Toxoplasma gondii is a category B biodefense pathogen that can be lethal in utero and in immunocompromised humans. This parasite is a candidate bioterrorism agent due to the extreme environmental stability of infective oocysts that can containate water or food supplies (1, 2). While infections in healthy humans are often benign, the identification of distinct Toxoplasma genotypes that are lethal in healthy adults (3) has changed the view of the bioterrorism potential of this pathogen and added to the urgency for the development of new chemotherapeutics and vaccines. Toxoplasma is unique among apicomplexans in its ability to infect, be transmitted by, and cause disease in all warm-blooded animals, a trait which has certainly contributed to its worldwide distribution (4). The genetic bases for this trait are unknown but are likely to be important given the clear link between host range expansion and increased virulence in pathogens (most clearly demonstrated in influenza virus [5]).

With the advent of whole-genome tiling arrays and, most importantly, next-generation sequencing technologies, it is now possible to examine structural differences in whole genomes both within and between closely related species. In humans, locus expansion and diversification have been linked to psychiatric disorders such as autism and schizophrenia (reviewed in reference 6) and to susceptibility to a variety of other diseases (reviewed in reference 7). Locus expansion can also be beneficial. In mammals, expansion and diversification of killer-cell immunoglobulin-like receptor genes are important for recognition of diverse pathogens (8). Laboratory studies with bacteria show that adaptation to selective conditions via gene expansion occurs much more frequently than that via point mutation (9), and in the field, copy number increases drive drug resistance in Drosophila melanogaster (10). Phenotypic impact can be driven by gene dosage, but gene duplication also allows the original copy to maintain its function.
while duplicated copies are free to change via mutation and selection (11).

Expanded and diversified gene families play important roles in pathogen virulence. Gene family expansions have been linked to virulence in *Candida* spp. (12) and *Rickettsia* spp. (13). The var family of genes is distributed throughout the *Plasmodium falciparum* genome and encodes erythrocyte membrane antigens (PfEMPs) that are under strong diversifying selection (14). Expanded genes have been linked to virulence, immune evasion (14), drug resistance (15), and host range (16) in *Plasmodium* spp.

Our recent work demonstrates a role for gene duplication and subsequent diversification in *Toxoplasma* host-pathogen interactions. The *T. gondii* ROP5 locus contains up to 10 copies depending on the strain, and this locus is essential for parasite virulence (17). Importantly, distinct isoforms from the ROP5 locus can have synergistic effects on parasite lethality, indicating that individual copies of the ROP5 gene have evolved subtly distinct functions. The ROP5 locus also exhibits isolate-specific copy number variation (CNV) (17). The surface antigen-1-related (SRS) and rhoptry protein 2 (ROP2) superfamilies have duplicated multiple times, and tandemly duplicated clusters of genes belonging to this family can be found throughout the genome (18). The SRS family has been implicated in immune evasion (19), and the single-copy ROP2 superfamily member ROP18 is a potent virulence factor in mice (20, 21). In *T. gondii*, expanded loci have impacts in other infection contexts, including the ROP4/7 locus, which has no dramatic impact on growth in vitro but affects cyst formation in vivo (23).

Less is known about CNV between species, although as early as 2007 it was postulated that differentially duplicated genes and genomic structural variations could contribute to phenotypic differences between chimps and humans and possibly have played a role in their speciation (24). Some support for this hypothesis is found in plants, where species-specific CNV is known to contribute in certain cases to reproductive isolation (25).

In this study, we used a genome-wide approach to compare the extents of locus expansion across the genomes of *T. gondii*, * Hammondia hammondi*, and *Neospora caninum*. These three species belong to the subfamily *Toxoplasmatinae* (26), and their genomes have been sequenced, revealing a high level of synteny (27, 28). *T. gondii* and *N. caninum* have distinct intermediate host ranges and different definitive hosts (felines and canines, respectively), while *T. gondii* and *H. hammondi* share the same definitive host (29). While *T. gondii* has a vast host range that includes birds and is virulent in mice (30), *H. hammondi* and *N. caninum* cannot infect birds and are avirulent in mice (31, 32). For three *T. gondii* strains (GT1, ME49B7, and VEG) and one *N. caninum* strain (NCLIV [27]), we used a manual pipeline to identify and curate all potentially expanded loci and to compare the degrees of overlap between them. This was facilitated by the fact that these genomes have been annotated. The *H. hammondi* reference genome assembly (GenBank accession no. AVCM00000000) has not yet been fully assembled into chromosomes or annotated (28). However, for a subset of expanded loci we were able to determine if they were similarly expanded in *H. hammondi*. Overall, we find that in contrast to the high level of synteny across these 3 species, there was a significant lack of overlap in their expanded loci. This suggests an important role for gene expansion in the evolution of these species since their divergence.

