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Short communication

Toward understanding the role of mitochondrial complex II in the intraerythrocytic stages of *Plasmodium falciparum*: Gene targeting of the Fp subunit

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ABSTRACT

Malaria parasites in human hosts depend on glycolysis for most of their energy production, and the mitochondrion of the intraerythrocytic form is acristate. Although the genes for all tricarboxylic acid (TCA) cycle members are found in the parasite genome, the presence of a functional TCA cycle in the intraerythrocytic stage is still controversial. To elucidate the physiological role of *Plasmodium falciparum* mitochondrial complex II (succinate-ubiquinone reductase (SQR) or succinate dehydrogenase (SDH)) in the TCA cycle, the gene for the flavoprotein subunit (Fp) of the enzyme, *pfsdha* (*P. falciparum* gene for SDH subunit A, PlasmoDB ID: PF3D7_1034400) was disrupted. SDH is a well-known marker enzyme for mitochondria. In the *pfsdha* disruptants, Fp mRNA and polypeptides were decreased, and neither SQR nor SDH activity of complex II was detected. The suppression of complex II caused growth retardation of the intraerythrocytic forms, suggesting that complex II contributes to intraerythrocytic parasite growth, although it is not essential for survival. The growth retardation in the *pfsdha* disruptant was rescued by the addition of succinate, but not by fumarate. This indicates that complex II functions as a quinol-fumarate reductase (QFR) to form succinate from fumarate in the intraerythrocytic parasite.

Many aerobic organisms, including humans, depend on oxidative phosphorylation for most of their energy metabolism. On the other hand, the intraerythrocytic malaria parasite synthesizes ATP by anaerobic glycolysis [1]. All the genes for the glycolytic pathway are found in the parasite genome, and pyruvate generated from glucose by glycolysis is converted to lactate with NAD^+ generation [2,3]. The role of mitochondria in parasite energy metabolism is unclear. β-oxidation is absent from the mitochondria and there is no biochemical evidence for a canonical and functional tricarboxylic acid (TCA) cycle in the intraerythrocytic form [2,4,5].

In mammals, mitochondrial complex II functions as a succinate-ubiquinone reductase (SQR) that catalyzes the oxidation of succinate in the TCA cycle and supplies electrons to the respiratory chain. Generally, complex II is composed of four subunits: a flavoprotein subunit (Fp) and an iron–sulfur protein subunit (Ip) as catalytic domains, and two hydrophobic subunits as membrane anchor domains. The genes for the Fp and Ip, *pfsdha* (PlasmoDB ID: PF3D7_1034400) and *pfsdhb*, have been cloned, and *P. falciparum* mitochondrial proteins show both succinate dehydrogenase (SDH) and SQR activities, indicating that complex II should have some role in parasite survival [6,7]. On the other hand, complex II functions as a quinol-fumarate reductase (QFR), the reverse action of SQR, for anaerobic respiration in various anaerobic organisms [8]. Thus, the direction of the reaction suggests the biological function of complex II.

Since mitochondrial complex II was potentially expected to be essential for parasite survival, a tetracycline analogue-regulated transgene expression system in *P. falciparum* was chosen to establish a conditional knockout strain for the analysis of this potentially essential gene [9]. Since this is a Tet-Off system, the target gene under control is expressed in the absence of the tetracycline analogue anhydrotetracycline (ATc), and the addition of ATc should repress the target gene expression. A conditional knockout of the gene for the Fp subunit in SQR from *P. falciparum* was tried with a pTGPI-GFP derived vector, by which the target gene expression could be controlled with ATc in the parasite (Fig. 1) [9].

As pTGPI-GFP is a 'Tet-Off' system, the transformants were cultured in a medium without ATc, to keep *pfsdha* expression. Stable recombinant parasites had been cloned by limiting dilution from pTSDHA-trunc and pTSDHA-full transformants. The genomic organization of the targeted loci was confirmed by Southern blotting (not shown).

To analyze the *pfsdha* transcription, total RNAs from the trophozoite/schizont-rich culture were used for semi-quantitative RT-PCR and Northern blot analysis. Unexpectedly, the *pfsdha* disruptant did not express *pfsdha* mRNA even in the absence of ATc (not shown). These results indicate that the established *pfsdha* disruptants were not conditional

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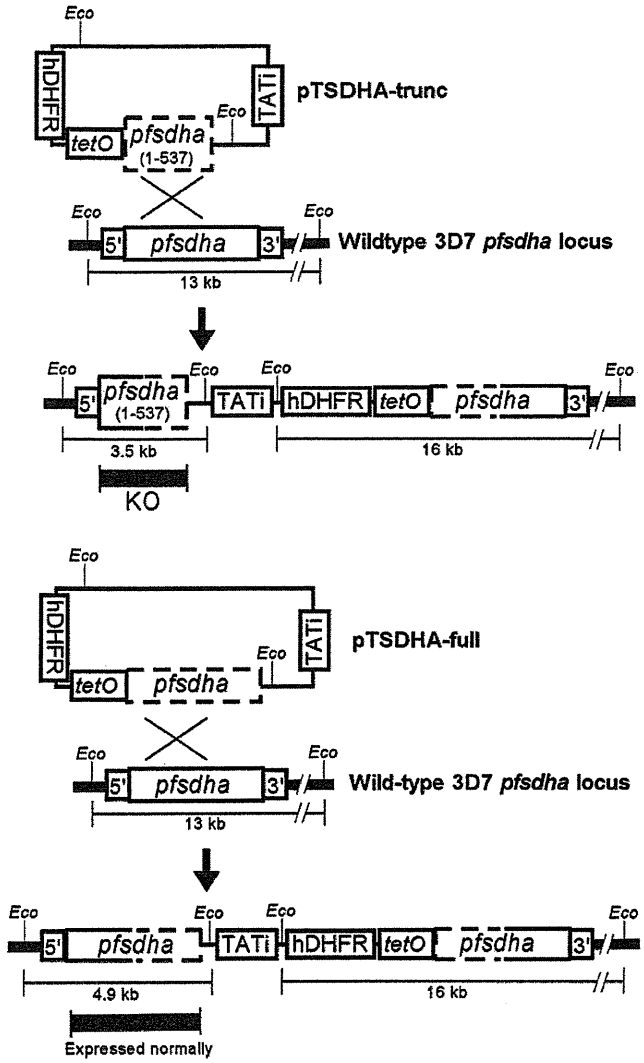

Fig. 1. (A) Schematic diagrams for '*pfsdha* disruption' and the control integration into the target locus. The *pfsdha* gene (1–537 and full-length), human dihydrofolate reductase (hDHFR), tetracycline repressor and transactivator fusion protein (TATi3), tetracycline operator sequence (*tetO*), and the *Eco*RV site (*Eco*) are labeled. Thin and thick solid lines indicate the backbone sequences of the plasmid and the 3D7 chromosomal DNA, respectively. Parasites were cultured following standard protocols [17]. *P. falciparum* (3D7 strain) were cultivated according to [10] with slight modifications. The parental plasmid, pTGPI-GFP, was a kind gift from Dr. B.S. Crabb (Walter and Eliza Hall Institute of Medical Research, Australia) [9]. Since the Rep20 element in pTGPI-GFP is a subtelomeric repeated region and localizes the plasmid to perinuclear chromosome-end clusters [18], and it was expected to potentially inhibit plasmid integration into the homologous chromosomal locus, pTGPI-GFPΔRep20 plasmid was obtained from pTGPI-GFP with *Bgl*II digestion and self-ligation. A DNA fragment of *pfsdha* (GenBank ID: XM_001347582, PlasmoDB ID: PF3D7_1034400) corresponding to nucleotide coding region 1–537 with additional sequences of *Sse*8387I and *Spe*I sites at each end, allowing for insertion between the *Pst*I and *Spe*I sites of pTGPI-GFP, was amplified by PCR from 3D7 genomic DNA prepared with DNAzol (Invitrogen, Life Technologies). The PCR product was digested with *Sse*8387I and *Spe*I and then inserted into the digested pTGPI-GFPΔRep20, resulting in plasmid pTSDHA-trunc. As a control, a DNA fragment of full-length *pfsdha* with additional sequences of *Sse*8387I and *Spe*I sites at each end was amplified by PCR from genomic DNA. The fragment was cloned between the *Pst*I and *Spe*I sites of pTGPI-GFPΔRep20 by the same method as above, resulting in plasmid pTSDHA-full. The primer sequences were available from the authors upon request. Plasmid DNA was electroporated to infected erythrocytes and transfected parasites were selected with 5 nM WR99210 (Jacobus Pharmaceutical Co., Inc., Princeton, NJ, USA, a kind gift of Dr. David Jacobs) [19]. Single-cell cloning was carried out by limiting dilution.

knockouts, but constitutively *pfsdha*-repressed strains. In a previous study, a similar phenomenon was observed in parasites cultured for prolonged periods (Dr. B.S. Crabb, personal communication). These mutants were useful for the study on the role of complex II in intraerythrocytic form as constitutively *pfsdha*-repressed mutants although the *pfsdha* disruptants were not expected mutants.

Following the analysis of the transcription of *pfsdha* gene, the expression of Fp and Ip peptides was examined by Western blot analysis with mitochondrial proteins prepared from trophozoite/schizont-rich culture [10]. The expression of Fp protein in the mitochondrial fraction from the *pfsdha* disruptant was significantly lower than that of the other controls (not shown). Interestingly, in spite of the normal expression of the *pfsdhb* transcript, repression of Ip protein was observed in the *pfsdha* disruptant (not shown). Although their expressions were not completely repressed, both of the signal intensities for the Fp and Ip proteins of the *pfsdha* disruptant should be low enough to evaluate its role.

The enzyme activities of complex II and dihydroorotate dehydrogenase (DHOD) were examined with mitochondrial proteins isolated from the parasites according to [10]. DHOD is the fourth enzyme of the *de novo* pyrimidine synthetic pathway. *P. falciparum* DHOD localizes on the mitochondrial membrane and transfers electrons to ubiquinone in the respiratory chain [11]. Both SDH and SQR activities in mitochondria from the *pfsdha* disruptant, examined according to [7,10], were repressed to undetectable levels, while mitochondria from the controls showed 3.84 to 6.15 nmol/min/mg of SDH and 3.11 to 5.45 nmol/min/mg of SQR activities. On the other hand, there was no difference in DHOD activities between the controls (13.9 to 18.9 nmol/min/mg) and the *pfsdha* disruptant (15.7 nmol/min/mg).

To examine the effect of Fp repression, parasite growth was analyzed after a 48-h culture, one intraerythrocytic growth cycle. As shown in Fig. 2A, the growth retardation was detected in experiments started at 0.2% parasitemia (gray bars). The parasitemia of the controls was $1.78 \pm 0.14\%$ (wild type), $1.73 \pm 0.15\%$ (control of plasmid transfection), and $1.70 \pm 0.15\%$ (control of integration). Meanwhile, the parasitemia of the *pfsdha* disruptant was $0.94\% \pm 0.09\%$, half of that for the controls (Fig. 2A).

