

TCCTCTAAATAGACTAAAATACCGCCAAATTCCTTTAAG  
TTTTAAGATTATAACTATTACTACTTTAATATATAAAA  
TTTACTTTTTAAATAATAGGGTATCTAATCCTGGTTA

LHmd: AAAAATTTTTCTTCTAATCTAATTTACTATGTAA  
ATCCTACTTTTTAAATTTATCAATATTTAAATAAATCCT

CCAAAATAATTAGATTTTTATTGTGACTCATTATTTTCT  
TAATTATAAACTGCACCTTGATCTGACATAATTATTAA  
TTAAAAATTTTGAAAATTATTATCTAATAAAAATATTC  
TTATGACGACGATATACAAATTAACATAAAAATTAAGT  
AGGGTAAATTGTGGGGTTATCATTTATTTAACAAGGTT  
CCTCTAAATAG.

*J. Parasitol.*, 92(3), 2006, pp. 668–670  
© American Society of Parasitologists 2006

## Nourseothricin Acetyltransferase: A Positive Selectable Marker for *Toxoplasma gondii*

Tam T. Van\*, Peggy J. Rooney\*, and Laura J. Knoll†, Department of Medical Microbiology and Immunology, University of Wisconsin-Madison, 1300 University Avenue, Madison, Wisconsin 53706; \*These authors contributed equally to this research note. †To whom correspondence should be addressed. e-mail: ljknull@wisc.edu

**ABSTRACT:** Molecular analysis of parasite genomes will require new molecular genetic tools. The *nat1* gene of *Streptomyces noursei* encodes nourseothricin acetyltransferase, conferring resistance to the aminoglycoside antibiotic nourseothricin. Electroporation of *nat1* cassettes into RH or Prugniaud strains of *Toxoplasma gondii* allows for selection of stable nourseothricin-resistant clones.

*Toxoplasma gondii* is an obligate intracellular protozoan parasite that is a member of the Apicomplexa. The *T. gondii* genome is organized into a searchable database (<http://www.toxodb.org>) including 10× coverage of the genome, tags from serial analysis of gene expression, and >72,000 expressed sequence tags. To fully capitalize on these data, sequence analysis must be complemented with functional genomics approaches, necessitating multiple positive selectable markers. Use of the tetracycline transactivator system requires 2 transformations, adding the tetracycline repressor and the tetracycline-controlled gene of interest (Meissner et al., 2002). Positive selectable markers chloramphenicol acetyltransferase and bleomycin-binding protein are available (Kim et al., 1993; Messina et al., 1995). Dihydrofolate reductase-based resistance to pyrimethamine and complementation of tryptophan auxotrophy produce parasites resistant to the front-line drug used to treat human infection, or interferon- $\gamma$ -resistant parasites, respectively (Donald and Roos, 1993; Sibley et al., 1994). Hypoxanthine-xanthine-guanine phosphoribosyltransferase (HXGPRT) gives resistance to mycophenolic acid (MPA) by activating exogenous xanthine, but requires use of an available functional HXGPRT knockout strain (Donald et al., 1996). Selection against HXGPRT has been achieved using 6-thioxanthine, allowing reuse of this marker (Donald and Roos, 1998), and the Cre recombinase has been used to remove the LacZ reporter between 2, directly repeated loxP sites (Brecht et al., 1999). The *sat1* gene encoding streptothricin acetyltransferase has been used as a positive selectable marker in *Leishmania major* by conferring resistance to nourseothricin, a secondary metabolite produced by *Streptomyces noursei* (Joshi et al., 1995). A similar gene encoding nourseothricin acetyltransferase (*nat1*) has been used to confer nourseothricin resistance to several fungi, including *Saccharomyces cerevisiae*, *Cryptococcus neoformans*, and *Candida albicans* (Goldstein and McCusker, 1999; McDade and Cox, 2001; Shen et al., 2005). Since the antibiotic nourseothricin is not used therapeutically because of nephrotoxicity, use of the *nat1* marker should not impede treatment regimes. Here, we describe use of the *nat1* gene to encode a selectable marker in *T. gondii*.