RESULTS

Fifty-three loci have increased sequence coverage in *T. gondii*. We used a manual identification and curation pipeline to identify putatively expanded loci using sequence read coverage in *T. gondii* (Fig. 1) and identified 53 loci of high sequence complexity in the nuclear genome of *T. gondii*. Average sequence coverage across the

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**FIG 1** Flow chart depicting the pipeline used to identify, curate, and annotate expanded loci in *Toxoplasma gondii*, *Neospora caninum*, and *Hammondia hammondi*. ESTs, expressed sequence tags.
entirety of the 3 currently available Sanger-sequenced genomes differed slightly (median values, 15×, 19×, and 14× for GT1, ME49, and VEG strains, respectively) due to different numbers of raw sequenced reads (see Table S1 in the supplemental material), with 95 to 98% of the raw reads mapping to the ME49B7 genomic assembly using BLAT (33). Normalized sequence coverage across entire chromosomes from all three queried *T. gondii* strains was typically homogeneous, with sporadic patches of increased coverage at certain locations and telomeric sites (Fig. 2A), indicating that gene duplication/expansion was relatively infrequent. We examined gene expansion at all loci across GT1, ME49, and VEG to estimate copy number across the three strains (as in Fig. 2B). Of the 53 expanded loci, only 1 (expanded locus 13, EL13; Fig. 3 and Table 1) appeared to be entirely missing in one of the three *T. gondii* strains, in this case the type I strain (GT1). Otherwise, the remaining 52 loci were conserved in their expanded state in all 3 queried *T. gondii* strains (see Table S2). However, based on sequence coverage analysis, 22 loci exhibited CNVs of ≥3 copies across the 3 queried *T. gondii* strains. This list included the ROP5 locus (EL47, Fig. 3 and Table 1), which, based on sequence coverage, has ~11 copies in ME49B7 and 6 and 4 copies in GT1 and VEG, respectively. This is similar to previously published analyses using high-molecular-weight Southern blotting of the ROP5 locus in *T. gondii* strains RH (type I), ME49 (type II), and CTG (type III) (17).

**Expanded *T. gondii* loci are enriched for secretory proteins with few exons.** Of the 53 expanded loci, 42 were predicted to contain protein-coding genes (http://www.toxodb.org). In addition, one locus (EL40) that did not have a predicted gene model within it does have evidence for being a coding sequence due to the
presence of expressed sequence tags that map to this locus (see Table S2 in the supplemental material). We anticipate that some of the expanded loci without an associated gene prediction will be transcribed to produce either coding or noncoding RNAs. It should be noted that, with few exceptions, the number of paralogs predicted in each of these three genome sequences greatly underestimated copy number (Table 1; see also Table S2). This was most certainly due to collapsing of the assembly in regions containing tandemly duplicated clusters of genes that are similar in sequence, as has been observed in other genomes (e.g., Homo sapiens [34] and Trichomonas vaginalis [35]).

We used existing annotations of the T. gondii genome to characterize the nature of the 42 T. gondii loci containing predicted genes. We found a significant enrichment for genes predicted to contain N-terminal signal sequences (29 out of 42 compared to the entire predicted proteome; hypergeometric distribution [HGD], $P = 5.1 \times 10^{-11}$ [Table 2]). In addition, these 42 genes have fewer exons (mean, 2.1; median, 1) than the rest of the predicted genes in the genome (mean, 5.2; median, 4). Kolmogorov-Smirnov (KS) analysis revealed a significant ($P = 1.4 \times 10^{-7}$) difference in the exon distribution between these two gene sets reflected by their cumulative distributions (see Fig. S1 in the supplemental material). In fact, 26 of the 42 expanded gene-containing loci in T. gondii have only 1 exon (a significant enrichment compared to the genome as a whole; $P = 8.9 \times 10^{-7}$; Table 2).

### TABLE 1 Properties of 18 expanded loci in T. gondii, N. caninum, and H. hammondi

| Locus | Chr. | Gene | Annotation | T. gondii copy no. by strain | No. of predicted T. gondii orthologs by strain | N. caninum copy no. | H. hammondi copy no. |
|-------|------|------|------------|-----------------------------|-----------------------------------------------|--------------------|---------------------|
|       |      |      |            | I | II | III | I | II | III | Chrb | Pos. (MB) | Copy no. | Chrb | Pos. (MB) | Copy no. | Chrb | Pos. (MB) | Copy no. |
| EL1   | Ia   | 0.41 | ROP47      | 5 | 5 | 5   | 1 | 1 | 1   | 1   | 1.79 | 3 | 7–8 |
| EL2   | Ib   | 1.16 | ROP42      | 7 | 7 | 7   | 2 | 2 | 2   | 1   | 1.54 | 1 | ND  |
| EL3   | II   | 0.36 | KRUF family| 4 | 4 | 4   | 2 | 2 | 2   | 1   | NA  | 0 | 1   |
| EL4   | III  | 2.48 | GRA11      | 1 | 1 | 1   | 0 | 1 | 1   | 1   | 2.05 | 1 | 1   |
| EL5   | VI   | 0.26 | SRS22G     | 0 | 0 | 0   | 1 | 1 | 1   | 1   | 0.25 | 1 | ND  |
| EL6   | VI   | 1.68 | Hypothetical| 1 | 1 | 1   | 1 | 1 | 1   | 1   | 1.47 | 2 | 1   |
| EL7   | VI   | 1.88 | ROP38      | 2 | 3 | 3   | 2 | 2 | 2   | 1   | 1.65 | 18 | 2   |
| EL8   | VIib| 2.79 | SRS26A     | 1 | 2 | 1   | 3 | 3 | 3   | 1   | 2.67 | 1 | ND  |
| EL9   | VIII| 6.74 | MIC17      | 3 | 3 | 3   | 2 | 2 | 2   | 1   | 6.49 | 2 | 7–8 |
| EL10  | IX   | 1.3  | Hypothetical| 6 | 6 | 6   | 1 | 1 | 1   | 1   | NA  | 0 | ND  |
| EL11  | X    | 3.86 | SRS250     | 9 | 9 | 9   | 0 | 0 | 0   | 1   | 3.69 | 1 | ND  |
| EL12  | X    | 7.09 | ROP2/8     | 6 | 6 | 6   | 1 | 1 | 1   | 1   | NA  | 0 | ND  |
| EL13  | X    | 7.27 | SRS48      | 5 | 5 | 5   | 0 | 0 | 0   | 1   | 6.75 | 2 | ND  |
| EL14  | XI   | 6.57 | Hypothetical| 4 | 4 | 4   | 2 | 2 | 2   | 1   | 6.08 | 8 | 6–7 |
| EL15  | XII  | 0.54 | ROP56      | 3 | 4 | 4   | 1 | 1 | 1   | 1   | 0.35 | 2 | 8–9 |
| EL16  | XII  | 5.67 | ROP2/8     | 6 | 6 | 6   | 1 | 1 | 1   | 1   | 5.32 | 1 | 2–3 |
| EL17  | XII  | 6.66 | NTPase II  | 3 | 3 | 3   | 2 | 2 | 2   | 1   | 6.24 | 2 | 2–3 |
| EL18  | XII  | 6.68 | NTPase I   | 2 | 2 | 2   | 1 | 1 | 1   | 1   | 6.29 | 1 | 4–5 |

