NOTES

Toxoplasma gondii and Cryptosporidium parvum Lack Detectable DNA Cytosine Methylation\textsuperscript{\textdagger}

Mathieu Gissot,\textsuperscript{1} Sang-Woon Choi,\textsuperscript{2} Reid F. Thompson,\textsuperscript{3} John M. Greally,\textsuperscript{3} and Kami Kim\textsuperscript{1}\textsuperscript{*}

Departments of Medicine (Infectious Diseases) and Microbiology & Immunology, Albert Einstein College of Medicine, Bronx, New York 10461\textsuperscript{1}; Jean Mayer USDA Human Nutrition Center on Aging at Tufts University, 711 Washington Street, Boston, Massachusetts 02111\textsuperscript{2}; and Departments of Medicine (Hematology) and Molecular Genetics, Albert Einstein College of Medicine, Bronx, New York 10461\textsuperscript{3}

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Epigenetic factors play a role in the expression of virulence traits in Apicomplexa. Apicomplexan genomes encode putative DNA cytosine methylation enzymes. To assess the presence of cytosine methylation of Toxoplasma gondii and Cryptosporidium parvum DNA, we used mass spectrometry analysis and confirmed that these organisms lack detectable methylecytosine in their DNA.

Mechanisms of gene regulation are poorly understood in Apicomplexa, a phylum that encompasses deadly human pathogens like Plasmodium, Toxoplasma, and Cryptosporidium. Sequence analysis of Toxoplasma gondii, Cryptosporidium parvum (19), and Plasmodium species (1) reveals few candidate proteins with canonical sequence-specific transcription factor function. More recently, studies have focused attention on the epigenetic control of gene expression in Apicomplexa (9).

In higher eukaryotes, the presence of 5-methylcytosine (5mC) at the promoter is associated with gene silencing (5). The presence of 5mC has also been reported in unicellular eukaryotes such as Dictyostelium (12) and Entamoeba (6) but is absent in the Saccharomyces cerevisiae genome. Recently, the P. falciparum genome has been shown to lack 5mC (4). The cytosine-5 DNA methyltransferases (DNMT) are enzymes able to catalyze the methylation of the cytosine in the DNA context. The Dnmt2 family is conserved in lower eukaryotes and catalyzes DNA cytosine methylation in Dictyostelium (12) and Entamoeba (6) mainly at repetitive elements and retroposons.

The current version of the T. gondii genome (www.toxodb.org) encodes two genes (49.m03360 and 42.m03580) containing a motif signature of the Dmnt2 family of DNMT. The transcript corresponding to 49.m03360 (on chromosome VI) is expressed during all stages of the life cycle (according to expressed sequence tag [EST] data) and at similar levels in tachyzoites of the type I (RH) and induced bradyzoite cultures of the type II (PLK) strains, as assayed by real-time quantitative reverse transcription (RT)-PCR (data not shown). RH is a type I strain that does not make bradyzoites, whereas PLK is a type II strain that makes bradyzoites in vitro and in vivo. In contrast, the gene 42.m03580 (chromosome X) may not be expressed, as no ESTs have been sequenced and we did not detect expression by real-time quantitative RT-PCR (data not shown).

The C. parvum genome encodes one protein with similarity to the proteins of the Dmnt2 family of DNMT. No EST data are available for the gene that encodes that protein. Therefore, the T. gondii and C. parvum genomes encode a putative protein of the same family as EtMeth, the DNMT responsible for DNA methylation at cytosine in Entamoeba. The apicomplexan DNMT proteins could be orthologues of Entamoeba DNA mtDNMT; act as tRNA methyltransferases, as observed in humans (10); or have other unique biological functions.

To examine the presence of cytosine methylation in T. gondii at CpG dinucleotides across the genome, we surveyed the T. gondii genome by using the HELP (HpaII tiny fragment enrichment by ligase-mediated PCR) assay (15). For this, we digested T. gondii DNA to completion (after overnight digestion with an excess of enzymes and as verified on an agarose gel) with the methylation-sensitive restriction enzyme HpaII and with its methylation-insensitive isoschizomer MspI and hybridized labeled DNA to a tiled T. gondii genomic microarray (Nimblegen). Individual probes were mapped to the HpaII fragments in which they were contained and were then summarized on a per-fragment basis using a 20\% trimmed mean of the raw log intensities. The data for each chip were then adjusted for fragment size by sliding-window quantile normalization, a within-chip extension of the approach described in reference 11. Figure 1 shows that the hybridization profiles of MspI and HpaII are essentially identical, in contrast to rat liver DNA and mouse sperm DNA, which are both methylated at CpG sites. For moderately (liver) or highly (sperm) methylated DNA, HpaII-sensitive bands will be larger and therefore underrepresented in the pool of small genomic DNA fragments used for hybridization to the genomic array (15), resulting in a negative log ratio of HpaII to MspI. In contrast, T. gondii HpaII/MspI ratios were positive, with uniform hybridization throughout the genome. Therefore, analysis of the hybridiza-
tion patterns indicated that *T. gondii* is not methylated at CpGs (Fig. 1).