Nourseothricin was tested for its effect on *T. gondii* growth as well as toxicity to the human foreskin fibroblast (HFF) host cells in which the parasites are maintained. When intracellular parasites were treated at 500  $\mu\text{g/ml}$  nourseothricin (Werner BioAgents, Jena, Germany, or Fisher Scientific, Pittsburgh, Pennsylvania), growth of *T. gondii* strains RH (type I) and Prugniaud (type II) was unaffected; however, this concentration was toxic to HFF monolayers (data not shown). Extracellular RH strain parasites were incubated at  $10^7$  cells/ml in 100  $\mu\text{l}$  of culture medium (Dulbecco's minimal essential medium with 10% fetal bovine

serum) with 0, 50, 200, 500, or 1,000  $\mu\text{g/ml}$  nourseothricin for 4 hr at 37 C in 5% CO<sub>2</sub> and added directly to 5 ml of HFF cultures (Fig. 1). This 50-fold dilution of drug (maximum final concentration of 20  $\mu\text{g/ml}$ ) was not toxic to HFFs during 4 days of incubation (data not shown). After selection, parasite viability was measured both by counting the number of plaques formed after 5 days and by an 18-hr [<sup>3</sup>H]uracil incorporation, initiated after an overnight incubation, allowing the parasites to invade and begin replicating (Pfefferkorn and Pfefferkorn, 1977). At 500  $\mu\text{g/ml}$ , there was a substantial decrease in *T. gondii* viability (Fig. 1).

To create vectors for *T. gondii* transformation, the *nat1* open reading frame (ORF) was amplified from pAG25 (obtained from the European *Saccharomyces cerevisiae* Archive for Functional Analysis, Frankfurt, Germany) by using primers NsiNAT-F (5'-AAAATGCATACCACTCTTGACGACACGGCTTAC) and PacNAT-R (5'-TTTAAATTAAGATTAAGGGCAGGGCATGCTCATG), creating 5' NsiI and 3' PacI sites. The 578-bp polymerase chain reaction (PCR) product was cloned and sequenced. Plasmid pTUB1nat was created by replacing the CAT ORF of pTUB1/CAT with the NsiI-PacI *nat1* fragment (Soldati and Boothroyd, 1993). Similarly, the pGRA1nat plasmid was constructed by replacement of the NsiI-PacI green fluorescent protein (GFP) fragment in pGRA1-GFP5S65T with the *nat1* ORF (Kim et al., 2001). Transcription of *nat1* from pTUB1nat and pGRA1nat is under the control of the strong, commonly used *T. gondii*  $\alpha$ -tubulin (*TUB1*) and dense granule protein 1 (*GRA1*) promoters, respectively. Derivative vectors were constructed when a NotI-XhoI fragment from pminiHXGPRT-I containing HXGPRT was added into the NotI site of pTUB1nat and pGRA1nat by

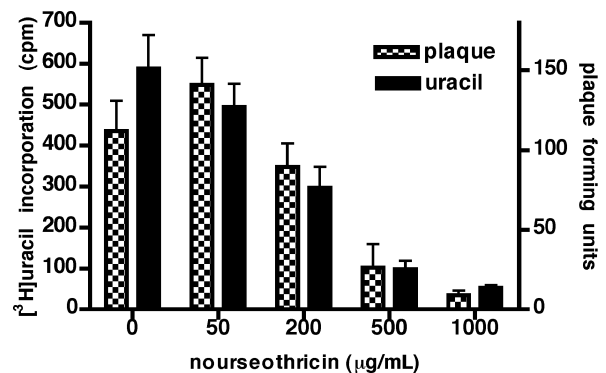


FIGURE 1. Survival of extracellular *T. gondii* (strain RH) after incubation with nourseothricin. Parasites were treated with 0, 50, 200, 500, or 1,000  $\mu\text{g/ml}$  nourseothricin (Ns) for 4 hr. Survival was measured by incorporation of [<sup>3</sup>H]uracil (left axis, checked bars) and plaque number (right axis, solid bars). Data represent the mean of 2 experiments  $\pm$  SD.