### TABLE 2 Bioinformatic properties of duplicated/expanded loci in Toxoplasma gondii and Neospora caninum

| Property | T. gondii | N. caninum |
|----------|-----------|------------|
|          | With property | Total | With property | Total | HGD | P value | With property | Total | With property | Total | HGD | P value |
| Signal peptide | 29 | 42 | 1,756 | 8,103 | 5.10E−11 | 34 | 45 | 1,596 | 7,227 | 2.60E−14 |
| Single-exon genes | 26 | 42 | 2,121 | 8,103 | 8.90E−07 | 31 | 45 | 1,514 | 7,227 | 4.70E−12 |
| Rhoptry proteins | 5 | 29 | 1,756 | 3.0E−04 | 2 | 34 | 30 | 0.11 |
| SAG-related surface antigens | 5 | 29 | 87 | 0.010 | 22 | 34 | 218 | 4.10E−12 |
| Developmentally regulated: tachyzoite/bradyzoite | 13 | 42 | 1,093 | 8,059 | 0.0020 | 10 | 42 | 1,337 | 8,059 | 0.07 |

a. HGD, hypergeometric distribution. Values in bold are significant.
b. Based on version 8.2 annotation, http://toxodb.org.
c. Based on analysis of complete life cycle transcriptional profile of T. gondii strain M4 (38).
Expanded *T. gondii* loci are dominated by genes of unknown function or localization but also include predicted rhoptry proteins and surface antigens. We examined the degree of annotation of the 42 putatively protein-coding expanded loci using ToxoDB and our own protein family searches. Of these, 29 were annotated only as hypothetical or conserved hypothetical proteins (see Table S2 in the supplemental material) and showed little similarity to previously characterized proteins from *T. gondii* or other eukaryotic species based on domain and BLAST analyses available on ToxoDB. We screened all 42 loci for PFAM domains (both A and B) and found 27 with a PFAM-A hit with an expect value of ≤1 (see Table S3). There were also multiple loci in this group that encode proteins with unannotated PFAM-B matches. Of those with PFAM-B hits, there were 6 PFAM-B domains that matched to multiple expanded loci, suggesting that genes with similar protein architectures have expanded into multilocus, multigene families (including EL17 and EL25 [PFAM-B 2112] and EL6 and EL50 [PFAM-B 3349]; see Table S3). Regardless, the majority of expanded *T. gondii* genes have yet to be characterized in terms of their function or subcellular localization and contain protein domains that have yet to be annotated and/or characterized.

Of those that were annotated, 5 were predicted rhoptry proteins, some of which have been previously characterized. These are EL1, EL3, EL5, EL6, and EL7 (ROP4/7, ROP42, ROP38, ROP2/8, and ROP5, respectively [18]; Table 1). Based on the hypergeometric distribution (HGD) (36), this was a significant enrichment in predicted rhoptry proteins over the current annotation of the genome (Table 2; $P = 7.3 \times 10^{-4}$), further implicating the rhoptry proteome as a target for locus expansion (18). Four expanded loci were annotated as members of the SAG1-related family of surface antigens. These are EL13, EL22, EL37, and EL38 (SRS22, SRS26, SRS48, and SRS59, respectively [37]). One was annotated as a dense granule protein (EL12; GRA11), another as a microneme protein (EL23; MIC17), and 2 were previously characterized bradyzoite-specific nucleoside triphosphatase (NTPases) that had been determined to be found in tandem in the genome (EL52 and EL53; NTPases II and I).

