infection and in experimental models. *S. venezuelensis* has been used as a tool and model for strongyloidiasis research [9,10]. Elimination of *S. venezuelensis* adult worms from mice has been particularly associated with proliferation and activation of intestinal mast cells and eosinophils [11-15].

Several studies have demonstrated > 90% reduction in worm count and fecundity of worms in rats [16] and mice [10,17,18] that were infected and challenged with live-larvae of *S. venezuelensis* or *S. ratti* compared to only primary infected animals. It has been suggested that eosinophils, neutrophils and parasite-reactive antibodies were associated with destruction of *Strongyloides* larvae [19-22].

The role of B-cells in primary and challenge infections of larval *S. stercoralis* in mice had been studied [21] and the authors concluded that B-cells are not required in the primary response, yet they are required in the secondary immune response.

To date, a detailed investigation of the role of B-cells in the protective mechanism against adult *Strongyloides* infection in experimental animals has not been reported in the literature. The use of the immunodeficient animals helps to understand the checkpoints in host immunity. Therefore, the present study was carried to investigate the role of B-lymphocytes in immunity against adult *S. venezuelensis* infection using mice with a targeted deletion of the JH locus. This phenotype results in the absence of B-cells and subsequently antibody production [23].

Methods

Parasites and animals

Male Balb/c mice and Wistar rats were purchased from Kyudo (Kumamoto, Japan). JHD knockout mice on a Balb/c background [23] have been purchased from Taconic (Hudson, NY, US).

S. venezuelensis has been maintained in male Wistar rats in the Division of Parasitology, Department of Infectious Diseases, University of Miyazaki, Japan [24]. Mice were infected by surgical implantation of adult *S. venezuelensis* worms in the small intestine. For adult worm implantation, the upper half of the small intestine of Wistar rats, 8–10 days post-infection was opened longitudinally and washed with phosphate-buffered saline (PBS), followed by incubation in PBS at 37°C for 80 min. Adult worms that emerged from the intestine were washed with sterile PBS and adjusted to the appropriate number. Adult worms suspended in 500 μl of PBS were inoculated into the duodenum of the ether-anesthetized mice (1500/mouse) [25]. All experimental animals were kept and handled under the guidelines of the Animal Experiment Committee, University of Miyazaki, Japan.

Fecal egg count

Feces were collected daily, starting 2 days after surgical implantation of *S. venezuelensis* worms. Individual feces were weighed separately, and suspended in water. Eggs in small portions of each sample were counted under a microscope, and the number of eggs per gram of feces (EPG) was determined for each sample [9].

Recovery of adult worms from the intestine

Worms were recovered at day 5 and 11 from the small intestine of each infected mouse according to the method described before [14]. Briefly, the upper half of the small intestine from each infected mouse was removed after sacrifice, washed, cut open longitudinally, and incubated in PBS at 37°C for 4 h. Worms that emerged from the intestinal tissue were quantified by stereomicroscopy.

Histology

Mucosal mast cells and eosinophils were counted at the time of worm expulsion in wild type mice. For JHD knockout mice, a group of 5 mice were sacrificed for histological examination at day 11 (same day of wild type scarification), and the rest were sacrificed at the end of the observation period (day 107).

For mucosal mast cells, tissues of the small intestines were fixed with Carnoy's fixative, and paraffin-embedded sections were stained with Alcian blue, pH 0.3, and Safranin-O, pH 0.1 [26]. The number of intra-epithelial mast cells were counted in 50 villus-crypt units (VCU) and expressed as mast cell numbers per 10 VCU.

For eosinophils, tissues were fixed in acetone, and paraffin-embedded sections were stained with hematoxylin followed by 1% water soluble Biebrich Scarlet (Sigma) for 5 min [27]. The number of eosinophils in the small intestine was counted in 50 VCU and expressed as eosinophil number per 10 VCU.

Statistical analysis

Experiments consisted of five mice per group and all experiments described were performed at least twice. SPSS software was used for data analysis. Descriptive statistics including the mean ± standard deviation (SD), and median values were used. A non-parametric Mann–Whitney test was used to test for significant differences between groups. The data were considered significant if P values were less than 0.05.

Results

In this study, we surgically implanted adult worms to examine the mucosal protection against adult *S. venezuelensis*. After the same number of adult worms (1500/mouse) was implanted in the small intestine, both groups of mice started to lay eggs from the 2nd day. The fecal egg count

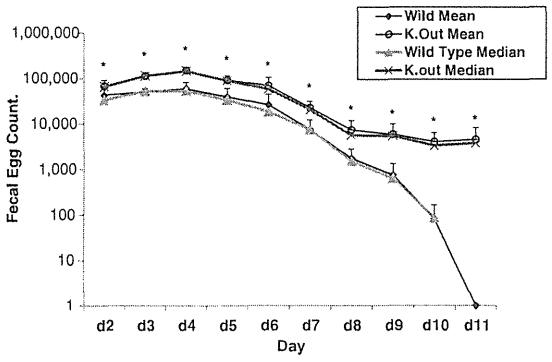

Figure 1 Fecal egg count after surgical implantation of *S. venezuelensis* adult worms in wild-type and JHD knockout mice. Mice were infected with 1500 adult worms, and EPG were counted from 2nd day after implantation. All values are mean ± SD, and median values were also included. *$P < 0.05$.

was significantly higher in JHD knockout mice compared to wild-type mice. Moreover, parasites from wild-type mice stopped laying eggs by day 11 after implantation, while parasites from JHD knockout mice continued to lay eggs till the end of the observation period, day 107 (Figure 1).

The kinetics of *S. venezuelensis* infection in mice revealed that there was a statistical difference in the numbers of worms recovered from the small intestines of knockout mice at day 5 after surgical implantation compared to wild type mice (Table 1). Worm fecundity was comparable in both groups of mice (Table 1). Day 11 post infection no worms were recovered from wild type mice.

At day 11, all wild type mice and one group of JHD knockout mice were sacrificed for histological examination. The number of mucosal mast cells (MMC) and eosinophils were comparable in both types of mice (Table 2).

By the end of the observation period (day 107), MMC and eosinophils in JHD knockout mice were still present,

Table 1 The number of worm recovery, fecal egg count and fecundity 5 days after surgical implantation of *S. venezuelensis* adult worms in wild-type and JHD knockout mice

	Wild type mice	JHD knockout mice
Worms recovered	325 ± 45	749 ± 50*
	(317)	(747)
FEC	39383 ± 20397	91896 ± 23824*
	(33645)	(88398)
Eggs/worm/gm feces	117 ± 47	121 ± 24
	(106)	(118)

All values are mean ± SD.
Numbers given between parentheses indicate the median value.
*$P < 0.05$.
FEC fecal egg count.