To our knowledge, there are no remnants of retroposon elements in the *T. gondii* genome (14) that might be regions of selective silencing via DNA methylation as seen in *Entamoeba histolytica* or *Dictyostelium* (6, 16). We reasoned that if DNA methylation was a significant mechanism by which genes were silenced, we might see significant methylation at genes whose expression was stage specific. No evidence of cytosine methylation at any stage-specific locus across the genome was observed in the HELP assay or by bisulfite sequencing (two bradyzoite gene loci tested; data not shown) in both the RH and PLK strains.

Although in mammalian cells, 5mC residues are predominantly localized at CpG dinucleotides, this may not be the case for lower eukaryotes, as reported for *E. histolytica* (17). We therefore decided to use a high-sensitivity approach able to identify 5mC independent of the surrounding nucleotide sequence. This method, based on liquid chromatography (LC)-mass spectrometry (MS), for the measurement of 5mC residues in genomic DNA allows the quantitative determination of genomic DNA methylation status (7). Genomic DNA was extracted from *T. gondii* tachyzoites and *C. parvum* excysted oocysts purified from infected cattle (obtained from Saul Tzipori, Tufts University) and hydrolyzed by sequential digestion with three enzymes, nuclease P1, venom phosphodiesterase I, and alkaline phosphatase. The DNA solution is directly delivered onto the analytical column in isocratic mode, allowing the separation and identification of cytosine and 5mC after electrospray ionization (ESI)-MS analysis of chromatographic peaks (4, 7). The ESI condition causes the separation of the pentose moiety from the pyrimidine ring of both 2′-deoxycty-
DNA. (B, C, D, and E) Two peaks correspond to 5mC (m/z = 112) and its stable isotope (m/z = 115) for a representative experiment detecting cytosine in T. gondii DNA. (B, C, D, and E) Two peaks correspond to 5mC (m/z = 126) and its stable isotope (m/z = 130). A 1:1 mixture of T. gondii and human genomic DNAs has a 5mC peak (B). No 5mC is detected in T. gondii (C), C. parvum (D), or P. falciparum (E) DNA.

The chromatograms from LC-ESI-MS analysis of all DNA samples showed the elution of peaks between 4 and 5 min corresponding to cytosine (m/z 112) and its stable isotope (m/z 115) (Fig. 2A and data not shown). Various mixtures of human and T. gondii DNAs showed the expected 5mC peak (Fig. 2B). In contrast, T. gondii (Fig. 2C), C. parvum (Fig. 2D), and P. falciparum (Fig. 2E) DNAs showed no peak of 5mC (m/z 126) eluting between 6 and 7 min with its stable isotope (m/z 130).

The range of DNA methylation of mammalian DNA is typically 4 to 6% (3, 18). When it is represented as an absolute amount of methylcytosine per microgram of DNA, the reported range of DNA methylation in human or rodent DNA is between 1 and 6 ng/μg of DNA (2, 8, 13), in agreement with the values we obtained for samples containing a mixture of human and T. gondii DNAs (0.83 ng/μg or 2.22% for a 50% human DNA sample; 0.76 ng/μg or 1.26% for a sample with 20% human DNA). LC-ESI-MS is reported to detect 5mC on the order of picograms per microgram of DNA (7), and the methodology used here can detect less than 0.04% methylated cytosine. Although P. falciparum DNA is highly AT rich and C. parvum DNA is somewhat AT rich, the T. gondii genome has a GC content of approximately 50% (14).

We have used a variety of methods to examine the cytosine methylation status of T. gondii DNA. The HELP assay identified no T. gondii loci with evidence of CpG dinucleotide methylation. Using LC-ESI-MS, we could not detect 5mC in T. gondii DNA or C. parvum DNA, and we confirmed prior findings for P. falciparum (4). In conclusion, we have found that T. gondii and C. parvum do not have detectable levels of 5mC within their genomes. While it is possible that cytosine methylation is present in other developmental stages less amenable to analysis, our studies suggest that in apicomplexan parasites, cytosine methylation is not a major regulator of epigenetic processes.

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