Succinate and fumarate are substrates for SQR and QFR, respectively. The direction of the complex II reaction was examined by the addition of the substrates to the *pfsdha* disruptant. The maximum concentration of succinate and fumarate used was 5 mM, because the intraerythrocytic parasite cannot survive in a culture medium with ≥ 50 mM succinate or fumarate (not shown). As a result, 5 mM of succinate rescued the growth retardation of the *pfsdha* disruptant, but fumarate did not (Fig. 2B). The growth of the controls was not affected by the substrate addition. To examine the dose response, the parasites were incubated with between 5 mM and 50 nM of succinate or fumarate. Growth retardation rescue was observed in the *pfsdha* disruptant with ≥ 50 μM of succinate, but fumarate did not rescue the parasite growth retardation (Fig. 2B). These findings suggest that complex II catalyzes succinate production by fumarate reduction in the intraerythrocytic forms.

If the volume of cell and protein concentration [12] is taken account, the concentrations of succinate and fumarate in human cell [13] could be equivalent to those in the parasite (10^{-1} mM) [14]. Thus, under physiological condition, growth retardation of the disruptant in the intraerythrocytic stages may not be observed.

Complex II appears to function as a QFR, and the produced succinate is required in other metabolic pathways. Heme biosynthesis might be a potential pathway that needs the produced succinate, because succinate could be a precursor of succinyl-CoA for the first step of heme biosynthesis. Moreover, QFR has been proposed to couple with protoporphyrinogen IX oxidase in the heme biosynthesis pathway of *P. falciparum* [15]. Our previous observation showing functional link between dihydroorotate-dependent respiration and QRF [7] is consistent with this idea. Considering that a recent report suggested branched TCA cycle metabolism in the parasite [16], further biochemical analysis is indispensable to understand the real nature of the energy metabolism of the parasite.

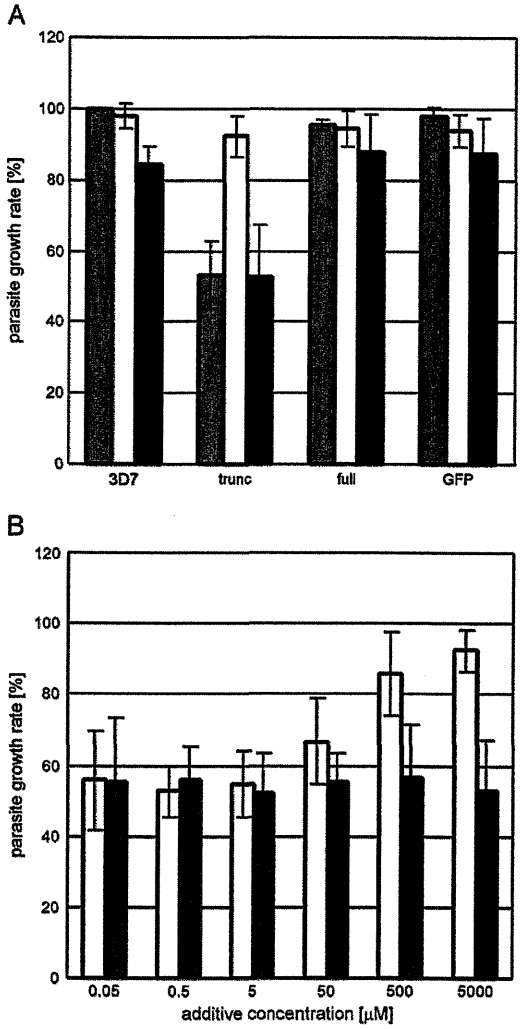

Fig. 2. (A) Growth retardation of the *pfsdha* disruptant and growth rescue by substrates. Gray, white, and black bars indicate no additives, 5 mM of succinate, and 5 mM of fumarate, respectively. (B) Dose dependency of succinate on growth rescue in the *pfsdha* disruptant (pTSDHA-trunc clonal line). White and black bars indicate succinate (Suc) and fumarate (Fum) addition to the *pfsdha* disruptant, respectively. 3D7, wild-type 3D7 strain; trunc, pTSDHA-trunc clonal line ('*pfsdha* disruptant'); full, pTSDHA-full clonal line; GFP, pTGPI-GFPΔRep20 transformant. Synchronized parasites, prepared according to [20], at 0.2% starting parasitemia were triplicated with 1 ml each of complete medium containing 0.5% (w/v) AlbuMAX I (Gibco, Life Technologies). Giemsa-stained smears were prepared after a 48-h incubation without media change, and the parasitemia was evaluated by optical microscopy.

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Identification of a Bacteria-Like Ferrochelatase in *Strongyloides venezuelensis*, an Animal Parasitic Nematode

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Abstract

Heme is an essential molecule for vast majority of organisms serving as a prosthetic group for various hemoproteins. Although most organisms synthesize heme from 5-aminolevulinic acid through a conserved heme biosynthetic pathway composed of seven consecutive enzymatic reactions, nematodes are known to be natural heme auxotrophs. The completely sequenced *Caenorhabditis elegans* genome, for example, lacks all seven genes for heme biosynthesis. However, genome/transcriptome sequencing of *Strongyloides venezuelensis*, an important model nematode species for studying human strongyloidiasis, indicated the presence of a gene for ferrochelatase (FeCH), which catalyzes the terminal step of heme biosynthesis, whereas the other six heme biosynthesis genes are apparently missing. Phylogenetic analyses indicated that nematode FeCH genes, including that of *S. venezuelensis* (SvFeCH) have a fundamentally different evolutionally origin from the FeCH genes of non-nematode metazoa. Although all non-nematode metazoan FeCH genes appear to be inherited vertically from an ancestral opisthokont, nematode FeCH may have been acquired from an alpha-proteobacterium, horizontally. The identified *Sv*FeCH sequence was found to function as FeCH as expected based on both *in vitro* chelatase assays using recombinant *Sv*FeCH and *in vivo* complementation experiments using an FeCH-deficient strain of Escherichia coli. Messenger RNA expression levels during the *S. venezuelensis* lifecycle were examined by real-time RT-PCR. SvFeCH mRNA was expressed at all the stages examined with a marked reduction at the infective third-stage larvae. Our study demonstrates the presence of a bacteria-like FeCH gene in the *S. venezuelensis* genome. It appeared that *S. venezuelensis* and some other animal parasitic nematodes reacquired the once-lost FeCH gene. Although the underlying evolutionary pressures that necessitated this reacquisition remain to be investigated, it is interesting that the presence of FeCH genes in the absence of other heme biosynthesis genes has been reported only for animal pathogens, and this finding may be related to nutritional availability in animal hosts.

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Introduction

Heme is essential for the vast majority of life serving as a prosthetic group for many hemoproteins such as catalase, cytochrome, hemoglobin, myoglobin, and peroxidase [1]. Although most aerobic organisms possess a complete biosynthetic pathway for this compound [2], certain organisms are deficient in heme biosynthesis, lacking some or all genes for the hemebiosynthetic pathway. Some anaerobic protists, such as *Giardia intestinalis*, *Trichomonas vaginalis*, *Entamoeba histolytica*, *Cryptosporidium parvum*, *Blastocystis hominis*, and *Encephalitozoon cuniculi* do not possess any heme biosynthetic genes [3]. Members of the family Trypanosomatidae lost some or the entire set of heme biosynthesis genes. They acquire heme or heme precursors from their diet [3,4]. In Trypanosomatidae, members of the genus *Trypanosoma* lack all the heme biosynthesis genes [3,5,6,7], whereas other members such as *Leishmania* spp. possess the genes for the last three steps which were horizontally acquired from a gamma–proteobacterium [3]. Insect trypanosomatid species (*Blastocrithidia culicis* and *Crithidia oncopelti*) cannot synthesize heme by themselves but harbor bacterial endosymbionts that generate and donate heme or heme precursors to the host (trypanosomatid) cells [4,8]. More peculiar is the case of *Phytomonas serpens*, a plant kinetoplastid [9]. This organism lacks most of the known hemoproteins including respiratory cytochromes and does not require heme for viability despite its dependence on oxidative metabolism [9]. The draft genome of *P. serpens* does not appear to contain heme biosynthesis genes other than ferrochelatase (FeCH, EC 4.99.1.1) [9].

Another important and interesting group of organisms that lack the ability to synthesize heme is the nematodes. Nematodes, or roundworms, are typically small, diverse, and highly abundant metazoan organisms [10]. Although free-living species are found

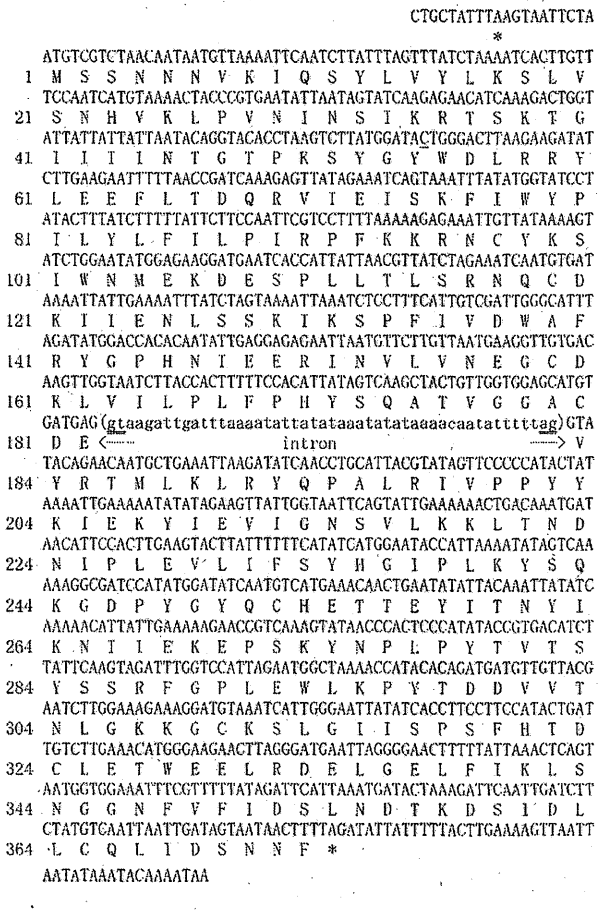

Figure 1. Genomic DNA and cDNA sequences of the *Strongyloides venezuelensis* FeCH gene. Both sequences were identical excluding the intronic region, which existed only in the genomic DNA, and a nucleotide at the 54th codon (single-underlined), which was cytosine in the cDNA sequence but was thymidine in the genomic DNA sequences (silent mutation). The deduced amino acid sequence is shown below the nucleotide sequence. In-frame stop codons are indicated by asterisks. 5′- and 3′-splice junction sites that obey the GT-AG rule of eukaryotic introns are indicated by double lines.
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in nearly all habitats (marine, freshwater, and soil), nematodes are also parasites of vertebrate and invertebrate animals as well as plants. Molecular phylogenetics have defined five major nematode clades (I through V), within which parasitism has arisen multiple times [11]. The genome of *Caenorhabditis elegans*, which was the first metazoan genome to be completely sequenced [12], appears to lack all seven genes necessary to synthesize heme from 5-aminolevulinic acid [13].