Table 2 The number of mucosal mast cells and eosinophils 11 days after surgical implantation of *S. venezuelensis* adult worms in wild-type and JHD knockout mice

Cell count	Wild-type mice	JHD knockout mice
MMC/10 VCU	142 ± 21	123 ± 15
	(151)	(120)
Eosinophils/10 VCU	38 ± 4	34 ± 3
	(38)	(33)

All values are mean ± SD.
Numbers given between parentheses indicate the median value.
MMC mucosal mast cells.
VCU villus-crypt units.

with insignificantly lower counts compared to day 11 counts of the same group (Table 3).

Discussion

Despite the high prevalence and chronic morbidity produced by nematodes, immunoprotective mechanisms involved in the response against these parasites are not completely understood. In this study, we utilized mice with well-characterized mutations that disabled humoral immunity, in order to determine its role in host protection against adult *S. venezuelensis*.

In the absence of functional B-cells, JHD mice excreted significantly higher FEC compared to wild-type mice up to day 11 after infection. Parasites from wild-type mice stopped egg laying by day 11, while parasites from JHD mice continued to excrete eggs till the end of the observation period, day 107. Although FEC is the only parasitological parameter of immunity that can be obtained sequentially and regularly in the same animal over the course of an infection, it does not strictly reflect the fecundity of the female worm population [28] as many other factors may affect the FEC. Determining the number of eggs in utero is a better index of decreased fecundity. Worm fecundity is determined by dividing the total eggs by the total number of adult worms recovered from the small intestine. Since *S. venezuelensis*-infected

Table 3 The number of mucosal mast cells and eosinophils in JHD knockout mice 11 and 107 days after surgical implantation of *S. venezuelensis* adult worms

Cell count	JHD knockout mice	JHD knockout mice
	Day 11	Day 107
MMC/10 VCU	123 ± 15	0.7 ±0 .4*
	(120)	(0.7)
Eosinophils/10 VCU	34 ± 3	0.5 ± 0.4*
	(33)	(0.6)

All values are mean ± SD.
Numbers given between parentheses indicate the median value.
*Significant difference vs. day 11 ($P < 0.05$).
MMC mucosal mast cells.
VCU villus-crypt units.

hosts have only female worms in the small intestine, worm fecundity can be estimated by dividing the number of eggs eliminated in feces by the number of worms recovered from the intestine of each mouse. At day 5 post infection, the kinetics of *S. venezuelensis* revealed that there was statistical difference in the numbers of worms that get established in the small intestines of knockout mice compared to wild type mice. At day 11 post infection, no worms were recovered from wild type mice indicating that all worms established had been expelled. From these data, it is clear that the higher number of parasite eggs excreted in the feces of JHD knockout infected mice was a consequence of higher worm burden in the small intestine of these animals. On the other hand worm fecundity was comparable in both groups of mice.

It is well known that in *S. venezuelensis* infection, mastocytosis and eosinophilia are associated with worm expulsion [11,12,14,15]. Worm expulsion was impaired in mice deficient in the IL-3 gene [11,29]. In these mice, MMC were completely absent and *S. venezuelensis* continued to parasitize the intestine for more than 50 days.

In the current study, both B-cell-deficient mice and wild-type mice, showed an influx of MMC and eosinophils. The absolute numbers in JHD knockout mice were lower than those seen in wild-type mice at day 11, but not to the level of significance.

JHD knockout mice could not recover from infection despite recruitment of both types of cells and their persistence until the end of the observation period. Therefore, it is clear that mucosal mastocytosis and eosinophilia are not solely responsible for worm expulsion and other effector mechanisms must be involved in the expulsion process. It is possible that the defect in worm expulsion following surgical implantation of adult *S. venezuelensis* results from the failure of mast cells and eosinophils to degranulate and release their effector mediators. We previously suggested a role of secretory IgA in conjugation with eosinophils in immunity against adult worm invasion and expulsion [14]. Furthermore, the role of IgE in mast cell degranulation remains equivocal; some authors claim IgE plays a role [30], while others deny any role for IgE [31]

To the best of our knowledge, we are the first to report a role for B cells in mucosal immunity against primary invasion of adult *S. venezuelensis* and in its expulsion. It appears that B cells play a critical role in the elimination of adult *S. venezuelensis* by antibodies or other mechanisms that remain to be fully investigated.

Conclusion

Our findings highlight a role of B cells in mucosal immunity against invasion of adult S. venezuelensis and in its expulsion. Therefore, we conclude that B-cells together with mucosal mast cells and eosinophils, contribute to immunity against adult S. venezuelensis by mechanism(s) to be investigated.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ME, HM, SA and NO designed the experiment. ME, SA and SE performed lab work and drafted the manuscript. All authors read and approved the final version of the manuscript.

Acknowledgement

Mohamed EL-Malky was awarded a grant by the Short-term Invitation Program of Japan Student Services Organization (JASSO).

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Received: 14 February 2013 Accepted: 22 May 2013
Published: 24 May 2013

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doi:10.1186/1756-3305-6-148
Cite this article as: EL-Malky *et al.*: The role of B-cells in immunity against adult *Strongyloides venezuelensis*. *Parasites & Vectors* 2013 **6**:148.

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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.

Citation: Nagayasu E, Ishikawa SA, Taketani S, Chakraborty G, Yoshida A, et al. (2013) Identification of a Bacteria-Like Ferrochelatase in *Strongyloides venezuelensis*, an Animal Parasitic Nematode. PLoS ONE 8(3): e58458. doi:10.1371/journal.pone.0058458

Editor: Stuart Alexander Ralph, University of Melbourne, Australia

Received August 1, 2012; **Accepted** February 5, 2013; **Published** March 13, 2013

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Funding: This study was supported by Grant-in-Aid for Young Scientists (B) (23790461) from Japan Society for the Promotion of Science (JSPS), Grant-in-Aid for Scientific Research C (21590466), Grant-in-aid for Scientific Research on Priority Areas 'Matrix of Infection Phenomena' (21022041), Health Labor Sciences Research Grant (H23-Shinko-Ippan-014, H21-kokui-shitei-004), and Grant-in-Aid for Scientific Research on Innovative Areas "Genome Science" from MEXT. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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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 [?], 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