Expanded *T. gondii* loci are enriched for developmentally related genes. We used previously published microarray expression data for *T. gondii* strain M4 (38) to quantitatively assess gene expression across multiple *T. gondii* genes during parasite development, focusing on the oocyst-sporocyst and tachyzoite-bradyzoite transitions. We found that 13 of the 42 expanded loci contained genes that were significantly up- or downregulated at the transcriptional level during the tachyzoite-to-bradyzoite transition in vitro and/or in vivo (Fig. 4). Upregulated genes included those for the rhoptry proteins ROP42 and ROP2/8, and downregulated genes included those for NTPase I and a paralog belonging to the SRS22 family. This enrichment was significant (HGD, $P = 0.0019$; Table 2) compared to the entire predicted transcriptome assayed by the microarray. Ten of the 42 genes were developmentally regulated during the oocyst-sporozoite transition, but this difference was not significant (HGD, $P = 0.07$; Table 2).

Multiple *T. gondii* expanded loci exhibit within-lineage copy number variation. Twenty-two of the 53 expanded loci in *T. gondii* showed differences in sampling frequencies between the 3 representative genome strains, suggestive of copy number variation (as seen previously for *T. gondii* ROP5 [17]). Consistent with this, using CNV-seq to statistically assess copy number variation at all 53 loci (see Fig. S4 in the supplemental material), we identified 23 that significantly varied in copy number between GT1/VEG and ME49 (6 loci), GT1 and ME49 (12 loci), and VEG and ME49 (5 loci). This list included the ROP5 locus, for which copy number has been determined previously in multiple *T. gondii* strains using high-molecular-weight Southern blotting (17). We also conducted whole-genome comparative genomic hybridization (CGH) for two distinct members of each of the 3 major lineages of *T. gondii*. Of the 41 protein-coding expanded loci that could be surveyed by microarray, 24 had significantly higher hybridization intensity across the *T. gondii* strains queried (see Materials and Methods for statistical analyses). In addition to this, we observed a general correlation between copy number as estimated by sequencing coverage analysis and our CGH data. For example, EL5 is predicted to have 4 to 5 copies in the type I strain GT1 and only 1.
Southern blotting for 
To address this further, we performed high-molecular-weight 
were distinct between

remaining loci queried by the microarray, 18 had sufficient CGH 
data but did not show a significant increase in hybridization in-

of the sequence coverage plots. Of the 
were not represented on the microarray (see Fig. S2C).

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We also identified some loci with CGH intensity profiles sug-
esting a difference in copy number between members of the same 
clanage, most notably EL30, for which CGH intensity values 
were distinct between T. gondii strains ME49 and PRU (Fig. 5B).

To address this further, we performed high-molecular-weight 
Southern blotting for EL30 as well as EL3 and EL45 across 6 T. gon-
dii strains using restriction enzymes predicted to cut outside the 
entire expanded locus. For all 3 loci, we observed differences in 
estimated copy number both between and within lines. We 
observed intralineage variation in locus size for EL3 (ROP42) be-
tween GT1 and RH as well as between ME49 and PRU, and we 
estimated that GT1 and RH have 6 and 9 copies, respectively, and 
that ME49 and PRU have 8 and 6 copies, respectively. We esti-

mated that VEG and CTG have 7 copies (Fig. 5C). We detected 
intralineage variation for EL30 and EL45, where ME49 and PRU 
had different-size loci (Fig. 5C). Southern blot data for these three 
loci are generally consistent with the CGH intensity values, al-
though there are some exceptions. For example, for EL3 strain 
GT1 has a higher CGH intensity than would be predicted based on 
the Southern blot. This could be due to as-yet-unidentified cryptic 
restriction sites in the locus that are specific to strain GT1. In 
comparison, for the single-copy locus AMA1 we did not detect inter-
or intralineage variation in sequence read coverage, CGH 
probe intensity, or locus size as estimated by Southern blotting 
(see Fig. S3).

Three T. gondii expanded loci are not essential for in vitro 
growth. We successfully knocked out 3 expanded loci (EL3 [ROP42], EL6, and EL23) in a virulent type I background 
(RHΔku80Δhxgprt [39]). Parasite lines with deletions at each of 
these loci (see Fig. S5 in the supplemental material) exhibited no 
obvious defects in in vitro growth, and neither RHΔku80Δel3 or 
-Δel23 parasite clones showed any defects in acute virulence as 
measured by survival time in mouse infections with 100 
tachyzoites. We did not test RHΔku80Δel6 in mouse virulence 
assays.

N. caninum has a markedly different set of expanded genes 
that is enriched for members of the SAG1-related surface anti-
gen family. In order to compare gene expansion between T. gondii 
and its close relative N. caninum, we first examined gene expan-
sion in N. caninum using approaches identical to those for T. gon-
dii. We identified 65 expanded loci in N. caninum (Liverpool 
strain; http://www.toxodb.org), 45 of which contained predicted 
protein-coding genes. These loci are listed in Table S4 in the sup-
plemental material. The set of N. caninum expanded genes was 
also enriched for genes encoding proteins with signal peptides 
(34/45) and single-exon genes (31/45) compared to the genome as 
a whole (HGD, P = 2.6 × 10^{-14} and 4.7 × 10^{-12}, respectively; 
Table 2). Remarkably, nearly half (22/45; 49%) were found to 
contain a SAG PFAM domain, suggesting that they belong to the 
SRS family, and this is a significant enrichment over the annotated 
genome (P = 4.1 × 10^{-12}; Table 2). Of the remaining 23 protein-
coding expanded N. caninum loci, 3 were previously annotated 
(ROP4/7 and NTPases 1 and 11), 17 had at least one recognized 
PFAM domain, and 4 were completely unannotated and had no 
recognizable PFAM domains. While the increased number of SRS 
family genes in N. caninum has been reported previously (27), our 
data indicate that these loci have also been subject to multiple 
rounds of tandem (i.e., local) duplication.