Some hemoproteins of animal parasitic nematodes are particularly well studied because of the interests in their roles in low-oxygen environment (host intestine). One such protein is perienteric hemoglobin of *Ascaris lumbricoides* (parasitic nematode of humans), which has an extraordinary high oxygen affinity, approximately 10,000-fold higher than that of the host's globin [14]. The proposed functions of this oxygen-avid hemoglobin include oxygen detoxification by a reaction driven by nitric oxide [15] and maintenance of body wall O_2 tension by creating an inward-decreasing O_2 gradient that is considered important for oxygen unloading from body wall myoglobin, another heme-containing protein [16]. Another example of well-studied nematode hemoproteins is cytochrome *b* in the mitochondrial respiratory complex II of *Ascaris suum* (swine parasitic nematode). *A. suum* larvae utilize classic mammalian-type respiration, expressing a small subunit of larval cytochrome *b* ($CybS^L$) [17]. In contrast, adult worms live in the host small intestine, where oxygen tension is low and utilize an anaerobic NADH-fumarate reductase system expressing a different small subunit of cytochrome *b* ($CybS^A$) instead of $CybS^L$ [17]. Given the important roles played by the hemoproteins in animal parasitic nematodes, it is interesting to know how heme molecules are synthesized or acquired from the animal hosts.

Strongyloides is a genus of obligate gastrointestinal parasites of vertebrates that belong to nematode clade IV [18]. Among more than 50 documented species, two are known to cause human infections, namely *Strongyloides stercoralis* and *Strongyloides fuelleborni* [18]. It is estimated that 30–100 million individuals are infected with *Strongyloides* worldwide primarily in tropic and subtropic regions [19]. Symptoms are usually absent or mild in immunocompetent hosts. However, in impaired host immunity, severe manifestations can develop, and fatalities may ensue [20].

To study strongyloidiasis, *Strongyloides venezuelensis*, which is native to rats but can also infect mice, has been widely used as a model [21]. In a transcriptome sequencing project of this *Strongyloides* species, we identified a partial cDNA sequence that most likely encodes a gene for FeCH [22]. FeCH catalyzes the terminal step of heme biosynthesis [23]. The existence of FeCH sequences was noticed in the genomes of *Brugia malayi* (another animal parasitic nematode belonging to nematode clade III) and its bacterial endosymbiont, (*Wolbachia*). However, further analysis was conducted only on the FeCH gene in the endosymbiont genome [24,25].

In the present study, we cloned the entire cDNA sequence of the FeCH gene from *S. venezuelensis* (*Sv*FeCH). Our BLAST search on publically available databases revealed that only a fraction of nematode species possesses the FeCH gene. Interestingly, all these species were parasites of mammals. Surprisingly, in our phylogenetic analysis, nematode FeCH formed a distinctive clade, and it was placed distantly from the clade that contains non-nematode metazoan FeCH, suggesting that the origin of nematode FeCH genes are different from those of non-nematode metazoan FeCH. The chelatase activity of the *Sv*FeCH was confirmed by an *in vitro* assay using recombinant protein and a gene complementation assay using an FeCH-deficient *Escherichia. coli.*

Nematode genes for heme biosynthesis have not been cloned or characterized to date, essentially because of the nonexistence of these genes in species commonly used in laboratories such as *C. elegans*. Thus, the present study represents the first report of a cloned active FeCH from organisms in the phylum *Nematoda.*

Although the biological significance of carrying only the FeCH gene among other heme biosynthesis genes is unclear, the presence of this gene only in animal parasites suggests a possible role for this gene in nutritional adaptation to the animal host environment.

Materials and Methods

Ethics Statement

S. venezuelensis has been maintained over serial passages in male Wistar rats purchased from Kyudo Co. Ltd. (Kumamoto, Japan). The animals were housed and handled in the Division of Parasitology, Department of Infectious Diseases, University of Miyazaki [26]. All animal studies were conducted under the applicable laws and guidelines for the care and use of laboratory animals in the University of Miyazaki and approved by the Animal

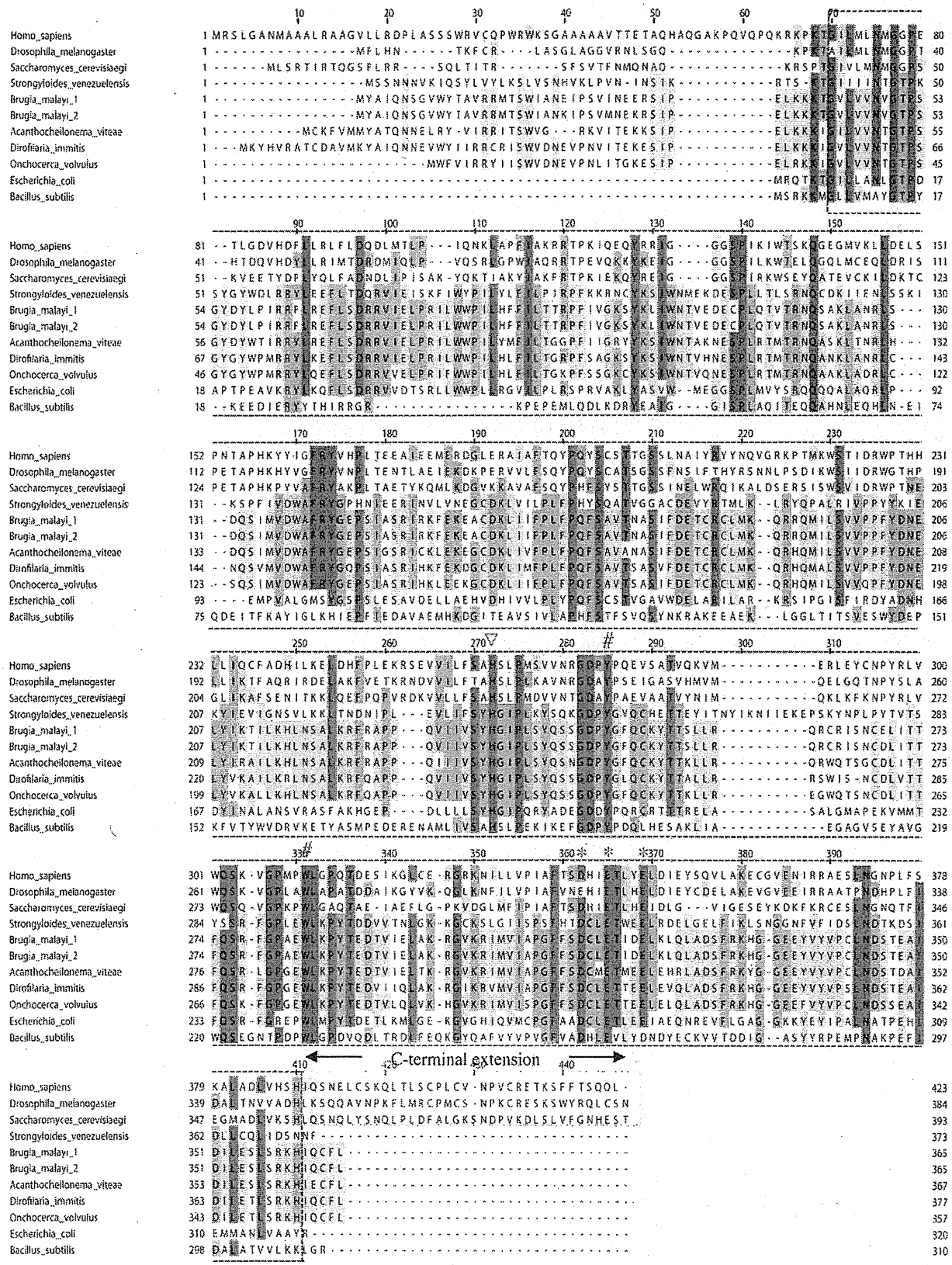
Homo_sapiens
Drosophila_melanogaster
Saccharomyces_cerevisiaegi
Strongyloides_venezuelensis
Brugia_malayi_1
Brugia_malayi_2
Acanthocheilonema_viteae
Dirofilaria_immitis
Onchocerca_volvulus
Escherichia_coli
Bacillus_subtilis
C-terminal extension

Figure 2. Multiple sequence alignment of FeCH sequences. The FeCH sequences were taken from the NCBI protein database together with the *S. venezuelensis* sequence (this study); *Homo sapiens* (CAB65962), *Drosophila melanogaster* (AAC26225), *Saccharomyces cerevisiae* (EDV10759), *Brugia malayi* 1 (ADI33748), *Brugia malayi* 2 (ADI33749), *Acanthocheilonema viteae* (ADI33750), *Dirofilaria immitis* (ADI33752), *Onchocerca volvulus* (ADI33751), *Escherichia coli* (AP_001124), and *Bacillus subtilis* (NP_388894). The sequences were computationally aligned by the ClustalX program [55]. The catalytic core and the C-terminal extension are boxed by red and green dotted lines, respectively. A histidine residue reported to be critical for metal substrate binding (H263, human sequence numbering) is indicated by an inverted triangle. A cluster of three acidic residues are marked with asterisks. Two residues at the active site that were reported to be identical in all known FeCH sequences (Y276 and W310) [56] are indicated by number (#) marks.
doi:10.1371/journal.pone.0058458.g002

Experiment Committee of the University, as specified in the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, Japan, 2006.

5′- and 3′-rapid Amplification of cDNA Ends (RACE)

To determine the sequences of the 3′- and 5′-ends of FeCH cDNA, RACE experiments were performed [27,28]. The priming sites used for these experiments were based on a contig sequence obtained from our *S. venezuelensis* transcriptome sequencing project [22]. For 3′-RACE, a PrimeScript RT-PCR kit (Takara, Japan) was used with oligo(dT) adaptor primers to synthesize cDNA from total RNA prepared from parasitic adult worms. Using this 3′-RACE-ready cDNA as a template, hemi-nested PCR was performed first with primer pairs ENM059/ENM008, followed by ENM060/ENM008. The primer sequences used in this study are summarized in Table S1. The resultant PCR products were cloned into pCR2.1 TOPO (Invitrogen, Carlsbad, CA, USA) for DNA sequencing.

For 5′-RACE, a gene specific-primer (reverse) ENM070 was used to synthesize cDNA from total RNA prepared from adult worms. The addition of a homopolymeric A-tail to the 3′-end of the synthesized first-strand cDNA was performed using dATP and terminal transferase. The dA-tailed cDNA was used as a template for hemi-nested PCR first with primers ENM5_6_7, and ENM008/ENM071, then with primers ENM008/ENM072. The resultant PCR products were cloned into pCR2.1 TOPO for DNA sequencing.

Based on the sequence information obtained from the 5′- and 3′-RACE experiments, a PCR primer pair (ENM073/ENM074) was designed to amplify the entire ORF of the *Sv*FeCH gene. The PCR products obtained using an adult-stage cDNA sample as a template were cloned into pCR2.1 TOPO vectors to determine the sequence. The resultant full-length ORF sequence was deposited into DNA Data Bank of Japan under the accession number AB710465, which can be accessed through GenBank (http://www.ncbi.nlm.nih.gov/genbank/), and used to deduce the amino acid sequence of the *Sv*FeCH.

