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論　文　名	「*Cryptosporidium parvum*: From Distinct Molecular Features of the Parasite Metabolism to an Improved Anti-protozoan Drug Testing Assay」 (*Cryptosporidium parvum* に関する研究：寄生虫代謝の分子生化学的解析を利用した抗原虫薬評価法の改良)
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論文要旨

Introduction

Cryptosporidium parvum is a unicellular parasite that can cause severe diarrhea in humans and animals. Based on host specificity, *C. parvum* used to be separated into two types: Type I isolates were primarily transmitted among humans, while Type II isolates were zoonotic. In 2002, Type I *C. parvum* was renamed to *C. hominis*. In addition to *C. parvum* and *C. hominis*, at least 12 other *Cryptosporidium* species have been generally accepted as valid species that infect a wide range of animals, including mammals, birds, fishes, reptiles and amphibians. In domestic animals, cryptosporidiosis may cause death or severely affect the growth and development in calves and foals. In humans, this parasite can cause prolonged, life threatening infection in immunocompromized individuals. Therefore, *C. parvum* and *C. hominis* are also a significant opportunistic pathogen in AIDS patients. Also because the environmental oocysts are highly resistant to almost all disinfectants used in community and recreational waters, *Cryptosporidium* is a significant water- and food-borne pathogen.

Cryptosporidium parvum belongs to the Phylum Apicomplexa, in which all the members are parasites and many of them are of medical or veterinary importance (e.g., *Plasmodium* [malaria],

Toxoplasma [toxoplasmosis], *Babesia* [Babesiosis] and *Eimeria* [coccidiosis]). Like other coccidia, the life cycle of *C. parvum* consists of at least three distinct developmental stages: sporogony to produce infectious sporozoites within oocysts; two generations of intracellular merogony to form eight and four merozoites; and sexual gametogony to form micro- and macro-gametes that fuse to become zygotes. Zygotes will develop into immature oocysts, which in turn undergo a next generation of sporogony that produces infectious oocysts before releasing into the host digestive guts. All apicomplexan life stages except for the short-lasting zygotes are haploid. Both sporogony and merogony are types of cell multiplication differing from host somatic cell duplication.

Although globally important, effective treatments are limited in both human and animal cryptosporidiosis. Currently, only a single drug (i.e., nitazoxanide [NTZ]) has been approved for treating cryptosporidiosis in the United States, Central and South America. The slow drug development in treating cryptosporidiosis is mainly caused by the author's limited understanding of basic metabolism in *C. parvum*, which in turn is largely due to the technical difficulties in obtaining a large quantity of pure parasite material for molecular and biochemical studies, and to the lack of genetic transfection systems. Current knowledge on the metabolism of *Cryptosporidium* is largely acquired by gene discovery and more recently, the analysis of gene compositions based on complete genome sequences. This parasite has a compact genome (~ 10.5 megabases) that constitutes a highly streamlined metabolism. Many important pathways present in other apicomplexans are missing in *Cryptosporidium*. For example, *C. parvum* lacks an apicoplast and associated metabolic pathways, including type II fatty acid synthase (FAS) and iospropynoid synthesis. This parasite may contain a highly degenerated mitochondrion based on the presence of a number of mitochondrial-specific genes (e.g., heat shock protein 70 [hsp70], ferredoxin [fdx], alternative oxidase [AOX], and the headpiece of the F-ATPase alpha-subunit. However, it lacks Krebs cycle and cytochrome-based respiration. It has lost almost all *de novo* biosynthetic capacity, including these for amino acid, nucleosides, and fatty acids. Instead, it apparently scavenges nutrients from the host using a highly expanded set of transporters, such as those for sugars and amino acids.

All these molecular and biological features make *Cryptosporidium* unique among apicomplexans. Indeed, recent molecular phylogenetic reconstructions have consistently placed the *Cryptosporidium* genus at the base of the Apicomplexa, or even as a sister to the gregarines (a Class of parasites of invertebrates), which is in contrast to the conventional taxonomy that considers *Cryptosporidium* as a sister to the Coccidia. As mentioned above, *C. parvum* is also divergent from other apicomplexans at both molecular and biochemical levels. Therefore, one cannot simply learn or deduce the fundamental biology of *Cryptosporidium* species from the knowledge generated on other model apicomplexans (e.g., *Toxoplasma* and *Plasmodium*). It is necessary to thoroughly investigate its own molecular biology and biochemistry of *Cryptosporidium* in order to gain insight into the fundamental biology of this parasite.