Distinct sets of genes are expanded in T. gondii and N. cani-
um. To determine the degree of overlap between genes that are 
expanded in T. gondii and N. caninum, we used BLASTN to iden-
tify the syntenic location for each of the 53 T. gondii loci described 
above and then examined that region of the genome for signatures 
of gene expansion in N. caninum. We found that only 16 of the 53 
T. gondii loci also had evidence of expansion in N. caninum (≥2-
fold-higher sequence coverage than background; Fig. 6A). This 
lack of overlap between T. gondii and N. caninum at these loci is in 
contrast to the overall gene-by-gene synteny between the T. gondii 
and N. caninum genomes, and this lack of overlap is significant 
(HGD, 16/53 versus 6,463/8,103 for T. gondii: P = 7 × 10^{-15}; 
HGD, 16/64 versus 6,463/7,227 for N. caninum; Fig. 6A). One of 
the shared expanded loci (locus EL15; annotated in T. gondii as 
ROP38) had higher sequence coverage in N. caninum, while the 
remaining 15 had higher sequence coverage in T. gondii. Of the 37 
loci that were uniquely expanded in T. gondii, 19 had syntenic orthologs in N. caninum, but these loci showed no evidence of 
expansion (sequence coverage was ~1×, e.g., EL3 and EL30; 
Fig. 5A). The remaining 18 loci did not have a syntenic ortholog 
based on the current T. gondii annotation based on the clusters of 
orthologous groups database implemented in ToxoDB (40).

T. gondii and H. hammondi share 16 of 27 expanded loci. The 
H. hammondi genome has not been annotated or assembled into 
chromosomes, preventing a de novo analysis of gene duplication 
and expansion. However, we did use the recently published H. hammondi 
genome sequence and raw sequence reads (28) to 
determine which T. gondii loci were similarly expanded in H. hammondi. 
Of the 42 protein-coding loci, 27 had a perfect 
reciprocal-best-BLAST hit between T. gondii and H. hammondi. 
Of these 27 putative orthologous sequences, we estimated that 16 
of these (59%) had more than 1 copy in H. hammondi, while data 
for the remaining 11 loci suggested that they were single-copy 
genomes in H. hammondi (Fig. 6C).

DISCUSSION
Our previous work has demonstrated a clear and important role 
for gene duplication in the pathogenesis of Toxoplasma gondii (17); we showed that the ROP5 locus was tandemly expanded in 
multiple T. gondii strains and that this expansion led to diversifi-
cation of individual copies within the locus. We were therefore 
interested in identifying other T. gondii loci that were tandemly 
expanded to determine (i) what features were shared among ex-
panded genes and (ii) whether these loci were differentially ex-
panded both within the T. gondii species and in comparison with 
its nearest sequenced relatives, Neospora caninum (27) and 
H. hammondi (28). Stretches of increased copy number were rel-
atively rare in these genomes, and based on the currently released 
genome assemblies, all of the 42 protein-coding loci harbored 
multiple tandem duplications of the same gene.

An important finding of this work was that expanded loci ex-
hibit copy number variation even between members of the same
FIG 5  Sequence read, comparative genomic hybridization, and Southern blot analysis for EL3, EL30, and EL45. (A) Sequence read analysis for three strains of T. gondii (top) and N. caninum Liverpool (bottom). Data for each strain and locus are normalized to the read coverage in the 20 kb flanking the expanded locus to the left. (B) Comparative genomic hybridization (CGH) across 6 T. gondii strains, 2 from each of the 3 canonical lineages. Only microarray probes with perfect matches in GT1, ME49, and VEG were used in the calculations. Boxes span the first and third quartiles and contain the median value for all useful probes. P values for significant differences in hybridization intensity compared to the single-copy gene AMA1 (gray horizontal line) are at the top of the graph, and individual values are shown in the bee swarm plots. (C) Southern blots for 6 T. gondii strains using DNA digested with restriction enzymes predicted to cut outside the repeat locus. Restriction enzymes were BspEl, BgII, and NotI, respectively. A similar blot for the single-copy gene AMA1 can be found in the supplemental material. Numbers at left are molecular masses in kilobases.
clonal lineage. In Europe and North America, \textit{T. gondii} isolates are dominated by members of 3 main lineages (types I, II, and III), and isolates from within the same lineage appear to be clonal. However, based on Southern blot analysis we show that 3 loci exhibit CNV between members of the same clonal lineage, and other candidate loci with similar within-lineage variation can be identified from our CGH data. We do not assert that members of the same lineage are genetically identical; however, based on whole-genome comparisons, “true” members of the same clonal lineage are more genetically similar to one another than to other strains. For example, RH and GT1 strains have only 1,394 single-nucleotide polymorphisms (SNPs) that distinguish them, representing a polymorphism rate of ~0.002% (41), compared to a polymorphism rate between lineages that ranges from 1 to 5% (42). Therefore, we find that differences in copy number at these loci are in contrast to the overall genetic identity of the clonal strains, suggesting that these loci are changing more rapidly than the rest of the genome. However, we do not discount the impact of single-nucleotide polymorphisms in determining differences between members of the same strain type, which have been identified in RH and GT1 (41).

