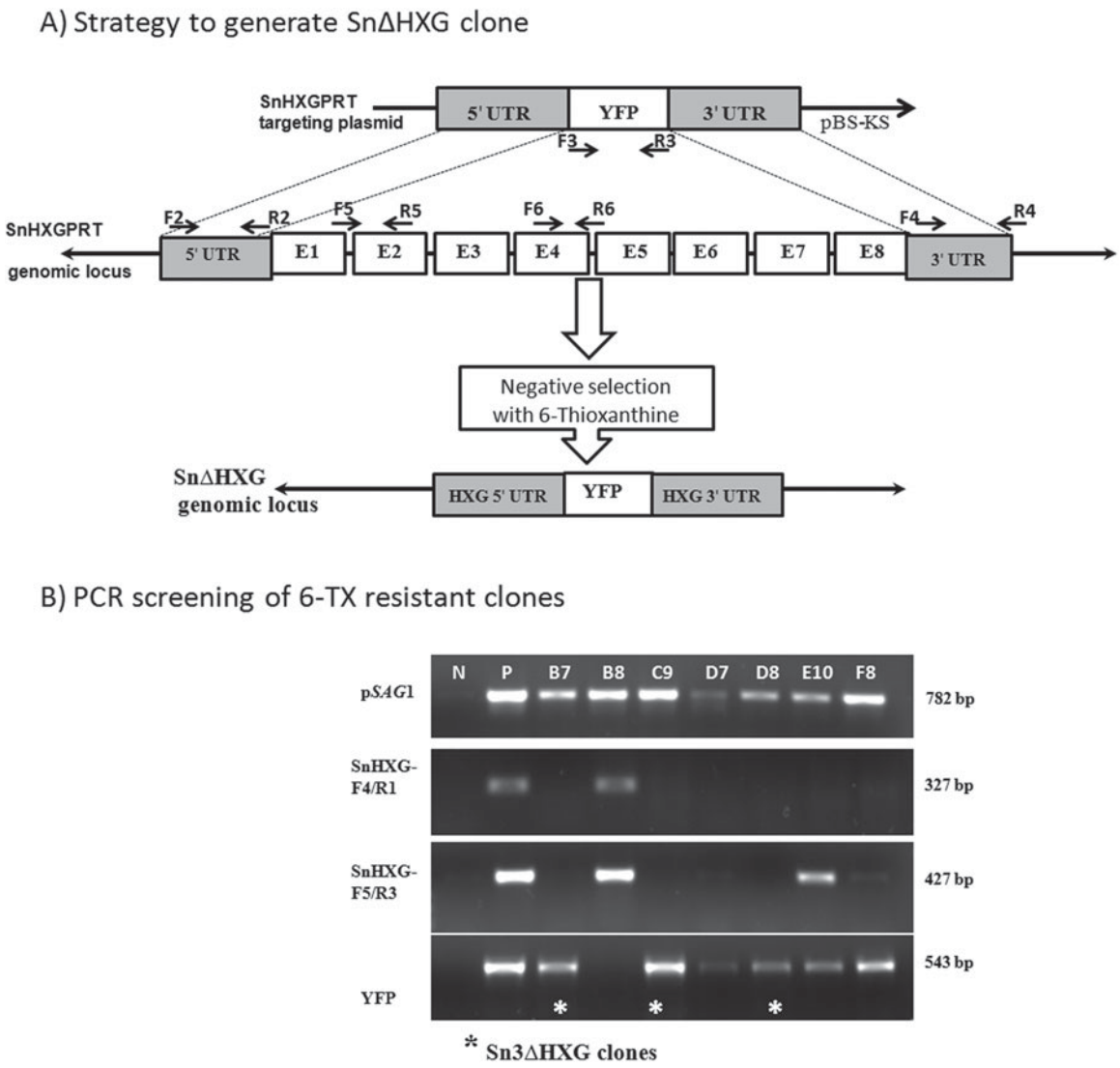

Fig. 1. Generation of *S. neurona* SnΔHXG clones. (A) A linearized gene-targeting plasmid, pSnHXG-UTRs-YFP, consisting of the YFP gene flanked by ~4 kb UTRs of Sn*HXGPRT* gene was used to target the native HXGPRT locus in the *S. neurona* genome. E1 to E8 represent exons of the Sn*HXGPRT* gene. Sn*HXGPRT*-deficient parasites were selected by growing the transfected parasites in culture medium containing 160 μg mL^{-1} 6-TX. The diagram also shows the location of primers used to generate the knockout construct and to screen the 6-TX-resistant clones. Primer information is provided in supplemental table S1. (B) Positive amplification of the YFP gene and no amplification of the native HXGPRT locus helped identify the HXGPRT knockout clones. * indicates *S. neurona* single-cell clones with disrupted HXGPRT locus. A primer pair targeting the promoter region of the *S. neurona* surface antigen SnSAG1 was used as a positive control.

readily in the presence of MPA but were inhibited by 6-TX (data not shown), thus suggesting that *S. neurona* possess a functional HXGPRT enzyme.