BLAST Homology Search

To search for heme biosynthesis genes, BLAST homology searches [29] were performed against predicted protein sequence data from published nematode genome projects (*Caenorhabditis briggsae* (nematode clade V) [30], *C. elegans* (V) [12], Pristionchus pacificus (V) [31], Meloidogyne incognita (IV) [31], Meloidogyne hapla (IV) [32], *Bursaphelenchus* xylophilus (IV) [33], B. malayi (III) [25], Ascaris suum (III) [34], and Trichinella spiralis (I) [35]) and nematode expressed sequence tags (ESTs) from NEMBASE4 [36], and *S. venezuelensis* genome (obtained by the Roche-454 pyrosequencing platform [37] with an estimated coverage of more than 20, unpublished), and transcriptome [22] datasets, using human sequences as queries with cutoff value of 1×10^{-4}. For the FeCH gene, the *S. venezuelensis* protein sequence, deduced from the cDNA sequence, was also used as a query to search for potential orthologs against the aforementioned set of nematode genome and EST datasets, as well as the NCBI nonredundant protein database. Similarly, nematode heme biosynthesis gene sequences identified during these database searches were used as queries, instead of the human sequences, to search for potential orthologs in our S. venezuelensis genome and transcriptome datasets.

Phylogenetic Analyses

We retrieved the gene sequences encoding FeCH of 71 bacterial and 65 eukaryotic species from the GENBANK nonredundant protein database (note that some eukaryotes possess more than two FeCH homologs). These amino acid sequences and those of the *S. venezuelensis* homolog were firstly aligned using MAFFT [38], and the resultant alignment was edited manually. After the exclusion of ambiguously aligned positions, the final FeCH alignment containing 71 eukaryotic and 71 bacterial homologs with 177 amino acid positions was subjected to phylogenetic analyses, as described below. Taxonomic affiliation and accession numbers for the sequences considered in our FeCH alignment are listed in Table S2.

Maximum likelihood (ML) phylogenetic analyses were performed using RAxML 7.2.8 [39]. The substitution model used was the LG model incorporating the among-site rate variation approximated with a discrete gamma distribution with four categories (LG+Γ). This particular substitution model was selected as the most appropriate model for the FeCH alignment using Aminosan [40]. The ML tree was selected from heuristic tree search initiated from 20 distinctive parsimonious trees. In ML bootstrap analysis (with 100 replicates), a single tree search was performed per replicate.

Bayesian analysis based on the LG+Γ model was also conducted using MrBayes 3.2.1 [41]. Four parallel Metropolis-coupled Markov chain Monte Carlo runs, each consisting of one cold and seven heated chains with a chain temperature of 0.1, were run for 5,000,000 generations. Log-likelihood scores and trees with branch lengths were sampled every 1000 generations. The first 1,250,000 generations were excluded as burn-in, and the remaining trees were summarized to obtain Bayesian posterior probabilities.

Bacterial Expression of Recombinant *Sv*FeCH and Measurement of Porphyrin-metal Chelatase Activity

A cDNA sequence corresponding to the entire catalytic core region of *Sv*FeCH (amino acid positions 29–373) was obtained by PCR using the primer pair TKT001/TKT002. The PCR product was cloned into pET-21a (+), an *E. coli* expression vector (Merck, Darmstadt, Germany), and the plasmid obtained was transferred to *E. coli* BL21. The bacteria were grown in LB medium for 16 h, and then the culture medium was diluted by 10-fold in fresh LB medium. The enzyme was expressed with 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 30°C for 2 h.

The cells were harvested by centrifugation and suspended in 20 mM Tris-HCl (pH 8.0), 10% glycerol, 1 mM DTT, 0.1%

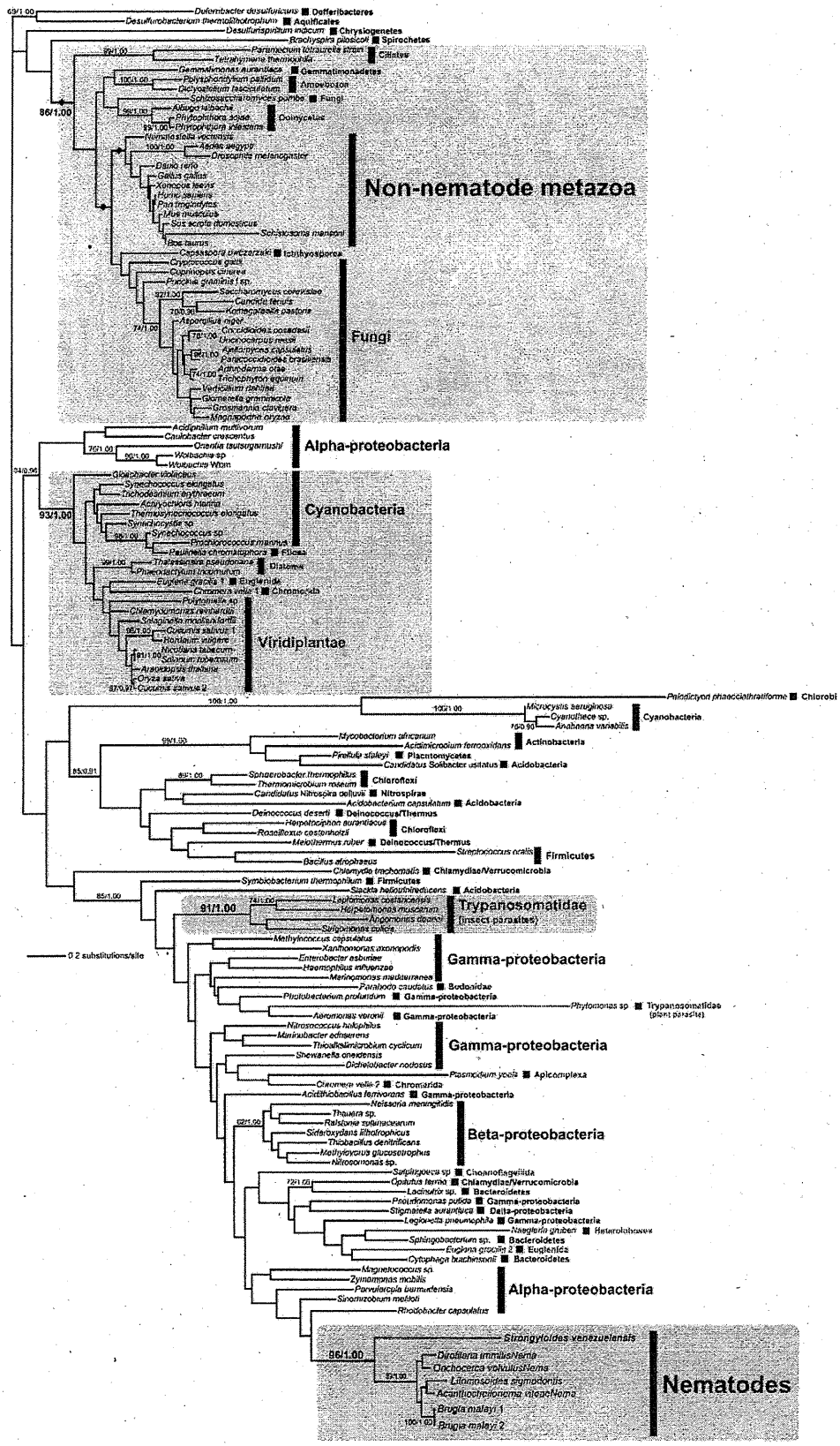
Non-nematode metazoa
Fungi
Alpha-proteobacteria
Cyanobacteria
Viridiplantae
Cyanobacteria
Actinobacteria
Chloroflexi
Firmicutes
Tryparosomatidae
(insect parasites)
Gamma-proteobacteria
Gamma-proteobacteria
Beta-proteobacteria
Alpha-proteobacteria
Nematodes
0.2 substitutions/site

Figure 3. Phylogenetic analysis of FeCH sequences. The ML phylogeny inferred from FeCH amino acid sequences from 71 bacteria and 65 eukaryotes. Numerical values at the nodes represent MLBPs and BPPs. Only MLBPs greater than 60% are shown. The majority of the FeCH homologs sampled from eukaryotes (colored in red) were separated into four clades shaded in blue, green, pink, and orange. The homologs from *Strongyloides venezuelensis* identified in this study formed the 'orange' clade with those of other nematodes. We compared the ML tree and the alternative hypotheses for the origin of the nematode FeCH genes by pruning and regrafting the entire nematode clade (shaded in orange) to the branches marked by diamonds in the 'blue' clade.
doi:10.1371/journal.pone.0058458.g003

Tween 20, and 0.3 M NaCl. Cells were disrupted by sonication and centrifuged at 5000×g at 4°C for 10 min. The supernatants were used for the enzyme assay.

The FeCH activity was determined by measuring the insertion of zinc ions into mesoporphyrin, as described previously [42]. After incubation at 30°C for 30 min, the protoporphyrin or zinc-protoporphyrin formed was measured fluorophotometrically.

Genetic Complementation Assay of hemH (Bacterial FeCH) Deficient *E. coli*

E. coli strain VS200 (ΔhemH), a deletion mutant for hemH gene [43] was provided by the National Bioresource Project of MEXT, Japan.

The entire ORF of *Sv*FeCH, obtained by RT-PCR with the primer pair ENM089/ENM098, was cloned into the *Xho*I/*Bgl*II restriction site of pFLAG-CTC plasmid, an *E. coli* expression vector containing a tac promoter (Sigma-Aldrich, St. Louis, MO, USA). The resultant plasmid pFLAG-CTC-*Sv*FeCH was tested as a gene complementation vector. The original pFLAG-CTC plasmid served as a control.

ΔhemH was transformed with pFLAG-CTC-*Sv*FeCH or with pFLAG-CTC. The transformed and untransformed *E. coli* ΔhemH strains were cultured overnight in LB medium supplemented with hemin (10 μg/ml).

For the culture of the transformed ΔhemH, ampicillin was also added at a concentration of 50 μg/ml. The bacteria from the overnight culture were pelleted by centrifugation and washed thrice with LB medium. After washing, the bacteria pellets were resuspended to give an OD_{600} of 0.1 in hemin-containing (10 μg/ml) or hemin-free LB medium with (for the transformed ΔhemH) or without (for the untransformed ΔhemH) ampicillin, and incubated at 37°C with rocking. O.D. 600 of each culture was measured every hour up to 20 h.