We have shown previously that the raw number of copies does not necessarily track with its impact on a particular phenotype (17). \textit{ROP5} alleles from type I and III \textit{T. gondii} strains have a higher contribution to virulence than the type II alleles, yet the type II parental strain (ME49) has ~10 copies while types I (RH) and III (CTG) are estimated to harbor 6 and 4, respectively (17). In this case, the sequence of an individual copy is more important, and therefore, the presence or absence of even a single copy at an expanded locus could have a phenotypic impact. While we cannot yet demonstrate conclusively that these changes are driven by selection, the fact that individual structural changes in the genome are much more rare than individual mutations (6) certainly points to the possibility that this may be a selection-driven process. Data emerging from the \textit{Toxoplasma gondii} GSCID project (https://sites.google.com/site/Toxoplasmagondiigscidproject/) will allow us to rapidly identify other expanded loci that differ between members of the same clonal lineage, since a number of these will be sequenced. We also do not know if within-lineage gene expansion occurs during asexual reproduction (which occurs in all intermediate hosts infected by \textit{T. gondii}), sexual reproduction (which occurs only in felines), or both. In the wild, \textit{T. gondii} is capable of self-mating (43), and these expansions could occur during genetic recombination.

We also successfully knocked out three expanded loci (\textit{EL3}, \textit{EL6}, and \textit{EL23}) in a highly virulent \textit{T. gondii} strain (RH\text{Δ}Ku80 [39]) and found no defects in their ability to replicate \textit{in vitro} or, for 2 of these loci (\textit{EL3} and \textit{EL23}), no defects in parasite virulence. To date, a number of expanded loci encoding secretory proteins such as those encoded in these loci have been deleted, including \textit{ROP2/8} (22) and \textit{ROP5} (17, 44), without any consequences for \textit{in vitro} tachyzoite growth. The fact that these parasite lines show no defects \textit{in vitro} or \textit{in vivo} is not surprising given that all three of these loci are upregulated during the tachyzoite-to-bradyzoite transition (Fig. 4). Our data, however, show that these loci can indeed be deleted, facilitating future studies on their role during

![](https://mbio.asm.org/images/mbio/mbio.01003-13.f06b.png)

**FIG 6** (A) Overall genomic synteny between \textit{T. gondii} and \textit{N. caninum} predicted genes. (Top) All predicted genes; (middle) all predicted genes with predicted signal peptides; (bottom) expanded loci (53 for \textit{T. gondii} and 64 for \textit{N. caninum}). The top 2 Venn diagrams are based on gene-by-gene synteny, while the bottom panel for expanded loci is based on whether the locus is expanded in both \textit{N. caninum} and \textit{T. gondii} (16 loci) or whether it is absent or present as a single copy in one species (37 and 48 for \textit{T. gondii} and \textit{N. caninum}, respectively). (B) Presence and expanded state of all 53 \textit{T. gondii} expanded loci compared to \textit{N. caninum}. Eighteen of the 53 \textit{T. gondii} loci do not have a syntenic ortholog in \textit{N. caninum}, a significant enrichment compared to the entire genome. (C) Comparison of the expanded state of \textit{T. gondii} loci for which a clear ortholog was identified in \textit{H. hammondi} HhCatGer041. Of the 27 loci, 11 are uniquely expanded in \textit{T. gondii} compared to \textit{H. hammondi}, while we estimated that \textit{H. hammondi} has at least 2 predicted copies for the remaining 16 loci.
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the chronic phase of infection where they are most highly expressed.

There was a statistically significant lack of overlap between tandemly duplicated loci in *T. gondii* and *N. caninum*. While it is tempting to hypothesize that these differentially expanded gene clusters may be responsible for the phenotypic differences between these species (such as differences in virulence in mouse and the different definitive hosts), our data cannot directly validate this claim. However, there is support for this hypothesis from other pathogenic species. In fungi, comparisons between species with different levels of pathogenesis in humans identified gene duplication and expansion as an important mechanism in the evolution of pathogenicity (reviewed in reference 12). For example, genomic comparisons between *Candida albicans* and *Candida dubliniensis* found that these species were most highly distinguished by the presence of two highly expanded gene families in pathogenic *C. albicans* compared to nonpathogenic *C. dubliniensis* (45), and these loci are under investigation as virulence factors. Findings from these studies also suggested that gene duplication and subsequent expansion may play a more important role than other mechanisms such as horizontal gene transfer in the evolution of novel traits in eukaryotic pathogens (12).