SnHXGPRT *can be manipulated for positive-negative selection*

Transfection of *S. neurona* wild-type merozoites with the pSnHXG-UTRs-YFP gene-targeting plasmid resulted in isolation of *S. neurona* that were resistant to 6-TX. Analysis of single-cell clones with PCR primers that target *SnHXGPRT* or *YFP* (Supplemental Table S1) confirmed disruption of the native locus and replacement with the *YFP* transgene in several 6-TX-resistant clones (Fig. 1). The SnΔHXG parasite clones were susceptible to MPA treatment, suggesting that these parasite lines indeed lack functional HXGPRT. Although the *YFP* gene was amplified from these SnΔHXG parasite clones, YFP expression was not detected by fluorescence microscopy or western blot analysis of either merozoite or schizont stages (Supplemental Fig. S1). Clone SnΔHXG.B7 was maintained in culture for further analyses.

TgHXGPRT restores HXGPRT-mediated purine salvage in S. neurona

To assess whether the *T. gondii* TgHXGPRT minigene would complement SnHXGPRT-deficient

https://doi.org/10.1017/S0031182014000687 Published online by Cambridge University Press

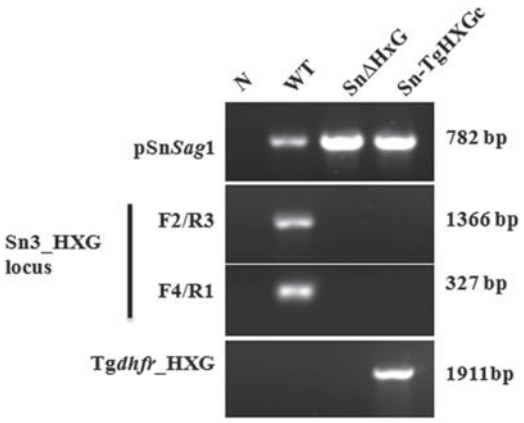

Fig. 2. Heterologous complementation of SnΔHXG parasites. The *T. gondii* HXGPRT minigene driven by the Tg*dhfr* promoter was transfected into SnΔHXG merozoites, and the transformants were selected in culture medium containing 25 μg mL^{-1} MPA and 50 μg mL^{-1} xanthine. Primer pairs targeting the native SnHXGPRT locus and the Tg*dhfr*_HXGPRT of the transfected plasmid were used in PCR to confirm that the MPA-resistant clones lacked the native SnHXGPRT locus but carried Tg*dhfr*_HXGPRT minigene. Primers targeting the promoter region of *SnSAG*1 were used as a positive control. Primer information is provided in supplemental table S1.

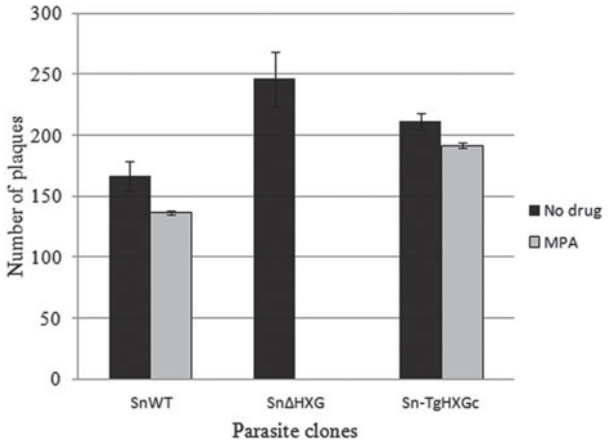

Fig. 3. Plaque assay to determine growth efficiencies of HXGPRT-deficient (SNΔHXG) and TgHXGPRT-complemented (Sn-TgHXGc) *S. neurona*. Approximately 1000 merozoites of wild-type SN3 and the transgenic clones were inoculated in triplicate into wells of a 24-well plate with confluent BT cell monolayers and were grown either in the absence or presence of MPA. Plaque counts at 10 days post-inoculation revealed that MPA completely inhibited the growth of SNΔHXG parasites (no plaques observed) while TgHXGPRT was able to complement the HXGPRT-deficient parasites.