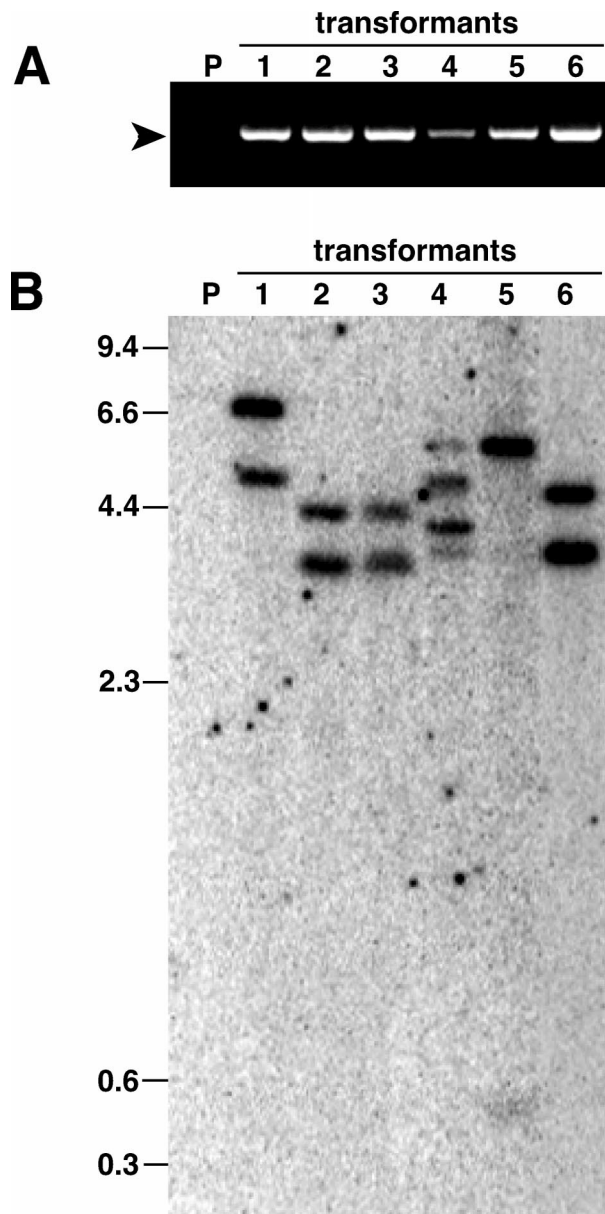


FIGURE 2. Analyses of *T. gondii* transformants. Prugnau $\Delta$ -HXGPRT was transformed with pGRA1nat+HPT, and stable transformants were cloned by limiting dilution. (A) PCR detection of transforming DNA. Prugnau (P) and transformant clones (1–6) were subjected to amplification using NsiNAT-F and PacNAT-R primers. The arrow points to the 578-bp product shown on an ethidium bromide-stained agarose gel. (B) Southern analysis of StyI-digested genomic DNA from P and transformant clones (1–6), probed with a radiolabeled NsiI-PacI *nat1* fragment. StyI cuts the 6.5-kb linearized transforming DNA once within the *nat1* ORF, producing fragments that are 2.8 and 3.7 kb. Transformants 2 and 3 are siblings, and number 4 has 2 insertions. Numbers on the left correspond to sizes of the DNA ladder in kilobases.

blunt ligation, generating pTUB1nat+HPT and pGRA1nat+HPT (Donald et al., 1996). HXGPRT was added to allow rapid screening for transforming DNA in  $\Delta$ HXGPRT strains by testing for MPA resistance in the presence of exogenous xanthine.