In both *T. gondii* and *N. caninum*, the expanded gene sets were statistically significantly enriched for genes predicted to encode secreted proteins with fewer exons compared to the genome as a whole. While secreted proteins make up the vast majority of *T. gondii* effectors, the significance of the increased propensity of genes with few exons to duplicate and expand is unknown. One possible explanation could be the fact that introns are free to mutate more freely and that while a single locus could be duplicated, subsequent distinct mutations in the introns of both copies may prevent further expansion of the locus during recombination or genome replication. The other, and not mutually exclusive, possibility is that genes with fewer exons are subjected to stronger selection, since all of the previously characterized *Toxoplasma* secretory proteins are known to play roles in pathogenesis, including the single-exon effector genes *ROP18* (20, 21), *ROP5* (17, 44), *ROP16* (46), and *GRA15* (47).

The minimal overlap between the loci that have expanded in *T. gondii* and *N. caninum*, and a similar lack of overlap between genes expanded in *T. gondii* compared to *H. hammondii*, is consistent with what has been reported for other closely related species with highly syntenic genomes (35). To our knowledge, this is the first comparative analysis of gene expansions across multiple apicomplexan species. In *T. gondii*, the majority of the uniquely expanded loci are of no known function based on primary sequence, while in *N. caninum*, the vast majority of the expanded loci are predicted to encode surface antigens belonging to the SRS superfAMILY. This was reported previously (27), although the phenotypic impact of this expansion is unknown.

**MATERIALS AND METHODS**

Sequence read alignments. Raw sequence reads for *T. gondii* strains GT1, ME49, and VEG were downloaded from the NCBI trace archive in fasta format. *T. gondii* and *N. caninum* reads were aligned to reference genomes using BLAT with the following settings: -fastMap -minIdentity = 95, -minScore = 90 (33). Following conversion of the BLAT output file (psl format) to SAM format using the ps2sam.pl script within the BLAT distribution, the SAM file was converted to a sorted BAM file using Samtools (48). Sequence coverage was determined in each 500-bp window using coverageBed, distributed with BEDtools (49). Raw *H. hammondii* sequence reads were aligned to the assembled contigs for *H. hammondii* strain HhCatGer041 using bowtie2 (50), and sequence coverage was determined in 500-bp windows across each contig using Samtools mpileup (48) and a custom Perl script.

Output was uploaded in R statistical software as well as a locally run genome browser to generate whole-chromosome and locus-specific plots and to facilitate manual curation of the expanded loci. For locus-specific plots, data for each strain were normalized to the local sequence coverage for that strain of the 20 kb upstream of the putatively expanded locus. Reads for all three strains were aligned to the ME49 genomic assembly (version 7.0; ToxoDB).

Coverage analysis and curation. Genome coverage plots were generated using the data above to construct chromosome-specific files that linked directly to ToxoDB or our own in-house genome browser. For visual inspection, peaks of coverage that were at least 3-fold above background were curatred as follows. We removed loci containing highly repetitive sequence (such as di- and trinucleotide repeats). To begin determining whether the locus was tandemly expanded or if the increased sequence coverage was due to the presence of an identical (or nearly identical) gene somewhere else in the genome, we parsed the BLAT output to identify sequence reads that mapped to different chromosomes or to a location on the same chromosome that was at least 25 kb away from the putatively expanded locus. For the remaining loci, the chromosomal region was examined for the presence or absence of predicted genes using ToxoDB. For those regions containing predicted genes in *T. gondii* or *N. caninum*, we examined the current annotation of the locus in ToxoDB and our own genome browser and collected evidence for gene duplication based on the occurrence of multiple predicted genes in the same locus with high identity. For the curated expanded gene sets, we performed enrichment studies for a variety of features, including the number of predicted exons and the presence/absence of a predicted signal peptide. PFAM domain analyses on predicted proteins from each locus were run locally.

Comparative genomic hybridization. For each of the 6 strains tested, parasites were grown in human foreskin fibroblasts (HFFs), released from host cells by needle passage, washed once by centrifugation at 800 × g, and filtered through 5.0-μm polyvinylidene difluoride (PVDF) syringe filters (Millipore). Genomic DNA (gDNA) was harvested from purified parasites using DNAzol (Invitrogen) according to the manufacturer’s protocol and treated with RNase A to remove RNA contamination. After confirmation of purity by gel electrophoresis, gDNA was sheared as follows: 1 μg of gDNA was added to 750 μl of shearing buffer (Tris-EDTA [TE], pH 8.0, and 10% glycerol) and 1 μl of 20-μg/μl glycogen in a prechilled nebulizer (Invitrogen) on ice. Nebulization was performed at 40 lb/in2 for 3 min using pressurized nitrogen. The size range of the resultant gDNA fragments was confirmed to be 200 to 600 bp by gel electrophoresis. DNA fragments were precipitated using 100% isopropanol. Biotin labeling of DNA fragments was performed using the BioPrime Array CGH genomic labeling system (Invitrogen) according to the manufacturer’s protocol. The 10× dCTP nucleotide mix was used with biotin-dCTP. Purification of labeled fragments was performed with the BioPrime purification module. For each strain, 2 μg of labeled DNA was hybridized to the Affymetrix ToxoGeneChip.