S. neurona, the ptubXFLAG::HX plasmid was transfected into the SnΔHXG.B7 clone. The transfected population yielded parasites that were resistant to MPA, thus indicating that purine salvage was restored via the heterologous HXGPRT from *T. gondii*. PCR amplification performed on single-cell clones of the Sn-TgHXGc parasites confirmed the presence of the TgHXGPRT minigene (Fig. 2). Plaque assays performed to compare the growth efficiencies of SnHXGPRT-deficient and TgHXGPRT-complemented *S. neurona* revealed that MPA completely inhibited the growth of SnΔHXG parasites while there was no significant reduction in the plaque counts of Sn-TgHXGc parasites (Fig. 3). Although there was no difference in the number of plaques when grown in the presence of 6-TX, the plaques for the Sn-TgHXGc and wild-type parasites were noticeably smaller in size and schizonts exhibited delayed development (data not shown).

DISCUSSION

Purine salvage is crucial for survival of apicomplexans, and enzymes involved in this process have been successfully exploited as therapeutic targets as well as selection markers for molecular genetics studies (Donald and Roos, 1998; Gherardi and Sarciron, 2007). Apicomplexans utilize complementary purine salvage pathways involving HXGPRT and/or AK as key enzymes. Interestingly, however, not all members of the Apicomplexa encode both HXGPRT and AK (Chaudhary *et al*. 2004). Bioinformatic searches of the various apicomplexan genomes indicated that *Cryptosporidium* and *Theileria* species encode AK alone. Similarly, *Plasmodium* species rely solely on HXGPRT for purine salvage. *Toxoplasma gondii* and *Eimeria* species use both HXGPRT and AK for purine salvage. In this paper, we report *in silico* identification of purine salvage enzymes of *S. neurona*, which suggested that purine salvage in this parasite can occur by either pathway involving HXGPRT or AK. These *in silico* findings were supported by the ability of wild-type *S. neurona* to grow in the presence of MPA, which requires functional HXGPRT. Likewise, HXGPRT-deficient *S. neurona* were viable and could be propagated in cell culture without any apparent reduction in growth efficiency, suggesting that the complementary purine salvage pathway involving AK is functional.

The failure to identify a PNP homologue in the *S. neurona* genome is significant. PNP is an important enzyme that is involved in converting inosine to hypoxanthine and guanosine to guanine (Berens *et al*. 1995), both of which serve as substrates for HXGPRT. In *T. gondii*, hypoxanthine is reported to be the major purine source, next only to adenosine (Chaudhary *et al*. 2004). The potential absence of PNP in *S. neurona* questions the capability of this parasite to generate inosine and guanosine-derived substrates for HXGPRT, and further suggests that this parasite must heavily rely on AK for purine salvage and/or is limited to xanthine as the primary

https://doi.org/10.1017/S0031182014000687 Published online by Cambridge University Press

substrate for HXGPRT. This analysis of extensive transcriptome data from merozoite and schizont stages of *S. neurona* also supports the absence of PNP in *S. neurona* (S. Dangoudoubiyam, unpublished data). Nevertheless, it is possible that the *S. neurona* PNP sequence has diverged significantly from its sister genera, making it difficult to identify by standard bioinformatic approaches.

In this study, we have used a gene-targeting plasmid for disruption of the native HXGPRT locus, thus demonstrating the feasibility of double-homologous recombination in *S. neurona*. It was intended that YFP expression would be driven by the native HXGPRT promoter following integration at this locus. Although proper integration of the YFP gene was confirmed in the SnΔHXG clones, expression of YFP could not be detected either by fluorescence microscopy or western blots. Moreover, sequencing of the PCR products from one of the SnΔHXG clones revealed that the YFP gene was in frame and contained no mutations. Point mutations were observed in the Sn*HXGPRT* 5′-UTR within 1 kb upstream of the YFP gene, but it is not known if this was responsible for silencing YFP expression. Despite the lack of YFP expression in the SnΔHXG clones, this will not hinder their use as a molecular genetics tool for positive-negative selection in *S. neurona*.