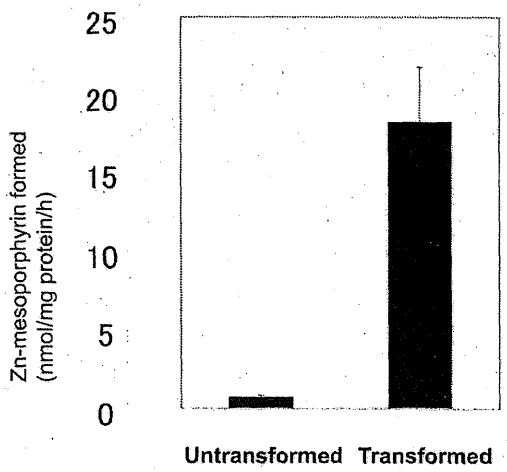

Figure 4. Chelatase assay using bacterially expressed recombinant *Sv*FeCH. The cell extracts were incubated with 20 mM Tris-HCl, pH 8.0, 0.1% Tween 20, 15 μM mesoporphyrin IX, and 40 μM zinc acetate in a final volume of 200 μl at 30°C for 60 min. The formation of zinc mesoporphyrin was measured. Data are expressed as the mean ± SD of triplicate experiments.
doi:10.1371/journal.pone.0058458.g004

Real-time RT-PCR Analysis

Total RNA samples were prepared from eggs, a mixture of first- and second-stage larvae (L1/L2), third-stage infective larvae (L3i), lung third-stage larvae (LL3), mucosal larvae (ML) and parasitic adult stages. Eggs were obtained by the floatation method with saturated salt solution from rat feces. L1/L2 and L3i were prepared from fecal culture. LL3 and ML were collected from infected male ICR mice 72 and 85 h after infection, respectively. Parasitic adults were collected from the small intestine of rats 10 days after infection. Eggs and worms were washed extensively with PBS, pelleted by centrifugation and stored at −80°C until used.

Frozen eggs or worms were crushed with a crushing device (SK-200) purchased from Tokken, Japan. Trizol (Invitrogen) was used for total RNA preparation following the manufacturer's instructions. After DNase I treatment, cDNA was synthesized using PrimeScript RT-PCR kit. Real-time RT-PCR was performed by the GoTaq qPCR system (***Promega***, Madison, WI, USA) using specific primer pairs (ENM056/ENM057 for *Sv*FeCH and 377F/501R for 18S ribosomal RNA genes). The real-time RT-PCR analyses were performed using biological triplicate samples.

Results

Initially, we identified an EST contig that appeared to represent a transcript from *Sv*FeCH gene [22]. The entire cDNA sequence was determined by 3′- and 5′- RACE experiments. This sequence could be mapped to the genomic DNA sequence of this organism obtained from our genome sequencing project, the details of which will be published elsewhere. The genomic and cDNA sequences of the *Sv*FeCH gene are presented in Figure 1. The length of the coding region was 1122 bp including the stop codon. There was one short (49 bp) intron. The deduced amino acid sequence had a length of 373 residues and an expected molecular mass of 43.3 kDa.

In our search for the presence of other heme biosynthesis genes, BLAST homology searches were performed against nematode genome and EST databases, using human heme biosynthesis gene sequences as queries (Tables S3 and S4). Overall, many nematodes appeared to lack all the heme biosynthesis genes, as reported for *C. elegans* [13]. However, some exceptions were also noticed, including the presence of the aminolevulinic acid dehydrogenase (ALAD) gene in several species and the uroporphyrinogen decarboxylase (UROD) gene in *Meloidogyne paranaensis*, the coproporphyrinogen oxidase (CPOX) gene in *Ancylostoma caninum*, and the FeCH gene in *B. malayi* and *Strongyloides ratti*. No heme biosynthesis gene other than FeCH was found in our *S. venezuelensis* genome and transcriptome data using the human sequences as queries. We did not obtain any significant hit from the BLAST analyses for *S. venezuelensis* genome and transcriptome datasets using the ALAD, UROD, and CPOX gene sequences identified in the nematode genome/EST datasets (see above) as queries. When the *Sv*FeCH protein sequence was used as a query for the BLAST analysis, two additional species were found to carry FeCH gene

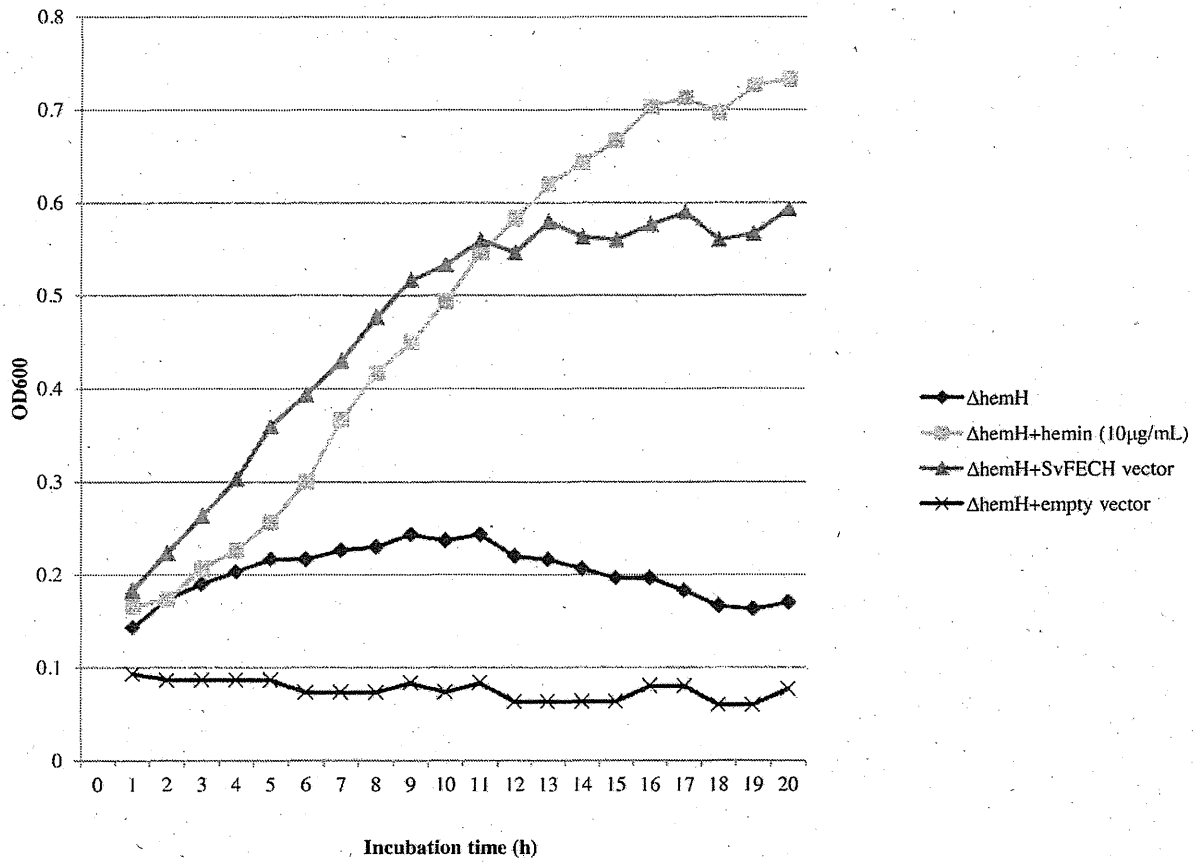

Figure 5. Genetic complementation assay of ΔhemH *E. coli*. An untransformed ΔhemH strain of *E. coli* was grown in the absence (diamond) or presence 10 μg/ml hemin (square). In the same experiment, a transformed ΔhemH strain of *E. coli* either with *Sv*FeCH gene expression vector (triangle) or with empty vector (x-mark) was cultured in the absence of hemin. OD_{600} was measured every hour up to 20 h to monitor bacterial growth.
doi:10.1371/journal.pone.0058458.g005

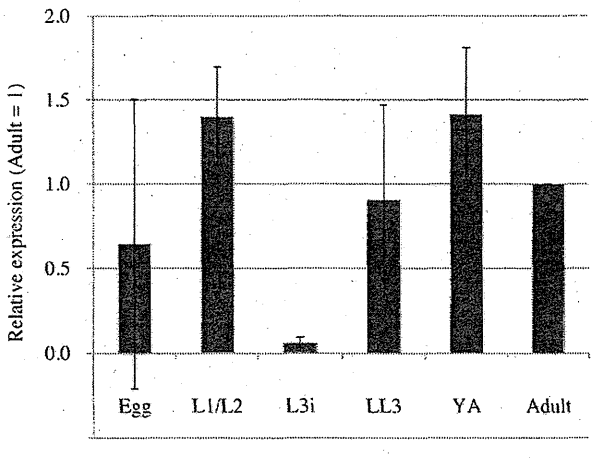

Figure 6. Expression analysis of *Sv*FeCH gene by real-time RT-PCR. mRNA abundance is shown ***relative*** to the expression level at the adult stage, after normalizing to 18S rRNA expression levels. The bars represent the means and standard deviations (±) of biological triplicates. Real-time RT-PCR was performed in triplicate wells for each biological replicate.
doi:10.1371/journal.pone.0058458.g006

(Table S5), namely *Litomosoides sigmodontis* and *Onchocerca volvulus*. The *S. venezuelensis* sequence was also used for BLAST searches against NCBI non-redundant protein database, which led to the identification of two more nematode species that carry FeCH, namely *Dirofilaria immitis* and *Acanthocheilonema viteae*. These results are interesting because all the species found to carry the FeCH gene were animal parasites (filarial nematodes in clade III and *Strongyloides* in clade IV).

A multiple sequence alignment of FeCH protein sequences from selected organisms is presented in Figure 2. Amino acid residues in the catalytic core (boxed by a red dotted line) displayed moderate similarity. Key residues for FeCH activity, such as H263 (human sequence numbering), which was proposed to be involved in metal substrate binding [23,44], were well conserved. Characteristically, nematode (*S. venezuelensis* and *B. malayi*) FeCH lacked a protein region called the "C-terminal extension," a short (approximately 30–50 amino acid residues) stretch of sequences at the C-terminus of the protein that is commonly present in the FeCH of non-nematode opisthokonts [23,45] (boxed by a green dotted line in Figure 2). To measure the similarities of these selected sequences, BLAST scores and amino acid identities were retrieved by the BLASTP program (Table S6). All the nematode FeCH sequences had higher BLAST scores and percent similarity values to the *E. coli* sequence (BLAST score: 202–221; similarity: 33.6%–36.8%)