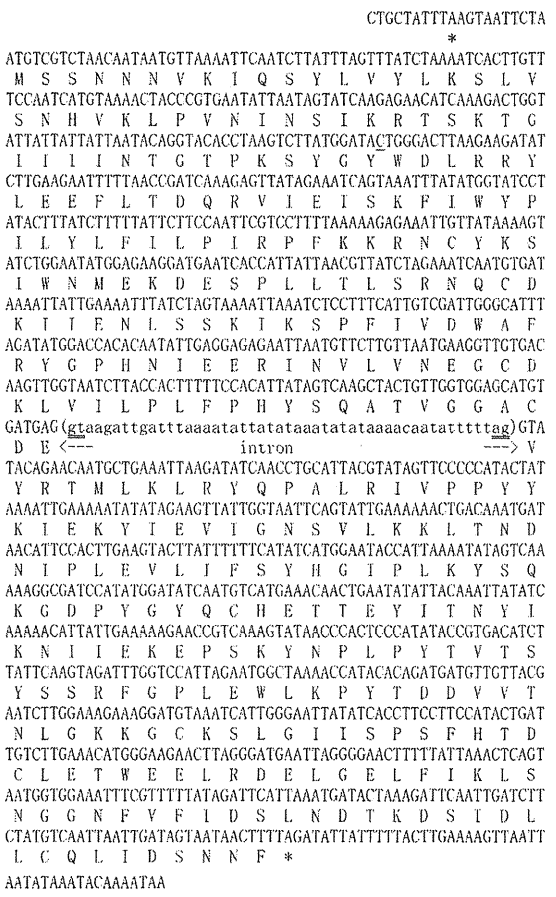


Figure 1. Genomic DNA and cDNA sequences of the *Strongyloides venezuelensis* FeCH gene. Both sequences were identical excluding the intronic region, which existed only in the genomic DNA, and a nucleotide at the 54th codon (single-underlined), which was cytosine in the cDNA sequence but was thymidine 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.
doi:10.1371/journal.pone.0058458.g001

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*.

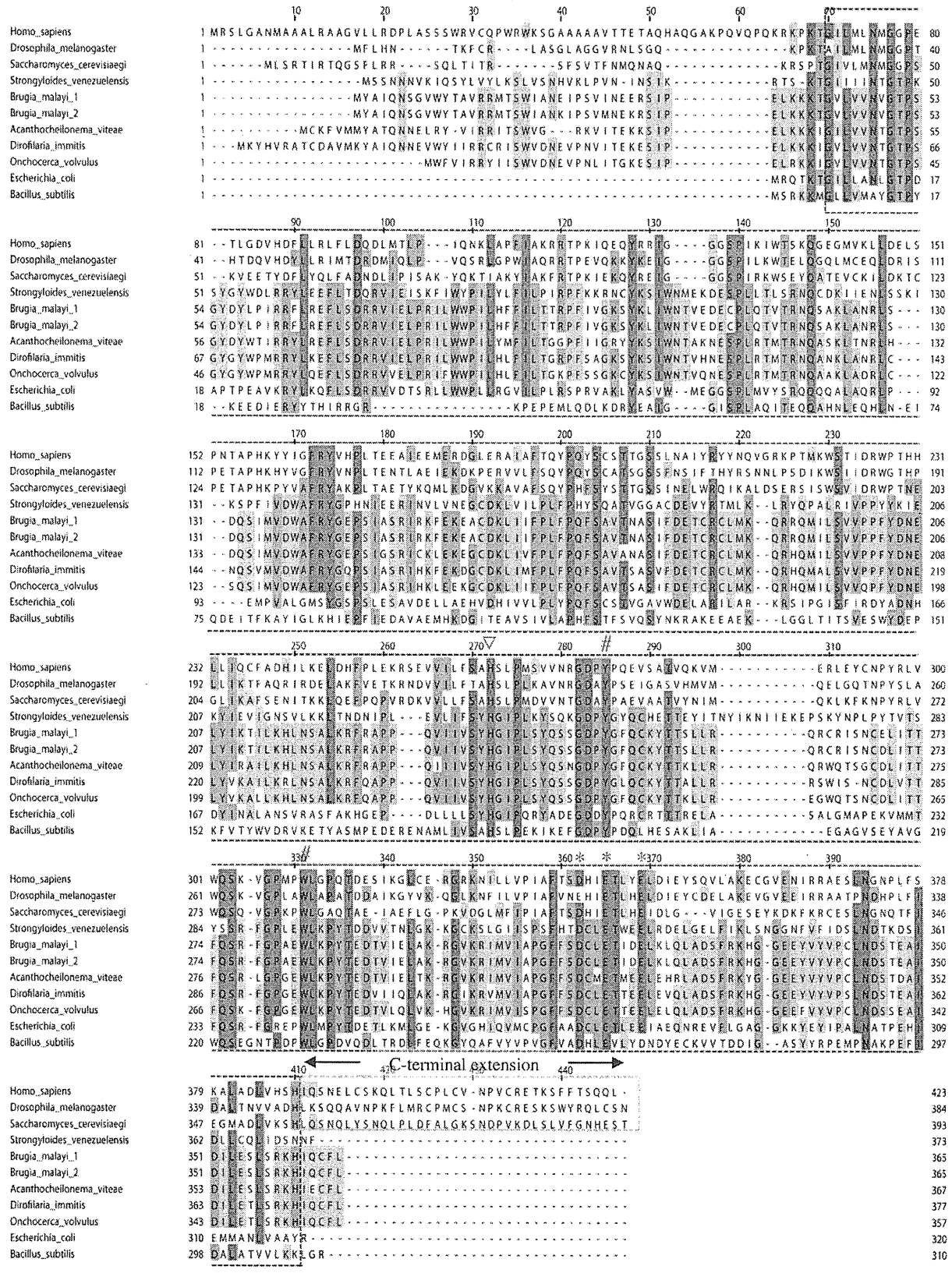
Nematode genes for heme biosynthesis have not been cloned or characterized to date, essentially because of the nonexistence of these genes in species commonly used in laboratories such as *C. elegans*. Thus, the present study represents the first report of a cloned active FeCH from organisms in the phylum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