Comparative genomic hybridization data analysis. Raw Affymetrix data files for each strain were analyzed in R statistical software using the “affy” module ([http://cran.r-project.org](http://cran.r-project.org)). Individual probe-level data were normalized using the “constant” method, and raw intensity values were exported using the expression chip definition file for the ToxoGeneChip (51). For each probe sequence from the *T. gondii* Affymetrix GeneChip, the number of occurrences of that 24-bp sequence in the raw genomic sequence reads for *T. gondii* GT1, ME49B7, and VEG was calculated, and probes not present in a particular strain were not used in subsequent calculations. This resulted in some strains having no useful probes for a given expanded locus (e.g., *EL48* [see Fig. S2B in the supplemental material]). To determine whether a locus showed significantly
higher hybridization intensity, which is indicative of locus duplication/ expansion, from all 6 strains were pooled and compared to data from the single-copy gene AMA1 using a Bonferroni-corrected Student t test (52). For display purposes and visual inspection of between- and within-lineage variation in CGH hybridization intensity, values for each probe were mean centered.

Identification of loci with strain-specific copy number variation using CNV-seq. We used CNV-seq (53) to compare sequence coverage at the 53 curated loci for all 3 T. gondii strains. In addition to default settings, we used the following parameters: –genome-size = 65,000,000; minimum-windows-required = 1. CNV-seq empirically calculated the minimum window size based on sequence coverage for each strain, which was 6,158 bp for GT1 versus ME49B7 and 6,607 bp for VEG versus ME49B7. Copy number variation was determined at a significance threshold of P < 0.0001.

High-molecular-weight Southern blotting. Genomic DNA was isolated from 6 T. gondii strains (2 representative strains from each of the 3 clonal lineages: types I, II, and III). For each strain, 20 μg of genomic DNA was digested overnight (EL3, BspEII; EL30, BglII; EL45, NotI; and AMA1, Saci) and resolved by pulsed-field gel electrophoresis using the CHEF-DR III system (Bio-Rad; run parameters, 6.0 kV, 120°, 1.3 s for 8 h and 2.7 s for 7 h). Resolved fragments were transferred onto a nylon membrane (Bio-Rad) and probed with digoxigenin (DIG)-labeled (Roche), locus-specific probes made from PCR-generated DNA fragments per the manufacturer’s protocol. Primers used for generating locus-specific probes are listed in Table S5 in the supplemental material.

Genetic knockouts and mouse infections. To knock out loci EL3, EL6, and EL23 in the RHΔhxgprtΔku80 strain (39), 1- to 2-kb flanking regions were amplified using primers shown in Table S5 in the supplemental material. For EL3, these were cloned sequentially into plasmid pTKO-mCherry (a gift from John Boothroyd) flanking a hypoxanthine phosphoribosyltransferase minigene (HXGPRT [54]) and transfected into RHΔhxgprtΔku80. Knockout constructs for EL6 and EL23 were created using splicing by overlap extension PCR (SOE PCR [55]) as follows: the HXGPRT minigene was amplified from pTKO-mCherry with 20- to 25-bp 5′ extensions that overlapped flanking regions from the locus. Regions flanking the expanded locus were amplified from RHΔhxgprtΔku80 genomic DNA using primers with 5′ extensions identical to those of the HXGPRT primers, except that they were reverse complemented. For each locus, individual PCRs were performed, and products were gel purified and then used in equal amounts in a second PCR. Controls consisted of reaction mixtures lacking at least one of the 3 fragments. For EL23, multiple SOE reactions were performed and ethanol was precipitated and transfected directly into RHΔhxgprtΔku80. For EL6, the SOE PCR fragment was cloned into pCR2.1-Topo and then transfected into RHΔhxgprtΔku80. Strains were selected using mycophenolic acid and xanthine medium (MPA/X) as described previously (54), and individual clones were isolated by limiting dilution. Knockouts were validated using PCR for (i) integration of the plasmid into the correct location in the genome and (ii) absence of amplification from a gene within the locus (see Fig. S5). For each knockout clone, 3 to 4 mice were infected with 100 tachyzoites (along with an empty-vector control strain) to determine the effect of the knockout on parasite lethality. Mice were monitored daily for signs of morbidity according to approved IACUC protocol 12010130.

Animal experiments. All animal experiments and procedures met the standards of the American Veterinary Association and were approved locally under IACUC protocol 12010130.

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01003-13/-/DCSupplemental. Figure S1, TIF file, 0.1 MB.
Figure S2, PDF file, 0.4 MB.
Figure S3, TIF file, 2.1 MB.
Figure S4, TIF file, 1.1 MB.
Figure S5, TIF file, 3.2 MB.
Table S1, DOCX file, 0.1 MB.
Table S2, XLSX file, 0.1 MB.
Table S3, XLSX file, 0.1 MB.
Table S4, XLSX file, 0.1 MB.
Table S5, DOCX file, 0.1 MB.

ACKNOWLEDGMENTS
We acknowledge Vern Carruthers for providing the RHΔhxgprtΔku80 strain.

This work was supported by NIH scholar development award K22 AI080977 and a Pew Scholarship in the Biomedical Sciences to J.P.B. and University of Pittsburgh Biological Sciences summer and academic year fellowships funded by the Howard Hughes Medical Institute to H.E.W. and A.L.B.

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