An earlier study that used pTgSAG1 to drive expression of luciferase in *S. neurona* suggested that heterologous promoters were not efficient in this parasite (Gaji and Howe, 2009). In contrast to this, HXGPRT function in SnΔHXG parasites was successfully restored using HXGPRT of *T. gondii* driven by the TgDHFR promoter, thereby demonstrating the feasibility of complementation using heterologous promoters and genes. Recently, other promoters such as pTgTUB and pTgIMC have also been found to be functional in *S. neurona* (S. Dangouboubiyam, unpublished data), which further indicates that certain *T. gondii* promoters are capable of driving expression in *S. neurona*. This could prove advantageous for studies in comparative apicomplexan biology by diminishing the need for generating transgene constructs driven by *S. neurona* promoters.

HXGPRT and AK exist as single copy genes in apicomplexans, and prior work has shown that either enzyme alone is sufficient for parasite survival with no obvious fitness defects (Chaudhary *et al*. 2004). Plaque assays of SnΔHXG and Sn-TgHXGc parasites in MPA were as anticipated, but 6-TX in the growth medium only delayed development of Sn-TgHXGc parasites, as was evident by the smaller plaque sizes. This finding is consistent with 6-TX being a parasititstatic drug and not parasiticidal (Pfefferkorn *et al*. 2001).

Previously developed methods for DNA transfection, expression of reporter molecules, and selection of stable transformants (Gaji *et al*. 2006) have established *S. neurona* as a practical model for comparative studies of the Apicomplexa (Vaishnava *et al*. 2005). The SnΔHXG clones described herein will facilitate positive-negative selection of transgenes and are a valuable addition to the molecular genetic tools available for manipulating *S. neurona*. Moreover, the recently sequenced genome of *S. neurona* further enhances investigation of this parasite. Collectively, these new resources are anticipated to accelerate gene discovery and functional studies in *S. neurona*, while also enabling additional comparative analyses that will contribute to the understanding of apicomplexan biology.

SUPPLEMENTARY MATERIAL

To view supplementary material for this article, please visit http://dx.doi.org/S0031182014000687.

ACKNOWLEDGEMENTS

We thank Dr Anthony Sinai for providing ptubXFLAG::HX plasmid for use in heterologous complementation of HXGPRT in *S. neurona*. The *S. neurona* Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession JAQE00000000. The version described in this paper is version JAQE01000000. Published as Kentucky Agricultural Experiment Station Article No. 14-14-026 with approval of the Director.

FINANCIAL SUPPORT

This work was supported by a grant from the United States Department of Agriculture National Institute of Food and Agriculture (award 2009-65109-05918 to D. K. H.) and funds from the Amerman Family Equine Research Endowment.

REFERENCES

Beech, J. (1974). Equine protozoan encephalomyelitis. *Veterinary Medicine, Small Animal Clinical* **69**, **1562**, 1564–1566.

Berens, R. L., Krug, E. C. and Marr, J. J. (1995). Purine and pyrimidine metabolism. In *Biochemistry and Molecular Biology of Parasites* (ed. Marr, J. J. and Müller, M.), pp. 323–336. Academic Press, London, UK.

Bhatti, M. M. and Sullivan, W. J., Jr. (2005). Histone acetylase GCN5 enters the nucleus via importin-alpha in protozoan parasite *Toxoplasma gondii*. *Journal of Biological Chemistry* **280**, 5902–5908.

Chaudhary, K., Darling, J. A., Fohl, L. M., Sullivan, W. J., Jr., Donald, R. G., Pfefferkorn, E. R., Ullman, B. and Roos, D. S. (2004). Purine salvage pathways in the apicomplexan parasite *Toxoplasma gondii*. *Journal of Biological Chemistry* **279**, 31221–31227.

Cheadle, M. A., Yowell, C. A., Sellon, D. C., Hines, M., Ginn, P. E., Marsh, A. E., Dame, J. B. and Greiner, E. C. (2001). The striped skunk (*Mephitis mephitis*) is an intermediate host for *Sarcocystis neurona*. *International Journal for Parasitology* **31**, 843–849.

Cheadle, M. A., Ginn, P. E., Lindsay, D. S. and Greiner, E. C. (2002). Neurologic disease in gamma-interferon gene knockout mice caused by *Sarcocystis neurona* sporocysts collected from opossums fed armadillo muscle. *Veterinary Parasitology* **103**, 65–69.

Donald, R. G. and Roos, D. S. (1998). Gene knock-outs and allelic replacements in *Toxoplasma gondii:* HXGPRT as a selectable marker for hit-and-run mutagenesis. *Molecular and Biochemical Parasitology* **91**, 295–305.