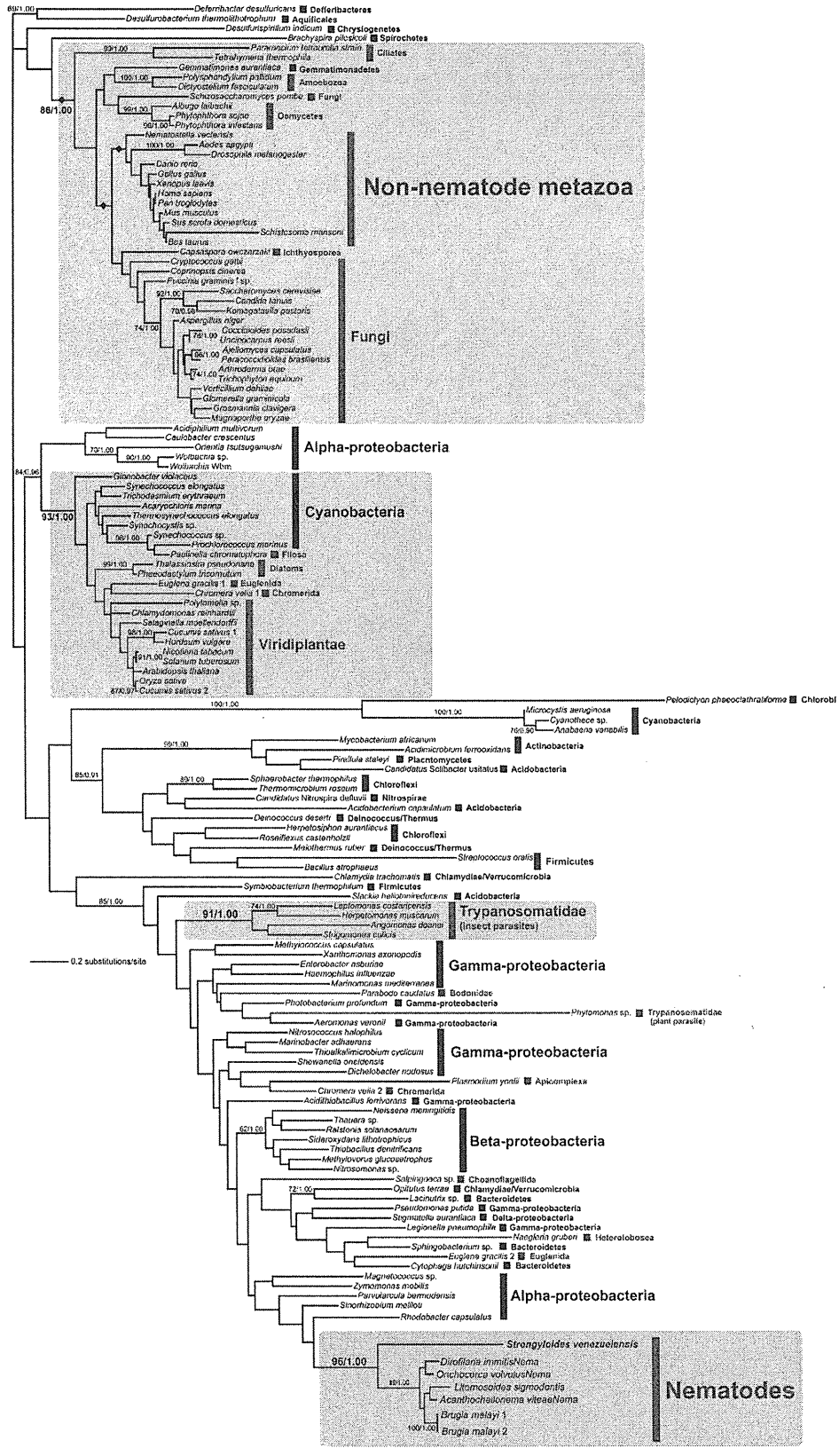
Deferribacter desulfuricans Deferribacteres
Desulfurobacterium thermolithotrophum Aquificales
Desulfurispirillum indicum Chrysiogenetes
Brachyspira pilosicoli Spirochetes
Paramecium tetraurelia strain Ciliates
Tetrahymena thermophila
Gemmatimonas aurantiaca Gemmatimonadetes
Polysphondylium pallidum Amoebozoa
Dictyostelium fasciculatum
Schizosaccharomyces pombe Fungi
Albugo laibachii Oomycetes
Phytophthora sojae
Phytophthora infestans
Nematostella vectensis Non-nematode metazoa
Aedes aegypti
Drosophila melanogaster
Danio rerio
Gallus gallus
Xenopus laevis
Homa sapiens
Pan troglodytes
Mus musculus
Sus scrofa domesticus
Schistosoma mansoni
Bos taurus
Capsaspora owczarzaki Ichthyosporea
Cryptococcus gattii Fungi
Coprinopsis cinerea
Puccinia graminis f sp.
Saccharomyces cerevisiae
Candida tanus
Komagataella pastoris
Aspergillus oryzae
Coccidioides posadasii
Uncinocarpus reesii
Ajellomyces capsulatus
Paracoccidioides brasiliensis
Arthroderma spp.
Trichophyton equinum
Verticillium dahliae
Glomerella graminicola
Grosmannia clavigera
Magnaporthe oryzae
Acidiphilium multivorum Alpha-proteobacteria
Caulobacter crescentus
Orientia tsutsugamushi
Wolbachia sp.
Wolbachia Wbm
Gloeobacter violaceus Cyanobacteria
Synechococcus elongatus
Trichodesmium erythraeum
Acaryochloris marina
Thermosynechococcus elongatus
Synechocystis sp.
Synechococcus sp.
Prochlorococcus marinus
Pavlovella chromatophora Plasmodiophora
Thalassiosira pseudonana Diatoms
Phaeodactylum tricornutum
Euglena gracilis 1 Euglenida
Chromera velia 1 Chromerida
Polytomella sp. Viridiplantae
Chlamydomonas reinhardtii
Selaginella moellendorffii
Cucumis sativus 1
Hordeum vulgare
Nicotiana tabacum
Solanum tuberosum
Arabidopsis thaliana
Oryza sativa
Cucumis sativus 2
Pelodictyon phaeoclathratiforme Chlorobi
Microcystis aeruginosa Cyanobacteria
Cyanothece sp.
Anabaena variabilis
Mycobacterium africanum Actinobacteria
Acidimicrobium ferrooxidans
Pirellula staleyi Placntomycetes
Candidatus Solibacter usitatus Acidobacteria
Sphaerobacter thermophilus Chloroflexi
Thermomicrobium roseum
Candidatus Nitrospira defluvii Nitrospirae
Acidobacterium capsulatum Acidobacteria
Deinococcus deserti Deinococcus/Thermus
Herpetosiphon aurantiacus Chloroflexi
Roseiflexus castenholzii
Meiothermus ruber Deinococcus/Thermus
Streptococcus oralis Firmicutes
Bacillus atrophaeus
Chlamydia trachomatis Chlamydiae/Verrucomicrobia
Symbiobacterium thermophilum Firmicutes
Slackia heliotrinireducens Acidobacteria
Leptomonas costaricensis Trypanosomatidae (insect parasites)
Herpetomonas muscarum
Angomonas deanei
Strigomonas culicis
Methylococcus capsulatus Gamma-proteobacteria
Xanthomonas axonopodis
Enterobacter asburiae
Haemophilus influenzae
Marinomonas mediterranea
Parabodo caudatus Bodonidae
Photobacterium profundum Gamma-proteobacteria
Phytomonas sp. Trypanosomatidae (plant parasite)
Aeromonas veronii Gamma-proteobacteria
Nitrosococcus halophilus
Marinobacter adhaerens
Thioalkalimicrobium cyclicum
Shewanella oneidensis
Dichelobacter nodosus
Plasmodium yoelii Apicomplexa
Chromera velia 2 Chromerida
Acidithiobacillus ferrivorans Gamma-proteobacteria
Neisseria meningitidis
Thauera sp.
Ralstonia solanacearum Beta-proteobacteria
Sideroxydans lithotrophicus
Thiobacillus denitrificans
Methylovorus glucosetrophus
Nitrosomonas sp.
Salpingoeca sp. Choanoflagellida
Opitutus terrae Chlamydiae/Verrucomicrobia
Lacinutrix sp. Bacteroidetes
Pseudomonas putida Gamma-proteobacteria
Stigmatella aurantiaca Delta-proteobacteria
Legionella pneumophila Gamma-proteobacteria
Naegleria gruberi Heterolobosea
Sphingobacterium sp. Bacteroidetes
Euglena gracilis 2 Euglenida
Cytophaga hutchinsonii Bacteroidetes
Magnetococcus sp.
Zymomonas mobilis
Parvularcula bermudensis Alpha-proteobacteria
Sinorhizobium meliloti
Rhodobacter capsulatus
Strongyloides venezuelensis
Dirofilaria immitisNema
Onchocerca volvulusNema
Litomosoides sigmodontis
Acanthocheilonema viteaeNema
Brugia malayi 1
Brugia malayi 2 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.