Recently, several important metabolic pathways in *C. parvum* have been more extensively investigated in the authors' laboratory, such as the fatty acid biosynthesis, glycolysis, DNA replication and transcription. The ultimate goal for studying the metabolism in *C. parvum* is to truly understand the fundamental biology of this parasite and build a solid ground for the development of drugs and/or means to control or treat cryptosporidiosis in humans and animals. Therefore, this dissertation will cover the studies ranging from the molecular and biochemical analysis of distinct parasite genes and proteins to the development of improved molecular assays for the drug testing against *C. parvum in vitro*.

Chapter 1: Functional Characterization of an Evolutionarily Distinct Phosphopantetheinyl Transferase in the Apicomplexan, *Cryptosporidium parvum*

Recently, two types of fatty acid synthases (FASs) have been discovered from apicomplexan parasites. Although significant progress has been made in characterizing these apicomplexan FASs, virtually nothing was previously known about the activation and regulation of these enzymes. In this study, we report the discovery and characterization of two distinct types of phosphopantetheinyl transferase (PPTase) that are responsible for synthesizing holo-acyl carrier protein (ACP) from three apicomplexan parasites: surfactin production element (SFP)-type in *Cryptosporidium parvum* (CpSFP-PPT), holo-ACP synthase (ACPS)-type in *Plasmodium falciparum* (PfACPS-PPT), and both SFP and ACPS types in *Toxoplasma gondii* (TgSFP-PPT and TgACPS-PPT). CpSFP-PPT and TgSFP-PPT are monofunctional, cytosolic, and phylogenetically related to animal PPTases. However, PfACPS-PPT and TgACPS-PPT are bifunctional (fused with a metal-dependent hydrolase), likely targeted to the apicoplast, and more closely related to proteobacterial PPTases.

The function of apicomplexan PPTases has been confirmed by detailed functional analysis using recombinant CpSFP-PPT expressed from an artificially synthesized gene with codon usage optimized for *Escherichia coli*. The recombinant CpSFP-PPT was able to activate the ACP domains from the *C. parvum* Type I FAS *in vitro* using either CoA or acetyl-CoA as a substrate, or *in vivo* when co-expressed in bacteria, with kinetic characteristics typical of PPTases. These observations suggest that the two types of fatty acid synthases in the Apicomplexa are activated and regulated by two evolutionarily distinct PPTases.

Chapter 2: Intron-containing β-Tubulin Transcripts in *Cryptosporidium parvum* Cultured *in vitro*

The genome of *Cryptosporidium parvum* contains a relatively small number of introns,

which includes the β-tubulin gene with only a single intron. Recently, we observed that the intron was not removed from some of the β–tubulin transcripts in the late life cycle stages cultured *in vitro*. Although normally spliced β-tubulin mRNA were detected in all parasite intracellular stages by RT-PCR (e.g., HCT-8 or Caco-2 cells infected with *C. parvum* for 12 to 72 hr), at 48 – 72 hr post infection unprocessed β-tubulin transcripts containing intact introns started to appear in parasite mRNA within infected host cells. The intron-containing transcripts could be detected by fluorescence *in situ* hybridization using an intron-specific probe.

The intron-containing β-tubulin transcripts appeared unique to the *in vitro* cultured *C. parvum*, since they were not detected in parasite-infected calves at 72 hr. It is yet unclear whether the late life cycle stages of *C. parvum* are partially deficient in intron-splicing, or the intron-splicing processes have merely slowed, both of which would allow the detection of intron-containing transcripts. Another possible explanation is that the decay in transcript processing might be simply due to the onset of parasite death. Nonetheless, the appearance of intron-containing transcripts coincides with the arrest of *C. parvum* development *in vitro*. This unusual observation prompts us to speculate that the abnormal intron-splicing of β-tubulin transcripts may be one of the factors preventing complete development of this parasite *in vitro*. Furthermore, the presence of both processed and unprocessed introns in β–tubulin transcripts *in vitro* may provide a venue for studying overall mechanisms for intron-splicing in this parasite.

Chapter 3: Application of Quantitative Real-Time Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) in Assessing Drug Efficacy Against the Intracellular Pathogen *Cryptosporidium parvum in vitro*

We report here a quantitative real-time RT-PCR (qRT-PCR) assay for assessing drug efficacy against the intracellular pathogen, *Cryptosporidium parvum*. The qRT-PCR assay detects 18S rRNA transcripts from both parasites ($C_{T[P18S]}$) and host cells ($C_{T[H18S]}$), and evaluates the relative expression between parasite and host rRNA levels (i.e., $\Delta C_T = C_{T[P18S]} - C_{T[H18S]}$) to minimize experimental and operational errors. The choice of qRT-PCR over qPCR in this study is based on the observations that: 1) the relationship between the logarithm of infected parasites (log[P]) and the normalized relative level of rRNA ($\Delta\Delta C_T$) is linear with a 4-fold dynamic range using qRT-PCR, but sigmoidal (non-linear) using qPCR; and 2) the level of RNA represents that of live parasites better than that of DNA, because the decay of RNA (99% in ~3 hr) in dead parasites is faster than that of DNA (99% in ~24 - 48 hr) under *in vitro* conditions.