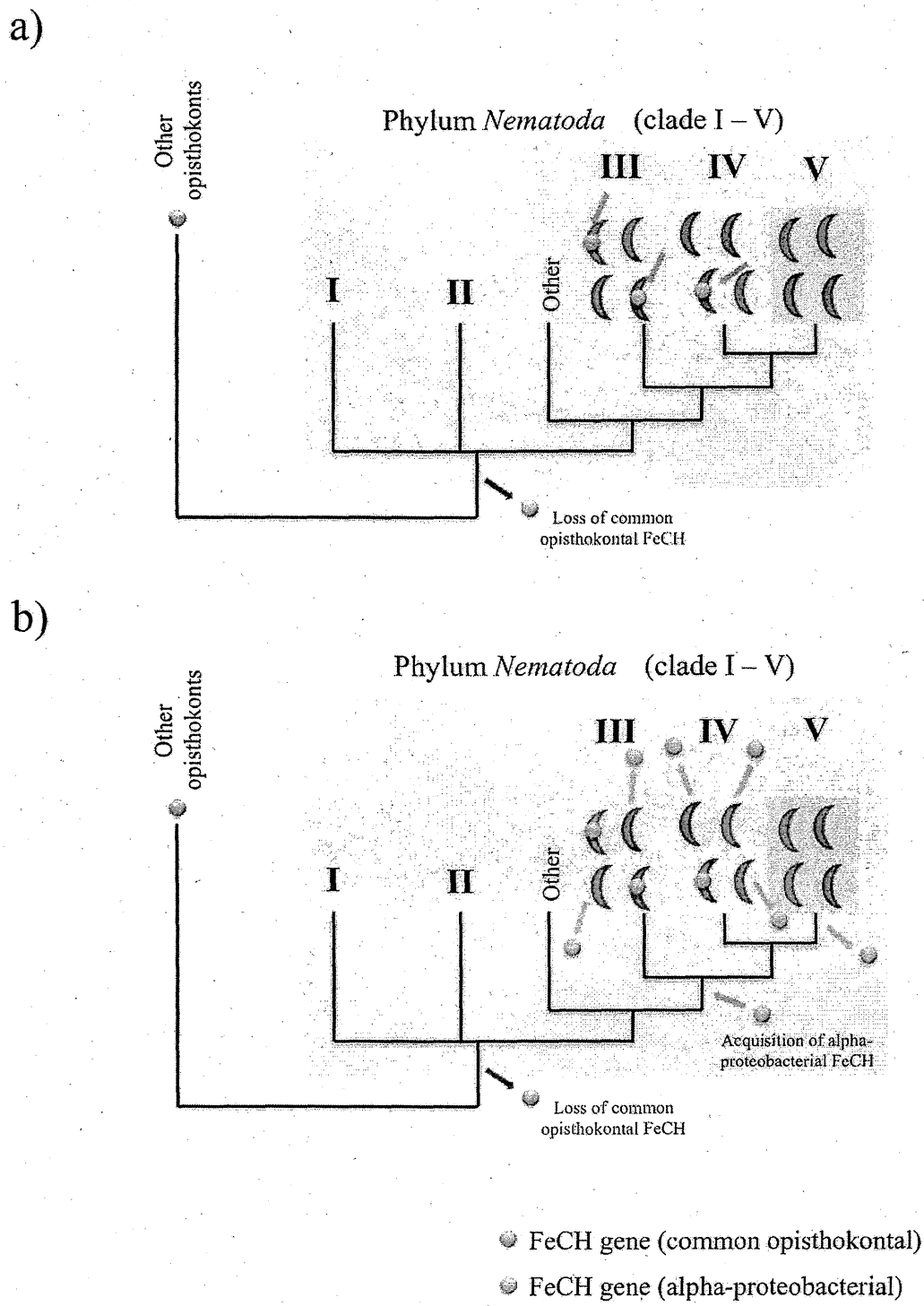

Figure 7. Proposed hypotheses for the loss of the original (common opisthokontal) FeCH gene and the re-acquisition of alpha-proteobacterial FeCH in the evolution of the phylum Nematoda. The initial loss of the common opisthokontal FeCH gene may have occurred at the common ancestor level (red arrows). (a) Scenario 1: The first scenario hypothesizes that alpha-proteobacterial FeCH was acquired independently by some species in clades III and IV (green arrows). b) Scenario 2: Reacquisition of FeCH from an alpha-proteobacterium may have occurred at the common ancestor level of clades III, IV and V (blue arrow) followed by a secondary loss in some species in clade III and IV and in the branch leading to clade V (pink arrows). The phylogenetic relationships of the nematode clades are based on Sommer and Streit [10].
doi:10.1371/journal.pone.0058458.g007

than to human (92–106 and 26.8%–27.5%, respectively), *Drosophila* (93–102 and 24.0%–25.9%, respectively) and *Saccharomyces* sequences (74–96 and 23.0%–27.2%, respectively). When BLAST homology searches were conducted using the *Sv*FeCH protein sequence as a query against the NCBI non-redundant protein as described above, virtually all the top hits were bacterial sequences excluding the sequences of filarial nematodes (data not shown). These findings prompted us to conduct a phylogenetic analysis to better clarify the evolutionary origin of nematode FeCH genes.

Phylogenetic Analysis

The amino acid alignment of FeCH sampled from 71 eukaryotic and 71 bacterial species was phylogenetically analyzed by ML and Bayesian methods (Figure 3). Overall, the FeCH trees inferred by the ML and Bayesian methods were concordant with each other as well as the results of previously published FeCH phylogenies [7,46,47,48]. Four major clades including the FeCH homologues sampled from eukaryotes were reconstructed with ML bootstrap support values (MLBPs) of 86%–96% and a Bayesian posterior probability (BPP) of 1.00 (shaded in blue, green, pink, and orange; Figure 3): (1) a 'blue' clade comprising a single bacterial homolog (*Gemmatimonas aurantiaca*) and those of eukaryotes–non-nematode metazoans, fungi, *Capsaspora owczarzaki*, oomycetes, amoebozoans, and ciliates; (2) a 'green' clade of the homolog of cyanobacteria including an obligate endosymbiont in the testate amoeba, *Paulinella chromatophora* [49], and putative plastid homolog in photosynthetic eukaryotes; (3) a 'pink' clade comprising the homolog of insect trypanosomatids [3]; (4) an 'orange' clade comprising the homolog of parasitic nematodes including *S. venezuelensis*. Other homologs sampled from eukaryotes were scattered amongst the bacterial homologues, and they exhibited no specific evolutionary affinity to other homologs.

The FeCH phylogeny suggested that the homologs from non-nematode metazoans nested in the 'blue' clade and those of nematodes forming the 'orange' clade were distantly related to each other. Although they received little support from the ML bootstrap and Bayesian analyses, the homologs from non-nematode metazoans, *Capsaspora*, and fungi were grouped together, corresponding to members of Opisthokonta, a well-established monophyletic assemblage [50]. Curiously, the nematode FeCH homologs formed a robust clade with an MLBP of 96% and BPP of 1.00, being distinct from other metazoan homologs. This tree topology can be rationalized by the vertical inheritance of FeCH genes from the ancestral opisthokont species to non-nematode metazoans and horizontal transfer of a FeCH gene between the ancestral nematodes and a non-metazoan organism. This conjecture was further supported by a topology test comparing the ML tree shown in Figure 3 with three alternative trees, in which the nematode homologs were enforced to branch at the base of (1) the non-nematode metazoan clade, (2) the clade of the opisthokont homologues (excluding that of *Schizosaccharomyces pombe*), and (3) the 'blue' clade composed of the eukaryotic and *Gemmatimonas* homologs (highlighted by diamonds in Figure 3). Importantly, all the alternative trees were successfully rejected with very small p values (2.0×10^{-78}–2.0×10^{-36}).

Chelatase Assay using Recombinant *Sv*FeCH

To determine whether the FeCH gene of *S. venezuelensis* identified in the present study encodes an active enzyme, we conducted a chelatase assay using a bacterially expressed recombinant *Sv*FeCH. We constructed an expression plasmid, pET-*Sv*FeCH, which was used to transform *E. coli* strain BL21. Protein expression was induced by incubation with 0.3 mM IPTG at 30°C for 2 h. The enzyme activity was measured using the cell extracts of untransformed and transformed bacteria. The FeCH activity in transformed bacteria, which was derived from over-expressed *Sv*FeCH and endogenous *E. Coli* FeCH, was much higher than that in the untransformed control, which originated solely from endogenous FeCH, indicating that the enzyme was active (Figure. 4).

Genetic Complementation Assay of hemH Deficient *E. coli*

The VS200 strain of *E. coli* K12, a hemH null-mutant, was used for the gene complementation assay, and the results are shown in Figure 5. VS200 could not grow in LB medium, unless hemin (10 μg/ml) was supplemented. The expression of *Sv*FeCH by pFLAG-CTC-*Sv*FeCH made the bacteria capable of growing in the LB medium in the absence of hemin. Transforming the bacteria with the control vector (pFLAG-CTC) did not have such an effect. Therefore, it was concluded that *Sv*FeCH is an active enzyme that can function as FeCH.

Expression of FeCH during the Life Cycle of *S. venezuelensis*

The relative expression levels of *Sv*FeCH mRNA were assessed by real-time RT-PCR analysis using RNA samples prepared from the six major developmental stages of *S. venezuelensis* (Figure 6). It was observed that although *Sv*FeCH mRNA expression was present throughout the stages, it was relatively low in L3i.

Discussion

We demonstrated that a gene for FeCH exists in the *S. venezuelensis* genome. Although the presence of the FeCH gene in the draft genome of *B. malayi* was reported previously [24,25], no further characterization was reported. The present study represents the first cloning and characterization of nematode FeCH, particularly in an evolutionary context.

Phylogenetic analyses revealed that nematode FeCH forms a distinct clade from that of non-nematode metazoans, indicating that the evolutionary origin of nematode FeCH is fundamentally different from that of the FeCH genes of other metazoan organisms. In the ML phylogeny, the nematode clade was placed within the homologs from a subset of alpha-proteobacteria, although the statistical support for this hypothesis is inconclusive. If the affinity between the nematode and alpha-proteobacterial FeCH homologs is genuine, then an as-yet-unknown alpha-proteobacterium was the source of the FeCH homologs working in the extant nematodes. This hypothesis is intriguing because replacement of the eukaryotic FeCH gene by a bacterial FeCH gene had been suggested only for unicellular eukaryotes, such as apicomplexan parasites (*Plasmodium falciparum, P. chabaudi, P. berghei, Eimeria tenella, Toxoplasma gondii,* and *Neospora caninum*) [47,48,51], the chromerid *Chromera velia* [47], rhodophytes (*Cyanidioschyzon merolae, Porphyra yezoensis,* and *Galdieria sulphuraria*) [48], and the euglenid *Euglena gracilis* [46].

BLAST analysis of the sequenced nematode genomes and transcriptomes revealed that the FeCH gene is present only in *Strongyloides* (clade IV) and filarial parasites (clade III). It is still not clear at which point of nematode evolution the proposed horizontal gene transfer event occurred. Regarding *B. malayi* and related filarial nematodes, horizontal gene transfer from *Wolbachia*, a bacterial symbiont, is known to have occurred [52]. However, the FeCH sequences present in nematode genomes do not appear to originate from *Wolbachia* based on the positions of the *Wolbachia* species in the phylogenetic tree (Figure 3).