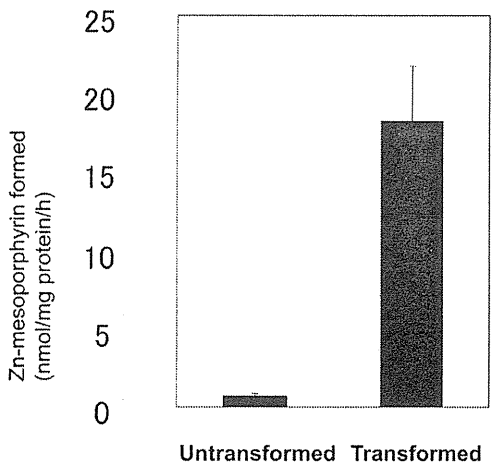

Figure 4. Chelatase assay using bacterially expressed recombinant *Sv*FeCH. The cell extracts were incubated with 20 mM Tris-HCl, pH 8.0, 0.1% Tween 20, 15 μM mesoporphyrin IX, and 40 μM zinc acetate in a final volume of 200 μl at 30°C for 60 min. The formation of zinc mesoporphyrin was measured. Data 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

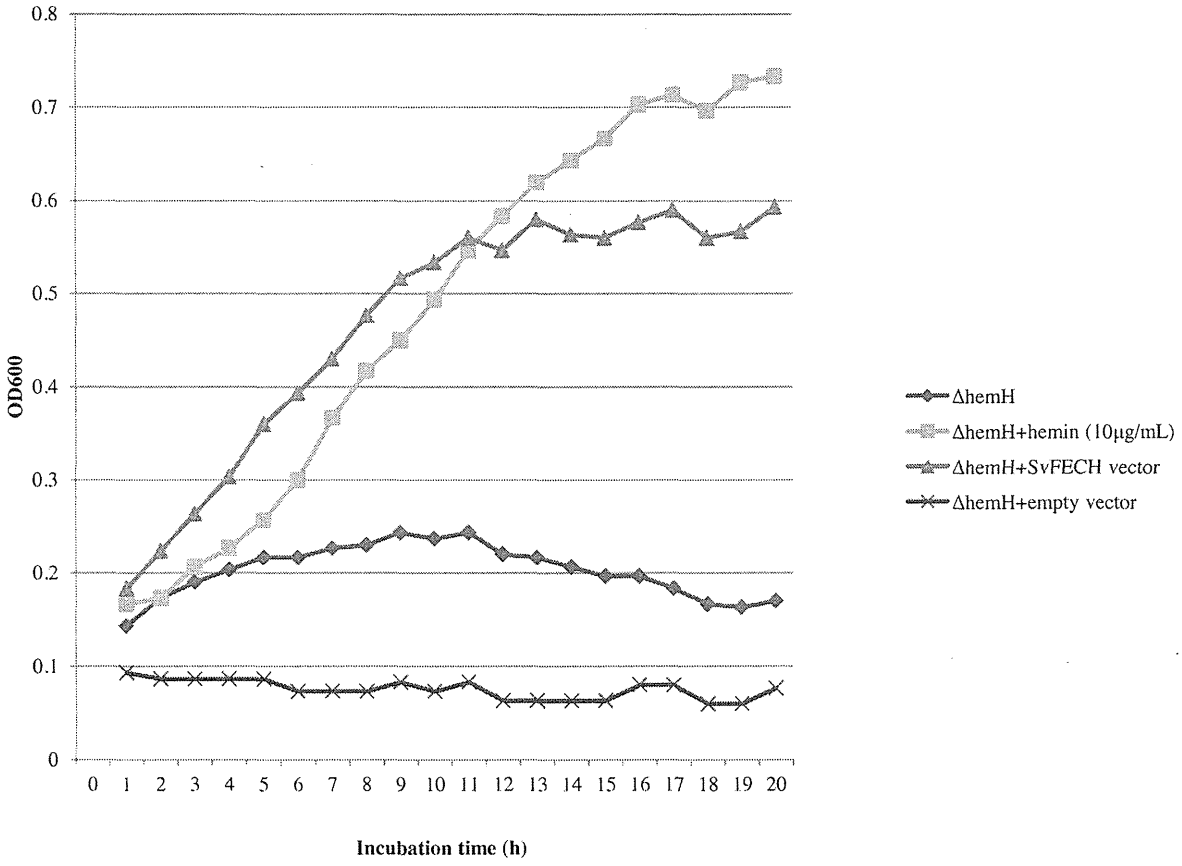

Figure 5. Genetic complementation assay of ΔhemH *E. coli*. An untransformed ΔhemH strain of *E. coli* was grown in the absence (diamond) or presence 10 μg/ml hemin (square). In the same experiment, a transformed ΔhemH strain of *E. coli* either with *Sv*FeCH gene expression vector (triangle) or with 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

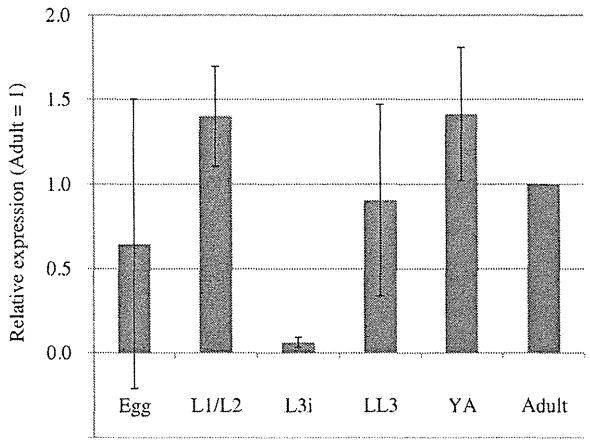

Figure 6. Expression analysis of *Sv*FeCH gene by real-time RT-PCR. mRNA abundance is shown ***relative*** to the expression level at the adult stage, after normalizing to 18S rRNA expression levels. The bars represent the means and standard deviations (±) of biological triplicates. Real-time RT-PCR 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%)