Transformants were generated by electroporating  $10^7$  RH or Prugnau parasites with 0, 10, 20, or 100  $\mu$ g of SacI-linearized vectors (Soldati and Boothroyd, 1993). Additionally, 100 units of NotI was

added to the electroporations for restriction enzyme-mediated integration (Black et al., 1995). Parasites were allowed to infect HFFs for 3 days (to reduce the number of transiently transformed parasites). From this population,  $10^6$  cells in 100  $\mu$ l underwent extracellular selection in 500  $\mu$ g/ml nourseothricin for 4–6 hr at 37 C in 5% CO<sub>2</sub>, and they were allowed to infect HFF for 3 days. Parasites were released by syringe lysis, and a second round of selection was applied. Control transformations without DNA showed fewer surviving parasites after the second selection. After the second round of selection, transformants were cloned by limiting dilution. Twenty-four of 29 Prugnau clones were positive for transforming DNA when analyzed by PCR (Fig. 2A; data not shown) by using primers NsiNAT-F and PacNAT-R. Nat-selected pGRA1nat+HPT and pTUB1nat+HPT transformants were treated with 50  $\mu$ g/ml MPA and 100  $\mu$ g/ml xanthine to confirm the presence of the HXGPRT marker. In these clones, resistance to MPA/xanthine correlated with nourseothricin resistance (data not shown). Southern analysis of Prugnau transformant genomic DNA demonstrated that all PCR-positive clones contained the transforming DNA integrated into the genome (Fig. 2B; data not shown). Most transformants contained a single copy of the transforming DNA, indicating that multiple copies are not needed to survive the selection, and 15 of 24 Prugnau clones possessed unique banding patterns in Southern hybridizations.

The selection is less stringent for the RH strain, because 7 of 56 RH clones were positive for *nat1* by Southern hybridization (data not shown). These differences were independent of DNA concentration, promoter controlling *nat1* expression, or presence or absence of the HXGPRT cassette (data not shown). A third round of selection did not enhance the percentage of positive RH clones obtained. Both RH and Prugnau clones surviving selection, but lacking transforming DNA, showed poor growth in nourseothricin-containing medium, as assessed by [<sup>3</sup>H]uracil incorporation in the presence or absence of nourseothricin (data not shown). This poor growth is in contrast to parasites bearing the *nat1* gene, because they grew at the same rate with or without nourseothricin. These results suggest that expression of Nat1 did not affect parasite growth and that nontransformed parasites surviving selection have not acquired resistance to the drug. Additionally, expression of Nat1 does not seem to affect in vitro bradyzoite cyst development, assessed using protocols described in Knoll and Boothroyd (1998) (data not shown). Together, these data indicate that the *nat1* marker is effective for selecting stable *T. gondii* transformants.

We are indebted to Thomas D. Sullivan for suggesting this study. This research was supported by the National Institutes of Health (NIH) Award A1054603 (to L.J.K.). T.T.V. is supported by NIH National Research Service Award GM072125, and P.J.R. is supported by the American Heart Association Postdoctoral Fellowship 0520062Z.

#### LITERATURE CITED

- BLACK, M., F. SEEGER, D. SOLDATI, K. KIM, AND J. C. BOOTHROYD. 1995. Restriction enzyme-mediated integration elevates transformation frequency and enables co-transfection of *Toxoplasma gondii*. *Molecular and Biochemical Parasitology* **74**: 55–63.
- BRECHT, S., H. ERDHART, M. SOETE, AND D. SOLDATI. 1999. Genome engineering of *Toxoplasma gondii* using the site-specific recombinase Cre. *Gene* **234**: 239–247.
- DONALD, R. G., D. CARTER, B. ULLMAN, AND D. S. ROOS. 1996. Insertional tagging, cloning, and expression of the *Toxoplasma gondii* hypoxanthine-xanthine-guanine phosphoribosyltransferase gene. Use as a selectable marker for stable transformation. *Journal of Biological Chemistry* **271**: 14010–14019.
- , AND D. S. ROOS. 1993. Stable molecular transformation of *Toxoplasma gondii*: a selectable dihydrofolate reductase-thymidylate synthase marker based on drug-resistance mutations in malaria. *Proceedings of the National Academy of Sciences USA* **90**: 11703–11707.
- , AND ———. 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.
- GOLDSTEIN, A. L., AND J. H. MCCUSKER. 1999. Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. *Yeast* **15**: 1541–1553.
- JOSHI, P. B., J. R. WEBB, J. E. DAVIES, AND W. R. MCMASTER. 1995.