Donald, R. G. K., Carter, D., Ullman, B. and Roos, D. S. (1996). Insertional tagging, cloning, and expression of the *Toxoplasma gondii* hypoxanthine-xanthine-guanine phosphoribosyltransferase gene. *Journal of Biological Chemistry* **271**, 14010–14019.

Dubey, J. P., Saville, W. J., Lindsay, D. S., Stich, R. W., Stanek, J. F., Speer, C. A., Rosenthal, B. M., Njoku, C. J., Kwok, O. C., Shen, S. K. and Reed, S. M. (2000). Completion of the life cycle of *Sarcocystis neurona*. *Journal of Parasitology* **86**, 1276–1280.

Dubey, J. P., Lindsay, D. S., Saville, W. J., Reed, S. M., Granstrom, D. E. and Speer, C. A. (2001*a*). A review of *Sarcocystis neurona* and equine protozoal myeloencephalitis (EPM). *Veterinary Parasitology* **95**, 89–131.

Dubey, J. P., Saville, W. J., Stanek, J. F., Lindsay, D. S., Rosenthal, B. M., Oglesbee, M. J., Rosypal, A. C., Njoku, C. J., Stich, R. W., Kwok, O. C., Shen, S. K., Hamir, A. N. and Reed, S. M. (2001*b*). *Sarcocystis neurona* infections in raccoons (*Procyon lotor*): evidence for natural infection with sarcocysts, transmission of infection to opossums (*Didelphis virginiana*), and experimental induction of neurologic disease in raccoons. *Veterinary Parasitology* **100**, 117–129.

Fenger, C. K. (1998). Treatment of equine protozoal myeloencephalitis. *Compendium on Continuing Education for the Practising Veterinarian* **20**, 1154–1157.

Gaji, R. Y. and Howe, D. K. (2009). The heptanucleotide motif GAGACGC is a key component of a cis-acting promoter element that is critical for SnSAG1 expression in *Sarcocystis neurona*. *Molecular and Biochemical Parasitology* **166**, 85–88.

Gaji, R. Y., Zhang, D., Breathnach, C. C., Vaishnava, S., Striepen, B. and Howe, D. K. (2006). Molecular genetic transfection of the coccidian parasite *Sarcocystis neurona*. *Molecular and Biochemical Parasitology* **150**, 1–9.

Gherardi, A. and Sarciron, M. E. (2007). Molecules targeting the purine salvage pathway in Apicomplexan parasites. *Trends in Parasitology* **23**, 384–389.

Hoane, J. S., Carruthers, V. B., Striepen, B., Morrison, D. P., Entzeroth, R. and Howe, D. K. (2003). Analysis of the *Sarcocystis neurona* microneme protein SnMIC10: protein characteristics and expression during intracellular development. *International Journal for Parasitology* **33**, 671–679.

Howe, D. K. and Sibley, L. D. (1997). Development of molecular genetics for *Neospora caninum:* a complementary system to *Toxoplasma gondii*. *Methods: A Companion to Methods in Enzymology* **13**, 123–133.

Krug, E. C., Marr, J. J. and Berens, R. L. (1989). Purine metabolism in *Toxoplasma gondii*. *Journal of Biological Chemistry* **264**, 10601–10607.

MacKay, R. J. (2006). Equine protozoal myeloencephalitis: treatment, prognosis, and prevention. *Clinical Techniques in Equine Practice* **5**, 9–16.

Pfefferkorn, E. R. and Borotz, S. E. (1994). *Toxoplasma gondii:* characterization of a mutant resistant to 6-thioxanthine. *Experimental Parasitology* **79**, 374–382.

Pfefferkorn, E. R. and Pfefferkorn, L. C. (1978). The biochemical basis for resistance to adenine arabinoside in a mutant of *Toxoplasma gondii*. *Journal of Parasitology* **64**, 486–492.

Pfefferkorn, E. R., Bzik, D. J. and Honsinger, C. P. (2001). *Toxoplasma gondii*: mechanism of the parasitostatic action of 6-thioxanthine. *Experimental Parasitology* **99**, 235–243.

Vaishnava, S., Morrison, D. P., Gaji, R. Y., Murray, J. M., Entzeroth, R., Howe, D. K. and Striepen, B. (2005). Plastid segregation and cell division in the apicomplexan parasite *Sarcocystis neurona*. *Journal of Cell Science* **118**, 3397–3407.

https://doi.org/10.1017/S0031182014000687 Published online by Cambridge University Press