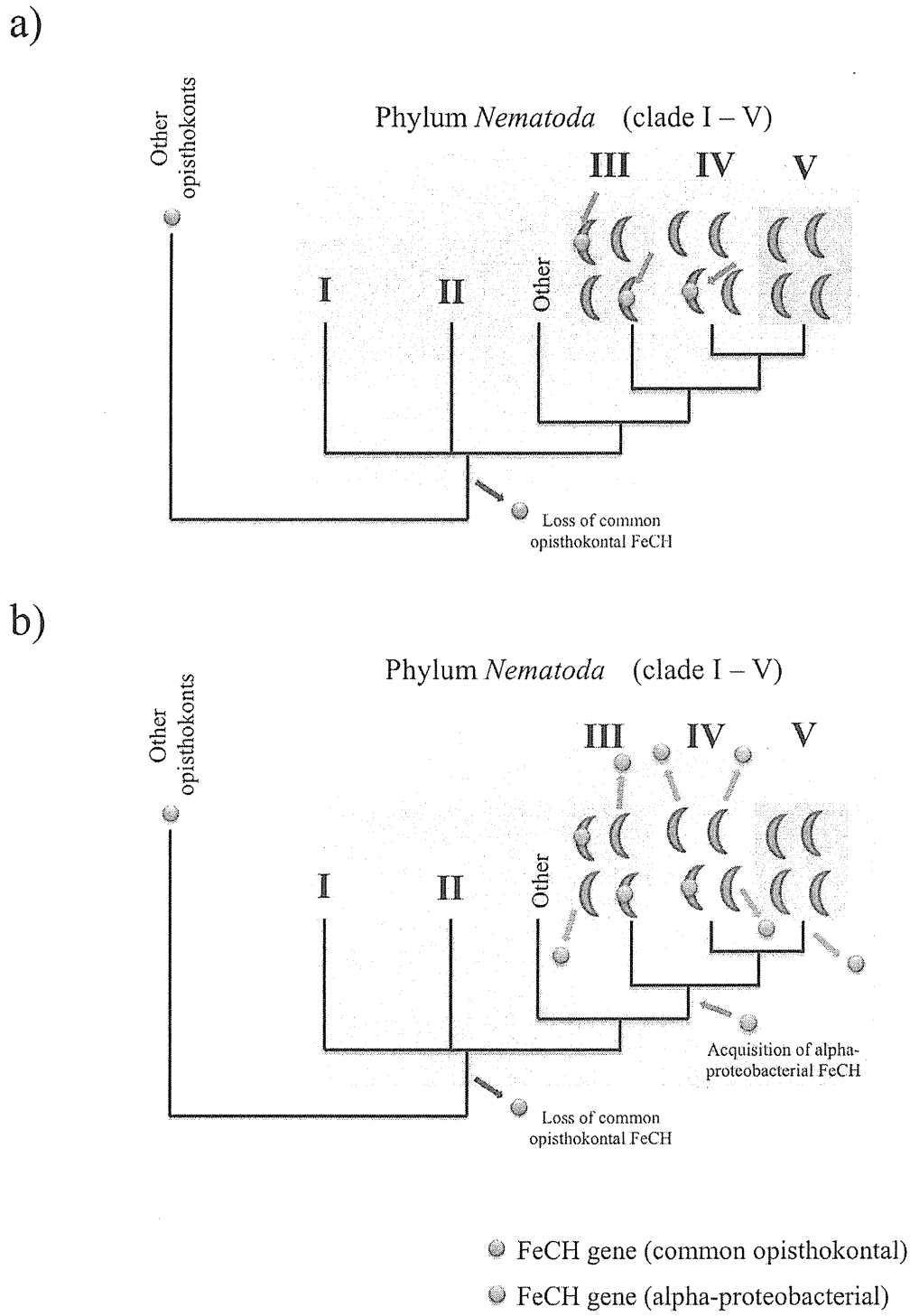

Figure 7. Proposed hypotheses for the loss of the original (common opisthokontal) FeCH gene and the re-acquisition of alpha-proteobacterial FeCH in the evolution of the phylum Nematoda. The initial loss of the common opisthokontal FeCH gene may have occurred at the common ancestor level (red arrows). (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)

Acknowledgments

SI is a research fellow supported by the Japan Society for Promotion of Sciences for Young Scientists (No. 00007). We thank Dr. Tadasuke Ooka (University of Miyazaki, Japan) for helpful discussions and Amy Hombu (University of Miyazaki) for critical reading of the manuscript.

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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動物由来回虫症に対するアルベンダゾールの有効性の検討

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Key Words: 動物由来回虫症，アルベンダゾール，血清診断，好酸球

はじめに

動物由来回虫症は，ヒト以外の動物が本来の宿主である回虫類による寄生虫感染症で，主な原因寄生虫は，イヌ回虫 *Toxocara canis*・ネコ回虫 *Toxocara cati*・ブタ回虫 *Ascaris suum* と考えられている[1,2]．好酸球性肺炎として発症する場合が多いが，ぶどう膜炎や脊髄炎が前面に出ることもある．

動物由来回虫症の薬物治療にはアルベンダゾールの内服が推奨されているが[3]，その有効性や副作用に関するまとまった研究はない．そこで今回，動物由来回虫症に対するアルベンダゾールの有効性等について，当研究室が診断に関与した症例を用いて解析した．

対象と方法

宮崎大学医学部寄生虫学分野で 2004 年から2012年に動物由来回虫症と診断された症例643例のうち，治癒効果判定のため抗体フォローアップの依頼があった症例 263 例を対象とした（表 1）．患者の性別，年齢，居住地，診断に至るまでの病歴や受診時の臨床所見，投与薬剤の種類と用量，治療後の経過等は，血清診断申込書に記載された情報を用いた．治療効果は，臨床症状，胸部異常陰影等の画像を含む検査所見，血清抗体濃度の推移から総合的に判断し，有効（軽快ないし治癒）と無効に分けた．

結果

動物由来回虫症のフォローアップ症例におけ

Assessment on the effectiveness of albendazole on the treatment of larva migrans syndrome caused by ascarid nematodes