- The gene encoding streptothricin acetyltransferase (*sat*) as a selectable marker for *Leishmania* expression vectors. *Gene* **156**: 145–149.
- KIM, K., M. S. EATON, W. SCHUBERT, S. WU, AND J. TANG. 2001. Optimized expression of green fluorescent protein in *Toxoplasma gondii* using thermostable green fluorescent protein mutants. *Molecular and Biochemical Parasitology* **113**: 309–313.
- , D. SOLDATI, AND J. C. BOOTHROYD. 1993. Gene replacement in *Toxoplasma gondii* with chloramphenicol acetyltransferase as selectable marker. *Science* **262**: 911–914.
- KNOLL, L. J., AND J. C. BOOTHROYD. 1998. Isolation of developmentally regulated genes from *Toxoplasma gondii* by a gene trap with the positive and negative selectable marker hypoxanthine-xanthine-guanine phosphoribosyltransferase. *Molecular and Cellular Biology* **18**: 807–814.
- MCDADE, H. C., AND G. M. COX. 2001. A new dominant selectable marker for use in *Cryptococcus neoformans*. *Medical Mycology* **39**: 151–154.
- MEISSNER, M., D. SCHLUTER, AND D. SOLDATI. 2002. Role of *Toxoplasma gondii* myosin A in powering parasite gliding and host cell invasion. *Science* **298**: 837–840.
- MESSINA, M., I. NIESMAN, C. MERCIER, AND L. D. SIBLEY. 1995. Stable DNA transformation of *Toxoplasma gondii* using phleomycin selection. *Gene* **165**: 213–217.
- PFEFFERKORN, E. R., AND L. C. PFEFFERKORN. 1977. Specific labeling of intracellular *Toxoplasma gondii* with uracil. *Journal of Protozoology* **24**: 449–453.
- SHEN, J., W. GUO, AND J. R. KOHLER. 2005. *CaNATI*, a heterologous dominant selectable marker for transformation of *Candida albicans* and other pathogenic *Candida* species. *Infection and Immunity* **73**: 1239–1242.
- SIBLEY, L. D., M. MESSINA, AND I. R. NIESMAN. 1994. Stable DNA transformation in the obligate intracellular parasite *Toxoplasma gondii* by complementation of tryptophan auxotrophy. *Proceedings of the National Academy of Sciences USA* **91**: 5508–5512.
- SOLDATI, D., AND J. C. BOOTHROYD. 1993. Transient transfection and expression in the obligate intracellular parasite *Toxoplasma gondii*. *Science* **260**: 349–352.

*J. Parasitol.*, 92(3), 2006, pp. 670–672  
© American Society of Parasitologists 2006

## ***Urotocus rossittensis* (Trematoda: Digenea: Leucochloriidae) in the Scarlet-Rumped Tanager, *Ramphocelus passerinii*, and Common Bush Tanager, *Chlorospingus ophthalmicus* (Passeriformes: Thraupidae), From the Área De Conservación Guanacaste, Costa Rica, with Taxonomic Revision of the Genus and Revised Key to the Leucochloridiid-Like Brachylaimoidea**

David Zamparo and Daniel R. Brooks, Department of Zoology, University of Toronto, Toronto, Ontario M5S 3G5, Canada. e-mail: zamparo@zoo.utoronto.ca

**ABSTRACT:** *Urotocus rossittensis* occurs in the bursa Fabricii of the scarlet-rumped tanager, *Ramphocelus passerinii*, and the common bush tanager, *Chlorospingus ophthalmicus*, from the Area de Conservación Guanacaste, Costa Rica. Morphological examination of type material of *U. fusiformis* and *U. kenyensis* and Costa Rican specimens suggests that *U. fusiformis* and *U. kenyensis* are indistinguishable from *U. rossittensis*. Confirmed accounts of *Urotocus* spp. refer to a single adult morphotype whose geographic distribution includes the Palearctic, Africa, Nearctic, and northern Neotropics.