We hypothesize two possible scenarios concerning the evolutionary histories of FeCH genes in nematodes, using a current view of the phylogenetic relationship of nematode clades [10]. Because no nematode species possesses the 'blue clade' FeCH commonly found in opisthokonts, it can be speculated that this type of FeCH was lost early in nematode evolution (Figure. 7). *Strongyloides* and the filarias may have acquired FeCH genes from alpha-proteobacteria independently. Alternatively, a common ancestral lineage leading to clades III, IV, and V may have received such an alpha-proteobacterial FeCH gene (scenarios 1 and 2, respectively: Fig. 7a and 7b). For scenario 1 to be true, the hypothetical alpha-proteobacterial species that provided FeCH genes to *Strongyloides* and filarias, need to be closely related to each other, because the nematode homologs were robustly grouped together in the FeCH phylogeny (Figure. 3). In scenario 2, the lateral transfer of a bacterial FeCH gene occurred through an ancestor leading to species that belong to clades III, IV, and V, and again, the FeCH gene disappeared in some species in clades III and IV such as *Ascaris* and *Meloidogyne* and in the branch leading to clade V (Figure. 7b).

Among the parasitic nematodes, the reason why only *Strongyloides* and filarias needed to reacquire (scenario 1) or retain (scenario 2) FeCH gene is unclear, particularly when the other six heme biosynthesis genes are still absent. This situation (the presence of FeCH gene in the absence of other heme biosynthesis genes) has been documented for a limited number of organisms, such as *Haemophilus influenzae* [53] and *P. serpens* [9]. As was suggested for *H. influenzae* [9,54], there may be a possibility that FeCH is used to obtain Fe^{2+} through its reverse activity rather than obtain heme from protoporphyrin IX using its forward activity.

Supporting Information

Table S1 List of primers used in this study.
(PDF)

Table S2 Taxonomic affiliation and accession numbers for the sequences considered in the phylogenetic analysis.
(PDF)

Table S3 BLAST homology search against predicted proteins from nematode genome projects.
(PDF)

Table S4 BLAST homology search against nematode EST database (NEMBASE 4).
(PDF)

Table S5 BLAST homology search against nematode EST database (NEMBASE 4) using *Strongyloides venezuelensis* ferrochelatase sequence as a query.
(PDF)

Table S6 Sequence similarities of FeCH proteins assessed by BLAST program.
(PDF)

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Author Contributions

Conceived and designed the experiments: EN HM. Performed the experiments: EN ST GC AY. Analyzed the data: SI YI. Wrote the paper: EN SI YI HM.

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Efficacy and safety of paromomycin for treating amebiasis in Japan

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ABSTRACT

The clinical management of amebiasis is a growing concern, particularly among human immunodeficiency virus (HIV)-infected individuals who are predisposed to severe illness. Treatment with a luminal amebicide is strongly recommended following acute-stage treatment with a nitroimidazole. In 2004, the Japanese Research Group on Chemotherapy of Tropical Diseases introduced paromomycin, which was not nationally licensed, and offered it to a number of patients. From 2004 to 2011, 143 case records of amebiasis (123 with amebic colitis, 16 with amebic liver abscess, and 4 with both) in which patients were treated with paromomycin, mainly 1500 mg/day for 9 or 10 days following metronidazole treatment, were submitted. Among 123 evaluable cases, 23 (18.7%) experienced possible adverse effects, the most common being diarrhea (17/123, 13.8%) and other gastrointestinal problems that were resolved after the completion or discontinuation of treatment. In addition, single cases of bloody stools associated with *Clostridium difficile* colitis, skin rash, and the elevation of liver enzymes were also reported, although the causal relationship was not clear. HIV infection did not appear to increase the incidence of adverse drug effects. Each of the 11 asymptomatic or mildly symptomatic amebic colitis cases became negative for stool cysts after paromomycin treatment. Paromomycin was shown to be safe and well tolerated, as well as effective in a special subset of amebic colitis cases.

1. Introduction

Amebiasis is a protozoan infection caused by *Entamoeba histolytica* that consists of two types, intestinal amebiasis (i.e., amebic colitis) and extraintestinal amebiasis, the latter including amebic liver abscess and rare manifestations such as pulmonary, cardiac, or brain involvement [1]. Both types can cause severe complications such as intestinal hemorrhage, ileus, intestinal perforation, or peritonitis in cases of amebic colitis, and rupture to the peritoneal, pleural, or pericardial cavity in cases of amebic liver abscess [2,3]. In developed countries, most amebiasis cases used to occur in travelers returning from developing countries [4,5]; however, recently, domestically infected cases among males who have sex with men (MSM) are increasing [6–8]. This is a concern given that MSM also constitute a risk group for human immunodeficiency virus (HIV) infection and amebiasis may develop into severe disease in HIV-infected individuals [9,10].

Currently, acute-stage treatment with a nitroimidazole (metronidazole or tinidazole) is followed by a luminal amebicide (paromomycin, diloxanide furoate, or iodoquinol), which is strongly recommended to eradicate the possibility of residual protozoan parasites [11,12]. This is because even after nitroimidazole treatment relieves acute symptoms and leads to negative stool tests for *E. histolytica*, a very small number of protozoa may survive in the intestine and can cause a relapse [1]. Luminal agents are poorly absorbed through the intestinal mucosa, thus attaining high intra-luminal drug concentrations. Apart from their use as anti-relapse therapy, luminal agents can be used as a monotherapy for treating asymptomatic (i.e., cyst passer) or mildly symptomatic amebic colitis [13].

The Japanese Research Group on Chemotherapy of Tropical Diseases, of which the authors are the principal members, imports nationally unlicensed medicines for tropical and parasitic diseases, and enables their use in patients in Japan when it is considered necessary and appropriate. This system is indispensable for the appropriate treatment of Japanese patients who contract exotic diseases against which there are only a limited number of nationally licensed medicines [14,15]. Previously, the research group had imported diloxanide furoate as a luminal agent and offered it to a number of patients with amebiasis. However, because diloxanide furoate became unavailable, the research group initiated the importation of paromomycin instead in 2004.

With the increased number of cases treated with this agent, we aimed to clarify the safety profile of paromomycin when used in Japan mainly as an anti-relapse amebicide, and also to evaluate its efficacy in asymptomatic or mildly symptomatic amebic colitis, although the

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cases might be few. These data have been obtained outside of Japan; however, the available Japanese data are very limited. Although this study was not a formal clinical trial, it could contribute to the delineation of how this agent could be utilized in Japan.

2. Patients and methods

2.1. The research group

The research group was established in 1980, and is currently funded by the Japanese Ministry of Health, Labour and Welfare. At present, the research group imports more than 15 nationally unlicensed medicines, among which 6 are antimalarial drugs. Imported drugs are stocked at the central storage facility, the Institute of Medical Science, University of Tokyo, Tokyo, and provided to 25 registered medical facilities upon request. Members of the registered medical facilities have obtained approval for participating in this program from the research ethics committee of each facility based on the approval obtained at the chief investigator's institution (M. Kimura, No. 09002). The drugs are used at those registered facilities after obtaining the patient's informed consent, which clearly states that the drugs are not licensed in Japan. In exceptional cases in which the patients cannot be referred to one of these medical facilities (e.g., due to severity of the disease), drugs are used outside the registered medical facilities on a humanitarian basis. Following treatments, physicians in charge fill in the patient records that were formulated by the research group and send them to us.

2.2. Treatments and analyses

Paromomycin in 250 mg capsules (Humatin®, manufactured by Parke-Davis, Germany) was purchased from the German pharmacy Paesel & Lorei. All of the moderately to severely symptomatic amebiasis cases were first treated with a nitroimidazole at the acute stage, followed by paromomycin for the prevention of relapse. The daily dose and duration of paromomycin administration were primarily determined by the physicians in charge, although in some cases advice was given from one of the authors.

Analysis was conducted using the patient records submitted by the physicians in charge. When necessary, direct contact with the physicians was made in order to gain more detailed information. Adverse effects (AEs) were evaluated by the physicians' descriptions, as well as by our own judgments on the laboratory data shown in the patient records. Cases that could not be followed up after treatment were excluded from the drug safety analysis.

2.3. Efficacy study

Asymptomatic or mildly symptomatic colitis cases were enrolled for the efficacy analysis if they seemed to be due to *E. histolytica*, but not due to *Entamoeba dispar*, which is morphologically undistinguishable from *E. histolytica* in the cyst form, but a non-pathogenic protozoan, and if they were treated with paromomycin, but no other amebicides, at least within 1 month. Specifically, cases were presumed to be amebic colitis if stool microscopy had previously shown typical features of *E. histolytica* trophozoites (e.g., ingestion of red blood cells), a colonoscopy had shown characteristic features with or without histology compatible with the disease, a stool had tested positive with a species-specific PCR, or serum antibodies had been raised [16]. Treatment success was defined mostly as the disappearance of cysts from the stool.

3. Results

3.1. Patients and treatments

From 2004 to 2011, 199 cases were treated with paromomycin, with 145 patient records being submitted and enrolled in this study.

Table 1
Characteristics of patients ($n = 145$).

Age[a]	Median: 45 (IQR: 37–57)		
Sex	Male	124	(85.5%)
	Female	19	(13.1%)
	Unknown	2	(1.4%)
Nationality	Japanese	141	(97.2%)
	Other	3	(2.1%)
	Unknown	1	(0.7%)
Illness	Amebic colitis alone	123	(84.8%)
	Amebic liver abscess alone	16	(11.0%)
	Both amebic colitis and liver abscess	4	(2.8%)
	Giardiasis	2	(1.4%)
HIV status	Reported to be positive	31	(21.4%)
	Not reported to be positive (i.e., negative or unknown)	114	(78.4%)

[a] Age was not reported in 2 cases. HIV, human immunodeficiency virus; IQR, inter-quartile range.

As shown in Table 1, the median age of the patients was 45 years, with 2 of them being under 20 years old (2 and 6 years old). The majority of patients were male, and most were of Japanese nationality. The majority were treated for amebic colitis alone, and a few received treatment for amebic liver abscess alone or for both diseases. Exceptionally, 2 cases were treated for giardiasis. Although the HIV status of the patients was not requested in the patient records, approximately 1 in 5 cases were reported to be HIV positive.

The majority (90/145, 62.1%) of patients were treated with a daily dose of 1500 mg for 8–10 days, 20 cases (20/145, 13.8%) with 1500 mg for 5–7 days, 4 cases (4/145, 2.8%) with 1500 mg for 1–4 days, 5 cases (5/145, 3.4%) with 1500 mg for unknown period, and 3 (3/145, 2.1%) cases with 750 mg for 3 days. In 11 cases, each received individually different combination of the daily dosage and the period (including cases treated for unknown period), and in the remaining 12 cases (12/145, 8.2%), daily dosages were not described. Among them, the 2-year-old child was treated with 360 mg/day for 10 days, and the 6-year-old child received 600 mg/day for an unknown period.

3.2. Safety of paromomycin

A total of 123 cases could be assessed for drug safety, among which 23 cases (23/123, 18.7%) were reported to have AEs (Table 2). Most of the reported AEs were gastrointestinal symptoms, mainly diarrhea (17/123, 13.8%), which were relieved after the completion or discontinuation of paromomycin administration (Table 3). Paromomycin was discontinued due to AEs in 7 cases (5.6%), including the 3 cases described below and another 4 diarrheal cases in which the diarrhea resolved soon after the discontinuation of the drug. No AEs were reported in either the 2- or 6-year-old child.