The reliability of the qRT-PCR method was validated by testing the efficacy of nitazoxanide and paromomycin on the development of two strains of *C. parvum* (IOWA and KSU-1) in HCT-8 cells *in vitro*. Both compounds displayed dose-dependent inhibitions. The observed MIC_{50} values for nitazoxanide and paromomycin were 0.30 - 0.45 μg/ml and 89.7 - 119.0 μg/ml, respectively,

comparable to the previously reported values. Using the qRT-PCR assay, we have also observed that pyrazole could inhibit *C. parvum* development *in vitro* (MIC_{50} = 15.8 mM), suggesting that the recently discovered *Cryptosporidium* alcohol dehydrogenases may be explored as new drug targets.

Conclusions

Although *Cryptosporidium* genus has been traditionally considered as a sister to the Class Coccidia, the present molecular and metabolic data indicate that this genus is highly divergent from other apicomplexans. This notion is also supported by a number of recent molecular phylogenetic reconstructions that have consistently placed *Cryptosporidium* at the base of the Apicomplexa.

Cryptosporidium possesses an SFP-type PPTase (CpSFP-PPT) that is responsible for activating the ACP domains in the multifunctional Type I CpFAS1 and CpPKS1. However, this parasite lacks an ACPS-type PPTase and Type II FAS that are present in *Toxoplasma* and *Plasmodium*. Recombinant CpSFP-PPT is able to activate recombinant ACP domains from CpFAS1 *in vitro* or *in vivo* when co-expressed in bacteria, with enzymatic kinetics characteristic to other characterized PPTases.

In vitro cultivated *C. parvum* may have a deficiency in intron-splicing (at least for the β-tubulin gene), which may be partially associated with the difficulties to complete the entire parasite life cycle under *in vitro* conditions. It may also provide a potential tool for dissecting the mechanism of intron-splicing in this parasite.

The present qRT-PCR is an improved method for evaluating the efficacies of drugs against *C. parvum in vitro*. It is a good alternative to the conventional methods and may be developed into high-throughput screening of compounds against *C. parvum in vitro*.

審査結果の要旨

クリプトスポリジウムは、ヒトと動物に重篤な下痢を引き起こすアピコンプレックス門の原虫であり、現在、十数種に分類されている。幼齢動物はクリプトスポリジウム症により死亡することがあり、免疫力が低下したヒトでは本症によって生命の危機にさらされる。一方でクリプトスポリジウムは、環境中のオーシストが一般的な浄水過程で使用される消毒薬に対して強い耐性を示すので、重要な飲水あるいは食物媒介性病原体である。このように非常に重要な感染性原虫であるにもかかわらず、ヒトや動物のクリプトスポリジウム症に対する効果的な治療法は限られており、現在、米国、中南米では主に病原性の強い *Cryptosporidium parvum* 治療薬として一種類の薬剤（nitazoxanide）が承認されているに過ぎない。

申請者は、クリプトスポリジウム属の代謝経路、例えば脂肪酸生合成、解糖系、DNA の複製と転写などについて広範囲な研究を行い、本原虫がアピコンプレックス門のなかで特異な性質を持つ

ことを見いだした。この論文では、*C. parvum* の遺伝子とタンパク質を分子生化学的に解析した結果を利用して *in vitro* で抗原虫薬を試験する改良型定量的リアルタイムRT-PCR（qRT-PCR）法を開発し、以下の内容を得た。