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表 1　動物由来回虫症の発生動向

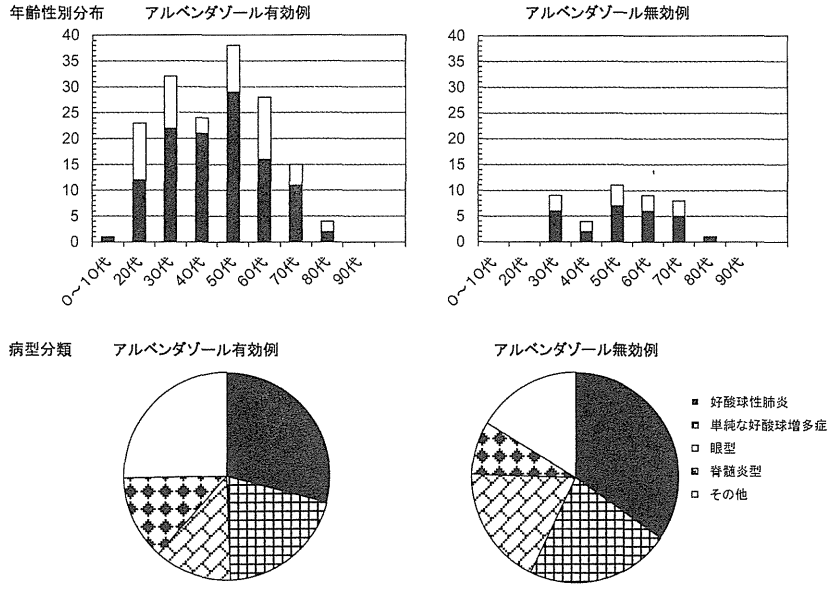
年	年間新規依頼件数	動物由来回虫症	
		新規症例数	フォローアップ症例数
2004	631	100	37
2005	597	103	56
2006	499	82	34
2007	498	101	29
2008	426	78	32
2009	380	49	22
2010	416	48	18
2011	419	52	19
2012	381	30	16
計	4,247	643	263

る使用薬剤は，アルベンダゾール単独使用が 231 例（87.8%），アルベンダゾールと他駆虫薬の使用が 9 例（3.4%），アルベンダゾール以外の駆虫薬の使用例が 18 例（6.9%），自然治癒が 5 例（1.9%）であった．

治療後の全体の転帰は，治癒ないし軽快の有効例が 203 例（77.2%），無効例が 60 例（22.8%）であった．これをアルベンダゾール単独使用例とアルベンダゾールと他剤の併用，アルベンダゾール以外の薬剤の使用例に分けて検討すると，アルベンダゾール単独使用での有効率は 78.8 %（182/231），アルベンダゾールと他剤の併用での有効率は 66.7%（6/9），そして他剤のみ使用での有効率は 61.1%（11/18）であった．ほとんどの症例で，アルベンダゾールの投与は，10～15mg/kg を 4 週間以上という熱帯病治療薬研究班の推奨用量にしたがっていた．

アルベンダゾール単独使用 231 例において，有効例と無効例の間の臨床的な違いの有無をみるため，年齢性別分布と病型について検討した．病型は，血清診断の検査申込書に記載された担当医師の所見をもとに，呼吸器型，神経型，眼型，単純好酸球増多等に分類した．しかしながら，有効例と無効例ではみられた病型の割合に有意差はなかった（図 1）．

アルベンダゾール投与による副作用では，何らかの副作用の記載があったものが 31 例あった．最も多かったのは肝障害の 28 例で，貧血，胃痛，嘔気が各 1 件ずつあった．アルベンダゾール以外の薬剤の有効性を検討する目的で，アルベンダゾール以外の薬剤を用いた治療例を調べたところ，イベルメクチン使用 9 例中，6 例（66.7%）が治癒または軽快したことが分かった（表 2）．



アルベンダゾール単独で治療した症例を有効例と無効例に分け，年齢性別分布と病型を検討した．どちらについても統計的な有意差はなかった．

図 1 アルベンダゾールの効果の有無と患者集団の特徴

表2　アルベンダゾール以外の薬剤による治療例

性別	年齢	居住地	臨床病型	使用薬剤	転帰
M	44	兵庫	脊髄炎	アルベンダゾールにより肝障害，メベンダゾール	軽快
M	57	不明	眼トキソカラ症	ジエチルカルバマジンとステロイド併用	治癒
不明	40	福岡	眼トキソカラ症	ジエチルカルバマジンとステロイド併用 さらにアルベンダゾール	治癒
M	60	京都	眼トキソカラ症	イベルメクチン	治癒
F	53	福岡	好酸球性心筋心膜炎	イベルメクチン	治癒
M	60	滋賀	好酸球性肺炎	イベルメクチン	治癒
F	62	兵庫	好酸球性肺炎	アルベンダゾールで肝障害，イベルメクチン	治癒
F	74	宮崎	好酸球性肺炎	アルベンダゾール無効にてイベルメクチン	治癒
F	52	大分	好酸球性肺炎	イベルメクチン	治癒
F	31	宮崎	好酸球性肺炎	アルベンダゾールで抗体価下がらずイベルメクチン	変化なし
不明	69	佐賀	頸髄障害	ジエチルカルバマジン	変化なし
M	27	東京	眼トキソカラ症	ジエチルカルバマジン	変化なし
F	71	長崎	好酸球性肺炎	イベルメクチン	変化なし
M	56	長崎	好酸球性肺炎	イベルメクチン	変化なし

考察

動物由来の回虫類幼虫による幼虫移行症は自然治癒傾向があるとされるが，放置した場合には好酸球増多が長期間続き，心筋等の障害が懸念される[4]．今回の分析では，ほぼ全ての症例で駆虫薬治療が選択され，263 例中駆虫薬の投与がなかったのは 5 例（1.9%）に過ぎなかった．

動物由来回虫症に対する第一選択薬は適応外薬のアルベンダゾールだが，有効性に関するまとまったデータはなかった．今回のわれわれの分析により，アルベンダゾール単独での治療成功例は軽快を含めて 231 例中 182 例で，有効率は 78.8% であった．一方，副作用は 240 例中 31 例（12.9%）であり，重篤なものはなかった．ただしこれらはあくまでも抗体フォローアップ依頼の申込書への記載に基づく分析であることに留意すべきである．

アルベンダゾール以外に使用できる薬剤としては，イベルメクチンが考えられた（有効率 66.7%）．症例数は少ないがアルベンダゾールの使用が難しいケースでは積極的にイベルメクチンを用いる価値があると考えられた．

謝辞

本研究は，厚生労働科学研究費補助金 医療技術実用化総合研究事業「わが国における熱帯病・寄生虫症の最適な診断治療体制の構築」（H25-医療技術-指定-012）の補助により実施された．

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寄生虫感染症

吸虫症

Fluke infections

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Key words：脳肺吸虫症，神経型住血吸虫症，痙攣，麻痺，プラジカンテル