The first discontinued case involved a 51-year-old HIV-positive male with amebic liver abscess who first received metronidazole, followed by 1500 mg/day of paromomycin. On day 5 of drug administration, he developed diarrhea and bloody stools. His stools tested positive for *Clostridium difficile* toxin A and a colonofiberscopy revealed features of pseudomembranous colitis. However, the patient had received other antibiotics including cefepime, ciprofloxacin, clindamycin, aztreonam, and cefmetazole in 1 month before the onset of the symptoms, and the physician in charge suggested that the illness might be due to those antibiotics. He was lost to follow-up. Second, a 59-year-old male with amebic

Table 2
Reported presence or absence of adverse effects ($n = 123$).

Adverse effects	*n*	%
+	23	18.7
−	100	81.3

Evaluation could not be performed in 22 cases.

Table 3
Reported adverse effects ($n = 123$).

	n	%
Diarrhea	17	13.8
Nausea	2	1.6
Anorexia	1	0.8
Abdominal bloating	1	0.8
Excessive flatulence	1	0.8
Heartburn	1	0.8
Bloody stools (*Clostridium difficile* colitis)	1	0.8
Headache	1	0.8
Drowsiness	1	0.8
Skin rash	1	0.8
Elevation of liver enzymes	1	0.8

Evaluation could not be performed in 22 cases.

colitis first received metronidazole, followed by 1500 mg/day of paromomycin. Before paromomycin treatment, abnormal liver function levels of 62 IU/L γ-GTP and 2.7 mg/dL total bilirubin were noted; on day 5 of paromomycin treatment, AST, ALT, and LDH levels were found to be 88 IU/L, 115 IU/L, and 263 IU/L, respectively. Paromomycin was discontinued and the liver enzymes became normalized after 10 days. Lastly, a 47-year-old male with amebic colitis first received metronidazole, followed by 1500 mg/day of paromomycin. On day 2 of paromomycin treatment, he developed erythema and uriticaria on the trunk and limbs, and was treated with a single injection of glycyrrhizin, followed by oral betamethasone and *d*-chlorpheniramine for 3 days, after which the rash resolved.

Among the evaluable 22 cases with AE, the daily dosages were 1500 mg in 21 cases and 750 mg in one case, while among the evaluable 88 cases without any AE, they were 1500 mg in 80 cases, 750–1250 mg in 4 cases, and 1750–2250 mg in 4 cases. The median daily dosages were not significantly different between individuals with and without AE ($p = 0.56$, Mann–Whitney's *U*-test).

Among the 22 evaluable cases that were reported to be HIV positive, 4 (4/22, 18.2%) patients had AEs. Among the 101 evaluable cases that were not reported to be HIV positive (i.e., the HIV status was negative or unknown), 19 (19/101, 18.8%) had AEs.

3.3. *Efficacy of paromomycin*

A total of 11 asymptomatic or mildly symptomatic cases of amebic colitis were analyzed for the efficacy of paromomycin when given alone (Table 4). Most cases were treated with 1500 mg/day for 9–10 days, and parasite clearance was achieved in all cases. The 6-year-old boy was also found to be negative for cysts in the stool after paromomycin treatment.

Exceptionally, 2 patients with giardiasis received paromomycin, with one of them being evaluable. The patient had previously received metronidazole twice unsuccessfully (500 mg/day for 10 days and 2250 mg/day for 21 days), and was reported to be cured with 750 mg/day of paromomycin given for 5 days.

4. Discussion

Since 2009, the Japanese Ministry of Health, Labour and Welfare has been taking action to dissolve the so-called "drug lag," and data obtained from studies other than formal clinical trials have become useful in making a previously unlicensed medicine licensed in Japan. This move prompted us to analyze the safety (and if possible the efficacy) of paromomycin, in an effort to contribute to licensing the medicine in Japan where the clinical management of amebiasis is increasingly recognized as important. From the 1960s to 1982 and 1998, this agent was licensed in Japan as 2 different brands for treating certain cases of bacterial enterocolitis, respectively. In fact, it was used most frequently for treating tapeworm infestations and most reports supported the safety of paromomycin when used for this purpose [17–19], although this may not guarantee the safety of the drug when used for the treatment of amebiasis for longer periods. Thus, in a Japanese multicenter case series, 118 cases of fish tapeworm received 50 mg/kg paromomycin either as a single dose or divided into 2 doses 30 minutes apart [19]. Four patients reported a total of 5 AEs, i.e., nausea in 2 patients, anorexia in one patient, and transient/slight tinnitus plus speech disturbance appearing in one patient. These patients were given almost concomitantly magnesium sulfate as a laxative to facilitate purging the tapeworm, and therefore drug-induced diarrhea could not be assessed properly in this study.

The current study was not conducted as a formal clinical trial and is, therefore, subject to some limits in interpretation. First, the evaluation of AEs and of the effectiveness of the drug may have not been conducted uniformly by the physicians in charge. Second, post-treatment follow-up periods may have varied between cases; for example, foreign visitors to Japan may have been observed only for a short period of time prior to returning to their original country. However, it is also plausible that the physicians established close relationships with their patients due to the unique nature of this trial, resulting in most of the unusual events, such as delayed AEs, being reported even after the patient record was completed and submitted. Thus, despite these limitations, the data seem to contribute to the efficacy and safety evaluation of paromomycin in Japan.

Studies on the efficacy and safety of paromomycin were reported mostly in the 1960s, with almost all reports showing mild and acceptable symptoms. These older studies may have included *E. dispar* infections, and thus, may not be adequate for analyzing drug efficacy; however, drug safety could be assessed. In an Ethiopian study, 96 patients with amebiasis aged 1–75 years received paromomycin, mostly either 30, 15, or 7.5 mg/kg/day for 5 days. Diarrhea occurred on days 2–3 of treatment, but most cases improved on day 5 [20]. In the US, 79 patients with amebiasis or *Dientamoeba fragilis* received paromomycin, 1,750 mg/day for 2, 3, 4, or 5 days, or 4 doses of 1,000 mg each for one day. Totally, diarrhea

Table 4
Asymptomatic or mildly symptomatic amebic colitis cases treated with paromomycin.

Age	Sex	Evidence of *E. histolytica* infection	Treatment	Findings	
				Before treatment	After treatment
53	M	Trophozoites in stool	1500 mg/day, 10 days	Stool cyst (+)	Stool cyst (−)
49	M	Colonoscopy	1500 mg/day, 10 days	Stool cyst (+)	Stool cyst (−)
42	M	Colonoscopy	1500 mg/day, 10 days	Trophozoites in colonic mucosa	Stool trophozoite (−), cyst (−), improved histology
32	M	Colonoscopy	1500 mg/day, 10 days	Stool cyst (+)	Stool cyst (−)
37	F	Trophozoites in stool	1500 mg/day, 9 days	Stool cyst (+)	Stool cyst (−)
65	M	Colonoscopy	1500 mg/day, 10 days	Stool cyst (+)	Stool cyst (−)
47	M	Colonoscopy, serum antibodies	1500 mg/day, 10 days	Stool cyst (+)	Stool cyst (−)
61	F	Colonoscopy	1500 mg/day, 9 days	Stool cyst (+)	Stool cyst (−)
ND	M	Colonoscopy	1500 mg/day, 9 days	Stool cyst (+)	Stool cyst (−)
47	M	Trophozoites in stool	1500 mg/day, 9 days	Stool cyst (+)	Stool cyst (−)
6	M	Stool PCR	600 mg/day, for unknown period	Stool cyst (+)	Stool cyst (−)

ND, not described.

was noted in 13 cases (16.4%), nausea in 5 cases (6.3%), headache in 3 cases (3.8%), dizziness or abdominal cramps/bloating each in 2 cases (2.5%), and itching, urticaria, heartburn, or insomnia each in 1 case (1.3%), and except for a lower frequency in the one-day treatment arm, frequencies of the AEs did not correlate with duration of the treatments [21]. Another US study on 114 MSM patients with amebic colitis, receiving 25–35 mg/kg daily in 3 divided doses for 7 days, reported a high incidence of soft stools/diarrhea (67%), with most of the cases being mild, as well as nausea in 2 cases and constipation or dizziness each in 1 case [22]. The symptoms were reported to disappear after the completion of paromomycin treatment. A Kenyan study randomly allocated 417 amebic colitis patients aged 6–80 years to several treatment groups, including a paromomycin group in which adults and children received daily doses of 1,000 mg and 30 mg/kg, respectively, in 2 divided doses for 5 days [23]. The overall tolerability was reported to be excellent in the paromomycin group, with poor tolerability found only in 1% of cases.

Our data were largely consistent with previous reports in that most of the AEs consisted of mild gastrointestinal symptoms, and were mainly diarrhea that was resolved after completion or discontinuation of the agent. The case of *C. difficile* colitis might be due to the multiple antibiotics that had been given before paromomycin. The patient with liver function disturbance might have had an underlying liver disorder, and to our knowledge, there are no other reports on drug-induced hepatotoxicity with paromomycin. We found only 1 report on skin eruption, which was urticaria, and thus the dermatological AE does not seem to be unacceptable [21]. It is reassuring that HIV infection did not appear to increase the risk of drug-related AEs, although this issue was not formally addressed in this study.

The excellent efficacy of paromomycin monotherapy observed in our asymptomatic or mildly symptomatic cases deserves mentioning. This is consistent with the study results comparing paromomycin and diloxanide furoate given randomly to patients with PCR-confirmed asymptomatic *E. histolytica* infections in Vietnam (both 500 mg t.i.d. for 10 days) that showed the superiority of paromomycin over diloxanide furoate, with cure rates of 85% and 51%, respectively [24]. Most of our patients received paromomycin for preventing a relapse after successful acute-stage treatment; however, such efficacy of the agent could not be addressed in our study due to lack of the adequate control subjects. In addition, there have been no other reports with conclusive results on the relapse-preventing effect of the drug. A recent study of Japanese HIV-positive men with amebiasis showed that the recurrence rates of amebiasis were not different between those who took a luminal amebicide (paromomycin or diloxanide furoate) and those who did not [25]. However, this may be influenced by reinfections occurring more frequently among MSM. Apart from its use in the treatment of amebiasis, paromomycin has been suggested to be effective in patients with giardiasis or cryptosporidiosis that are refractory to the first-line treatment, as shown in the giardiasis case in this study [26,27].

Just recently, in December 2012, paromomycin was licensed for the treatment of amebiasis in Japan and is expected to be commercially available in mid-2013. As this licensing was decided without a formal clinical trial and based exclusively on the data shown here, we must remain vigilant as to whether a very rare, but potentially serious AE may occur with wider use of the agent. We will also attempt to determine its efficacy in protozoan diseases other than amebiasis in an effort to have it approved for the treatment of such diseases in the near future.

In conclusion, paromomycin was shown to be safe and well tolerated, as well as effective when used in the treatment of asymptomatic or mildly symptomatic amebic colitis in Japan. This agent was licensed just recently, and we will continue to further monitor its efficacy and safety.

Conflict of interest

T.M. is currently affiliated with ViiV Healthcare K.K., which was established by GlaxoSmithKline and Pfizer. Others have no potential conflicts of interest.

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