第1章では、脂肪酸生合成過程において重要な役割を演じる脂肪酸シンターゼ(FAS)とその活性化を担うphosphophanotetheinyl transferase (PPTase)について解析し、以下の結果を得た。FASには、脂肪酸生合成に係わる複数の酵素が、アシルキャリアータンパク質(ACP)と結合した状態で複合体を形成して統合的に機能するType I FASと、複数の酵素がばらばらに存在して個々にACPと結合した状態のType II FASが存在する。アピコンプレックス門におけるFASに関する過去の報告では、*C. parvum*はType I を、 *Plasmodium falciparum*はType □を、*Toxoplasma gondii* はType I とType IIの双方を持つと報告されている。また、FASの活性化には、PPTaseが関与しており、Type I FASにはsurfactin産生因子 (SFP)-PPTが、Type II FASにはACPシンターゼ-PPT (ACPS-PPT)が作用すると考えられているが、アピコンプレックス門のPPTaseに関する詳細は不明であった。そこで、本章においては分子生物学的手法を用いて、上述の3種のアピコンプレックス門原虫のPPTaseを解析した。その結果、*C. parvum*はSFP-PPTを、*P. falciparum*はACPS-PPTを、*T. gondii*はSFP-PPTとACPS-PPTを保有していた。*C. parvum*においては、SFP-PPTのmRNAが全てのライフサイクルにおいて発現し、宿主細胞感染12時間後、次いで、感染前のスポロゾイトのステージにおいてその発現が高かった。さらに、*C. parvum*のSFP-PPTとACPの組換えタンパク質を作製し、組換えSFP-PPTが組換えACPのSer残基にphosphophanotetheinyl motifが付加されることをMALDI-TOF-mass spectrometry法により検証した。また、組換えSFP-PPTはMg^{2+}の存在下で酵素活性を示すことを確認した。最後に組換えSFP-PPTを用いてウサギ免疫血清を作製し、*C. parvum*のスポロゾイトおよびメロントでの局在を間接免疫蛍光法で観察し、SFP-PPTが細胞質全体に分布している可能性を示唆した。

第2章では、*C. parvum* の発育・増殖における β-tubulin 遺伝子に含まれる intron の役割について述べており、以下の結果を得た。*C. parvum* は他の寄生虫と比較してゲノム中の intron が少ないが、β-tubulin 遺伝子の 5'末端付近には1つの intron が存在することが報告されている。β-tubulin は、細胞内の微小管の主要構成タンパク質で、*C. parvum* の発育・増殖に深く関わっている。*C. parvum* は、宿主体内で感染 72 時間後に無数のオーシストを産生し、発育・増殖を繰り返すが、*in vitro* の培養細胞での発育・増殖は非常に悪いことが知られている。そこで、ヒト大腸腺癌由来培養細胞(HCT-8)とヒト結腸癌由来細胞（Caco-2）、および宿主である牛を用いて感染実験を行い、RT-PCR 法により intron の有無を検討した。培養細胞においては、HCT-8 では感染 48-72 時間後、Caco-2 では感染 30-60 時間後に採取したサンプルからは intron を含む配列が増幅されたが、感染後 72 時間後の牛の小腸上皮細胞から採取したサンプルからは intron を含む配列は増幅されなかった。ゲノムからのコンタミネーションを除外するために培養細胞から抽出した RNA サンプルを DNase で繰り返し処置したところ、同様の結果を得た。さらに RNase で処理した群では intron を含んだ配列は認められなかった。培養細胞における intron の存在をさらに確認するために、anti-intron および anti-exon プローブを用いた *in situ* ハイブリダイゼーション法を試みた結果、*C.*

*parvum*の虫体にのみanti-intronによる蛍光が観察された。以上の結果から*C. parvum*が培養細胞で発育・増殖が悪い原因の一つにβ-tubulinのintronが関与している可能性を示唆している。

第3章では、*C. parvum*に対する抗原虫薬の*in vitro*評価法としてのqRT-PCR法の応用について以下の結果を得た。*C. parvum*に対する薬剤の評価法は、従来、培養細胞を用いた*in vitro*法において、形態学的計測法や抗体を用いた測定法が用いられてきたが、感度と精度に大きな問題があった。近年、PCR法によりDNA量を測定することによる評価法が提唱されているが、死滅した寄生虫由来のDNAによって測定結果が影響を受けることが判明した。そこで、qRT-PCR法により*C. parvum*の 18S rRNAと宿主細胞の 18S rRNAを比較検討することによる測定法を開発した。qRT-PCRの信頼性は、*in vitro*でのHCT-8 細胞内*C. parvum*（IOWA株とKSU-1 株）の発育に関して、nitazoxanideとparomomycinの効果を試験することによって確認され、両薬剤は、用量依存的な抑制を示した。NitazoxanideとparomomycinのMIC_{50}値は、それぞれ 0.30-0.45μg/mlと89.7-119.0μg/mlであり、以前の報告と同様であった。さらに、pyrazoleが*in vitro*で*C. parvum*の発育を阻害する結果を得た。

本研究は、今後大きな社会問題になると予想されるクリプトスポリジウム感染症の治療と予防のために不可欠な薬剤を開発するための画期的な方法を提供するものである。一方で、感染症の中でも対応が遅れがちである原虫疾患への対処方法について分子生化学的手法を実践的に用いる点は、獣医学に大きく貢献するものと評価できる。したがって、本論文の審査および学力確認の結果とあわせて博士（獣医学）の学位を授与することを適当と認める。