はじめに

吸虫は，条虫類と同じく扁形動物門に属する寄生性の動物で，生活史は基本的に2つの中間宿主を必要とし，第1中間宿主は陸産・淡水産の巻貝，第2中間宿主は吸虫の種によって様々である．終宿主は脊椎動物で，ヒトは吸虫にとっては終宿主になる．吸虫感染症で神経症状をきたすことはまれだが，肺吸虫症と住血吸虫症で比較的多く報告されている．

1. 概念・定義

吸虫類の人体内での寄生部位は，消化管か消化管と解剖学的につながっている肺や胆道系が主で，神経系は本来の寄生部位ではない．吸虫症における神経症状は，虫体が神経系に‘迷入’するか，あるいは成虫が産卵した虫卵が血行性に中枢神経に到達することによって引き起こされる．

脳肺吸虫症は，肺吸虫の成虫が中枢神経系に迷入して起きる．かつて肺吸虫症が西日本を中心に多数みられたときには，小児を中心として大きな問題であった．一方，神経型住血吸虫症は，寄生部位である門脈系血管内に産卵された虫卵が中枢の小血管を塞栓し肉芽腫を形成するのが病態の中心をなす．

2. 疫学と病因

1) 肺吸虫症

肺吸虫属の中でこれまで人体寄生が確認されているのは7種で，そのうち神経系への迷入が報告されているのは，ウエステルマン肺吸虫(*Paragonimus westermani*)，宮崎肺吸虫(*P. miyazakii*)，*P. mexicanus*，*P. africanus*である．宮崎肺吸虫は今日ではスクリアビン肺吸虫(*P. skrjabini*)の亜種あるいは種内変異と見なされるようになったが，本稿では宮崎肺吸虫の用語を採用する．

肺吸虫症は多くの吸虫疾患と同様，食品由来の寄生虫感染症である．第2中間宿主の淡水産の甲殻類(我が国ではサワガニとモクズガニ，韓国ではザリガニが主)を加熱不十分な調理法で摂食すると，感染型の幼虫(メタセルカリア)が人体に取り込まれ感染が成立する．甲殻類の生食による感染以外に，イノシシ肉を刺身で食べてウエステルマン肺吸虫に感染する症例もみられる．イノシシが肺吸虫に感染したモクズガニやサワガニを食べると，メタセルカリアはイノシシの体内で成虫になれず，筋肉内でメタセルカリアないし幼若成虫のまま長期間とどまり感染源になる．

脳肺吸虫症の特徴として，理由は不明だが患者は子どもや若年層の男性に多いことが知られている．かつての我が国のウエステルマン肺吸

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0047-1852/13/¥60/頁/JCOPY

虫による脳肺吸虫症の報告では30歳以下が圧倒的に多く，特に10歳以下の小児が40-60％を占めていた[1]．メキシコ肺吸虫の報告でも5-8歳(3症例)の小児であり[2]，最近の中国における報告でも脳肺吸虫症は若年層男性に多い[3]．

2）住血吸虫症

人体に寄生する住血吸虫のうち神経症状が報告されているのは，ビルハルツ住血吸虫(*Schistosoma haematobium*)，マンソン住血吸虫(*S. mansoni*)，日本住血吸虫(*S. japonicum*)，メコン住血吸虫(*S. mekongi*)の4種類で，前2種では主に脊髄病変，後2種では主に大脳皮質の病変が報告されている．それぞれの種の分布域は，ビルハルツ住血吸虫がアフリカと中近東，マンソン住血吸虫がアフリカと中近東および中南米の一部，日本住血吸虫が中国の揚子江流域とフィリピンなどの東アジア，メコン住血吸虫はメコン川流域の東南アジアである．

住血吸虫類は，吸虫の中では例外的に第2中間宿主を必要とせず，第1中間宿主の貝から水中に遊出した感染型の幼虫(セルカリア)が，直接終宿主に経皮感染する．したがって住血吸虫症は食品由来ではなく，中間宿主貝が生息する河川や湖沼での農作業や漁撈，遊泳など，水との接触に伴って感染する．

3. 病　　態

1）肺吸虫症

病態の基本は，虫体そのものと虫体周囲の好酸球性炎症による組織破壊である．炎症の部位によって髄膜刺激症状や出血も起こしうる．食品とともに経口摂取されたメタセルカリアは消化管から腹腔内へ出て，腹壁の結合組織や筋肉内に入り込んでおよそ2週間ほどして再度腹腔に戻り，そこから次に肝臓/横隔膜を通過して胸腔に入る．そして肺の胸膜を破って肺実質へ侵入する．

このような体内移行経路をとるため，途中で様々な部位に侵入しうる．中枢神経系への侵入経路は必ずしも確定していないが，体内移行の途中に腹腔から血行性に脊髄腔へ入り頭蓋内に入る経路，縦隔の軟部組織を上行して頸静脈に沿って頭蓋腔へ入る経路，および頸静脈内を上行して横洞内を経て脳実質へ入るという経路が推測されている[4]．

2）住血吸虫症

住血吸虫のセルカリアは，経皮感染後に血流に乗って肺に至り，血管内を移動して成虫になる．寄生部位は，ビルハルツ住血吸虫は膀胱近傍の静脈系，マンソン住血吸虫や日本住血吸虫は，腸間膜静脈-肝門脈系である．

神経型住血吸虫症は感染者全体の2-4％で報告されるが，比較的報告数が多い日本住血吸虫症では大脳皮質の病変が中心で，ビルハルツ住血吸虫，マンソン住血吸虫症では，より下位の脊髄病変が多い．神経型住血吸虫症は，虫卵による病変が直接の病因となっている直接型と，肝性脳症による間接型に分けて考えると理解しやすい．

a. 直 接 型

住血吸虫卵による中枢神経系血管内の塞栓で生じるもの，虫卵塞栓を核にした肉芽腫によるもの，肉芽腫血管炎や虫体・虫卵の代謝産物による炎症性変化で生じるものなどに分けて考えることができる．前者では，脳血管障害でみられるような巣症状が中心となるが，後者では，髄膜炎・脳炎様症状が伴うことがある[5,6]．

大量の住血吸虫セルカリアに同時に感染した場合，片山症候群といわれる急激な発熱に続き，咳や腹部膨満感，頭痛・嘔吐・痙攣・意識障害といった急性髄膜脳炎の症状を示すことがある．非特異的な急性症状が中心で，好酸球増多を示す例が多くステロイド治療に反応することから，虫卵塞栓よりも免疫反応活性化の関与が大きいと思われる．

慢性期には，神経系の他の虚血性病変でもみられる多彩な巣症状と，病変の中心が皮質にある場合は，痙攣発作で始まることが多い．発作型は，大発作は20％程度で部分発作が残りの大半を占める[7]．初発作後に上肢の単麻痺や片麻痺，失語症や視野障害などを合併することが多く，局在性病巣による巣症状は，一過性のこともあれば継続する場合もある．発作の頻度は，病初期には多いが次第に減少していく．中枢神