The digenean genus *Urotocus* comprises 5 nominal species: *Urotocus rossittensis* Mühling, 1898, originally described from the bursa Fabricii of the fieldfare, *Turdus pilaris*, Linnaeus, 1758, from Rossitten East Prussia (now Rybachy, Karliningrad oblast, Russia) (Mühling, 1898) also has been reported in the rock pipit *Anthus petrosus* Montagu, 1798 from the U.K. (Williams, 1960); in the dunnock *Prunella modularis* Linnaeus, 1758 from Novgorod district of Russia (Strom, 1927); in the garden warbler *Sylvia borin* Boddaert, 1783, european robin *Erithacus rubecula* Linnaeus, 1758, song thrush *Turdus philomelos* Brehm, 1831, and eurasian blackbird *Turdus merula* Linnaeus, 1758 from Poland (Okulewicz, 1991); and in the varied thrush *Ixoreus naevius* Gmelin, 1789 from Vancouver, British Columbia (Ching, 1993); *Urotocus fusiformis* McIntosh, 1935, originally described from the bursa Fabricii of the mourning warbler, *Oporornis philadelphia*, Wilson, 1810, in Washington, D.C. (McIntosh, 1935) also has been reported in the white-eyed vireo *Vireo griseus* Boddaert, 1783, in the magnolia warbler *Dendroica magnolia* Wilson, 1811, and in the common yellowthroat *Geothlypis trichus* Linnaeus, 1766 from Washington, D.C. (McIntosh and McIntosh, 1935) and in the great-tailed grackle *Quiscalus (=Cassidix) mexicanus* Gmelin, 1788 from Louisiana (Yamaguti, 1971); Canaris (1965) described *Urotocus kenyensis* Canaris, 1965 in the grey woodpecker, *Mesopicos goertae* Mueller, P. L. S., 1776, from Njoro, Kenya; and finally, there are 2 nominal species of uncertain status, i.e., *Urotocus*

*inflatoceolum* Leonov & Tsimbaliuk, 1965 in the arctic warbler *Phylloscopus borealis* Blasius, 1858 from Elizovskii raion, Kamchatka oblast, Russia, and *Urotocus tholonetensis* Timon-David, 1955 in eurasian magpie *Pica pica* Linnaeus, 1758 from France. Williams (1960) suggested that the latter species was a synonym of *U. rossittensis*, but, not having seen type material, refrained from proposing a formal synonymy. Williams also suggested that *U. fusiformis* and *U. rossittensis* were synonyms, but this view has not been widely accepted (Canaris, 1965; Travassos et al., 1969; Yamaguti, 1971; but see Okulewicz, 1991).

Herein, we report finding 26 specimens of *U. rossittensis* inhabiting 1 of 9 scarlet-rumped tanagers, *Ramphocelus passerinii* Bonaparte, 1831, and a single specimen in a common bush tanager, *Chlorospingus ophthalmicus* Du Brus de Gisignies, 1847, from the Area de Conservación Guanacaste (<http://www.acguanacaste.ac.cr>) in northwestern Costa Rica. This is the first report of *Urotocus* in the Neotropics and constitutes new host and locality records. Our morphological examination of holotype, paratype, and voucher material of *U. fusiformis* (USNPC 3309, 44832, and 90947) and paratypes of *U. kenyensis* (USNPC 947-3, -4, -5, and -8) as well as voucher specimens of *U. rossittensis* (USNPC 76883 and 82755), and our own specimens (vouchers deposited as USNPC 95834 and 95835), lead us to agree with Williams (1960) and Okulewicz (1991) that *U. fusiformis* is synonymous with *U. rossittensis*, and we further suggest that *U. kenyensis* must be considered in that synonymy.

Mühling (1898) described *U. rossittensis* as 2.26 mm long by 300  $\mu$ m wide with an elongate body shape, whereas McIntosh (1935) contrasted his 2 specimens, from a mourning warbler, as 5.00 mm long by 1.45 mm wide, with a spindle-shaped body, proposing *U. fusiformis* for them. We have, however, discovered that McIntosh described only the holotype; the paratype of *U. fusiformis* is 2.5 mm long by 750  $\mu$ m wide. This difference may have led Canaris (1965), in part, to think that specimens in a grey woodpecker from Kenya, which he named *U. kenyensis*, were intermediate between *U. rossittensis* and *U. fusiformis*. However,